C-Terminal Mutations in the Chloroplast ATP Synthase γ Subunit Impair ATP Synthesis and Stimulate ATP Hydrolysis†

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ABSTRACT: Two highly conserved amino acid residues, an arginine and a glutamine, located near the C-terminal end of the γ subunit, form a "catch" by hydrogen bonding with residues in an anionic loop on one of the three catalytic β subunits of the bovine mitochondrial F_1-ATPase [Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628]. The catch is considered to play a critical role in the binding change mechanism whereby binding of ATP to one catalytic site releases the catch and induces a partial rotation of the γ subunit. This role is supported by the observation that mutation of the equivalent arginine and glutamine residues in the *Escherichia coli* F_1 γ subunit drastically reduced all ATP-dependent catalytic activities of the enzyme [Greene, M. D., and Frasch, W. D. (2003) *J. Biol. Chem*. *278*, 5194−5198]. In this study, we show that simultaneous substitution of the equivalent residues in the chloroplast F_1 γ subunit, arginine 304 and glutamine 305, with alanine decreased the level of proton-coupled ATP synthesis by more than 80%. Both the Mg^{2+}-dependent and Ca^{2+}-dependent ATP hydrolysis activities increased by more than 3-fold as a result of these mutations; however, the sulfite-stimulated activity decreased by more than 60%. The Mg^{2+}-dependent, but not the Ca^{2+}-dependent, ATPase activity of the double mutant was insensitive to inhibition by the phytotoxic inhibitor tentoxin, indicating selective loss of catalytic cooperativity in the presence of Mg^{2+} ions. The results indicate that the catch residues are required for efficient proton coupling and for activation of multisite catalysis when MgATP is the substrate. The catch is not, however, required for CaATP-driven multisite catalysis or, therefore, for rotation of the γ subunit.

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 transporter. The chloroplast F_O (CF_O)[1] contains four different polypeptide subunits (I−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−ϵ) with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$. The α and β subunits alternate to form a hexameric ring with three catalytic sites located one on each of the three β subunits at $\alpha\beta$ interfaces, and three noncatalytic sites located one on each of the three α subunits, at the alternate $\alpha\beta$ interfaces (*1*, *2*).

The three catalytic sites are structurally asymmetric, the result of differential interactions between each of the three $\alpha\beta$ pairs and the single-copy γ subunit (*1*, *3*, *4*). During ATP hydrolysis, cooperative ATP binding, hydrolysis, and ADP release result in directional rotation of the γ subunit (*1*, *3*, *5*, *6*). During ATP synthesis, the proton gradient is considered to drive rotation of the γ subunit in the opposite direction, forcing the catalytic sites through the reverse sequence of conformational states. This results in sequential high-affinity binding of ADP and P_i, ATP synthesis and then ATP release (*3*, *6*, *7*). The crystal structure of the bovine mitochondrial F_1 identified several points of contact or "catches" between the γ subunit and the $\alpha_3\beta_3$ hexamer that potentially represent some (or all) of the asymmetric contacts responsible for defining the different conformational states of the catalytic sites (*1*). One such contact involves two highly conserved residues, an arginine and a glutamine, near the C-terminal tip of the γ subunit, that form hydrogen bonds with residues on an anionic loop on one of the three β subunits. In two of the three β subunits, the anionic loops form interfacial contacts with the nucleotide phosphate-binding P-loops within the nucleotide binding pockets. The catalytic site associated with the third β subunit does not contain bound nucleotide. In this site, the catch in the C-terminus of γ appears to have pulled the anionic loop away from the P-loop by several angstroms (*1*). This observation implies that formation of the catch helps to reduce the affinity of the

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[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DTT, dithiothreitol; CF_1, EcF_1, MF_1, and TF_1, catalytic coupling factor 1 from chloroplasts, *Escherichia coli*, mitochondria, and thermophilic bacteria, respectively; $CF_1(-\delta\epsilon)$, CF_1 deficient in the δ and ϵ subunits; CF_O, proton-transporting chloroplast factor O; Tricine, *N*-[2-hydroxy-1-bis-(hydroxymethyl)ethyl]glycine; Tris, tris(hydroxymethyl)aminomethane; PMS, phenazine methylsulfate.

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nucleotide at this site (*8*), and forming and breaking the catch is considered to constitute an "escapement mechanism" that is a critical step in the cooperative binding change process (*9*). The importance of the catch residues for catalysis in EcF_1 was demonstrated by selective substitution of the equivalent residues, Arg268 and Gln269, with leucine which was expected to block their potential for hydrogen bonding to the anionic loops on the β subunits. Indeed, either mutation resulted in an almost complete loss of both ATP hydrolysis and synthesis (*9*).

In the full-length structure of the bovine MF_1 γ subunit, the last ~45 residues of the C-terminus extend from the base (membrane side) of the $\alpha_3\beta_3$ hexamer nearly to the top of the hexamer where the last 12 residues fit snugly into a tightly packed ring of structure formed by the N-termini of the alternating α and β subunits. Abrahams et al. (*1*) suggested that this structure may act as a bearing for rotation of the γ subunit, and this concept has been used in modeling rotational catalysis by several groups (*10*−*12*). To examine the idea that the γ C-terminus acts as a bearing during rotation, a series of mutant CF_1 γ subunits was constructed having between 6 and 20 amino acids deleted from the γ C-terminus (*13*). Remarkably, deleting up to 14 residues from γ, including all of the putative bearing-forming residues, stimulated rather than inhibited both the Ca^{2+}-dependent and Mg^{2+}-dependent ATP hydrolysis activities, indicating that the tip residues are not required as a rotational bearing in CF_1. Subsequent studies indicated that the last 14 residues of the EcF_1 (*14*) and TF_1 (*15*) γ subunits are likewise dispensable for ATP hydrolysis and γ rotation. However, deletion of residues beyond the last 14 in CF_1 γ, down to and including the putative catch residues Arg304 and Gln305, resulted in a decrease of more than 80% in the ATPase activity, and the remaining activity was insensitive to the allosteric inhibitor tentoxin, an indication that the residual activity was no longer a multisite cooperative process (*16*, *17*). This observation reinforced the structural data suggesting that the catch formed between the conserved arginine and glutamine may be a general feature of the F_1 enzymes and that it is required for the binding change process and for generation of rotational torque. This is, however, in contrast with results obtained more recently with TF_1 in which deletion of 21 residues from the γ C-terminus, although reducing the ATPase rate to less than 1% of that of the wild type, failed to abolish γ rotation, indicating that multisite catalytic cooperativity was retained (*15*).

