Studies of the C-terminal Region of the Gamma Subunit of the Chloroplast ATP Synthase

By

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Dedicated to my parents Qi He and Yong Xu, my wife Xin Wang and my soon to be born daughter Joyce He

Abstract

Two highly conserved amino acid residues, an arginine and a glutamine, 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 αβ hexamer of the F_1 segment. Forming the catch is hypothesized 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. A series of single and multiple amino acid substitutions of the two analogous residues, Arg304 and Gln305, in the chloroplast F_1 γ subunit, as well as the neighboring residue γArg302, were constructed utilizing site-directed mutagenesis. The mutations were introduced with the intention of eliminating any charge-charge or hydrogen bonding interactions between the catch residues and the anionic loop on the β subunit, thereby testing the hypothesis.

Each mutant γ subunit was assembled together with the 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 subunit rotation. 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 >90% inhibited, whereas

the CaATPase activities were inhibited by less than 50%.

In a second study, the γ catch mutants were assembled with the native CF_1 α and β subunits and, in some cases, with the ε and δ subunits followed by reconstitution with CF_1-free thylakoid membranes. The Mg^{2+}-dependent and Ca^{2+}-dependent ATP hydrolysis activities of the native assembled $\alpha_3\beta_3\gamma$ complex both increased by more than 3-fold as a result of the mutations; however, the sulfite-stimulated activity decreased by more than 60%. Proton-driven ATP synthesis of the thyloakoid-bound mutant enzyme also decreased by about 80% compared to the wild type enzyme. Nucleotide binding and exchange studies of CF_1 using TNP-ADP revealed two nucleotide binding sites with completely different properties. One site binds and exchanges nucleotides rapidly and is likely to be a catalytic site while the other site displays slow nucleotide binding and exchange and appears to be non-catalytic. The triple mutant containing three alanines in place of Arg302, Arg304 and Gln305 within the CF_1 γ subunit displayed higher exchange rates in the fast filling, catalytic site but failed to exchange the majority of the TNP-ADP in the slow filling, non-catalytic site even after 30 minutes of incubation with ATP hydrolysis substrates.

The results of these studies 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). However, contrary to the hypothetical role in orchestrating

catalytic cooperativity among the nucleotide binding sites, the catch is not essential for ATP hydrolysis. Instead, the catch appears to be involved primarily in the process of oxyanion activation of MgATP hydrolysis and in coupling the proton gradient to ATP synthesis. The results have led to the new hypothesis that the catch residues perform the critical function of substrate (MgADP) recruitment during photophosphorylation.

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Chapter One

Introduction

A. Adenosine TriPhosphate (ATP) ---- the Energy Carrier

Besides its genetic function of supplying the purine unit, adenine, for nucleic acid biosynthesis, ATP has another contribution to life on earth, to serve as an energy carrier or energy transmitter. The hydrolysis of ATP produces adenosine diphosphate (or ADP), inorganic phosphate (Pi), and approximately 7.3 kcal per mol of energy that can be coupled to energy dependent biological processes. Because ATP, ADP, and inorganic phosphate are highly mobile and easy to transport inside and outside of the cell, most living organisms rely heavily on the energy produced by ATP hydrolysis and on short term energy storage in the form of ATP. An average human can consume and replenish up to his / her body weight in ATP molecules every day. Therefore, ATP plays one of the most important roles in the cellular world.

B. The ATP Synthase

ATP synthases are found in bacteria, mitochondria in animal cells and chloroplasts in plant cells. The primary function of the ATP synthases is to

synthesize ATP using ADP and inorganic phosphate and store energy for other cellular work inside the cell. All of the ATP synthases share many similar properties both structurally and functionally. Every member of this protein family has a membrane-spanning unit that anchors the protein into the membrane (bacterial cell membrane, mitochondrial membrane, and chloroplast thylakoids membrane). The sophisticated membrane architecture of the ATP synthase allows it to extract the chemiosmotic energy resulting from a transmembrane electrochemical gradient generated by protons crossing the membrane, and to use the energy to synthesize ATP on one side of the membrane (Mitchell 1961).

1. General Structure and Subunit Stoichiometry of the ATP Synthases

The putative structure of the chloroplast ATP synthase is shown in Figure 1. The ATP synthase contains two distinct protein parts that can be separated both physically and functionally, factor O (F_o) and factor 1 (F_1). F_o contains a proton channel that spans the membrane. The stoichiometry of F_o subunits varies depending on the species. The F_o segment from the *Escherichia coli* ATP synthase (EcF_o) contains three different polypeptides, designated subunit a, subunit b and subunit c that are present in a stoichiometry of a_1, b_2, and c_{10}. The ten copies of subunits c form a transmembrane ring structure that acts as a proton channel while the a and b subunits function as a peripheral stalk structure connecting F_o to F_1. The chloroplast F_o (CF_o) has four subunits, labeled I to IV

