Mutations within the C-Terminus of the γ Subunit of the Photosynthetic F_1-ATPase Activate MgATP Hydrolysis and Attenuate the Stimulatory Oxyanion Effect†

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ABSTRACT: Two highly conserved amino acid residues near the C-terminus within the γ subunit of the mitochondrial ATP synthase form a "catch" with an anionic loop on one of the three β subunits within the catalytic $\alpha\beta$ hexamer of the F_1 segment [Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628]. Forming the catch is considered to be an essential step in cooperative nucleotide binding leading to γ subunit rotation. The analogous residues, Arg304 and Gln305, in the chloroplast F_1 γ subunit were changed to leucine and alanine, respectively. Each mutant γ was assembled together with α and β subunits from Rhodospirillum rubrum F_1 into a hybrid photosynthetic F_1 that carries out both MgATPase and CaATPase activities and ATP-dependent γ rotation [Tucker, W. C., Schwarcz, A., Levine, T., Du, Z., Gromet-Elhanan, Z., Richter, M. L. and Haran, G. (2004) *J. Biol. Chem. 279*, 47415−47418]. Surprisingly, changing Arg304 to leucine resulted in a more than 2-fold increase in the k_{cat} for MgATP hydrolysis. In contrast, changing Gln305 to alanine had little effect on the k_{cat} but completely abolished the well-known stimulatory effect of the oxyanion sulfite on MgATP hydrolysis. The MgATPase activities of combined mutants with both residues substituted were strongly inhibited, whereas the CaATPase activities were inhibited, but to a lesser extent. The results indicate that the C-terminus of the photosynthetic F_1 γ subunit, like its mitochondrial counterpart, forms a catch with the α and β subunits that modulates the nucleotide binding properties of the catalytic site(s). The catch is likely to be part of an activation mechanism, overcoming inhibition by free mg^{2+} ions, but is not essential for cooperative nucleotide exchange.

The ATP synthases of chloroplasts, mitochondria, and bacteria are composed of two physically and functionally separable components, F_1 (factor 1), a peripheral membrane component that contains the catalytic sites for ATP synthesis and hydrolysis, and F_O (factor O), an integral membrane component that transports protons across the membrane. Chloroplast F_1 (CF_1)[1] is comprised of five polypeptide subunits designated $\alpha-\epsilon$ in order of decreasing molecular weight. Chloroplast F_O (CF_O) is comprised of polypeptide subunits designated I−IV. The subunit stoichiometry of the CF_1F_O complex is $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1I_1II_1III_{14}IV_1$ (*1*). The α and β subunits alternate to form a hexamer with six nucleotide binding sites, one at each of the six $\alpha-\beta$ subunit interfaces. The β subunits contribute most of the structure for three of the nucleotide binding sites which are considered to be catalytic. The remaining three are formed principally by the three α subunits and are considered noncatalytic with a regulatory function. At any given instant in time, each of the three catalytic sites exists in a different conformational state, resulting from asymmetric binding interactions between the single-copy γ subunit and the $\alpha\beta$ hexamer (*2, 3*). Binding of a nucleotide to F_1 induces sequential and directional rotation of the γ subunit (e.g. ref 4), breaking and re-forming the $\gamma-\alpha\beta$ contacts as suggested in the cooperative binding change catalytic process (*5, 6*).

The published structure of bovine mitochondrial F_1 (MF_1) (*2*) shows the N- and C-termini of the γ subunit forming a twisted helical pair that extends through the center of the $\alpha_3\beta_3$ hexamer making several contacts or "catches" with different parts of the hexamer. In the cross section of the mitochondrial enzyme shown in Figure 1, the tip of the C-terminal helix interacts with the tightly packed N-terminal domains of all six $\alpha\beta$ subunits and has been proposed to act as a bearing for rotation of the γ subunit during catalysis (*2*). A second contact involves a close interaction between two highly conserved residues on the C-terminal tip on the γ subunit with conserved anionic loop structures on the α and β subunits immediately below the proposed bearing. Specifically, γArg256 and γGln257 (bovine heart MF_1 numbering) form hydrogen bonds with residues on an anionic loop of the β_E subunit, the β subunit that is not occupied by nucleotide.

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[1] Abbreviations: CF_1, EcF_1, RrF_1, TF_1, and MF_1, catalytic coupling factor 1 enzymes from chloroplast, *E. coli*, *R. rubrum*, a thermophilic PS3 bacterium, and bovine heart mitochondria, respectively; $CF_1(-\delta\epsilon)$, CF_1 deficient in the δ and ϵ subunits; CF_O, proton transporting chloroplast coupling factor O; Tricine, *N*-[2-hydroxy-1-bis(hydroxymethyl)ethyl]glycine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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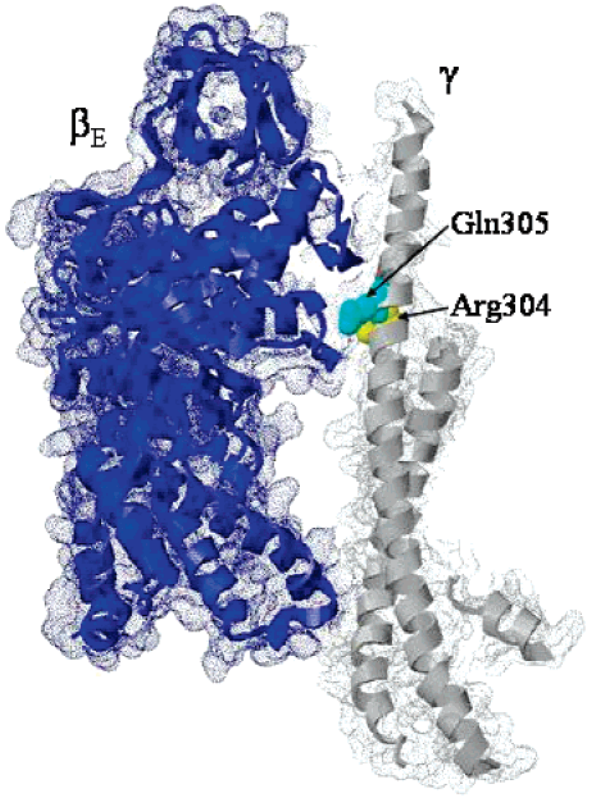

FIGURE 1: Proposed catch interactions between the CF_1 β and γ subunits. Cross section of part of CF_1 showing the putative catch between γGln305 (cyan), γArg304 (yellow), and β_E (blue). The homology model of CF_1 (kindly provided by S. Engelbrecht) was based on the crystal structure of the mitochondrial enzyme (*2*). Residues Gln305 and Arg304 on the γ C-terminal helix are denoted with arrows.