Interestingly, successive removal of residues from the C-terminal tip of the CF_1 γ subunit resulted in successive loss of the oxyanion activation of the MgATPase activity of CF_1 (*13*). Removing 20 residues from the C-terminus abolished oxyanion (sulfite) stimulation altogether (*13*). Free magnesium ions or MgADP strongly inhibits the ATP hydrolysis activities of F_1-ATPases by forming a stable MgADP−enzyme complex (*18*−*20*). The effect is particularly pronounced in the mitochondrial and chloroplast enzymes and is considered to be a physiologically important regulatory mechanism, preventing futile ATP hydrolysis which would otherwise deplete essential ATP pools when electron transport is inactive (*18*, *21*). Oxyanions such as bicarbonate or sulfite are thought to compete with metal-binding ligands within the nucleotide binding pocket leading to faster release of ADP (*13*, *18*, *21*). The loss of oxyanion stimulation in the deletion mutants provided the first evidence of the involvement of the γ C-terminus in oxyanion activation (*13*).

To examine the role(s) of individual catch residues in catalysis and oxyanion activation, mutant CF_1 γ subunits were prepared in which Arg304, Gln305, and neighboring Arg302 were substituted, singly, in pairs, or all together with alanine or leucine. The mutant γ subunits were assembled with recombinant α and β subunits from F_1 of the photosynthetic bacterium *Rhodospirillum rubrum* (RrF_1) (*21*−*24*). This hybrid enzyme has been extensively characterized as a model photosynthetic enzyme and was shown recently to be capable of ATP hydrolysis-driven rotational catalysis (*25*). The study resulted in two very interesting observations. The first was that single mutations of the catch residues not only failed to eliminate ATPase activity but also in some cases significantly stimulated catalytic turnover. In addition, significant turnover persisted even when all three putative catch residues were substituted together. The second observation was that mutation of Gln305 to alanine completely abolished the stimulatory effect of oxyanions on Mg^{2+}-dependent ATP hydrolysis. These observations clearly indicated that the catch residues affect catalysis in different ways in EcF_1, TF_1, and the photosynthetic F_1 and that they are not universally required for cooperative multisite catalysis. They also confirmed that catch residues are involved in oxyanion activation with the intriguing possibility that they are involved in communication between adjacent catalytic and noncatalytic sites (*8*).

In this study, we have examined the involvement of catch residues in proton-coupled processes in the photosynthetic enzyme by reconstituting the γ mutants at Arg302, Arg304, and Gln305 with an $\alpha_3\beta_3$ hexamer prepared from native CF_1 using previously established procedures (*4*). The reconstituted mutant enzymes exhibited several novel features. Most interestingly, the triple mutant, in which all three residues were substituted with alanine, showed a dramatically stimulated CaATPase activity (k_{cat} of 500 s^{-1} compared to a value of 140 s^{-1} for the wild-type enzyme), a partially stimulated MgATPase activity, and a markedly decreased response to stimulatory oxyanions. In contrast, the ATP synthesis activity of all of the mutants decreased in parallel with loss of oxyanion stimulation. In addition, the residual oxyanion-stimulated MgATPase activity of the triple mutant was insensitive to the allosteric inhibitor tentoxin in contrast to the CaATPase activity which remained fully sensitive. The results are consistent with the hypothesis that the formation of the catch is an essential step in proton-coupled ATP synthesis and hydrolysis but does not represent an essential step in the multisite cooperative binding change process or, therefore, in ATP-dependent generation of γ subunit rotation.

EXPERIMENTAL PROCEDURES

Materials. Intact CF_1 and CF_1 lacking δ and ϵ subunits were isolated from fresh market spinach as described elsewhere (*26*, *27*). Purified CF_1 was stored as an ammonium sulfate precipitate at 4 °C and desalted prior to use. A mixture containing approximately equal amounts of the native ϵ and δ subunits was isolated from CF_1 and stored for short periods (1−3 days) at 4 °C or for longer periods at −20 °C in a buffer containing 20% ethanol (v/v) and 30% glycerol (v/v)

(*27*−*29*). DEAE-cellulose, antibiotics (ampicillin, tetracycline, and chloramphenicol), Sephadex G50, and Ni-NTA resin were purchased from Sigma. Hydroxyapatite HTP gel was from Bio-Rad. Tryptone and yeast extract were obtained from DIFCO. ATP (grade II) and urea (ultrapure) were purchased from Fluka. Dialysis tubing (8000 molecular weight cutoff) was obtained from Biodesign Inc. (New York, NY). All other chemicals were of the highest quality reagent grade available.

Mutation of the γ Subunit. Mutant CF_1 γ subunits were constructed by enzymatic amplification of the pET8c-γ.BB1 expression plasmid (*13*). Primers were obtained from Integrated DNA Technologies with chemically phosphorylated 5′ termini. PCRs, plasmid DNA purification, and bacterial transformation were carried out as described in detail elsewhere (*24*, *30*). The resulting plasmid was transformed into competent *Escherichia coli* XL1-blue cells. Cloned plasmid was then isolated and transformed into the expression host *E. coli* BL21(DE3)/pLysS, in which mutant CF_1 γ proteins were expressed in insoluble inclusion bodies as described previously (*4*). The entire sequence of each mutant gene was confirmed by the Iowa State DNA Sequencing Facility.

Assembly and Purification of Recombinant $\alpha_3\beta_3\gamma$ Complexes. CF_1 γ subunits were solubilized in urea and folded as described in earlier reports (*4*, *13*, *30*). Native chloroplast $\alpha_3\beta_3$ hexamers were prepared and purified from CF_1 by hydroxyapatite chromatography (*4*). Folded γ subunits were reconstituted with $\alpha_3\beta_3$ hexamers and purified by DEAE anion exchange chromatography (*4*).

Reconstitution of $\alpha_3\beta_3\gamma$ Assemblies with Thylakoid Membranes Deficient in CF_1. Spinach chloroplast thylakoids were prepared and treated with NaBr to generate CF_1-deficient membranes (*31*, *32*). Purified reconstituted $\alpha_3\beta_3\gamma$ assemblies were incubated with the ϵ δ preparation (*27*, *28*) at a saturating molar ratio (15 μg of $\delta\epsilon$ per 10 μg of $\alpha_3\beta_3\gamma$) at room temperature in 40 mM Tris-HCl (pH 8.0) for 1 h. The assembly was passed through a Sephadex G50 centrifuge column to remove unbound ϵ and δ subunits. The purified assembly (100 μg) was reconstituted with CF_1-deficient thylakoid membranes (equivalent to 20 μg of chlorophyll) in a buffer containing 20 mM Tricine-NaOH (pH 8.0), 1 mM $MgCl_2$, and 0.2 mg/mL bovine serum albumin in a final volume of 0.5 mL on ice for 10 min .

