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The role of specific β–γ subunit interactions in oxyanion stimulation of the MgATP hydrolysis of a hybrid photosynthetic F_1-ATPase

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Abstract Pairs of cysteine residues were introduced into the twisted N- and C-terminal helices of the γ subunit of the chloroplast F_1-ATPase to test, via disulfide cross-linking, potential inter-helical movements involved in catalysis of ATP hydrolysis. The extent of disulfide cross-linking was determined by estimating the amount of free sulfhydryl available for labeling with fluoresceinyl maleimide before and after cross-linking. Significant disulfide formation (50–75%) was observed between cysteines introduced at positions 30 and 31 in the N-terminal helix and 276 and 278 in the C-terminal helix. Cross-linking had no apparent effect on catalysis, therefore eliminating the involvement of large-scale inter-helical movements within this region of the γ subunit in cooperative ATP hydrolysis. However, the presence of the two cysteines together in the γV31C/A276C double mutant, irrespective of whether or not they were cross-linked together, lowered the MgATPase activity by more than 70% and completely eliminated the well-known activating effect of the oxyanion sulfite. The CaATPase activity was unaffected. Similar but less pronounced effects were seen with the γK30C/A276C double mutant. The results indicate that residues at or near positions 31 and 276 within the twisted helical pair of the γ subunit are required to overcome Mg^{2+} inhibition of ATP hydrolysis. These residues are likely to be involved in forming a point of contact between the γ and β subunits that is responsible for this effect.

Keywords ATP synthase · Regulation · Chloroplast · Gamma–beta interaction · Oxyanion activation

Introduction

The ATP synthase enzymes of chloroplasts, mitochondria and bacteria are composed of two protein segments, F_O (factor O) and F_1 (factor 1). The F_O segment is a membrane-spanning proton channel. The chloroplast F_O (CF_O)[1] contains four different polypeptide subunits (I to IV) with a stoichiometry of $I_1II_1III_{14}IV_1$. The F_1 segment contains the catalytic sites for ATP synthesis and hydrolysis. The chloroplast F_1 (CF_1) is comprised of five different polypeptide subunits (α to ε) with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The primary structural features of ATP synthases from one organism to another are highly conserved (Cross 2000). The $\alpha_3\beta_3$ hexamer, comprised of alternating α and β subunits, contains six nucleotide binding sites located at each of the six α/β subunit interfaces. The three β subunits contribute most of the structure of three catalytic nucleotide binding sites whereas the three α subunits contribute most of the structure of three non-catalytic nucleotide binding sites. Each of the three catalytic sites exists in a distinct conformation as a result of asymmetric interactions between the $\alpha_3\beta_3$ hexamer and the single-copy γ subunit (Abrahams et al. 1994; Gao et al. 1995). Rotation of the γ subunit within the $\alpha_3\beta_3$

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[1] The abbreviations used are: ACMA, 9-Amino-6-chloro-2-methoxyacridine; CF_1, EcF_1, MF_1 and TF_1, the catalytic coupling factor 1 from chloroplasts, *Eschericia coli*, mitochondria and thermophilic bacteria respectively; $CF_1(-\delta\varepsilon)$, CF_1 deficient in the δ and ε subunits; CF_O, the proton transporting chloroplast coupling factor O; Tricine, *N*-(2-hydroxy-1-*bis*(hydroxymethyl)ethyl)glycine; Tris, tris(hydroxymethyl) amino-methane.

hexamer, driven by the cooperative binding change mechanism (Boyer 1993, 1997), results in the sequential alternation of the catalytic sites between the different conformational states.

The CF_1 γ subunit contains a special regulatory domain consisting of ~40 amino acids that is not present in other F_1 enzymes (Richter et al. 2005). This unique domain contains a critically placed vicinal dithiol that, upon oxidation, forms an intrachain disulfide bridge resulting in a partially inhibited form of the enzyme (Ketcham et al. 1984; Moroney et al. 1980, 1984). Oxidation of the dithiol also promotes a binding interaction between the C-terminal domain of the inhibitory ε subunit and the γ subunit that results in a fully inhibited, latent form of the enzyme (Cruz et al. 1995; Cruz et al. 1997; Richter and McCarty 1987; Richter et al. 1984; Richter et al. 1985). Physiological conditions that result in oxidation of the dithiol in CF_OF_1 are important to prevent futile hydrolysis of essential ATP pools in the dark when photosynthetic electron transport and photophosphorylation are inactive (Ort and Oxborough 1992; Wu et al. 2007).