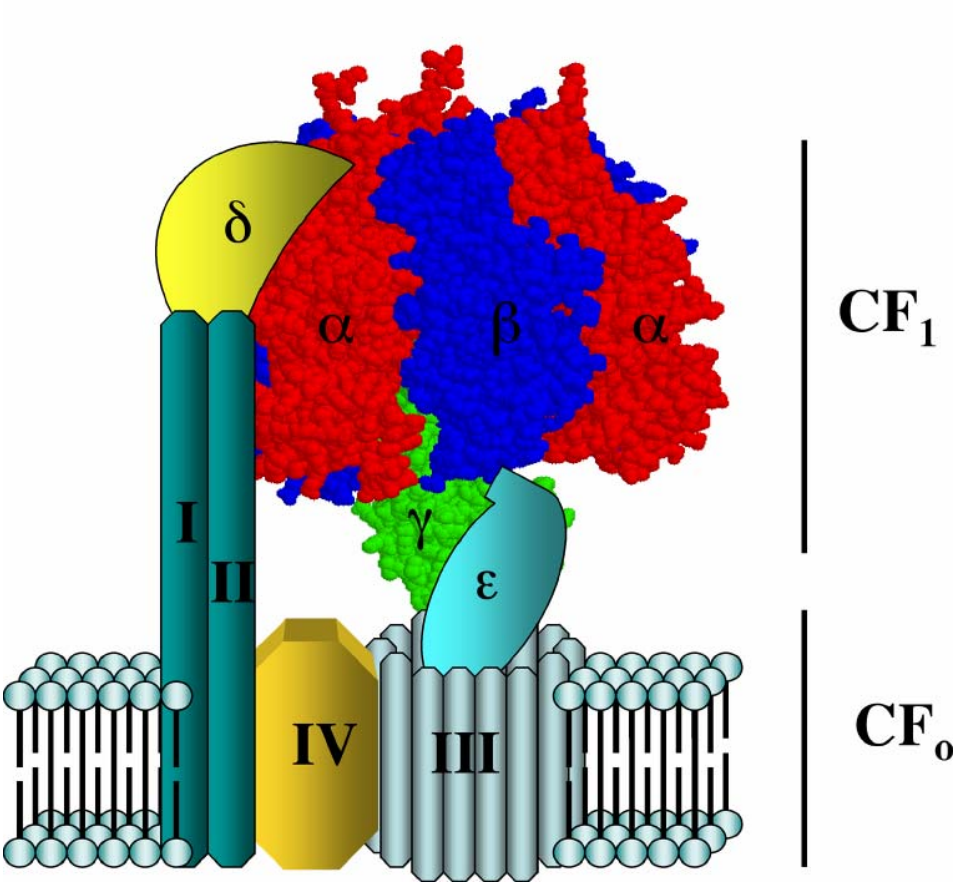

Figure 1. Proposed structural organization of the Chloroplast F_oF_1 ATP Synthase. Unsolved protein subunits are indicated in cartoon diagrams and a homology model of the chloroplast γ subunit is shown in green (courtesy of Dr. Engelbrecht).

with a stoichiometry of $I_1II_1III_{14}IV_1$ (Fillingame, Jones et al. 1998). Subunit III, which is equivalent to subunit c in EcF_o, forms the H^+ channel, and subunits I, II, and IV, equivalent to EcF_o subunits a, b and b^1 respectively, bind together, are partially embedded in the membrane, and hold the entire ATP synthase complex together. The mitochondrial F_o (MF_o) has a structure that is slightly more complicated than its counterparts in *E. Coli* and chloroplasts. MF_o is composed of nine different polypeptides, subunits a, b, c, d, e, f, g, F6, and A6L (Collinson, Runswick et al. 1994). Subunits a, b and c are equivalent to subunits a, b and c in EcF_o. Despite the complex nature, MF_o has a similar function to the other factor o segments (Rubinstein, Walker et al. 2003).

The factor 1 (F_1) portion of the ATP synthase is the catalytic unit of the enzyme. Chloroplast F_1 (CF_1) is comprised of five polypeptides that are named with Greek letters in order of decreasing molecular weight. These subunits are α (55.5 kDa), β (53.8 kDa), γ (35.9 kDa), δ (20.5 kDa), and ε (14.7 kDa). The subunit stoichiometry of CF_1 is $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The F_1 enzyme from *E. coli* (EcF_1) is similar to that of the chloroplast enzyme in structure while the mitochondrial F_1 (MF_1) has a δ subunit that corresponds to the ε subunit of CF_1 or EcF_1. MF_1 also contains an Oligomycin Sensitivity Coferring Protein (OSCP) that is similar to the δ subunit of CF_1 or EcF_1 and an 8 KDa ε subunit that has no counterpart in the bacterial and chloroplast enzymes (Walker, Fearnley et al. 1985).

Since the main focus of this dissertation is on the chloroplast ATP synthase,

CF_1 will be used as an example to explain how the F_1 enzyme is arranged (shown in Figure 1). The three copies of the α subunits and the three copies of the β subunits together form a hexametric ring with α and β subunits alternating. N-terminal and C-terminal helical elements of the γ subunit slice into the middle of the αβ hexamer and interact with αβ pairs from inside while the rest of the γ subunit, together with ε subunit, is sandwiched between the $\alpha_3\beta_3$ hexamer and the membrane. The ε subunit is also attached to the subunit III ring. The δ subunit links one α subunit to subunits I and II to form a stator structure and locks the peripheral portion of the enzyme (F_1) to the membrane anchor (F_o).

2. The Functional Core of the ATP Synthase ---- the F_1 Enzyme

Although the primary function of the ATP synthase is to harvest energy and synthesize ATP, the enzyme can also hydrolyze ATP to produce ADP and generate a proton gradient. For instance, some bacteria (like *E. coli*) use the ATP synthase to hydrolyze ATP to generate proton gradient needed for transporting other molecules across the plasma membrane (Padan, Bibi et al. 2005). Under most *in vivo* conditions, ATP synthases are closely regulated to prevent futile ATP hydrolysis. However, the peripheral F_1 enzyme can be dissociated from the membrane. Once isolated *in vitro*, the F_1 enzyme can only display ATPase activity (Walker, Fearnley et al. 1985; Murataliev and Boyer 1994; Gao, Lipscomb et al. 1995).

Catalytic sites are thought to be the same for both ATP hydrolysis and ATP synthesis. There are six nucleotide binding sites in total on the F_1 part of the enzyme (Shapiro, Gibson et al. 1991). Three of those sites are located at αβ interfaces on the α subunits and are considered to be non-catalytic while the other three, considered to be catalytic, are located at the alternative three αβ interfaces on the β subunits. Each of these sites is capable of binding one nucleotide in the presence or the absence of a metal ion. The three sites on the α subunits are expected to have regulatory functions although the regulatory mechanism is not understood (Boyer 1997). The γ subunit and the ε subunit together connect the F_o part with the F_1 part and function as a rotating spindle. During ATP synthesis, protons are captured on one side of the membrane by subunit III and released on the F_1 side of the membrane following a 360^o rotation of the subunit III ring. This rotation generates the torque required for the γ subunit to rotate. It was first demonstrated by Kinosita's group that the three-subunit F_1 enzyme ($\alpha_3\beta_3\gamma$), when attached to a glass surface, was able to carry out rotation of large (1-2 μm in length) actin filaments during ATP hydrolysis (Noji, Yasuda et al. 1997). Similar experiments with gold particles attached to the γ subunit further revealed that γ rotation occurs in discrete rotational "steps" of 120° with "sub-steps" of 90° and 30° (Yasuda, Noji et al. 2001).