ATP Hydrolysis Assays. Enzyme samples (5−20 μg) were preincubated with 10 mM dithiothreitol for a minimum of 10 min at room temperature in 25 mM Tris-HCl (pH 8) to reduce the regulatory disulfide bridge in the γ subunit prior to the assay (*27*). The Mg^{2+}- and Ca^{2+}-dependent ATP hydrolysis activities were measured by incubation with 2 μg of enzyme for 5 min at 37 °C in 1 mL of assay mixture containing 25 mM Tris-HCl (pH 8) and ATP, $MgCl_2$, or $CaCl_2$ at the concentrations indicated in the figure legends. An equal volume of 0.5 M trichloroacetic acid was added to stop the reaction, and the concentration of P_i was measured as described previously (*33*). In the tentoxin titrations of Figures 5 and 6, the enzyme was preincubated with tentoxin at the concentrations indicated in the figure legends for 5 min in 0.5 mL of 25 mM Tris-HCl (pH 8.0). The assay was initiated by addition of 0.5 mL of double-strength assay mixture. Tentoxin was dissolved in acetone, and compensating amounts of acetone were added to samples such that the final concentration was the same (≤2%, v/v) in all samples. Protein concentrations were determined using the Bradford method (*34*). Kinetic constants were obtained using SigmaPlot.

Proton Permeability Measurements. Reconstituted thylakoid membranes equivalent to 20 μg of chlorophyll were assayed for light-dependent pH gradient formation using 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching in an assay buffer with a total volume of 1 mL containing 40 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 50 μM phenazine methosulfate (PMS), 2 μM ACMA, and 2.5 mM ascorbate (pH 6.8). Quenching of the fluorescence of ACMA (excitation at 410 nm and emission at 450 nm) was measured as described previously (*31*, *35*, *36*). Actinic light was supplied by an LED spotlight emitting at 660 nm at an intensity of 200 μmol m^{-2} s^{-1}. The quenching signal was reported as a percentage of the ratio of $\Delta F/F$ of mutant assemblies relative to that of wild-type CF_1, where ΔF was the difference in fluorescence (dark minus light) and F the steady-state fluorescence in the dark (*36*).

ATP Synthesis Measurements. ATP synthesis was assessed in 1 mL of assay mixture containing 500 mM Tricine-NaOH (pH 8.0), 500 mM NaCl, 5 mM $MgCl_2$, 0.05 mM PMS, 2 mM potassium phosphate (pH 7.0), 1 mM ADP (ATP-free), 0.1 mM diadenosine pentaphosphate, and reconstituted thylakoid membranes equivalent to 20 μg of chlorophyll. The reaction was carried out under a light intensity of 2.5×10^6 ergs cm^{-2} s^{-1} for 2 min at 22 °C. Trichloroacetic acid was added to the reaction mixture to a final concentration of 0.5% (v/v) after the light was switched off, and the mixture was centrifuged at 30000*g* for 5 min at room temperature. After a 20-fold dilution, the resulting supernatant was assayed for ATP concentration using a Sirius II luminometer in which 100 μL of the supernatant solution was mixed in a Sarstedt tube with 100 μL of instantly injected assay buffer containing 25 mM Tris-acetate (pH 7.75), 2 mM EDTA, 50 mM DTT, 0.02 mM D-luciferin, 1.5 mg/mL BSA, 20 mM magnesium acetate, and 0.3 μg/mL luciferase. The luminescence was measured by integrating the signal for 5 s after injection. The same reaction mix was used to determine concentrations of ATP standards (10^{-11} to 10^{-5} M in 10-fold increments) dissolved in 25 mM Tris-acetate (pH 7.75).

RESULTS

Assembly Competency of Mutant Chloroplast γ Subunits. Mutant and wild-type chloroplast γ subunits were expressed, folded, and assembled with native chloroplast $\alpha_3\beta_3$ hexamers as described previously (*4*, *13*). The reconstituted assemblies were purified by gel filtration (*22*) and exhibited electrophoretic profiles on sodium dodecyl sulfate gels that were indistinguishable from that of the native $CF_1-\delta,\epsilon$ complex (not shown). The purified $\alpha_3\beta_3$ hexamer displayed specific activities for MgATP and CaATP hydrolysis of approximately 0.1 μmol min^{-1} mg^{-1}, and the MgATPase activity was not stimulated by addition of the oxyanion sulfite. In contrast, the purified, reassembled complex containing the wild-type γ subunit had a specific CaATPase activity of 20 μmol min^{-1} mg^{-1} and a MgATPase activity of 2.5 μmol min^{-1} mg^{-1} that was stimulated 23-fold upon addition of 50 mM sulfite. These latter properties of the reconstituted enzyme are essentially identical to those of the native $CF_1-\delta,\epsilon$ complex (*27*, *28*).

ATPase Activities of Mutant F_1 Assemblies. Three residues in the C-terminal region of the chloroplast γ subunit were targeted in this study. Residues equivalent to Arg304 and Gln305 of the CF_1 γ subunit were identified in the crystal structure of bovine MF_1 to form hydrogen bonds with residues on an anionic loop of one of the three β subunits. The contact was suggested to be necessary for creating the asymmetric nucleotide binding properties of the enzyme involved in the binding change process (*1*). A residue equivalent to Arg302 in the CF_1 γ subunit is sufficiently close to an anionic loop on an α subunit in the bovine MF_1 to form a salt link (*1*). Substitution of this residue with leucine resulted in a marked stimulation of the MgATPase activity of the hybrid photosynthetic F_1 assembly, indicating that it also plays a role in modulating catalytic function (*8*).

Arg302 and Arg304 were initially substituted with leucine to negate the positive charge while maintaining a side chain similar in size. Subsequently, Gln305, then Arg304 and Gln305 together, and finally all three residues together were substituted with alanine to eliminate the potential of these residues to form hydrogen bonds with residues on the α and β subunits. All of the mutant assemblies were exposed to 10 mM DTT in a preincubation step to reduce the regulatory disulfide bond that is present in the γ subunit. Reduction is necessary for maximum activation of the ATP hydrolysis activity (*27*). The ATPase activities of the F_1 assemblies containing the mutant γ subunits are shown in Figure 1, and the k_{cat} values are summarized in Table 1. Marked differences were observed between the Ca^{2+}- and Mg^{2+}-dependent activities. The CaATPase activity of the γR302L mutant decreased by 20%, but those of the γR304L and γQ305A mutants increased by 26 and 91%, respectively. The MgATPase activities of all three mutants increased significantly (Figure 1b), the greatest increase ($\sim$2-fold) being that of the γR304L mutant. Interestingly, the rates of MgATPase activity in the presence of the activating oxyanion sulfite decreased in each case, the greatest decrease ($\sim$50%) also being observed with the γR304L mutant (Figure 2 and Table 1).