Deletion of up to 14 residues from the C-terminus of the CF_1 γ subunit had the unexpected result of enhancing rather than abolishing the ATP hydrolysis activity, indicating that the C-terminal tip of γ is not required as a bearing for rotational catalysis (*7*). This result was confirmed in *Escherichia coli* F_1 (EcF_1) (*8*) and more recently in a thermophilic bacterial F_1 (*9*). Mutants in which the proposed bearing-forming residues were deleted retained their capacity for ATP-driven γ rotation (*8*, *9*). Deletion of more than 14 residues up to and including the catch residues, Arg304 and Gln305, which are 19 and 20 residues from the C-terminus, respectively, led to a successive loss in catalytic turnover in CF_1, indicating that the catch immediately below the proposed bearing contact is functionally important in CF_1 as well as in other F_1 enzymes. The residual activity of the mutant with all 20 C-terminal residues deleted was insensitive to the CF_1-specific inhibitor tentoxin, an indication that it was noncooperative and therefore not expected to involve rotation of the γ subunit (*7*). Selective mutation of γArg268 and γGln269 in EcF_1 (analogous to γArg304 and γGln305, respectively, in CF_1) reduced the k_{cat} for ATP hydrolysis by 88 and 99%, respectively, and the ATP synthesizing capacity of both mutants was likewise impaired (*10*). Successive deletion of residues from the C-terminus of the thermophilic PS3 F_1 (TF_1) γ subunit also resulted in successive reductions in k_{cat}, again indicating that the catch plays an important role in the catalytic process (*9*). However, a C-terminal mutant of the TF_1 γ subunit in which the last 21 residues, including residues equivalent to CF_1 γ subunit residues 303−305, reduced the k_{cat} to less than 1% of that of the wild-type enzyme but did not prevent rotation of the γ subunit (*9*). This surprising result indicated that the catch residues may not be required for γ rotation, although a lack of correspondence between the reduction of catalytic and rotational rates in this mutant indicates that more information is needed to confirm this result.

To examine the importance of the catch residues in CF_1 function and to elaborate on the functional role of these residues in rotational catalysis, we prepared γ mutants containing selective substitutions of the catch residues γArg304 and γGln305. Mutant γ subunits were assembled into the highly active hybrid photosynthetic F_1 complex comprised of α and β subunits from *Rhodosporillum rubrum* F_1 (RrF_1) and the γ subunit from CF_1 that has been described in detail in several recent publications (*4*, *11*−*13*). The value of this system in studying rotational catalysis in photosynthetic F_1 enzymes is several-fold. The hybrid exhibits catalytic properties very similar to those of the native CF_1 and RrF_1 complexes, including high CaATP hydrolysis activities and oxyanion-stimulated MgATPase activity (*12*, *13*). The hybrid is also subject to regulation by the γ dithiol, a property that is unique to the higher plant enzyme (*1*), and is fully sensitive to the inhibitory ϵ subunit (*12*, *13*). Importantly, the hybrid system allows incorporation of recombinant subunits into an active photosynthetic F_1 complex used recently to demonstrate CaATP-dependent rotation of the γ subunit (*4*). Remarkably, mutation of γArg304 to leucine, which is expected to eliminate its ability to hydrogen bond with the anionic loops on the β subunits, resulted in a large increase in the rate of catalytic turnover. Mutation of γGln305 to alanine, which was also expected to remove a critical hydrogen bonding interaction with the β subunits, had little effect on catalysis but completely abolished the stimulatory effect of oxyanions on the MgATPase activity of the enzyme. The results are thus consistent with observations of the TF_1 enzyme indicating that the catch, like the bearing, does not play an essential role in driving directional rotation of the γ subunit during ATP hydrolysis. The results have also provided evidence for a role of γ C-terminal residues in the process of oxyanion activation.

EXPERIMENTAL PROCEDURES

Materials. $RrF_1\alpha$-His_6 (six-histidine tag at the C-terminus), $RrF_1\beta$, and $CF_1\gamma$ were expressed in insoluble inclusion bodies as described elsewhere (*11*). DEAE-cellulose, antibiotics (ampicillin, tetracycline, and chromophinical), and Ni-NTA resin were purchased from Sigma. Tryptone and yeast extract were from DIFCO. ATP (grade II) and urea (ultrapure) were purchased from Fluka. Dialysis tubing (8000 MW cutoff) was obtained from Biodesign Inc. (New York, NY). All other chemicals were of the highest quality reagent grade available.

Generation of γ Subunit Mutants. Mutant subunits were constructed by enzymatic amplification of the expression plasmid pET8c-γ.BB1 (*7*, *14*). Primers (obtained from IDTDNA) were 25−37 nucleotides long and were chemically phosphorylated at the 5′-termini. Plasmid DNA for PCR was prepared using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). PCR was carried out in 50 μL of cloned *Pfu* DNA polymerase reaction buffer also containing 60 ng of the pET8cγbb1 plasmid as described in detail elsewhere (*7*,

14). PCR products were purified using the QIAquick gel extraction kit (Qiagen). The purified DNA was circularized by incubating 100−200 ng of the DNA with 3 units of T4 DNA ligase (Promega) in T4 DNA ligase buffer overnight at room temperature. The resulting plasmid was transformed into competent *E. coli* XL1-blue cells. Cloned plasmid was isolated and transformed into the expression host *E. coli* BL21(DE3)/pLysS (*15*). The entire sequence of each mutant gene was confirmed by the Iowa State DNA Sequencing Facility.