The regulatory γ dithiol is located over 60 Å away from the catalytic sites in the enzyme (Richter et al. 1985; Snyder and Hammes 1985). It is assumed that disulfide formation, together with the induced interaction with the ε subunit, blocks an essential conformational step in catalysis. Identification of this step is of considerable interest both in understanding the physiological process of regulation and in understanding the conformational steps that produce directional rotation of the γ subunit. The recently published homology model of the CF_1 γ subunit shown in Fig. 1, that is based on known structures of its mitochondrial and bacterial counterparts (Richter et al. 2005), has provided new insight into the structure of the regulatory domain. The model has also identified potential inter-domain movements within the γ subunit that may either be required for catalysis or responsible for its regulation (Richter et al. 2005). One possible conformational movement may involve the N- and C-terminal ends of the γ subunit that have been shown in MF_1 and EcF_1 to form helical elements that twist around each other to form the rotating spindle. Relative rotation of the two helical elements has been proposed as a possible mechanism of absorbing rotational strain to offset a symmetry mismatch between γ subunit rotation and the rotating ring of C subunits (Junge et al. 1997). In addition, a possible mechanism of dithiol regulation in CF_1 assumes that a relative rotation of the N- and C-terminal helices with respect to one another occurs during ATP hydrolysis and is impeded upon formation of the regulatory disulfide bond (Richter et al. 2005; Samra et al. 2006).

To examine the possibility that relative rotation of the N- and C-terminal helices occurs during ATP hydrolysis and/or regulation of this process by CF_1, we prepared mutant CF_1 γ subunits that contained pairs of cysteinyl residues introduced at select locations where, according to the previously published CF_1 γ subunit homology model, they would be expected to form disulfide bridges and thus short, covalent cross-links between the two helical elements. The mutant γ subunits were assembled into a hybrid photosynthetic F_1 complex containing the α and β subunits from *Rhodospirillum rubrum* F_1 (RrF_1). Assembly into the hybrid enzyme was chosen as a format for examining the γ

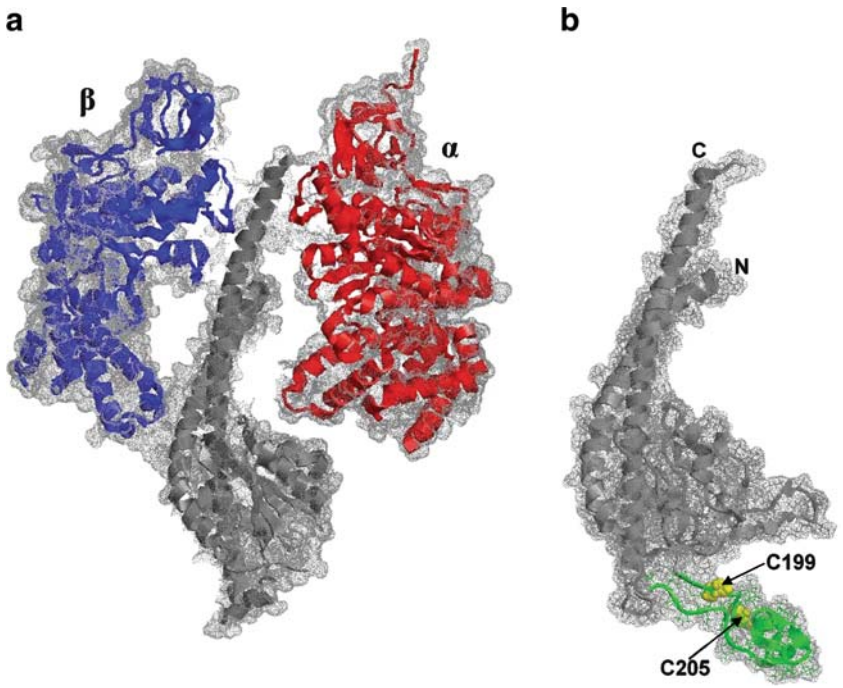

Fig. 1 F_1 subunit interactions. **a** A cross section of part of the bovine MF_1 showing the γ subunit (*grey*), one β subunit (*blue*), and one α subunit (*red*). **b** Side view of the CF_1 gamma homology model (*grey*) indicating the regulatory domain (*green*). Cys 199 and Cys 205, the two residues that comprise the regulatory dithiol, are space-filled in *yellow*

mutants in the event that it is necessary to show the effects of the mutations on γ rotation. The hybrid assembly is the only photosynthetic assembly that can be produced from recombinant subunits, a prerequisite for experiments in which actin filament rotation can be examined (Du and Gromet-Elhanan 1999; Du et al. 2001; Tucker et al. 2004).

There are two significant findings of this study. One is that cross-linking the N- and C-terminal helices together had no apparent effect on the ATPase activity of the hybrid enzyme indicating that large-scale inter-helical movements are not required for ATP hydrolysis. The second finding is that introducing a pair of cysteines in place of V31 and A276 in the γ subunit completely abolished the oxyanion-induced stimulation of the MgATPase activity that is characteristic of F_1 enzymes from eukaryotic sources. This latter observation has important implications for our understanding of the binding change process that leads to the cooperative nucleotide interactions that drive directional rotation of the γ subunit.