The effects of simultaneous substitution of γArg302 and γR304, γR304 and γQ305, or all three residues together on the ATPase activities are shown in Figure 3, and the k_{cat} values are summarized in Table 1. The γR302L/R304L double mutant was very similar to the γR302L single mutant in showing a partially reduced CaATPase activity and an enhanced MgATPase activity. The γR304A/Q305A double mutant and the triple mutant exhibited remarkably high CaATPase activities, the highest that we have ever recorded and approximately 3-fold higher than the activity of the wild-type enzyme. The MgATPase activities of the double and triple mutants were also elevated 3−4-fold over the wild-type activity. In contrast, the sulfite-activated catalytic rates were significantly reduced in all of the mutants (Figure 4 and Table 1).

Loss of Sensitivity to Tentoxin. The fungal phytotoxin tentoxin is a potent inhibitor of cooperative multisite catalysis by CF_1 (*16*). Maximum inhibition is reached at a concentration of approximately 1 μM. Higher concentrations of tentoxin lead to reactivation of the inhibited form, and significant stimulation occurs at still higher concentrations (*37*−*39*). The results of titration of the wild-type and mutant assemblies with tentoxin are shown in Figures 5 and 6. The

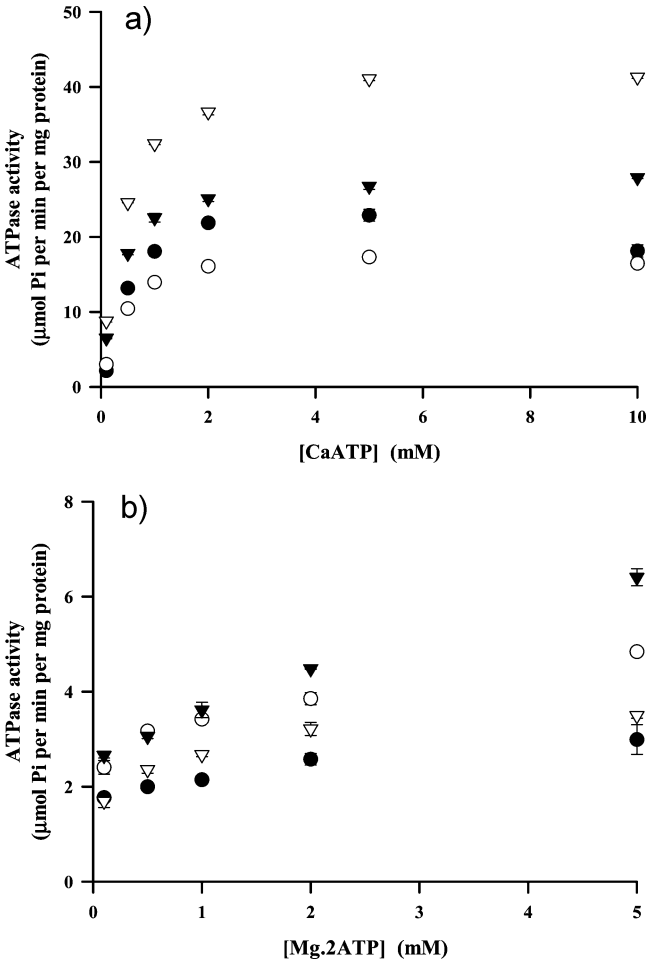

FIGURE 1: ATP hydrolysis by CF1 assemblies containing single C-terminal γ mutations. ATP hydrolysis assay mixtures contained a 1:1 Ca^{2+}:ATP ratio (a) or a 0.5:1 Mg^{2+}:ATP ratio (b) at the indicated concentrations: (●) $CF_1\gamma_{wild-type}$, (○) $CF_1\gamma_{R302L}$, (▼) $CF_1\gamma_{R304L}$, and (▽) $CF_1\gamma_{Q305A}$. Error bars represent standard deviations ($n = 3$).

data are representative titrations that were performed twice with the same outcome. The Ca^{2+}-dependent ATPase activities of the wild type and mutants exhibit full sensitivity to inhibition by tentoxin (Figure 5A). In contrast, the MgATPase activities of the mutants (Figure 5B) exhibit decreased sensitivity to tentoxin inhibition. The MgATPase activities of the γR304A/Q305A double mutant and the γR302A/R304A/Q305A triple mutant were unaffected. The effect of tentoxin on the sulfite-stimulated MgATPase activities of the mutants is shown in Figure 6. All of the mutants except the γR302L single mutant showed a decreased sensitivity to tentoxin inhibition, the maximum effect being obtained with the double γR304A/Q305A mutant and the triple mutant. Thus, the C-terminal mutations selectively interfered with tentoxin inhibition of the MgATPase activity of the enzyme. All of the mutant enzymes exhibited the typical reactivation and/or stimulation of both MgATPase and CaATPase activities that occur at higher concentrations of tentoxin, indicating that only inhibition of MgATPase activity was affected.

Effects of Mutations on Proton-Coupled Functions. The $\alpha_3\beta_3\gamma$ assemblies containing the recombinant γ subunits were first reconstituted with the δ and ϵ subunits and then reconstituted with CF_1-deficient thylakoid membranes (*28*, *40*). In the absence of CF_1, protons flow freely through CF_O and the thylakoid membranes are unable to maintain an

Table 1: Summary of k_{cat} Values for γ Mutant F_1 Assemblies

γ mutant	k_{cat} (s^{-1})[a]			R[c]
	Ca^{2+}	Mg^{2+}	Mg^{2+} and sulfite[b]	
$CF_1(\alpha\beta)_3\gamma_{wild-type}$	136.9 ± 13.7	15.6 ± 1.4	353.0 ± 24.2	22.6
$CF_1(\alpha\beta)_3\gamma_{R302L}$	109.1 ± 4.1	25.4 ± 2.3	264.8 ± 8.1	10.4
$CF_1(\alpha\beta)_3\gamma_{R304L}$	172.9 ± 2.0	35.2 ± 7.0	161.4 ± 9.7	4.6
$CF_1(\alpha\beta)_3\gamma_{Q305A}$	261.6 ± 3.0	19.9 ± 1.4	238.6 ± 7.8	12.0
$CF_1(\alpha\beta)_3\gamma_{R302L/R304L}$	101.6 ± 3.1	69.3 ± 12.5	196.2 ± 20.2	2.8
$CF_1(\alpha\beta)_3\gamma_{R304A/Q305A}$	441.6 ± 13.2	46.7 ± 6.3	138.8 ± 14.0	3.0
$CF_1(\alpha\beta)_3\gamma_{R302A/R304A/Q305A}$	499.8 ± 14.5	49.6 ± 15.2	167.2 ± 16.2	3.4

[a] k_{cat} values were calculated using the nonlinear regression tools in Sigma Plot 8.0. Errors are expressed as standard deviations ($n = 3$). [b] Sodium sulfite (50 mM) was added to the assay mixture. [c] Ratio of activity in the presence of sulfite to that in the absence of sulfite.