Reconstitution and Purification of Hybrid Assemblies. Slurries containing inclusion bodies of each subunit ($RrF_1\alpha_{his6}$, $RrF_1\beta$, and $CF_1\gamma$) were solubilized in an equal volume of 8 M urea and diluted to a final protein concentration of 2 mg/mL using reconstitution buffer containing 50 mM Tricine-NaOH (pH 7.8), 5 M urea, 50 mM DTT, 50 mM ATP, 50 mM $MgCl_2$, and 20% (v/v) glycerol. Solubilized proteins were incubated on ice for 1 h and centrifuged at 20000*g* and 4 °C to remove any insoluble material (*11*). Solubilized α, β, and γ subunits were mixed at concentrations of 50, 50, and 30 μg/mL, respectively, and dialyzed overnight at 4 °C against 8 volumes of dialysis buffer [50 mM Tricine-NaOH (pH 7.8) and 20% (v/v) glycerol]. Insoluble material was removed by centrifugation (20000*g* and 4 °C). The supernatant was diluted in 2 volumes of a buffer that contained 20 mM Tricine-NaOH (pH 7.8), 2 mM EDTA, and 1 mM ATP. The protein was purified by DEAE anion exchange chromatography (*3*) and applied to a Ni-NTA affinity column equilibrated with buffer containing 50 mM Tris-HCl (pH 7.8), 0.3 M NaCl, and 10 mM imidazole. The protein was eluted with the same buffer containing 250 mM imidazole. The reconstituted hybrid enzyme was further purified by size-exclusion chromatography on *Superdex 200* (Pharmacia) using a Biologic HR workstation FPLC system (Bio-Rad) at a flow rate of 0.5 mL/min. Protein was eluted with 50 mM Tricine-NaOH (pH 7.8) buffer containing 50 mM NaCl. Glycerol was added to the purified protein to a final concentration of 20% (v/v) prior to storage at −80 °C.

ATP Hydrolysis Assays. The ATP hydrolysis activities were measured with 2 μg of protein in 40 mM Tricine-NaOH (pH 8.0), varying concentrations of $MgCl_2$, or $CaCl_2$, and ATP (as indicated in the legends of Figures 2 and 4) for 2 min at 37 °C. For sulfite-dependent ATPase measurements, the assay buffers contained 40 mM Tricine-NaOH (pH 8.0), 1 mM $MgCl_2$, 2 mM ATP, and varying concentrations of Na_2SO_3 (as indicated in the legends of Figures 3 and 5). The ATP hydrolysis reaction was stopped by addition of 1 mL of 0.5 M trichloroacetic acid, and the concentration of inorganic phosphate was measured as described previously (*3*, *16*). Protein concentrations were determined by the Bradford method (*17*). Kinetics constants were obtained using the built-in nonlinear regression tools in SigmaPlot 8.0.

RESULTS

Mutant Assemblies Are Catalytically Active. Wild-type and mutant chloroplast γ (γ_C) subunits were assembled with recombinant *R. rubrum* F_1 α and β subunits ($\alpha_R\beta_R$), and the assemblies were purified by anion exchange and gel filtration as described previously (*11*−*13*) and with an additional nickel affinity chromatography step as described in Experimental Procedures. The catalytic properties of the RrF_1/CF_1 hybrid assembly were described in detail in earlier reports (*12*, *13*). The assembly was shown recently to be capable of CaATP-dependent γ rotation. MgATP-dependent rotation, however, required the presence of 100 mM of the oxyanion bicarbonate (*4*).

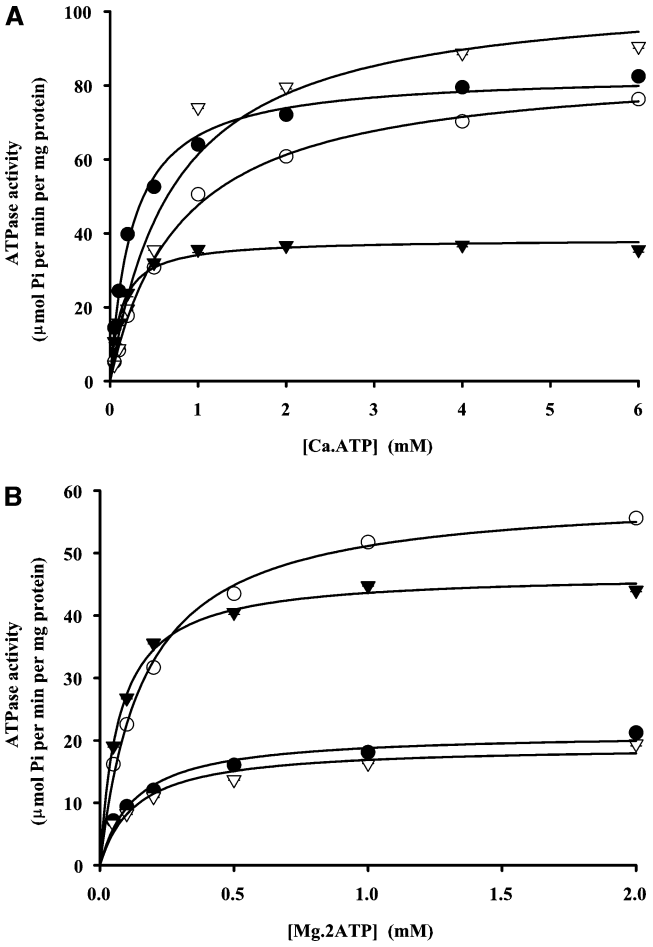

FIGURE 2: Substrate titrations of hybrid F_1 complexes containing single γ C-terminal mutations. The ATP hydrolysis activities of purified enzyme assemblies were determined as described in Experimental Procedures. (A) The CaATP concentration was varied as indicated while a constant molar ratio of $CaCl_2$ to ATP of 1:1 was maintained. (B) The MgATP concentration was varied as indicated while a constant molar ratio of $MgCl_2$ to ATP of 0.5:1 was maintained: (●) $\alpha_3\beta_3\gamma$wild type, (○) $\alpha_3\beta_3\gamma$R302L, (▼) $\alpha_3\beta_3\gamma$R304L, and (▽) $\alpha_3\beta_3\gamma$Q305A.