Experimental procedures

Materials DEAE cellulose, antibiotics (ampicillin, tetracycline, and chloramphenicol), Sephadex G-50 resin, and Ni-NTA resin were purchased from Sigma-Aldrich. Hydroxyapatite HTP gel was from BioRad. Tryptone and yeast extract were obtained from DIFCO. ATP (grade II) was purchased from Midwest Scientific and urea (ultra pure) was purchased from ICN Biomedicals Inc. Dialysis tubing (8,000 M.W. cut-off) was obtained from Biodesign Inc. (New York). All other chemicals were of the highest quality reagent grade available.

Production of γ subunit mutants Mutant CF_1 γ subunits were constructed by enzymatic amplification of the expression plasmid pET8c-gamma.BB1 (Sokolov et al. 1999). This plasmid contained the wild-type γ subunit gene with all four native cysteines mutated to alanines, resulting in a "Cysless" γ subunit. Primers were obtained from Integrated DNA Technologies (Iowa). PCR reactions, plasmid DNA purification, and recombinant plasmid ligation were carried out as described elsewhere (Tucker et al. 2001). The resulting plasmid was transformed into competent *E. coli* XL1-blue cells. The entire sequence of each mutant gene was confirmed by the Iowa State DNA Sequencing Facility. Sequenced plasmids were then transformed into the expression host *E. coli* BL21(DE3)/pLysS, and the mutant γ subunits were expressed in insoluble inclusion bodies as described earlier (Gao et al. 1995).

Assembly and purification of hybrid $\alpha_3\beta_3\gamma$ complexes Inclusion bodies from the three core subunits ($RrF_1\alpha_{6xhis}$, $RrF_1\beta$, and wild type and mutant $CF_1\gamma$) were solubilized in 8 M urea, diluted in refolding buffer, and assembled as previously described (He et al. 2007). Following an overnight dialysis, the assembled protein complexes were purified by DEAE anion exchange chromatography (Gao et al. 1995). Further purification was achieved by size-exclusion chromatography using a Superdex 200 (Pharmacia) column attached to a Biorad Biologic HR fast performance chromatography system running at a flow rate of 0.5 mL/min. The recombinant protein samples were eluted in 50 mM Tricine–NaOH (pH 7.8) and 50 mM NaCl. 20% (*v*/*v*) glycerol and 1 mM ATP were added to the eluted protein samples before storage at −80°C.

Fluorescent labeling of free sulfhydryls To determine labeling specificity, isolated inclusion bodies for each cysteine pair mutant were solubilized in 8 M urea and 50 mM Tricine (pH 8.0) for 20 min at 4°C. Approximately 1 mg of each solubilized mutant protein was treated with 100 μM $CuCl_2$ (thiol oxidizing conditions) for 30 min, and another 1 mg sample of each was treated with 25 mM DTT (thiol reducing conditions) for 30 min. The samples were passed through Sephadex G-50 centrifuge columns, equilibrated with 8 M urea 50 mM Tricine (pH 8.0), to remove any remaining DTT or $CuCl_2$. Reduced samples were passed through an additional Sephadex G-50 column to ensure all DTT was removed. Approximately 100 μg of each inclusion body sample was then incubated with 30 μM fluoresceine maleimide for 30 min. Following incubation, 100 μM DTT was added to quench any unreacted probe.

To examine the amount of free sulfhydryl following oxidizing or reducing treatment of γ subunits that had been assembled with the α and β subunits, 120–200 μg of each recombinant complex in 0.5 mL of solution containing 50 mM Tricine–NaOH (pH 8.0) was treated either with $CuCl_2$ or DTT, excess reagent was then removed by gel filtration, followed by labeling of 100 μg of mutant enzyme with fluorescein maleimide as described above for the free γ subunit. The labeled enzymes were precipitated with 40% (*v*/*v*) trichloroacetic acid and centrifuged at 10,000×*g* for 20 min. The protein pellets were dissolved in an SDS sample buffer that was devoid of reducing agent. Gel electrophoresis was performed under non-reducing conditions on pre-cast NuPage® (Invitrogen, San Diego) gels (4–20% acrylamide gradient) with each lane containing approximately 7.5–10 μg of protein. Protein concentrations were determined by the Bradford method (Bradford 1976).