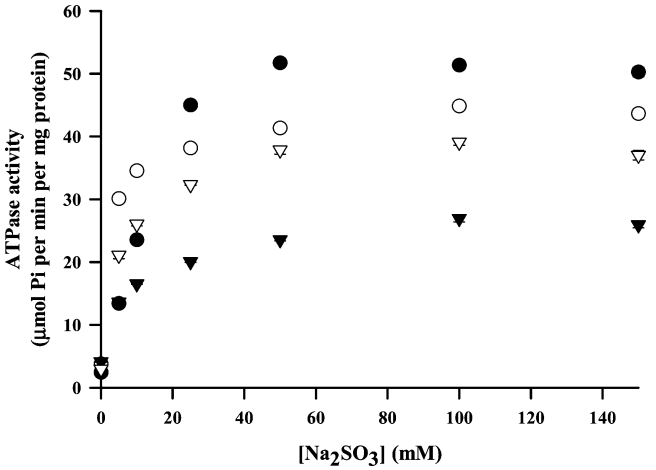

FIGURE 2: Sulfite activation of CF_1 assemblies that contain chloroplast γ subunits with single C-terminal mutations. The ATP hydrolysis activities were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$, and the indicated concentrations of sodium sulfite: (●) $CF_1\gamma_{wild-type}$, (○) $CF_1\gamma_{R302L}$, (▼) $CF_1\gamma_{R304L}$, and (▽) $CF_1\gamma_{Q305A}$. Error bars represent standard deviations ($n = 3$).

effective proton gradient. Addition of CF_1 blocks the free flow of protons across the membrane, restoring the ability to form and maintain a proton gradient (*26*, *28*, *32*).

The capacity of F_1 assemblies containing the mutant γ subunits to block the free flow of protons across the membrane was determined using ACMA fluorescence (*36*, *41*). Thylakoid membranes reconstituted with CF_1 assembled using the recombinant wild-type γ subunit showed a maximum light-dependent quenching of ACMA fluorescence of ~60%, the same as that observed in membranes reconstituted with native CF_1 (not shown). In contrast, the capacities of the assemblies containing the γ mutants to block proton flow through CF_O were significantly reduced (Table 2). This was not likely to be due to a reduced level of binding of mutant assemblies to the membranes. First, the degree of quenching did not increase with increasing concentrations of CF_1, indicating that saturation had been achieved; second, modifications of the γ subunit of CF_1 (e.g., reduction and specific cleavage by trypsin) do not affect the binding of CF_1 to CF_o (*42*), and third, generation of a measurable transmembrane pH gradient requires that 90% or more of the CF_o is complexed with CF_1 (*43*). Thus, the assemblies containing the γ mutants appeared to be proton-leaky.

The results shown in Table 2 further indicate a loss of ATP synthesis capacity by the mutant enzymes. The triple mutant was most affected, having only ~13% of wild-type ATP synthesis. Interestingly, the reduced ATP synthesis capacity of the mutants paralleled the reduction in sulfite-stimulated MgATPase activity (Table 1) which in turn paralleled the increase in CaATPase activity (Table 1).

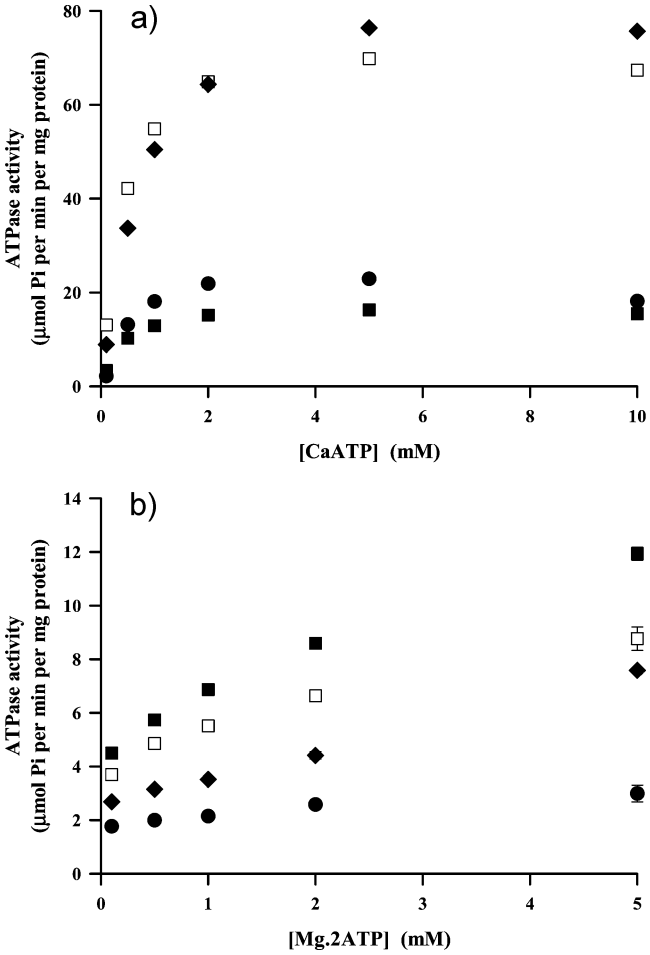

FIGURE 3: ATP hydrolysis by CF_1 assemblies containing multiple C-terminal γ mutations. ATP hydrolysis assay mixtures contained either a 1:1 Ca^{2+}:ATP ratio (a) or a 0.5:1 Mg^{2+}:ATP ratio (b) at the indicated concentrations: (●) $CF_1\gamma_{wild-type}$, (■) $CF_1\gamma_{R302L/R304L}$, (□) $CF_1\gamma_{R304A/Q305A}$, and (◆) $CF_1\gamma_{R302A/R304A/Q305A}$. Error bars represent standard deviations ($n = 3$).