Four residues within the γ C-terminus were substituted: γArg302, γArg304, γGln305, and γLys307. All four residues are located within two turns of the C-terminal α helix of the γ subunit and form a positively charged collar that interacts closely with the negatively charged anionic loops on the alternating α and β subunits within the $\alpha_3\beta_3$ hexamer of MF_1. Residues γArg304 and γGln305 are highly conserved, and the analogous residues in MF_1, γArg256 and γGln257, were identified as catch residues, bonding directly to anionic loop residues of one of the three β subunits (*2*) (Figure 1). The loop residues are also highly conserved. The MF_1 residue analogous to γArg302 in CF_1, although not previously identified as a catch residue, is sufficiently close to an aspartate residue on the adjacent α subunit to form a salt link, indicating that it may contribute to the catch. Thus, the three catch residues together form a bridge between the α

Table 1: Assembly of CF_1 γ Mutants with *R. rubrum* α and β Subunits

CF_1 γ subunit[a]	assembly competence[b]
γwild type	+
γR302L	+
γR304L	+
γQ305A	+
γK307A	−
γR302L/R304L	+
γR304A/Q305A	+
γR302A/R304A/Q305A	+

[a] CF_1 γ mutants within the C-terminal region were reconstituted with *R. rubrum* $\alpha_3\beta_3$ subunits as described in Experimental Procedures. [b] Assembly competence was judged by the yield of purified assembly relative to the wild-type yield: +, normal yield; −, insufficient yield to test enzymatic activity.

Table 2: Summary of Kinetic Rate Constants for Hybrid F_1 Mutant Assemblies

	k_{cat} (s^{-1})[b]	
F_1 assembly[a]	Ca^{2+}	Mg^{2+}
$\alpha_3\beta_3\gamma$wild type	499.4 ± 10.2	127.3 ± 6.2
$\alpha_3\beta_3\gamma$R302L	514.8 ± 11.6	357.0 ± 8.8
$\alpha_3\beta_3\gamma$R304L	230.3 ± 4.7	280.3 ± 4.6
$\alpha_3\beta_3\gamma$Q305A	635.9 ± 43.9	114.8 ± 7.2
$\alpha_3\beta_3\gamma$R302L/R304L	223.5 ± 5.0	67.5 ± 3.7
$\alpha_3\beta_3\gamma$R304A/Q305A	178.8 ± 2.2	23.1 ± 5.4
$\alpha_3\beta_3\gamma$R302A/R304A/Q305A	97.3 ± 1.2	13.3 ± 0.4

[a] Hybrid F_1 mutants were assembled, purified, and assayed as described in Experimental Procedures. [b] Catalytic rate constants (k_{cat}) were calculated using the nonlinear regression tools in SigmaPlot 8.0. Errors are expressed as standard deviations with $n = 3$.

and β subunits that are identified as α_T (the α subunit involved in forming the ATP binding catalytic site) and β_E (the β subunit involved in forming the empty catalytic site) in the MF_1 structure (*2*). Since formation of the catch is considered to play an essential role in nucleotide release during the cooperative interplay between catalytic sites and hence rotation of the γ subunit, all of the mutations mentioned above were expected to reduce or eliminate catalytic turnover by eliminating salt linkages or hydrogen bonding potential with the catch residues on the α and β subunits within the F_1 complex.

In initial experiments, the three positively residues at positions 302, 304, and 307 were mutated to leucine to remove the charge and therefore reduce the strength of their ionic interaction with the anionic loops on the α and β subunits, while maintaining a similar side chain bulk. In subsequent mutations, arginine 304 and glutamine 305 on γ were replaced with alanine to block the potential hydrogen bonding interactions with the anionic loops on the β subunits identified in the crystal structure of mitochondrial F_1 (*2*). The mutant forms of the γ subunit, with the exception of γLys307, assembled into stable enzyme complexes that remained intact following gel filtration chromatography (Table 1). The γLys307 mutant assembled poorly under the conditions used, and insufficient quantities of the purified enzyme could be obtained for functional assays. The calcium- and magnesium-dependent ATPase activities of the mutants containing single substitutions of γArg302, γArg304, and γGln305 to either leucine or alanine are shown in Figure 2. In contrast to the 1:1 Ca:ATP ratio, the Mg:ATP ratio was maintained at 0.5:1 to minimize the concentration of inhibitory free Mg^{2+} ions (*1*).

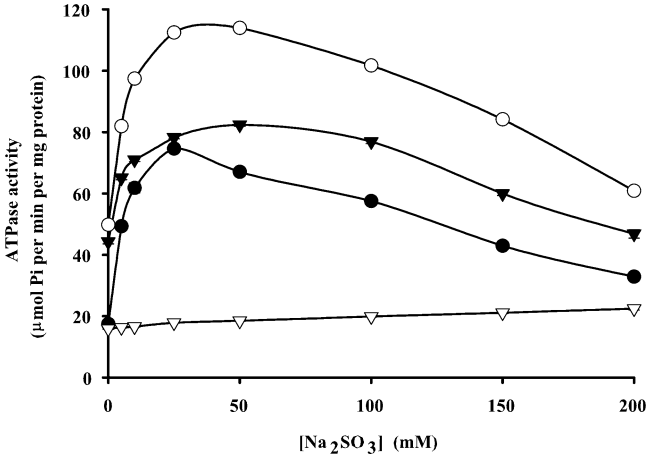

FIGURE 3: Effects of sulfite on hybrid F_1 complexes containing chloroplast γ subunits with single mutations in the C-terminus. The ATP hydrolysis activities of purified enzyme assemblies were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$, and the indicated concentrations of sodium sulfite as described in Experimental Procedures: (●) $\alpha_3\beta_3\gamma$wild type, (○) $\alpha_3\beta_3\gamma$R302L, (▼) $\alpha_3\beta_3\gamma$R304L, and (▽) $\alpha_3\beta_3\gamma$Q305A.

Interestingly, the γR302L mutation, while having little effect on the calcium-dependent activity, caused a nearly 3-fold increase in the rate of MgATP hydrolysis. The γR304L mutation had a similar effect on MgATPase activity but reduced the CaATPase activity to ~50% of that of the wild-type assembly. In contrast, the γQ305A mutant had no apparent effect on MgATPase activity and a small stimulatory effect on CaATPase activity. This result was surprising since the analogous mutation reduced the ATPase activity of the EcF_1 by 99% (*10*).

The magnesium-dependent activity of the hybrid was shown previously to increase significantly (>2-fold) in the presence of stimulatory oxyanions such as sulfite (*12*, *13*). The stimulatory oxyanion effect is well-known in the photosynthetic and mitochondrial F_1 enzymes and results from release of inhibition caused by stabilization of bound ADP by free magnesium ions (*18*−*20*). Oxyanions are required for high rates of magnesium-dependent catalytic turnover in both of these systems as well as in our assembled photosynthetic hybrid complexes (*12*, *13*) but not by EcF_1 which exhibits very high rates of magnesium-dependent ATPase activity in the absence of oxyanions where oxyanions are inhibitory (M. L. Richter, unpublished observation).