ATPase assays ATPase activities were determined by measuring phosphate release (Taussky and Shorr 1953) for 5 min at 37°C. The assay mixture for Ca^{2+}-dependent ATPase activity contained 50 mM Tricine–NaOH (pH 8.0),

5 mM ATP and 5 mM $CaCl_2$. That for Mg^{2+}-dependent ATPase activity contained 40 mM Tricine–NaOH (pH 8), 4 mM ATP, 2 mM $MgCl_2$ and in some cases 50 mM Na_2SO_3.

Results

Formation of inter-helical cross-links within the γ subunit Pairs of cysteine residues were introduced into the CF_1 γ subunit in an attempt to directly cross-link the putative N- and C-terminal helical elements of the γ subunit together via disulfide linkages. A recombinant "Cys-less" γ subunit was prepared in which all four native cysteines at positions 89, 199, 205, and 322 were changed to alanine. The Cys-less γ was used as a template to introduce pairs of cysteines at new positions as summarized in Table 1. The positions were chosen based on the published homology model of the CF_1 γ subunit as indicated in Fig. 2. Cysteines were introduced either at position 30 or 31 on the N-terminal helix and at position 276 or 278 on the C-terminal helix of the γ subunit. Formation of disulfide bonds between the introduced cysteine pairs would be expected to prevent or limit relative inter-helical rotation, thereby testing the requirement for such motion in the catalytic process.

Inclusion bodies containing wild type and mutant γ subunits were solubilized in urea (8 M final concentration) then exposed to either 100 μM $CuCl_2$ or 50 mM DTT to facilitate disulfide bond formation or reduction respectively. Following two successive passages through Sephadex G-50 columns to remove the oxidizing or reducing agent, each protein was treated with fluoresceinyl maleimide to label any free sulfhydryls. The proteins were subjected to SDS gel electrophoresis under non-reducing conditions and visualized first by fluorescence under ultra violet light (Fig. 3a, lower band) followed by Coomassie Blue staining (Fig. 3a, upper band). The Cys-less γ subunit (lanes 3 and 4) failed to label with the fluoresent probe demonstrating the specificity of the labeling process for cysteine residues. The wild type and all of the mutant γ subunits were labeled, confirming the presence of the introduced cysteine residues.

Wild type and mutant CF_1 γ subunits were assembled with recombinant α and β subunits from *R. rubrum* F_1 to form functional hybrid enzymes as described in the "Experimental procedures." The assembly conditions and the catalytic properties of the hybrid enzyme have been published previously (Du and Gromet-Elhanan 1999; Tucker et al. 2001). All mutant γ subunits assembled with the α and β subunits into catalytically active complexes (Table 1) that remained stable during purification and exhibited identical subunit ratios to those of the wild type complex as judged by SDS gel electrophoresis.

The purified mutant assemblies were exposed either to thiol oxidizing (100 μM $CuCl_2$) or reducing (50 mM DTT) conditions then treated with fluoresceinyl maleimide for 30 min. Figure 3b shows the bands corresponding to the α and γ subunits of the labeled mutant enzyme assemblies after separation by SDS gel electrophoresis. Equivalent amounts of enzyme were loaded into each lane. Images were captured with a minimal exposure time to mimic, as closely as possible, the relative fluorescence intensities that were observed with the naked eye. Each α subunit contains three cysteine residues and were heavily labeled by fluoresceinyl maleimide. The β subunits, which migrate between the α and γ subunits, contain no cysteine residues and therefore do not label. The γ subunit band of the wild type enzyme (lanes 1 and 2) showed the expected loss of labeling due to formation of the regulatory disulfide bond in the presence of $CuCl_2$ (lane 1). Large reductions in the extent of γ labeling were also observed with the γK30C/A276C, γV31C/A276C pair mutants indicating considerable disulfide bond formation under oxidizing conditions in each. The high intensity of labeling observed with both the γK30C/A276C and γV31C/A276C mutants after treatment with DTT indicated that, following reduction, at least one member of the cysteine pair became solvent accessible and strongly labeled. In contrast, both the oxidized and reduced samples of the γV31C/R278C pair (Fig. 3b, lanes 7 and 8) were labeled to a low extent although there was a slight increase apparent following reduction. The same was observed for the γK30C/R278C pair, however, the α subunit of this assembly was significantly proteolyzed during preparation for gel electrophoresis so the results are not shown.

The low labeling and the lack of difference between the oxidized and reduced samples in the γV31C/R278C pair mutant may have been due either to significant cross-linking or to poor solvent accessibility of the sulfhydryls or both. Since cross-linking within the γ subunit often leads to a small shift in the mobility of the γ band (Ketcham et al. 1984), the presence of a shift can be used to confirm cross-

Table 1 CF_1 gamma subunit mutants assembled with the *R.spirillum* α and β subunits

Hybrid protein[a]	Assembly competency[b]
$\alpha_3\beta_3\gamma$WT	+
$\alpha_3\beta_3\gamma$Cysless	+
$\alpha_3\beta_3\gamma$K30C/A276C	+
$\alpha_3\beta_3\gamma$V31C/A276C	+
$\alpha_3\beta_3\gamma$K30C/R278C	+
$\alpha_3\beta_3\gamma$V31C/R278C	+

[a] Hybrid F_1 assemblies were purified and assayed as described in "Experimental procedures."
[b] Assembly competency was determined by purification yield, catalytic competency and relative subunit staining on SDS-PAGE gels.