DISCUSSION

Catch Residues Are Not Essential for Cooperative Multisite Catalysis by CF_1. The results of this study confirm the importance of the catch residues in governing catalytic rates in CF_1. Single mutations of Arg304 or Gln305 led to increased rates of ATP hydrolysis; the Q305A mutation increased the rate of MgATP hydrolysis to a greater extent than the R304L mutant, whereas the R304L mutant increased the rate of CaATP hydrolysis to a greater extent than the

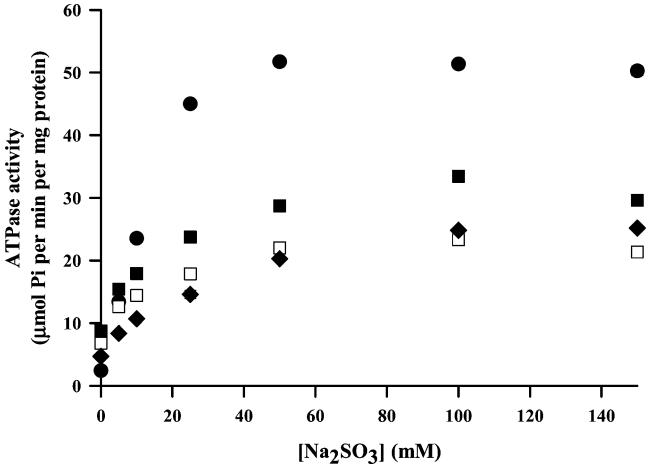

FIGURE 4: Sulfite activation of CF_1 assemblies with double and triple C-terminal γ mutations. The ATP hydrolysis activities of purified enzyme assemblies were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$, and the indicated concentrations of sodium sulfite: (●) $CF_1\gamma_{wild-type}$, (■) $CF_1\gamma_{R302L/R304L}$, (□) $CF_1\gamma_{R304A/Q305A}$, and (◆) $CF_1\gamma_{R302A/R304A/Q305A}$. Error bars represent standard deviations ($n = 3$).

Q305A mutant. Mutation of Arg302 to leucine resulted in a small decrease in CaATPase activity and an increase in MgATPase activity. The different degrees by which the mutations affect catalysis are assumed to result from different contributions of the three residues in binding the γ subunit to the α and β subunits. In general, it appears that reducing the amount of contact between γ and αβ subunits results in higher ATPase activity. This is in stark contrast to the effects of mutation of residues equivalent to γR304 and γQ305 to leucine in EcF_1 which drastically reduced the rate of catalytic turnover (*9*). Remarkably, the double γR304A/Q305A and triple γR302A/R304A/Q305A mutants showed a greater than 3-fold increase in CaATPase activity compared to the wild-type enzyme. The elevated rate of CaATP hydrolysis was fully sensitive to tentoxin, indicating that it resulted from normal cooperative multisite nucleotide interactions (*16*). The triple mutant contains four sequential alanine residues from position 302 through 305. These substitutions should eliminate any potential of this region of the γ subunit to form hydrogen bonds or salt links with the anionic loops on α and β subunits and therefore to form the catch. The fact that the triple mutant is more active as an ATPase than the native enzyme indicates that the catch residues are not critical for the cooperative multisite catalysis. From this result, we infer that forming the catch is not an essential step in driving unidirectional rotation of the γ subunit, although this remains to be demonstrated experimentally. This result is consistent with the results of the TF_1 γ deletion study (*15*).

The Catch Residues Are Required for Oxyanion Activation of the Mg^{2+}-Inhibited State of CF_1. Substitution of either Arg302, Arg304, or Gln305 with leucine or alanine significantly reduced the level of sulfite activation of ATP hydrolysis (Table 1). The γR304L mutant was most affected, having a ratio of sulfite-stimulated to basal ATP hydrolysis of ~5/1 compared to ~23/1 for the wild-type enzyme. A similar effect was observed previously when the γ mutants were assembled into the hybrid enzyme containing the α and β subunits from *R. rubrum* (*8*). In that case, replacement of Gln305 with alanine alone or in combination with

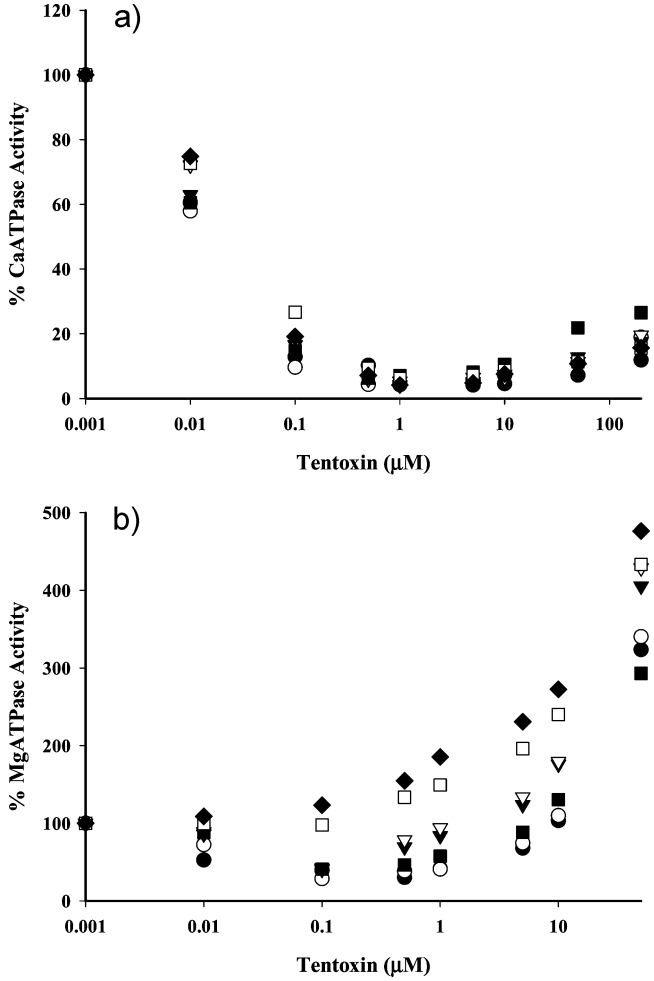

FIGURE 5: Titration of γ mutants with tentoxin. ATP hydrolysis assay mixtures contained either 5 mM ATP and 5 mM $CaCl_2$ (a) or 4 mM ATP and 2 mM $MgCl_2$ (b). Samples were preincubated with tentoxin at the indicated concentrations for 5 min prior to initiation of the assay: (●) $CF_1\gamma_{wild-type}$, (○) $CF_1\gamma_{R302L}$, (▼) $CF_1\gamma_{R304L}$, (▽) $CF_1\gamma_{Q305A}$, (■) $CF_1\gamma_{R302L/R304L}$, (□) $CF_1\gamma_{R304A/Q305A}$, and (◆) $CF_1\gamma_{R302A/R304A/Q305A}$. Error bars represent standard errors ($n = 2$).

mutations of Arg302 and Arg304 completely eliminated the sulfite-induced stimulation of MgATP hydrolysis.