The responses of the MgATPase activities of the single γ mutants to added sulfite are shown in Figure 3. The wild-type assembly exhibited a nearly 4-fold stimulation at the optimal sulfite concentration (25 mM). The stimulatory effect was reduced at higher concentrations due to a nonspecific inhibitory effect of ionic strength. Both the γR302L and γR304L mutants also exhibited further stimulation by sulfite (Figure 2). The k_{cat} of the γR302L mutant at the optimal sulfite concentration was 642 s^{-1}, the highest value ever obtained in our hands. Another striking observation was that the γQ305A mutation, while having little apparent effect on catalytic turnover, completely eliminated the sulfite-induced stimulation of MgATPase activity (Figure 3).

Double and Triple Mutants Reduce Catalytic Rates. Combination mutations in which both γArg302 and γArg304 or γArg304 and γGln305 were mutated to leucines or alanines, respectively, resulted in reduced rates of catalytic

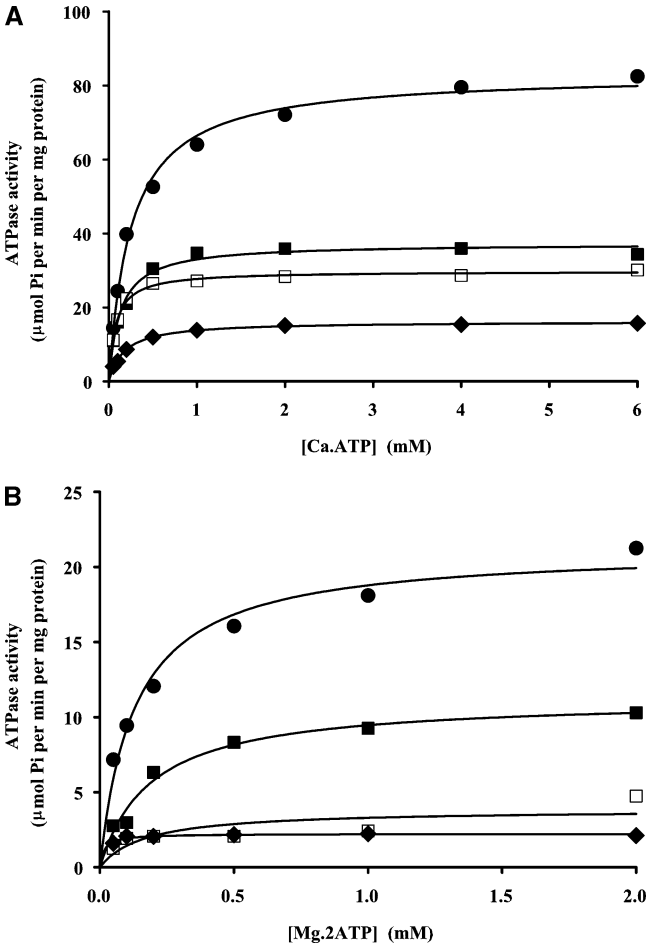

FIGURE 4: Substrate titrations of hybrid F_1 complexes containing chloroplast γ subunits with multiple mutations in the C-terminus. The ATP hydrolysis activities of purified enzyme assemblies were determined as described in Experimental Procedures. (A) The CaATP concentration was varied as indicated while a constant molar ratio of $CaCl_2$ to ATP of 1:1 was maintained. (B) The MgATP concentration was varied as indicated while a constant molar ratio of $MgCl_2$ to ATP of 0.5:1 was maintained: (●) $\alpha_3\beta_3\gamma$wild type, (■) $\alpha_3\beta_3\gamma$R302L/R304L, (□) $\alpha_3\beta_3\gamma$R304A/Q305A, and (◆) $\alpha_3\beta_3\gamma$R302A/R304A/Q305A.

turnover (Figure 4). Both the CaATPase and MgATPase activities of the γR302L/R304L double mutant were reduced to less than half of that of the wild-type assembly. The CaATPase activity of the γR304A/Q305A double mutant was ~40% of that of the wild type, whereas the MgATPase activity of this mutant was ~18% of that of the wild type, indicating that the MgATPase activity of this mutant was affected to a greater extent than the CaATPase activity. Similarly, the triple mutant, γR302A/R304A/Q305A, still retained ~25% of wild-type CaATPase activity but only ~10% of wild-type MgATPase activity. The effects of the combined mutations on the sulfite-stimulated MgATPase activity were even more pronounced (Figure 5), the MgATPase rate of the triple mutant being less than 3% of that of the wild-type assembly.

DISCUSSION

The hybrid enzyme used in this study has provided the means of genetically engineering F_1 subunits for structure−function analysis and rotation studies of F_1 enzymes derived from photosynthetic plant and bacterial sources. The k_{cat} for

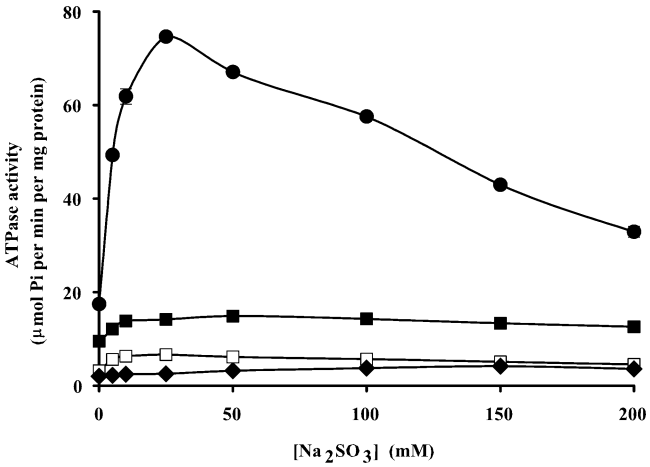

FIGURE 5: Effects of sulfite on double and triple hybrid F_1 mutants. The ATP hydrolysis activities of purified enzyme assemblies were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$, and the indicated concentrations of sodium sulfite as described in Experimental Procedures: (●) $\alpha_3\beta_3\gamma$wild type, (■) $\alpha_3\beta_3\gamma$R302L/R304L, (□) $\alpha_3\beta_3\gamma$R304A/Q305A, and (◆) $\alpha_3\beta_3\gamma$R302A/R304A/Q305A.