3. Crystal Structures of the ATP Synthase F_1 Enzymes

Many laboratories have spent decades trying to solve the structures of the ATP synthases. The first high resolution crystal structure was determined for the bovine heart mitochondrial F_1 by John Walker and his co-workers in 1994 (Abrahams, Leslie et al. 1994). The structure presented direct evidence that the three catalytic β subunits were in different conformations with a different nucleotide occupancy as predicted by Paul Boyer's binding change hypothesis discussed in the following section (Boyer 1993). The structure showed that the three non-catalytic sites each contained one ATP molecule and the three catalytic sites contained ATP, ADP and no nucleotides, respectively (colored differently in Figure 2). It was shown previously (Gao, Lipscomb et al. 1995) that the γ subunit was responsible for forming the contacts with α and β subunits necessary for asymmetric nucleotide binding. It is assumed that these contacts switch between different αβ pairs as the γ subunit rotates, sequentially changing the structures and nucleotide binding properties of the catalytic sites. Only 40% of the structure of the γ subunit was resolved in the original MF_1 crystal structure and the structures of the δ and ε subunits were not resolved. The MF_1 structure was later refined to a 2.4 Å resolution by the same research group (Gibbons, Montgomery et al. 2000). In this structure, the F_1 ATP synthase crystals were grown in the presence of the inhibitor dicyclohexylcarbodiimide (DCCD). The crystals revealed the entire structure of the mitochondrial γ subunit and confirmed the close interaction between the C-terminal region of γ and the catalytic domains

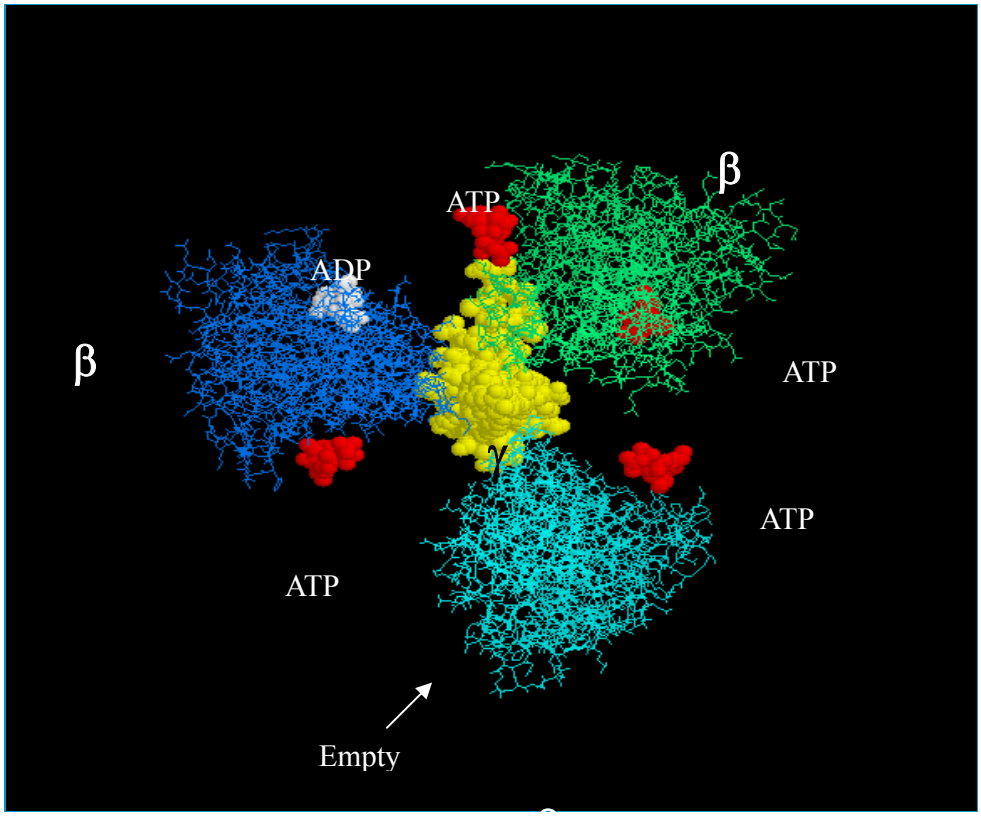

Figure 2. Top view of the crystal structure of the mitochondrial ATP synthase F_1 enzyme from bovine heart (adapted from Protein Data Bank ID: 1BMF). The three α subunits have been deleted for convenience. The three β subunits are shown in blue, cyan and green. The γ subunit is indicated in yellow. Four ATP molecules are present (red) and one molecule of ADP (white).

on the αβ ring. The structure further showed that part of γ interacts with the c subunits of MF_o to form the rotating stalk.

A Crystal structure of the *E. coli* F_1 has also been published but showed less asymmetry than MF_1 and only the structures of the α and β subunits were resolved (Hausrath, Gruber et al. 1999; Hausrath, Capaldi et al. 2001). Other partial structures solved include the F_1 from the thermophilic bacterium PS3, or TF_1, (Shirakihara, Leslie et al. 1997) and that from the rat liver (Bianchet, Hullihen et al. 1998). Those two structures also displayed a high degree of symmetry in the hexamer ring and very little structural information on γ subunit was obtained. In 2001, Groth and coworkers published a 3.2 Å resolution crystal structure of the chloroplast F_1 (Groth and Pohl 2001). The crystals were prepared without Mg^{2+} ions, which are believed to dictate, at least in part, the asymmetric features of the enzyme (Shapiro and McCarty 1988; Shapiro and McCarty 1990; Shapiro, Gibson et al. 1991; Shapiro, Huber et al. 1991). The structure of α and β proteins were solved by averaging all three copies of the same subunits and the overall structure was symmetrical. Despite the nucleotides being present during crystal preparation, no nucleotides could be seen in the structure.

The structure of the εδ complex of the *E. coli* F_1 (Rodgers and Wilce 2000) and the ε subunit alone (Wilkens, Dahlquist et al. 1995; Uhlin, Cox et al. 1997) were both published. Structures of the membrane spanning c subunits have also

been revealed in EcF_1 (Girvin, Rastogi et al. 1998; Rastogi and Girvin 1999).

4. The Binding Change Mechanism and Rotational Catalysis of the ATP Synthase

Isotopic exchange studies of ^{18}O and ^{32}P revealed that the γ phosphate bond of the nucleotide is broken and reformed with oxygen from water during oxidative phosphorylation (Boyer, Falcone et al. 1954). The reversible formation of the tightly bound ATP at a catalytic site on F_1 was recognized as a critical step in catalysis that occurred in the absence of energy input (Boyer, Cross et al. 1973). It was thus proposed that the energy required was not used to form ATP, but instead, to release the already formed ATP molecule (Boyer, Cross et al. 1973). These observations lead Paul Boyer to propose a new mechanism for energy coupling in the ATP synthase, referred as the Binding Change Mechanism (shown in Figure 3) (Boyer 1989; Boyer 1993).