It was noted previously (*8*) that residues equivalent to Arg302, Arg304, and Gln305 in the bovine MF_1 structure (Arg252, Arg254, and Gln255) form a bridge across a noncatalytic αβ interface, linking the adjacent α and β subunits together. Freshly isolated CF_1 contains two very tightly bound molecules of ADP (*44*), one in a catalytic site and one in a noncatalytic site (*45*−*47*). The enzyme is latent with respect to MgATP hydrolysis due to the presence of MgADP bound tightly in a noncatalytic site on the enzyme (*45*, *46*, *48*). Pretreatment of CF_1 with MgATP results in activation of the MgATPase activity by filling one or more noncatalytic sites with MgATP (*49*). Once activated, the ADP that is tightly bound in the catalytic site is assumed to rapidly exchange with MgATP via the cooperative binding change process (*50*). Oxyanions such as sulfite decrease the binding affinity of nucleotides at noncatalytic sites and increase the extent of nucleotide exchange at catalytic sites (*51*). In view of these properties, and by analogy to the MF_1 structure, the observed effects of mutating the catch residues on the sulfite-stimulated ATP hydrolysis activity of the hybrid enzyme led

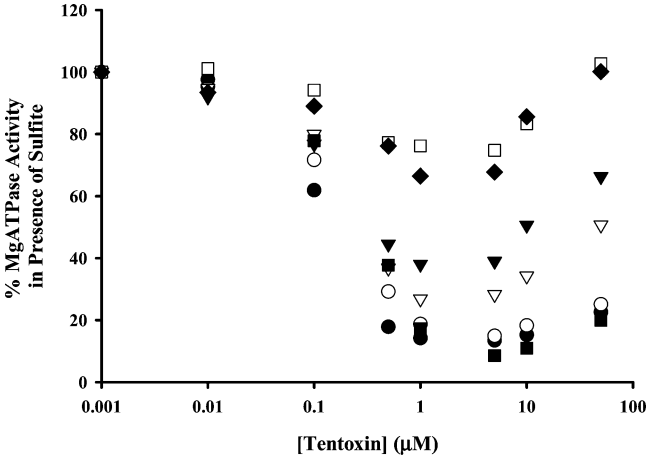

FIGURE 6: Tentoxin inhibition of sulfite-stimulated MgATP hydroysis. ATP hydrolysis assay mixtures contained 4 mM ATP, 2 mM $MgCl_2$, and 25 mM Na_2SO_3. Samples were preincubated with tentoxin at the indicated concentrations for 5 min prior to initiation of the assay: (●) $CF_1\gamma_{wild-type}$, (○) $CF_1\gamma_{R302L}$, (▼) $CF_1\gamma_{R304L}$, (▽) $CF_1\gamma_{Q305A}$, (■) $CF_1\gamma_{R302L/R304L}$, (□) $CF_1\gamma_{R304A/Q305A}$, and (◆) $CF_1\gamma_{R302A/R304A/Q305A}$. Error bars represent standard errors ($n = 2$).

Table 2: Proton Gradient Formation and ATP Synthesis by Mutant γ F_1 Assemblies

γ mutant	relative $\Delta F/F$ (%)[a]	ATP synthesis [μmol of P_i h^{-1} (mg of chl)$^{-1}$]	% of WT
$CF_1(\alpha\beta)_3\gamma_{wild-type}$	100	66.0 ± 3.4	100
$CF_1(\alpha\beta)_3\gamma_{R302L}$	64	30.6 ± 1.9	46
$CF_1(\alpha\beta)_3\gamma_{R304L}$	39	15.9 ± 3.2	24
$CF_1(\alpha\beta)_3\gamma_{Q305A}$	55	23.8 ± 1.2	36
$CF_1(\alpha\beta)_3\gamma_{R302L/R304L}$	40	18.6 ± 2.0	28
$CF_1(\alpha\beta)_3\gamma_{R304A/Q305A}$	42	12.3 ± 1.2	19
$CF_1(\alpha\beta)_3\gamma_{R302A/R304A/Q305A}$	36	8.5 ± 1.1	13

[a] ACMA quenching and ATP synthesis by purified F_1 assemblies were assessed as described in Experimental Procedures. Errors are expressed as standard deviations ($n = 4$).

to the hypothesis that the activating effect of oxyanions results from stimulation of exchange of MgATP for MgADP in a noncatalytic site and that occupancy of the noncatalytic site by MgATP is communicated to the catalytic site via the catch residues (*8*). The effects of the γ mutations on the sulfite-stimulated MgATPase activity of the homogeneous chloroplast enzyme described in this study are consistent with this hypothesis. The selective loss of sensitivity to tentoxin of the MgATPase activity further suggests that loss of connectivity across the noncatalytic interface that occurs with the γ triple mutant results in the loss of the cooperative exchange of the ADP tightly bound in one of the three catalytic sites. The rate of release of the tightly bound ADP from the catalytic site limits the rate of MgATP hydrolysis (*18*).

Tentoxin is known to block the cooperative exchange of nucleotides among the three catalytic sites that leads to high rates of multisite catalysis (*16*). One explanation for the loss of tentoxin sensitivity in the triple mutant that is consistent with the hypothesis is that, in the absence of the residues necessary to form the catch, cooperative exchange of the ADP that is tightly bound to a catalytic site is either blocked or greatly inhibited. If this is indeed the case, the residual tentoxin-insensitive activity must then result from single-site or two-site catalysis at the remaining site or sites. A thorough analysis of exchange of tightly bound ADP from catalytic and noncatalytic sites in the triple mutant is currently in progress to determine if the nonexchangeable ADP is located in a catalytic or noncatalytic site.

The observation that cooperative multisite hydrolysis of CaATP is unaffected or enhanced by removal of the catch residues is consistent with past studies indicating that free Ca^{2+} ions at concentrations of several millimolar are not inhibitory (*21*). Thus, Mg^{2+} ions but not Ca^{2+} ions specifically induce the inhibited conformational state of the enzyme. Since Ca^{2+} is a substrate for multisite ATP hydrolysis and can drive γ subunit rotation (*25*), it follows that the Mg^{2+}-induced state defined in the MF_1 structure may not represent an essential conformational intermediate in the binding change process that leads to rotation of the γ subunit.