hydrolysis of CaATP by the hybrid $\alpha_R\beta_R\gamma_C$ enzyme is substantially higher than those of the native RrF_1 and CF_1 enzymes (*12*, *13*). The k_{cat} for hydrolysis of MgATP by the hybrid in the absence of stimulatory oxyanions is 40-fold higher than that of RrF_1 and 8-fold higher than that of CF_1. The maximum sulfite-stimulated k_{cat} of the hybrid is identical to that of CF_1 and about 5-fold higher than that of RrF_1. The differences in catalytic rates between the hybrid and natural enzymes result from differences in the interactions between the γ subunit and the nucleotide binding α and β subunits. The differences are, however, likely to be subtle ones since the hybrid is capable of all normal functions, including rotational catalysis (*4*), stimulation by reduction of the γ disulfide, inhibition by CF_1 ϵ subunit, and coupled ATP synthesis (F. He, R. E. McCarty, and M. L. Richter, unpublished observations).

If formation and release of the catch between the γ C-terminus and the anionic loop on the β subunit is a general feature of F_1 enzymes that is required for generating rotational torque, possibly as an escape mechanism for nucleotide-driven γ rotation (*10*), then substitution of CF_1 γR304 and γQ305 with leucine or alanine would be expected to slow or eliminate catalytic turnover by preventing these residues from forming salt links or hydrogen bonds with catch residues on the β subunit. Indeed, single-point mutations of either of the analogous residues, or of the predicted interacting catch residues on the β anionic loop in EcF_1, drastically reduced the rate of catalytic turnover (*10*). In stark contrast, however, the γR304L mutant hybrid greatly stimulated MgATPase activity while preserving significant rates of CaATPase activity. Similarly, substitution of the nearby arginine residue at position 302 led to a significant stimulation of MgATPase activity, indicating, for the first time, that this residue also plays a role in modulating catalytic function.

While the double and triple mutations reduced the rate of catalysis, significant activity, particularly CaATP hydrolysis, remained. In the triple mutant, the stretch of residues between positions 302 and 305 is comprised of four consecutive alanine residues, virtually eliminating any potential for specific ionic or hydrogen bonding interactions between this segment and the anionic loops on the α and β subunits. The

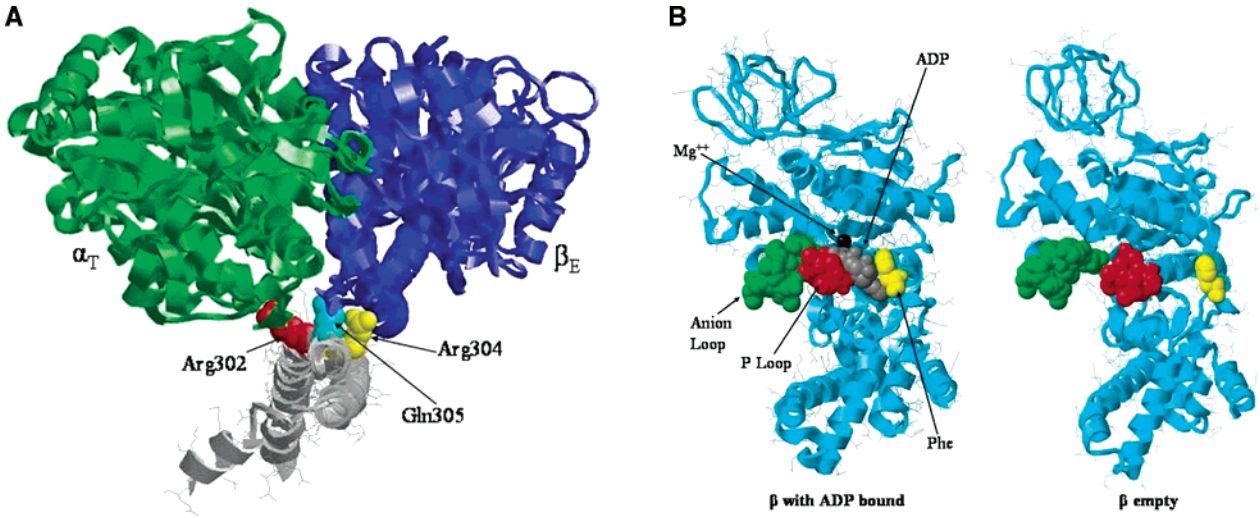

FIGURE 6: Modulation of catalytic sites by the catch interaction. (A) Top view of the proposed catch region extending across the noncatalytic interface formed between the β_E and α_T subunits in CF_1. The model (*31*) indicates that γArg304 (yellow) and γGln305 (cyan) contact an anionic loop on β_E in the same manner as in the MF_1 structure (*4*). γArg302 is predicted to form a salt link with Asp326 on the α_T subunit. (B) Partial structures of the empty and ADP sites on MF_1 comparing the relative positions of the p-loop (red), the anionic loop (green), the nucleotide (gray), and Phe424 (yellow) on the β_D and β_E subunits.

results thus indicate that γArg302, γArg304, and γGln305 are not essential for catalysis and raise doubts about the importance of the catch in cooperative catalysis and γ rotation. It is possible that neighboring residues are able to compensate for the loss of these three residues by providing alternative hydrogen bonding or charge interactions with the anionic loops on the β and α subunits. One such residue is γLys307. Since the analogous residue in EcF_1 is serine, which would not be expected to participate in forming a salt bridge with residues on the α or β subunits, this could explain why the mutations at positions 304 and 305 in the EcF_1 were so effective in blocking catalysis. Unfortunately, attempts to substitute γLys307 for alanine in this study have so far been unsuccessful, so we have been unable to test this possibility.