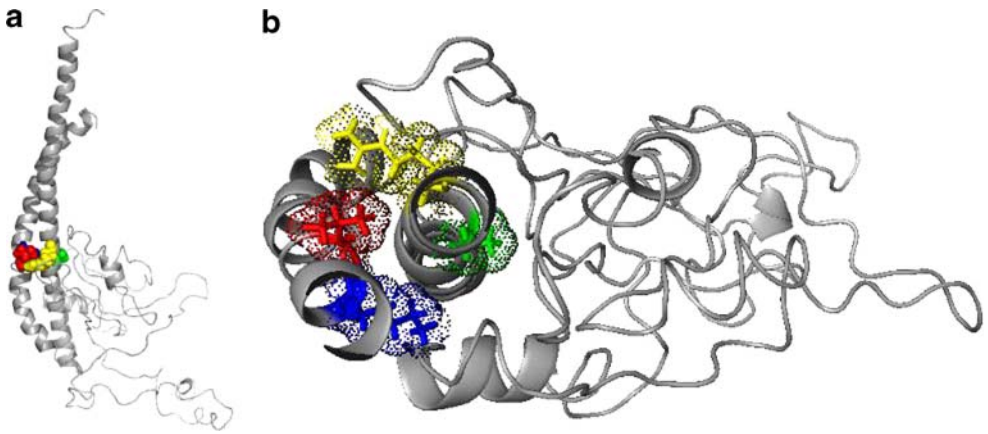

Fig. 2 Placement of potential cross-linking dithiols. **a** The homology model of the γ subunit of the chloroplast ATP synthase is shown in *grey*. The residues mutated to cysteines in this study are in ball and stick configurations: γK30 (*blue*), γV31 (red), γA276 (*green*), γR278 (*yellow*). **b** Through-space view of the chloroplast γ subunit model along the axis of the twisted helical pair, indicating the positions of the mutated residues. The figures were generated using PyMOL

linking. The absence of a shift, however, is not necessarily a reliable indicator of lack of cross-linking. There was indeed a shift observed in the mobility of both the γK30C/A276C and γV31C/A276C double mutants following coomassie staining of the gel shown in Fig. 3b (not shown). No shift was observed with either the γK30C/R278C or γV31C/R278C mutant pairs consistent with a lack of cross-linking. Either these residues are largely buried and poorly accessible to the labeling agent or they are not redox active. From the fluorescence and coomassie stained γ bands the extent of cross-linking was estimated to be greater than 75% in the γK30C/A276C mutant and greater than 50% in the γV31C/A276C mutant. The cross-linking experiments described in Fig. 3 were repeated at least once with the same outcome.

Effects of cross-linking on ATPase activity The wild type and mutant assemblies that were exposed to conditions that either promote disulfide formation or reduction were examined for their ATPase activities under saturating substrate concentrations as summarized in Table 2. Both the Ca- and Mg-dependent ATPase activities of the enzyme containing the wild-type γ subunit that still possesses the regulatory dithiol, showed the expected increase in activity (~1.5-fold) resulting from reduction of the disulfide. In contrast, the Cys-less mutant used as the template for inserting cysteine pairs showed the maximum activity and did not respond to oxidizing or reducing conditions. Since the mutant enzymes are deficient in the regulatory dithiol forming cysteines they should be maximally activated and any difference in activity resulting from exposure to oxidizing or reducing conditions should reflect the effect of formation of a disulfide bond between the introduced cysteine pairs. However, none of the mutants exhibited a significant difference in their Ca- or MgATPase activities following oxidation or reduction despite evidence of