The Binding Change Mechanism states the following features of the ATP synthase: 1) three catalytic sites are equally involved in a single catalytic cycle; 2) at any given time during a catalytic cycle, the three catalytic sites are always in different conformations indicated by the nucleotide occupancy, that is, one site has ADP and Pi bound (loose site), one site has tightly bound ATP (tight site), and one site is not filled with any nucleotide (open site); 3) during a single catalytic cycle, the events happening in each site must follow a fixed series of steps: a) an open

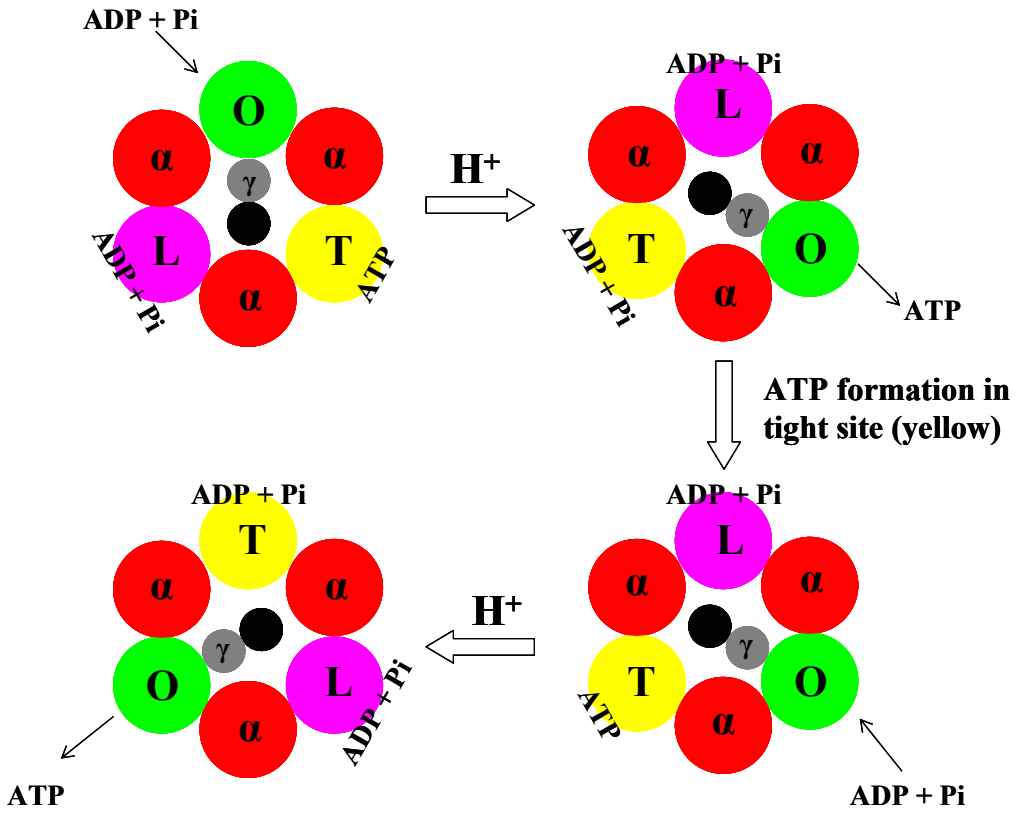

Figure 3. The Binding Change Mechanism. Three non-catalytic sites on α subunits are labeled in red while three catalytic sites on β subunits are in pink (loose site), green (open site), and yellow (tight site) respectively. The C-terminal helix of γ is in grey and the N-terminal helix is in black. Binding change events in the direction of ATP synthesis are shown (Boyer 1993).

site must become loose to attract substrates; b) a loose site binds substrates ADP and Pi and then catalyzes the formation of ATP when the site becomes a tight site; c) a tight site must release the product, ATP, to become open; 4) the catalytic events in each site are induced by those in the other two sites via a cooperative process mediated by the cooperatively interaction of the γ subunit with the catalytic β subunits; 5) the energy coupled through proton movement is used to release the tightly bound ATP (rather than in making ATP) and the cooperativity among all three sites results in a large increase in catalytic turnover.

The Binding Change mechanism was based on the hypothesis that all three β subunits must be in different conformations at any given time (Boyer 1993). The later published crystal structure of the MF_1 ATP synthase clearly supported this theory (Abrahams, Leslie et al. 1994). Based on the hypothesis that all three catalytic sites follow an identical and sequential order during a single cycle (Kayalar, Rosing et al. 1977; Hackney and Boyer 1978; Hackney, Rosen et al. 1979; Rosen, Gresser et al. 1979; Kohlbrenner and Boyer 1983; Ketcham, Davenport et al. 1984), Boyer suggested the concept of rotational catalysis that seemed to be the only possible way of explaining how the enzyme might work (Gresser, Myers et al. 1982).

The first direct observation of rotational catalysis was presented by Noji et al. in 1997 (Noji, Yasuda et al. 1997). In that experiment, a unidirectional rotation by a recombinant assembly of TF_1 was seen in the presence of low

concentrations of ATP but not in its absence. The rotation of the γ subunit displayed discrete 120^{o} steps in an anticlockwise direction, when viewed from the perspective of the membrane (Yasuda, Noji et al. 1998). The same type of experiment was later repeated using gold beads instead of actin filaments to reduce the friction (Yasuda, Noji et al. 2001). It was found that the 120^{o} rotation steps are composed of 90^{o} and 30^{o} substeps, which later were resolved as 80^{o} (triggered by ATP binding) and 40^{o} (following ATP hydrolysis and ADP release) substeps (Shimabukuro, Yasuda et al. 2003). The γ rotation has also been observed in EcF_1 (Omote, Sambonmatsu et al. 1999) and hybrid F_1 that contained α and β subunits from the bacterium *Rhodospirillum rubrum* and γ subunit from spinach chloroplast F_1 (Tucker, Schwarz et al. 2004). Recently, Noji and his group demonstrated a new technique using magnetic tweezers to generate the directional torque that is normally produced via proton transport in F_1, causing it to rotate in the opposite direction to that of ATP hydrolysis. In this case ATP was synthesized by TF_1 (Hirono-Hara, Ishizuka et al. 2005; Rondelez, Tresset et al. 2005).

All of these results indicate that the γ subunit undergoes stepwise rotation during catalysis, forming and breaking interactions with different αβ pairs. Thus, the behavior of each catalytic site at any given time is determined by the position of the γ subunit. The γ subunit, together with the ε subunit also connects F_1 to the c subunits (III subunits in chloroplast) of F_o and coverts the rotational torque

generated by membrane components into mechanical movement inside the F_1 portion, through which the energy is used to synthesize ATP.

C. The Chloroplast ATP Synthase

1. Special Properties of the CF_oCF_1 Enzyme

The chloroplast ATP synthase is located on the thylakoid membranes found in plant chloroplasts with the CF_1 portion facing the stromal side (N side). Constituting almost 10% of the thylakoid membrane mass, CF_oCF_1 is the key enzyme involved in the photosynthetic chain composed of a series of proteins which are in charge of harvesting light energy. Protons accumulated on the lumen side (P side) of thylakoids are moved across the membrane by CF_o, coupling the energy resulting from the H^+ gradient to ATP synthesis.

The two parts of the chloroplast ATP synthase, CF_o and CF_1, are partially linked by Mg^{2+} ions that bridge the two negatively charged protein surfaces. Treatment with chelating agents, such as ethylene-diamine tetra-acetic acid (EDTA), can extract the metal ions and cause CF_1 to dissociate from the membrane (McCarty and Racker 1966). The isolated CF_1 proteins are soluble to more than 100 mg/ml in aqueous solutions at room temperature but are labile at cold temperatures (McCarty and Racker 1966; McCarty and Racker 1968).