In general, the results of this study in which mutants were assembled with the native α and β subunits agree with those of the previous study in which the mutants were assembled with the hybrid enzyme (*8*). There are, however, some differences worth noting. First, in the hybrid enzyme, while single mutants stimulated the CaATPase activity to very high levels ($k_{cat} \sim 640\ s^{-1}$), the double and triple mutants partially inhibited CaATPase activity (50−80%) in contrast to the large stimulation observed in this study. One possible explanation for the difference is that the combined mutations partially destabilized the hybrid enzyme. The catalytic activities of the hybrid containing the wild-type γ subunit are significantly higher than those of the native enzyme which may result from fewer contacts between the γ subunit and the $\alpha_3\beta_3$ hexamer (*13*). Thus, a further reduction could lead to destabilization and loss of activity. Second, differences were observed in the extent of sulfite stimulation by the single mutations. For example, the γR304L mutant had no apparent effect in the hybrid but reduced sulfite-stimulated activity by ∼40% in this study. The reverse was true for the Q305A mutant which eliminated oxyanion stimulation in the hybrid but only partially inhibited this activity in the native enzyme. This points to subtle differences in the binding interactions among the catch residues in the different enzyme assemblies, and more studies are needed to identify these differences.

The Catch Residues Are Important for Mg^{2+}-Dependent Proton-Coupled ATP Synthesis. The effects of mutation of the catch residues on proton-coupled functions (Table 2) paralleled the loss of sulfite-stimulated MgATP hydrolysis (Table 1). The triple mutation in the γ subunit caused CF_OF_1 to become proton-leaky and reduced the ATP synthesis capacity to ∼13% of that of the wild type. The ratio of sulfite-stimulated to unstimulated MgATPase activity was similarly reduced by ∼85% in the triple mutant compared to that of the wild-type enzyme. This relationship is very significant. Light-dependent activation of ATP synthesis or hydrolysis by the CF_OF_1 complex has been shown to coincide with conversion of the enzyme from a form which has MgADP or MgATP tightly bound to catalytic and noncatalytic sites which is nonexchangeable with medium nucleotides to a form in which the tightly bound nucleotides can be rapidly exchanged with medium nucleotides (*20*, *47*, *52*). In contrast, adding MgADP to the enzyme in the dark following light activation results in the rapid conversion of the enzyme to the latent, inhibited form (*53*).

Activation of the latent ATPase activity of membrane-bound CF_OF_1 is a two-step process. Formation of a trans-

membrane pH gradient (ΔpH) induces a conformation in which a disulfide bridge between two vicinal thiols within a special regulatory domain of the γ subunit is reduced by thioredoxin (*54*). Disulfide reduction has been shown to result in a shift in the conformation of the inhibitory ϵ subunit releasing its inhibitory effect (*55*, *56*). Addition of a small amount of trypsin following reduction of the disulfide results in cleavage within the regulatory domain of γ and permanent loss of ϵ inhibition (*27*, *42*, *56*). However, the MgATPase activity of the enzyme remains latent even after disulfide reduction and trypsin cleavage of the γ subunit, requiring either the presence of a ΔpH or addition of oxyanions as a second activating step (*42*). In this respect, oxyanions mimic the proton gradient in overcoming the inhibition by free Mg^{2+} or MgADP. We have shown in this study that the γ catch residues play an important role in both the ΔpH and oxyanion activation processes. At least one step in this mechanism involves exchange of MgADP for MgATP into a noncatalytic site and communication of this exchange to catalytic sites. Mutation of the catch residues in the hybrid enzyme resulted in the complete loss of the oxyanion stimulation of catalysis (*8*). In CF_1, however, mutating the catch residues slowed, but did not eliminate, oxyanion stimulation. One possible explanation for the difference is that while oxyanions stimulate release of MgADP from catalytic sites as well as exchange of nucleotides in noncatalytic sites in CF_1 (*25*), they effect only noncatalytic nucleotide exchange in the hybrid. This explanation is supported by the observation that the MgATPase activity of the hybrid is already partially activated in the absence of activating oxyanions (*22*).

In the crystal structure of bovine MF_1, the β_{DP} (with ADP bound) and β_{TP} (with ATP bound) subunits form additional catches with the γ subunit that involve the conserved DELSEED loop sequences on the β subunits with residues in the C-terminus of the γ subunit from Met23 to Val26 and from Leu77 to Lys87, and from Arg228 to 230 on the N-terminus of the γ subunit (*1*). The catches are thought to assist in closing the catalytic sites upon nucleotide binding by forcing an upward motion of the helical structure that contains the DELSEED loop which is connected to residues located in the nucleotide binding pocket. The DELSEED loop is extended downward in the β_E subunit in the absence of bound nucleotide (*1*). Interestingly, an earlier study with EcF_1 (*57*) indicated that mutation of one of the catch residues, Met23, to lysine strongly affected proton coupling and ATP synthesis activities but had a much weaker effect on ATPases activity. In a more recent study in TF_1 (*58*), the entire DELSEED loop (DELSDED in PS3 F_1) was substituted with alanine residues. The substitutions were expected to eliminate the $\gamma-\beta$ catch interactions involving the DELSEED loop segment of the β subunit. Interestingly, the ATPase activity of the mutant was partially inhibited (~60%), yet the mutant was still fully capable of generating the same amount of rotational torque as the wild-type enzyme. These results are consistent with our observations of the effects of mutations within the upper catch and strongly support the idea that at least two of the catches identified in the crystal structure of MF_1 are primarily involved in coupling interactions and less important for the binding change process leading to γ rotation.

In summary, the residues identified in the crystal structure of MF_1 to form a catch between the C-terminus of the γ subunit and anionic loops on one of the three $\alpha\beta$ subunit pairs are not required for the binding change process in CF_1 and, therefore, are not required for generating rotational torque. Instead, they are specifically required for an activating mechanism involving exchange of inhibitory ADP for ATP in one or more noncatalytic sites on CF_1 that leads to rapid release of product ADP from catalytic sites via the binding change process during MgATP hydrolysis. Inhibitory binding of MgADP to CF_1 serves to prevent futile ATP hydrolysis under physiological conditions in the dark when electron transport is no longer active. The proton-driven conformational steps that lead to rapid exchange of inhibitory MgADP and subsequent activation of the enzyme cannot be ascertained from existing crystal structures of F_1 and F_OF_1 enzymes and will require new experimental approaches for their elucidation.

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