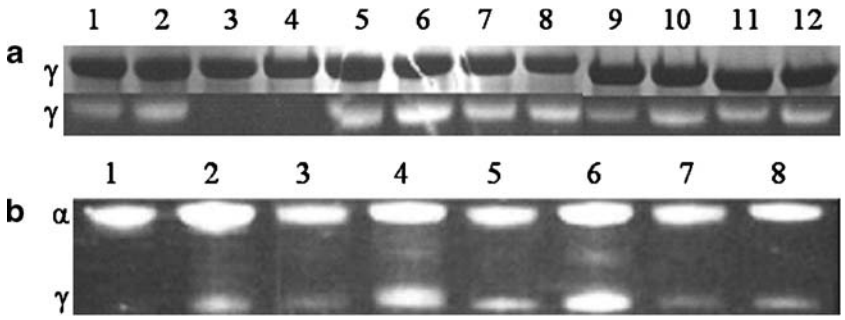

Fig. 3 Labeling of free sulfhydryls by fluoresceinyl maleimide. **a** Urea-solubilized γ mutants. Inclusion bodies containing the mutant γ subunits were solubilized in urea, treated with $CuCl_2$ (oxidized) or DTT (reduced), labeled with fluoresceinyl maleimide and subjected to SDS-PAGE as described in "Experimental procedures." The *upper portion* of the figure shows the gel stained with Coomassie brilliant blue, while the *lower portion* shows the fluorescence of the same gel prior to Coomassie staining. *Lane 1*, γWT oxidized; *Lane 2*, γWT reduced; *Lane 3*, γCys-less oxidized; *Lane 4*, γCys-less reduced; *Lane 5*, γK30C/A276C oxidized; *Lane 6*, γK30C/A276C reduced; *Lane 7*, γV31C/A276C oxidized; *Lane 8*, γV31C/A276C reduced; *Lane 9*, γV31C/R278C oxidized; *Lane 10*, γV31C/R278C reduced; *Lane 10*, γV30C/R278C oxidized; *Lane 11*, γV30C/R278C reduced. **b** hybrid enzyme assemblies containing fluoresceine-labeled γ mutants. Purified hybrid enzymes assembled with the mutant gamma subunits were labeled with fluoresceinyl maleimide before and after treatment with $CuCl_2$ (oxidizing) or DTT (reducing) as described in "Experimental procedures." The *upper band* is the α subunit which contains exposed cysteinyl residues that label under all conditions and is shown for comparison. The *lower band* is the γ subunit. *Lane 1*, $\alpha_3\beta_3\gamma$WT, oxidized; *Lane 2*, $\alpha_3\beta_3\gamma$WT, reduced; *Lane 3*, $\alpha_3\beta_3\gamma$K30C/A276C, oxidized; *Lane 4*, $\alpha_3\beta_3\gamma$K30C/A276C, reduced; *Lane 5*, $\alpha_3\beta_3\gamma$V31C/A276C, oxidized; *Lane 6*, $\alpha_3\beta_3\gamma$V31C/A276C, reduced; *Lane 7*, $\alpha_3\beta_3\gamma$V31C/R278C, oxidized; *Lane 8*, $\alpha_3\beta_3\gamma$V31C/R278C, reduced

Table 2 ATPase activities of mutant gamma subunit assemblies

Hybrid protein[a]	Ca^{2+}ATPase activity	Mg^{2+}ATPase activity
	(μmole per min per mg protein)	
	Oxidized[b]/reduced[b]	
$\alpha_3\beta_3\gamma$WT	31.5±4.0/47.8±2.1	8.3±0.2/14.0±0.9
$\alpha_3\beta_3\gamma$Cysless	46.0±1.6/45.8±2.2	13.8±1.5/13.4±1.6
$\alpha_3\beta_3\gamma$K30C/A276C	42.1±5.1/45.6±0.6	3.9±0.8/4.1±1.1
$\alpha_3\beta_3\gamma$V31C/A276C	42.1±2.1/44.7±1.5	4.3±1.0/3.9±1.7
$\alpha_3\beta_3\gamma$K30C/R278C	42.1±3.6/46.3±2.9	7.2±0.9/8.3±1.6
$\alpha_3\beta_3\gamma$V31C/R278C	37.5±5.2/37.0±2.3	6.1±1.2/5.8±0.9

[a] Hybrid F_1 assemblies were purified and assayed as described in "Experimental procedures."
[b] The enzyme assembly was pre-treated with either 25 mM DTT (reduced) or 100 μM $CuCl_2$ (oxidized) for 30 minutes prior to assay. Errors are expressed as standard deviations with n=4.

significant cross-linking under oxidizing conditions. There was, however, a decrease of approximately 40% to 70% in the MgATPase activities of all of the mutant enzymes with respect to the Cys-less control irrespective of the pre-treatment. The most marked effect, however, was on the γK30C/A276C and γV31C/A276C mutants which, in addition to loss of MgATPase activity, were much less responsive than the enzymes containing the wild type or Cys-less γ subunits to the stimulatory effect of the oxyanion sulfite (Table 3). All of the mutants except γK30C/A276C and γV31C/A276C were stimulated to near maximum MgATPase levels at saturating concentrations of sulfite. The γK30C/A276C mutant was partially stimulated (to ~37% of the Cys-less control) whereas the γV31C/A276C mutant showed no stimulation at all by sulfite. Since the single point mutation γA276C had no effect on MgATP hydrolysis or on sulfite stimulation (data not shown) it is concluded that the reduction in MgATPase activity and the loss of oxyanion stimulation resulted from the introduction of two cysteine residues together into the γ subunit in close proximity to each other.