Isolated CF_1 enzymes can be protected against denaturing conditions by addition of substrate nucleotides (ADP and/or ATP) and/or glycerol or in a precipitated form by ammonium sulfate beyond the saturating point (~50%). When studied *in vitro*, CF_1 displays ATP hydrolysis activity but no ATP synthesis activity without the membrane F_o component (McCarty and Racker 1966; Gao, Lipscomb et al. 1995). In contrast to the bacterial and mitochondrial F_1 enzymes, CF_1 is a latent ATPase requiring activation to exhibit appreciable ATP hydrolysis activity. This is achieved either by removing the inhibitory ε subunit or by reducing a critically-placed disulfide bond in the γ subunit as described in the following section.

2. Regulation of the Catalytic Activity of CF_oCF_1

The ε subunit is a regulatory protein in CF_1. This subunit forms a linkage between the γ subunit in CF_1 and the subunit III ring in CF_o (Schulenberg, Wellmer et al. 1997). The interaction between ε and γ is thought to be the key element that allows the rotational catalysis to proceed in a directional manner (Richter, Hein et al. 2000). The ε protein on membrane bound CF_1 functions as a door lock, that is, when there is not enough energy built by proton movement, ε interacts with γ closely to prevent the spindle from rotating to the next catalytic site or from rotating in a backwards direction. After enough torque is generated through subunit III rotation and H^+ pumping, the ε subunit undergoes a large

conformational change and loosens its binding to γ to set off the rapid stepwise rotation (Richter, Hein et al. 2000; Richter 2004). The ε subunit is also critical for proton coupling. Thylakoid membranes containing ε-deficient CF_1 (CF_1-ε) failed to establish a proton gradient (ΔpH) during illumination and the gradient could be restored by the reconstitution of the ε subunit (Richter, Patrie et al. 1984). Furthermore, an antibody study demonstrated that CF_1 on thylakoid membranes could not react with ε-specific antibodies until a light-dependent H^+ gradient was formed (Richter and McCarty 1987). This suggests that ε changes its conformation and relative position to other subunits during proton transport and activation of CF_1 (Evron, Johnson et al. 2000).

In vitro, the ε subunit inhibits ATP hydrolysis by CF_1. Studies have shown that isolated ε can rebind to the ε-deficient form of CF_1 and inhibit the ATPase activity by up to 95% (Richter, Patrie et al. 1984; Samra, Gao et al. 2006). The ε subunit also acts as an ATPase inhibitor in *E. coli* F_1 (Weber and Senior 1997) and in a recent rotation study of the EcF_1 enzyme, the addition of the ε subunit strongly inhibited γ rotation (Nakanishi-Matsui, Kashiwagi et al. 2006).

Activation of the ATPase activity of CF_1 can be achieved by: 1) heating CF_1 to about 65ºC, conditions that specifically denature the ε subunit (Patrie and McCarty 1984; Wang, Freire et al. 1993); 2) methanol, alcohol or detergent treatment that induce a reversible dissociation of the ε subunit from CF_1 (Sakurai, Shinohara et al. 1981; Pick and Bassilian 1982; Yu and McCarty 1985; Anthon

and Jagendorf 1986); 3) trypsin that selectively cleaves part of the γ subunit reducing its affinity for ε binding (Soteropoulos, Suss et al. 1992; Samra, Gao et al. 2006).

The chloroplast ATP synthase is also directly regulated by a disulfide bond on the γ subunit formed between Cys199 and Cys205, a unique feature that is only found in CF_1 proteins of higher plants (Arana and Vallejos 1982). The reversible oxidation of the dithiol in γ can keep the enzyme in an inactive state in the dark when the light intensity is not enough to carry out photosynthesis. In the light, under physiological conditions, the disulfide bond is reduced by the protein thioredoxin (McCarty 2005). Treatment with thiol reducing agents such as dithiothereitol (DTT) can artificially reactivate the enzyme as an ATPase (Nalin and McCarty 1984). The sophisticated built-in disulfide bond is thought to keep the ATP synthase from wasting energy on hydrolysis when the conditions are not suitable for ATP synthesis (Richter, Samra et al. 2005).

The γ dithiol is located in a segment of amino acids located between residue 196 and 242 that form a regulatory domain important for CF_1 γ function (Miki, Maeda et al. 1988). This 47-residue long domain (dithiol domain) is not found in MF_1 or EcF_1 γ subunits but is highly conserved in higher plants (Richter, Samra et al. 2005). Molecular dynamics simulations of the dithiol domain suggested that it undergoes a large conformational change in response to oxidation or reduction of the disulfide bond (Richter, Samra et al. 2005). The oxidation of

the disulfide bond in γ also leads to a decreased binding affinity (~ 20 fold) of ε for CF_1 (Soteropoulos, Suss et al. 1992). A recent study of γ-ε interactions showed that deletion of the regulatory domain resulted in a reduced maximum inhibition of ATPase activities by ε indicating a direct interaction between ε and the regulatory domain (Samra, Gao et al. 2006).

Labeling studies of a conserved residue in the chloroplast γ subunit, Cys 89, have demonstrated that this residue becomes exposed to the medium in the presence of a light-induced ΔpH (Moroney and McCarty 1979; Moroney, Fullmer et al. 1984; Komatsu-Takaki 1996; Evron and Pick 1997). This and further observations have indicated the following: 1) in the dark, the disulfide bond stays oxidized and the ε subunit is tightly bound to γ, keeping CF_1 rigid and latent; 2) upon illumination and formation of a proton gradient both ε and γ undergo significant conformational rearrangement and CF_1 becomes partially activated; 3) when the disulfide bond is reduced, the ATPase and ATP synthesis activities are fully activated and the threshold proton gradient, $\Delta\mu H^+$, for ATP formation is lowered (Ketcham, Davenport et al. 1984; Evron, Johnson et al. 2000; Richter 2004; Richter, Samra et al. 2005).

3. Metal Ion Specificity and Oxyanion Effect

Divalent cations play an important role in the functions of ATP synthases. In oxidative phosphorylation, MgADP, instead of free ADP, is the substrate for

ATP synthesis (Zhou and Boyer 1992). Mg^{2+} ions are the natural co-substrate for ATP synthesis (Boyer, Falcone et al. 1954; Petrack, Craston et al. 1965; Vambutas and Racker 1965). Mg^{2+} ions bind to the nucleotide binding site and stabilize the nucleotide by linking the β and / or γ phosphates to several negatively charged amino acid side chains. Mg^{2+} ions can also attract water molecules and help orient them during the nucleophilic attack that results in hydrolysis (Hochman, Gong et al. 2000). However, free Mg^{2+} ions in excess are potent inhibitors of ATP hydrolysis by both soluble and membrane-bound ATP synthases. This inhibition results from excess stabilization of bound product ADP in the catalytic site which limits catalytic turnover (Zhou and Boyer 1992).