Another striking result of this study was the loss of sulfite stimulation in the γQ305A mutant. Oxyanion stimulation of the MgATPase activity of F_1 enzymes results from the release of an inhibitory effect of free Mg^{2+} ions originally thought to bind and stabilize binding of inhibitory ADP to catalytic sites (*5*, *6*−*8*, *18*−*20*). This is supported by mutations of a conserved threonine residue in the conserved p-loop segment of the catalytic site on the β subunit. The p-loop threonine is involved in coordinating the metal ion which bridges the enzyme surface and the nucleotide phosphates (*2*). This residue has been mutated to serine in several respiratory F_1 ATPases (*21*, *23*, *24*), in *Chlamydomonas reinhardtii* (*25*), and in the hybrid photosynthetic F_1 used in this study (*13*), in each case resulting in elevated MgATPase activity and a reduced level of oxyanion stimulation. Recent studies with spinach CF_1 (*26*, *27*), however, have indicated that oxyanions act by blocking binding of ADP or ATP to noncatalytic sites. The extent of inhibition of nucleotide binding observed with different oxyanions closely mirrored the extent of stimulation of MgATPase activity (*26*). This apparent contradiction may be resolved by taking into account some earlier studies with yeast MF_1 (*21*) that indicated that the rate stimulation resulting from the substitution of the p-loop threonine with serine and the stimulatory effect of added sulfite are additive. Thus, they are distinct effects, each acting on a different part of the catalytic process. Magnesium ions may, in fact, stabilize binding of inhibitory ADP to both catalytic and noncatalytic sites.

A Possible Mechanism of Oxyanion Activation. The structural organization of the $\alpha\beta$ pair that is associated with the catch residues in the bovine MF_1 is shown in Figure 6. In panel A, γArg302, γArg304, and γGln305 (CF_1 numbering) are shown to interact with anionic loops on the β_E (empty site) and α_T (ATP site) subunits that form a close interaction at one of the three noncatalytic site interfaces. γArg304 and γGln305 bind to the β_E subunit, whereas γArg302 is positioned to form a salt link with a nearby aspartate residue on the α_T subunit, thereby bridging the two subunits across the noncatalytic interface. In panel B, two of the three catalytic site configurations of the bovine MF_1 enzyme are shown, the empty site on β_E and the site with ADP bound on β_D. In β_D, the anionic loop is closely associated with the p-loop which in turn is closely associated with the bound nucleotide which, together with the adjacent phenylalanine residue, forms a compact arrangement in which the nucleotide is tightly bound. In β_E, the anionic loop, which is associated with the γ catch residues, appears to have been pulled away from the p-loop residues. Similarly, the phenylalanine residue which is directly connected to the "foot" segment containing the DELSEED sequence of the β_D subunit (*2*) and which forms a separate catch with residues farther from the C-terminus in the γ subunit has also pulled away from the p-loop. It is assumed (*2*) that concurrent formation of the two catch interactions results in release of bound nucleotide from this site during γ rotation.

The structural organization of the β_E site suggests a possible explanation for the effects of mutation of catch residues 302−305 on catalysis by the hybrid enzyme. When isolated after ammonium sulfate precipitation, chloroplast F_1 contains two very tightly bound ADP molecules, one in a catalytic site and the other in a noncatalytic site (*28*). Tight binding of ADP to one of the three noncatalytic sites, as it does for the three catalytic sites, requires the sites to be asymmetric. Since asymmetric nucleotide binding and formation of tight nucleotide binding sites on CF_1 require an

asymmetric interaction between the γ subunit and the α and β subunits (*3*), it is a reasonable assumption that the noncatalytic site on CF_1, analogous to that formed between α_T and β_E in MF_1, is occupied by a tightly bound molecule of ADP. Since activation of the MgATPase activity of CF_1 requires filling of noncatalytic sites with MgATP (*29*), replacement of the tightly bound noncatalytic ADP is likely to be a prerequisite for optimal catalytic turnover. Thus, an inhibitory effect of free Mg^{2+} may arise from stabilization of ADP binding at the noncatalytic interface. The stimulatory effect of oxyanions such as sulfite would result from destabilization of noncatalytic ADP, allowing it to be rapidly exchanged with MgATP.

In this model, mutations in the γ subunit that weaken the catch interaction (γR302L and γR305A) would be expected to result in an increased rate of activation of the MgATPase activity and a weakened reliance on oxyanions as observed. The fact that the γQ305A mutation selectively eliminated the oxyanion effect would indicate that the interaction between this residue and the anionic loop on β_E is responsible for communicating the activating effect of ATP binding to the noncatalytic site. This explanation, although remaining hypothetical, is consistent with observations by Malyan's group (*26*−*28*) and is strongly reinforced by an earlier observation that the presence of sulfite dramatically increases the rate of exchange of tightly bound noncatalytic ADP for MgATP in CF_1 (N. Hu and M. L. Richter, unpublished experiments). Experiments aimed at examining the effects of oxyanions on binding of nucleotide to the mutant hybrid enzymes are underway and thus test this hypothesis.

In summary, the results of this study have shown that the highly conserved catch residues identified in the MF_1 structure as interacting with the anionic loops on the β subunits are neither universally nor obligatorily essential for catalytic turnover in F_1 enzymes. Instead, replacing γArg304 with leucine more than doubled the MgATPase activity as did replacement of nearby γArg302 with leucine. The results are opposite from those obtained with *E. coli* F_1 in which replacement of residues equivalent to either Arg304 or Gln305 drastically reduced the rate of catalytic turnover (*10*). Replacement of γGln305 with alanine in this study essentially eliminated the oxyanion stimulation of MgATP hydrolysis, indicating for the first time that this residue may play a pivotal role in this important regulatory process. Oxyanion activation of MgATP hydrolysis, which involves release of inhibition by MgADP, occurs in photosynthetic and mitochondrial enzymes and is replaced by the transmembrane proton gradient when F_1 is membrane-bound (*30*, *31*). The results thus indicate a functional link between proton-coupled activation and formation of the catch.

NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on February 9, 2007 with errors in Table 2. The corrected version was published February 27, 2007.