Discussion

Although the fluorescence accessibility study indicated that significant cross-linking occurred between two of the introduced cysteine pairs, cross-linking within these enzyme assemblies did not lead to a significant reduction in ATP hydrolysis rates. This observation eliminates an involvement of large-scale movements between the N- and C-terminal helical elements of the γ subunit in ATP hydrolysis (Richter et al. 2005). Remarkably, however, introduction of pairs of cysteines at positions 31 (or 30) and 276 resulted in differential effects on the CaATPase and MgATPase activities. MgATPase activities are low relative to CaATPase activities due to the effect of Mg^{2+} ions in stabilizing binding of inhibitory ADP (Du and Boyer 1990). Presumably the pair mutants enhance this effect. The most striking effect, however, was on the sulfite stimulation in the γV31C/A276C mutant in which the rate of MgATP hydrolysis was selectively reduced and the stimulatory effect of the oxyanion sulfite on the MgATPase activity was completely eliminated.

The region of contact between the N- and C-terminal helices in the vicinity of γK30/V31 and γA276 is close to two of the major points of contact, or "catches" between the γ subunit and the β_E (empty) and β_{DP} (ADP-containing) subunits originally identified in the first published crystal structure of the bovine MF_1 (Abrahams et al. 1994). The catch interactions involve the highly conserved "DELSEED loop" region on the β subunit (residues 380 to 386) and residues 21 to 31 on the N-terminal helix of the γ subunit and residues 278 to 284 in the C-terminal helix of the γ subunit. Sequentially forming and breaking of the two catches during catalysis is thought to result in the up and down motion of the DELSEED loop resulting in opening and closing of the catalytic site. Mutation of one of the catch residues, γM23, in EcF_1 γ to lysine greatly reduced proton-coupled ATP synthesis in EcF_OF_1 without strongly effecting ATP hydrolysis (Nakamoto et al. 1995). Interestingly, the effects of the mutation were reversed by several second site mutations, one of which was mutation of γR242 to cysteine (Al-Shawi et al. 1997). The equivalent residues to γM23 and γR242 in MF_1 are adjacent to each other in the MF_1 structure and interact with the DELSEED loop on the β subunit. The interpretation of these effects was that replacing M23 with lysine provided an additional bonding interaction between the twisted helical element of the γ subunit and the DELSEED loop resulting in tightening of the catch interaction. The effect was reversed

Table 3 MgATPase activities of mutant gamma subunit assemblies

Hybrid protein[a]	Mg^{2+}ATPase plus sulfite activity (μmole per min per mg protein)	
	Oxidized[b]	Reduced[b]
$\alpha_3\beta_3\gamma$WT	21.3±1.5	39.5±3.8
$\alpha_3\beta_3\gamma$Cysless	40.1±1.2	40.9±0.9
$\alpha_3\beta_3\gamma$K30C/A276C	14.9±1.0	15.1±0.9
$\alpha_3\beta_3\gamma$V31C/A276C	4.24±2.5	3.8±3.0
$\alpha_3\beta_3\gamma$K30C/R278C	36.5±1.0	38.2±2.6
$\alpha_3\beta_3\gamma$V31C/R278C	32.3±1.7	31.4±2.0

[a] Hybrid F_1 assemblies were purified and assayed as described in "Experimental procedures" and assayed for MgATP hydrolysis in the presence of Na_2SO_3 at the predetermined maximum stimulatory concentration of 50 mM.
[b] The enzyme assembly was pre-treated with either 25 mM DTT (reduced) or 100 μM $CuCl_2$ (oxidized) for 30 minutes prior to assay. Errors are expressed as standard deviations with n=4.

by removing a nearby bonding interaction by substituting γR242 with cysteine (Al-Shawi et al. 1997). Thus the effects observed in the γV31C/A276C double mutant described in this study, by analogy to the EcF_OF_1, may have resulted from the combined effect of removing a positively charged residue from the catch region while at the same time introducing two cysteine residues. Since the effect on catalysis was independent of the oxidation state of the dithiol, the potential negative charge on the cysteine sulfhydryls did not appear to be an important factor in inhibition of MgATPase activity.