Another divalent cation, the Ca^{2+} ion, can replace Mg^{2+} as a co-substrate for ATP hydrolysis and also increases the rate of ATP hydrolysis (Sokolov and Gromet-Elhanan 1996; Tucker, Du et al. 2000; He, Samra et al. 2007). However, Ca^{2+} ions are not effective co-substrates for ATP synthesis (Hochman, Gong et al. 2000). This is likely due to small, but important, differences in the binding geometry of the two metal ions (Hochman, Gong et al. 2000).

Ca^{2+} but not Mg^{2+} ions were shown to drive ATP dependent rotation by hybrid photosynthetic F_1 ATP synthase (Tucker, Schwarz et al. 2004). The catalytic rate constants for ATP hydrolysis by CF_1 in the presence of Ca^{2+} are usually much higher than those in the presence of Mg^{2+} due to the inhibitory effect of free Mg^{2+} ions (McCarty and Racker 1968). However, Mg^{2+} is the only cation

required for ATP synthesis on thylakoid membrane (Frasch, Green et al. 1989) and Ca^{2+} can, in fact, inhibit ATP synthesis competitively (Zhang and Jagendorf 1995). In one study, pre-incubation with Mg^{2+} increased the binding of ADP to CF_1 (Hisabori and Mochizuki 1993). CF_oCF_1 must be able to capture ADP to synthesize ATP while the concentration of extra-cellular ADP is more than 100 fold lower than that of ATP. This huge disadvantage is overcome by strong binding of ADP possibly stabilized by free Mg^{2+} ions that helps lock the substrate inside the catalytic pocket. When occupied by MgADP, the catalytic site is no longer capable of binding ATP. The binding of ADP to CF_oCF_1 thus serves the additional role of preventing the enzyme from carrying out wasteful ATP hydrolysis.

Early discoveries of enhancers of ATPase activity by isolated CF_1 include organic acids such as succinate or maleate (Jagendorf and Uribe 1966) and oxyanions such as bicarbonate (Batra and Jagendorf 1965; Nelson, Nelson et al. 1972) or sulfite ions (Du and Boyer 1990). Among all of them, sulfite seems to be the most effective (Malyan 2003). The exact mechanism of sulfite stimulation is yet to be determined. There is considerable evidence that sulfite destabilizes the tight binding of MgADP in the catalytic site(s), increasing its rate of release (Dou, Grodsky et al. 1997; Malyan and Vitseva 2001; Senior, Nadanaciva et al. 2002; Malyan 2003). Sulfite ions, as well as other oxyanions, facilitate the release of MgADP, presumably by competing with liganding amino

acid side chains inside the catalytic site (Weber, Hammond et al. 1998; Senior, Nadanaciva et al. 2002). Sulfite may also affect nucleotide binding to non-catalytic sites. Malyan's group have found that sulfite reduces nucleotide binding to non-catalytic sites while increasing nucleotide binding / exchange at catalytic sites (Malyan and Vitseva 2001; Malyan 2003; Malyan 2006).

4. Nucleotide Binding Sites on CF_1 and Inter-site Cooperativity

Six nucleotide binding sites are located on the $\alpha_3\beta_3$ ring, three of which do not release bound nucleotides during catalysis, making them non-catalytic (Xue, Miller et al. 1987). The three non-catalytic sites are located primarily on the three α subunits although a few amino acid side chains on the adjacent β subunits also are part of the structure to those sites. Conversely, the catalytic sites are mainly on the β subunits with some structural contribution from the α subunits. A highly conserved "Walker motif" GXXXXGKT/S is found within each one of the catalytic and non-catalytic sites that is known as the 'P' loop as it binds to the nucleotide phosphates (Senior, Weber et al. 2000; Senior, Nadanaciva et al. 2002).

Three catalytic sites of the F_1 ATP synthase work together in a coorperative manner. Both MF_1 and EcF_1 enzymes exhibit very low rate of ATP hydrolysis at a single site observed at very low MgATP concentrations (Xiao and Penefsky 1994; Senior, Nadanaciva et al. 2000). The ATP hydrolysis activity can be stimulated a thousand fold by binding and hydrolysis of MgATP at a second

site at higher MgATP concentrations as a result of "positive cooperativity" (Fromme and Graber 1990; Senior, Nadanaciva et al. 2000). The maximum hydrolysis rate is reached when all three sites are occupied by nucleotides (Weber, Wilke-Mounts et al. 1993).

Studies of MF_1 (Du and Boyer 1990), EcF_1 (Weber, Wilke-Mounts et al. 1993) and CF_1 (Shapiro, Gibson et al. 1991) have also shown that binding of one nucleotide to one catalytic site induces release of nucleotide from a second catalytic site, the phenomenon referred to as "negative coorperativity".

When freshly isolated from chloroplasts, CF_1 protein has two tightly bound ADP molecules (Shapiro, Huber et al. 1991; Gao, Lipscomb et al. 1995), one in a catalytic site and one in a non-catalytic site (Malyan and Vitseva 2001; Malyan 2002; Malyan 2005; Malyan 2006). The tightly bound ADP can be exchanged non-cooperatively for ADP (Shapiro and McCarty 1990), TNP-ADP (Hein and Richter unpublished results) or AMP-PNP (Hu, Mills et al. 1993) and at least one site can be exchanged cooperatively by ATP binding and hydrolysis at another site (Shapiro and McCarty 1988; Shapiro, Gibson et al. 1991; Shapiro, Huber et al. 1991). Extensive studies have shown that it is only possible to fill five of six sites on CF_1 (Shapiro, Huber et al. 1991). The properties of the sixth site awaits discovery but the identity of the site is probably equivalent to the "open" site on MF_1 revealed in the crystal structure (Abrahams, Leslie et al. 1994).

D. Focus of this Research

The published crystal structure of MF_1, used extensively in enzyme modeling studies (Abrahams, Leslie et al. 1994), identified three major regions of γ making direct contacts with αβ pairs. A homology model of CF_1 based on the MF_1 structure is shown in Figure 4. The three contact regions are identified in the Figure. These points of contact have been used extensively to model the steps involved in the catalytic cycle and in generating unidirectional rotation of the γ subunit.