REFERENCES

1. Richter, M. L., Samra, H., He, F., Giessel, A., and Kuczera, K. (2005) Coupling proton movement to ATP synthesis in the chloroplast ATP synthase, *Bioenerg. Biomembr. 37*, 467−473.
2. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria, *Nature 370*, 621−628.
3. Gao, F., Lipscomb, B., Wu, I., and Richter, M. L. (1995) In vitro assembly of the core catalytic complex of the chloroplast ATP synthase, *J. Biol. Chem. 270*, 9763−9769.
4. Tucker, W. C., Schwarz, A., Levine, T., Du, Z., Gromet-Elhanan, Z., Richter, M. L., and Haran, G. (2004) Observation of calcium-dependent unidirectional rotational motion in recombinant photosynthetic F1-ATPase molecules, *J. Biol. Chem. 279*, 47415−47418.
5. Boyer, P. D. (1993) The binding change mechanism for ATP synthase: Some probabilities and possibilities, *Biochim. Biophys. Acta 1140*, 215−250.
6. Boyer, P. D. (1997) The ATP synthase: A splendid molecular machine, *Annu. Rev. Biochem. 66*, 717−749.
7. Sokolov, M., Lu, L., Tucker, W., Gao, F., Gegenheimer, P. A., and Richter, M. L. (1999) The 20 C-terminal amino acid residues of the chloroplast ATP synthase γ subunit are not essential for activity, *J. Biol. Chem. 274*, 13524−13829.
8. Müller, M., Pänke, O., Junge, W., and Engelbrecht, S. (2002) F1-ATPase, the C-terminal end of subunit γ is not required for ATP hydrolysis-driven rotation, *J. Biol. Chem. 277*, 23308−23313.
9. Hossain, M. D., Furuike, S., Maki, Y., Adachi, K., Ali, M. Y., Huq, M., Itoh, H., Yoshida, M., and Kinosita, K., Jr. (2006) The rotor tip inside a bearing of a thermophilic F1-ATPase is dispensable for torque generation, *Biophys. J. 90*, 4195−4203.
10. Greene, M. D., and Frasch, W. D. (2003) Interactions among γR268, γQ269, and the β subunit catch loop of *Escherichia coli* F1-ATPase are important for catalytic activity, *J. Biol. Chem. 278*, 51594−51598.
11. Du, Z., and Gromet-Elhanan, Z. (1999) Refolding of recombinant α and β subunits of the *Rhodospirillum rubrum* F_0F_1 ATP synthase into functional monomers that reconstitute an active $\alpha_1\beta_1$ dimer, *Eur. J. Biochem. 263*, 1432−1327.
12. Tucker, W. C., Du, Z., Hein, R., Gromet-Elhanan, Z., and Richter, M. L. (2001) Formation and properties of hybrid photosynthetic F1-ATPases. Demonstration of different structural requirements for stimulation and inhibition by tentoxin, *Eur. J. Biochem. 268*, 2179−2186.
13. Du, Z., Tucker, W. C., Richter, M. L., and Gromet-Elhanan, G. (2001) Assembled F1-($\alpha\beta$) and Hybrid F1-$\alpha_3\beta_3\gamma$-ATPases from *Rhodospirillum rubrum* α, wild type or mutant β, and chloroplast γ subunits. Demonstration of Mg^{2+} versus Ca^{2+}-induced differences in catalytic site structure and function, *J. Biol. Chem. 276*, 11517−11523.
14. Samra, H. S., Gao, F., He, F., Hoang, E., Chen, Z., Gegenheimer, P. A., Berrie, C. L., and Richter, M. L. (2006) Structural analysis of the regulatory dithiol-containing domain of the chloroplast ATP synthase γ subunit, *J. Biol. Chem. 281*, 31041−31049.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
16. Taussky, H. H., and Shorr, E. (1953) A microcolorimetric method for the determination of inorganic phosphorus, *J. Biol. Chem. 202*, 675−685.
17. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
18. Malyan, A. N. (1981) Chloroplast ATPase (CF): Allosteric regulation by ADP and Mg^{2+} ions, *Photosynthetica 15*, 474−483.
19. Malyan, A. N., and Vitseva O. I. (1990) Kinetic analysis of ADP and Mg^{2+} dependent inactivation of CF1 ATPase, *Photosynthetica 24*, 613−622.
20. Murataliev, M. B., and Boyer, P. D. (1994) Interaction of mitochondrial F1-ATPase with trinitrophenyl derivatives of ATP and ADP. Participation of third catalytic site and role of Mg^{2+} in enzyme inactivation, *J. Biol. Chem. 269*, 15431−15439.
21. Muller, D. M. (1989) A mutation altering the kinetic responses of the yeast mitochondrial F1-ATPase, *J. Biol. Chem. 264*, 16552−16556.
22. Malyan, A. N., and Vitseva, O. I. (2001) Interaction of sulfite with the noncatalytic and catalytic sites of chloroplast coupling factor cf1, *Biochemistry (Moscow) 66*, 410−414.
23. Omote, H., Maeda, M., and Futai, M. (1992) Effects of mutations of conserved Lys-155 and Thr-156 residues in the phosphate-binding glycine-rich sequence of the F1-ATPase β subunit of *Escherichia coli*, *J. Biol. Chem. 267*, 20571−20576.
24. Jault, J. M., Dou, C., Grodsky, N. B., Matsui, T., Yoshida, M., and Allison, W. S. (1996) The $\alpha_3\beta_3\gamma$ subcomplex of the F1-ATPase from the thermophilic bacillus PS3 with the βT165S

substitution does not entrap inhibitory MgADP in a catalytic site during turnover, *J. Biol. Chem. 271*, 28818−28824.
25. Hu, D., Strotmann, H., Shavit, N., and Leu, S. (1998) The *C. reinhardtii* CF1 with the mutation βT168S has high ATPase activity, *FEBS Lett. 421*, 65−68.
26. Malyan, A. N. (2003) Interaction of oxyanions with thioredoxin-activated chloroplast coupling factor 1, *Biochim. Biophys. Acta 1607*, 161−166.
27. Malyan, A. N., and Allison, W. S. (2002) Properties of noncatalytic sites of thioredoxin-activated chloroplast coupling factor 1, *Biochim. Biophys. Acta 1554*, 153−158.
28. Malyan, A. N. (2006) ADP and ATP binding to noncatalytic sites of thiol-modulated chloroplast ATP synthase, *Photosynth. Res. 89*, 9−18.
29. Murataliev, M. B., and Boyer, P. D. (1992) The mechanism of stimulation of MgATPase activity of chloroplast F1-ATPase by non-catalytic adenine-nucleotide binding. Acceleration of the ATP-dependent release of inhibitory ADP from a catalytic site, *Eur. J. Biochem. 209*, 681−687.
30. Du, Z. Y., and Boyer, P. D. (1990) On the mechanism of sulfite activation of chloroplast thylakoid ATPase and the relation of ADP tightly bound at a catalytic site to the binding change mechanism, *Biochemistry 29*, 402−407.
31. Zhang, S., Letham, D. D., and Jagendorf, A. T. (1993) Inhibition of Thylakoid ATPase by Venturicidin as an Indicator of CF1-CF0 Interaction, *Plant Physiol. 101*, 127−133.

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