An indication as to why interference with the catch interaction has a greater effect on magnesium-dependent catalytic functions than on calcium-dependent functions has been provided by a recent study in which it was shown that mutations further along the C terminus of the CF_1 γ subunit caused a similar effect to that of the γV31C/A276C double mutant in reducing the oxyanion induced stimulation of MgATPase activity (He et al. 2007). The mutations at γ residues 302–305 interfere with a third catch between the γ subunit and the β_E subunit identified in the bovine MF_1 structure, indicated as the upper catch in Fig. 4. Both the upper and lower catch interactions are thought to be involved in forming the open conformation of the catalytic site so it is not unexpected that interference of either catch would have similar effects on catalytic activity. The selective loss of sulfite stimulation of the MgATPase activity observed with the γ302–305 mutants was attributed to the fact that the upper catch is specifically involved in activating the Mg-inhibited form of the enzyme. Since CaATPase activity remained high even after combined mutations that were expected to completely eliminate the catch interaction, and since CaATPase activity is assumed to result from the same binding change process (Tucker et al. 2004), it was argued that catch formation is not an essential step in the binding change process (He et al. 2007). In a follow-up study (He et al. manuscript submitted for publication) it was shown that mutation of γ residues 302 to 305 also resulted in loss of proton coupling and ATP synthesis. The fact that elimination of the upper catch interaction paralleled the effect of the γM23 mutation in EcF_1, both resulting in loss of proton coupling and ATP synthesis, further argues that both the upper and lower catches are involved in the same process. A study with the thermophylic F_1 (Hara et al. 2000) indicated that replacing β subunit DELSEED loop residues with alanine, individually or all five acidic residues at once, did not effect ATP hydrolysis-driven rotation of the γ subunit, indicating that the lower catch interaction is not required for rotational torque generation and is, therefore, not a critical component of the binding change process.

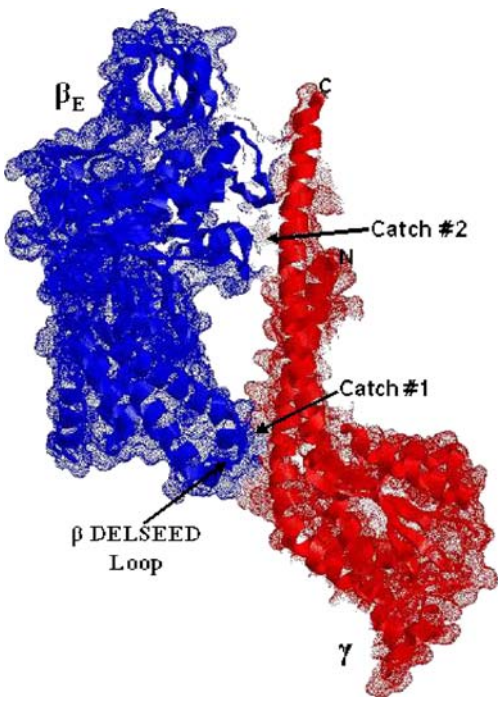

Fig. 4 Catch interactions between the γ subunit and the empty β subunit of MF_1. The figure represents a cross-section of part of the bovine MF_1 indicating the two main catch regions formed between the β_E subunit (*blue*) and the γ subunit (*red*)

Oxyanion stimulation of the MgATPase activity of F_1 enzymes results from alleviation of inhibition by free Mg^{2+} ions which stabilize bound, inhibitory ADP in nucleotide binding sites on CF_1 (He et al. 2007; Malyan 2003) and MF_1 (Malyan 2003; Murataliev and Boyer 1994). The catch interactions leading to the open configuration of the β subunit are clearly necessary for this activation process and it has been argued that this is the principle role of these interactions (He et al. 2007). The fact that they are also necessary for effective proton coupling and ATP synthesis indicates that tight binding of ADP leading to the so-called ADP-inhibited conformation is a necessary step in selective binding of ADP during ATP synthesis.

In summary, the results of this study have shown that cross-links can be formed between cysteines introduced at specific sites in the twisted N- and C-terminal helices of the γ subunit, confirming that these residues are in close proximity to one another as predicted by the modeled structure (Richter et al. 2005). However, cross-linking the two helices together had no apparent effect on catalysis, arguing against the requirement for significant relative rotation of the helices with respect to each other during ATP hydrolysis (Richter et al. 2005). The unexpected observation that the combined substitution of γV31 and γA276 with cysteinyl residues resulted in complete loss of oxyanion stimulation of the MgATPase activity of CF_1 without affecting Ca^{2+}-dependent ATP hydrolysis fits well with other observations suggesting that the interactions between the γC- and γN-terminal helices that lead to the

open conformation of the catalytic site on the β_E subunit that were identified in the crystal structure of bovine MF_1 (Abrahams et al. 1994) are not crucial for cooperative catalysis leading to γ rotation. The observations support instead the hypothesis that these β–γ contacts are required specifically to overcome Mg^{2+} ion inhibition, allowing expression of high rates of MgATP hydrolysis. Considerably more work is needed to properly identify the binding change conformations that result in γ subunit rotation.

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