One of the contacts is formed between the extreme C-terminal tip of γ that contains an α helical element made up of about ten mostly hydrophobic residues and a compact ring of structure composed of the N-terminal β barrel domains of the three α and three β subunits (sequence alignment of C-terminal regions of different γ subunits is shown in Figure 5). This contact was originally proposed to form a "bearing" structure that can hold the C-terminal tip of γ in place while the twisted N- and C- terminal helices rotate. The highly conserved nature of the C-terminus also suggested a similar important functional role in all F_1 proteins. To examine the "bearing" hypothesis, C-terminal amino acids were deleted sequentially and in pairs from the CF_1 γ subunit and the mutant γ proteins were folded and reconstituted with the native CF_1 $\alpha_3\beta_3$ (Sokolov, Lu et al. 1999).

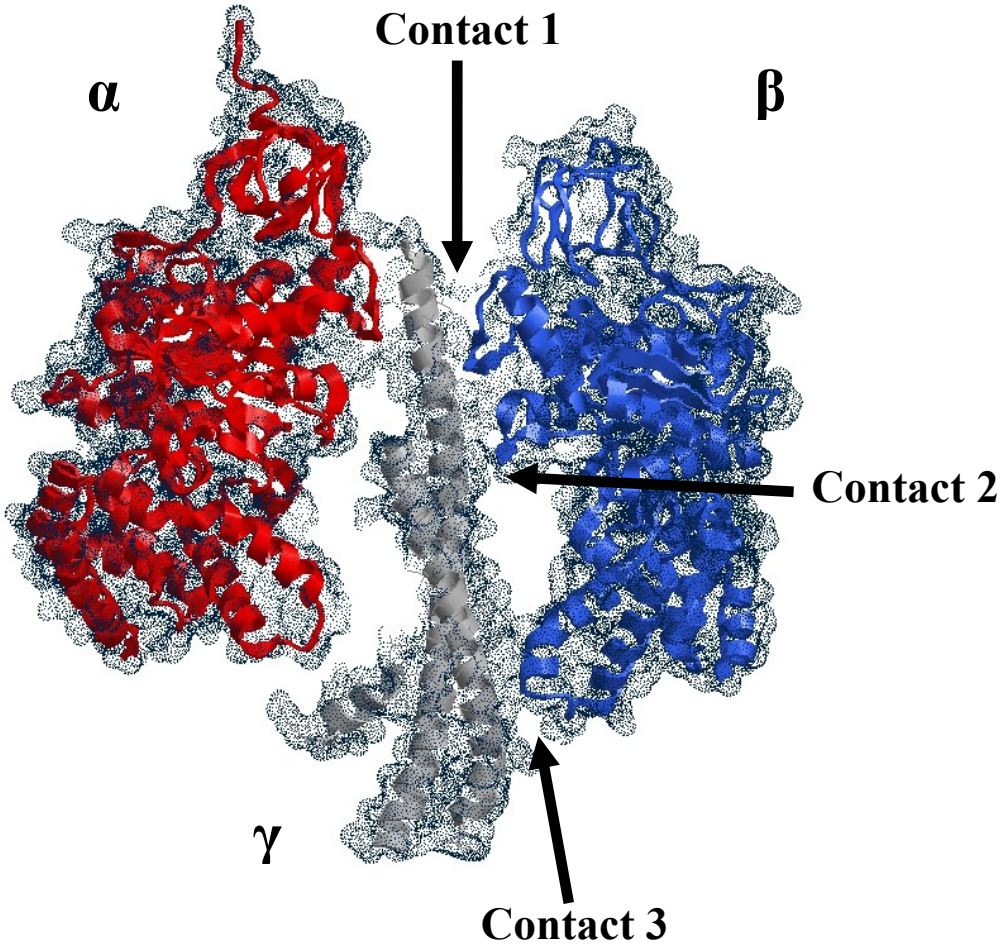

Figure 4. Positions of three major contact regions on the γ subunit. Homology modeled CF_1 structure is shown (courtesy of Dr. Engelbrecht). One α subunit (red) and one β subunit (blue) are shown for simplicity. The γ subunit is colored grey.

EF_1	261	- QLVYN	KARQA	SITQE	LTEIV	SGAAA	V	286
CF_1	297	- SINYN	RARQA	KITGE	ILEIV	AGANA	CV	323
TF_1	257	- TLSYN	RARQA	AITQE	ITEIV	AGANA	LQ	283
MF_1	247	- TLTFN	RTRQA	VITKE	LIEII	SGAAA	L	273

Figure 5. Comparison of the C-terminal amino acid sequences of the γ subunits from different species. The fragment in contact 1 (the bearing) is shown in blue and the fragment responsible for contact 2 in red.

Surprisingly it was observed that up to 20 residues could be deleted from the C-terminal tip of γ without eliminating ATP hydrolysis activity. Similar results were obtained recently in EcF_1 and TF_1 where proteins missing the C-terminal tip of γ were still able to carry out ATP hydrolysis as well as γ rotation (Muller, Panke et al. 2002; Hossain, Furuike et al. 2006).

In the C-terminal deletion study (Sokolov, Lu et al. 1999), deletion mutants missing up to 14 residues from the C-terminus of γ showed stimulated CaATPase and MgATPase activities, while deletion of 15 to 20 consecutive amino acids resulted in decreased calcium and magnesium ATP hydrolysis. Loss of activity may have resulted from interference with the second catch which may be important for CF_1 function. When examined for oxyanion effect, the C-terminal deletion mutants displayed a progressive loss of sulfite-stimulated MgATP hydrolysis.

The second contact, contact 2, is the main focus of this research. Two highly conserved amino acids, γArg304 and γGln305 in CF_1 (shown in Figure 5), together with another positively charged residue, γArg302, are located in this region and form a "catch" with anionic loop structures found in both α subunits and β subunits. In the C-terminal deletion study, deleting Gln305 and Arg304 resulted in a loss of ATPase activity and insensitivity to the inhibitor tentoxin, indicating that this contact is important for catalytic cooperativity (Sokolov, Lu et al. 1999). The research presented in this dissertation is designed to discover the

physiological role(s) of this "catch" in the chloroplast ATP synthase and to gain further insight into the catalytic and regulatory mechanisms governing ATP synthase function. The studies are summarized as follows:

1) The C-terminal region of the CF_1 γ subunit that is equivalent to the MF_1 region responsible for making the second "catch" was genetically modified to reduce its potential for forming salt links or hydrogen bonds with anionic loops on the α and β subunits. Functional analysis of mutant γ proteins was carried out using a hybrid protein system and revealed that the catch is not required for ATP hydrolysis but is critical for oxyanion stimulation of MgATP hydrolysis. Kinetic studies have led to a novel hypothesis for the role of contact 2 in selective binding of ADP to CF_oCF_1 during coupled ATP synthesis.
2) To further identify the natural role of contact 2, the same series of CF_1 mutant γ subunits were reconstituted with the native CF_1 $\alpha_3\beta_3$ hexamer to investigate proton coupled ATP synthesis on thylakoid membranes. The results demonstrate that forming contact two represents an essential step in proton coupling, supporting the hypothesis that this catch is closely involved in activation of CF_1 as part of the regulatory mechanism.
3) The effects of γ catch residue mutations on the exchange of nucleotides from both catalytic and non-catalytic sites were examined using the fluorescent nucleotide analog TNP-ADP. The results of this study

indicated that ‘catach two’ is required for communication between non-catalytic and catalytic sites resulting in enzyme activation.

Chapter Two

Mutational Studies of the C-terminal Region of the Gamma Subunit of the Chloroplast F_1-ATPase Using a Hybrid Protein Complex

A. Introduction

The published structure of the bovine MF_1 (Abrahams, Leslie et al. 1994) 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 6, the tip of the C-terminal helix interacts with the tightly packed N-terminal domains of all six αβ subunits and has been proposed to act as a bearing for rotation of the γ subunit during catalysis (Abrahams, Leslie et al. 1994). 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 unoccupied by nucleotide.

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

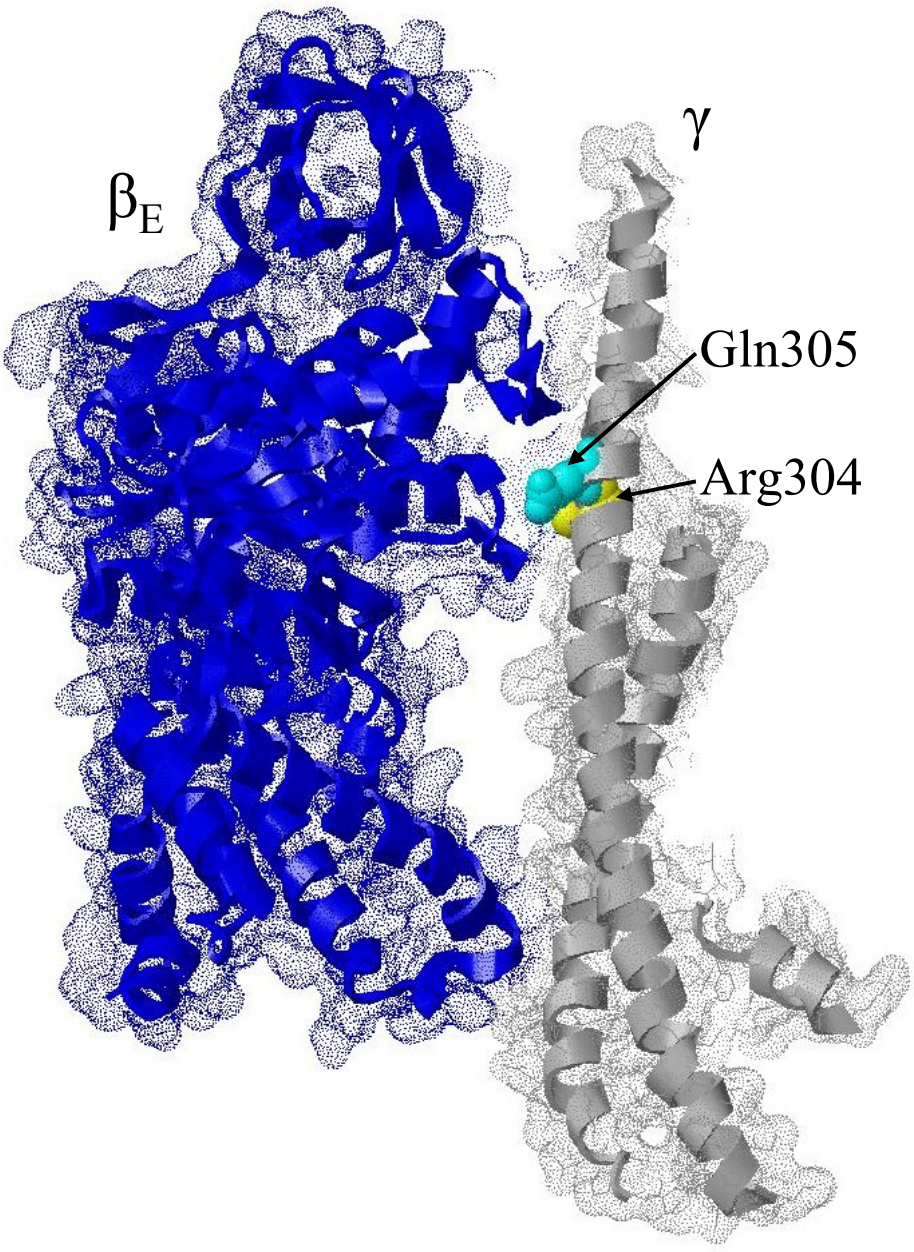

Figure 6. Proposed catch interactions between the CF_1 β and γ subunits. Cross-section of part of CF_1 showing the putative catch between γGln305, γArg304 and β_E. The homology model of CF_1 (courtesy of Dr. Engelbrecht) was based on the crystal structure of the mitochondrial enzyme (Abrahams, Leslie et al. 1994).

activity, indicating that the C-terminal tip of γ is not required as a bearing for rotational catalysis (Sokolov, Lu et al. 1999). This result was confirmed in EcF_1 (Greene and Frasch 2003) and more recently in TF_1 (Hossain, Furuike et al. 2006). In both cases it was directly demonstrated that mutants in which the proposed bearing-forming residues were deleted retained their capacity for ATP-driven γ rotation. 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 of γ, lead 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 non-cooperative and therefore not expected to involve rotation of the γ subunit (Sokolov, Lu et al. 1999). Selective mutation of γArg268 and γGln269 in EcF_1 (analogous to γArg304 and γGln305 in CF_1) reduced k_{cat} or ATP hydrolysis by 88 and 99% respectively and the ATP synthesizing capacity of both mutants was likewise impaired (Greene and Frasch 2003). Successive deletion of residues from the C-terminus of the 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 (Hossain, Furuike et al. 2006). However, deletion of 21 residues from the C-terminus of TF_1 γ, although reducing k_{cat} to less than 1% that of the wild type, only reduced the maximum rate of

rotation of the γ subunit to 30% that of wild type. This surprising result indicated that the catch residues do not play a critical role in γ rotation (Hossain, Furuike et al. 2006).

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 in which γ rotation was recently demonstrated (Tucker, Schwarz et al. 2004). Remarkably, mutation of γArg304 to leucine that is expected to eliminate its ability to hydrogen bond with the anionic loops on the β subunits resulted in a large increase in catalytic turnover. Mutation of γGln305 to alanine, which was also expected to remove a critical hydrogen bonding interaction with the β subunits, had no effect on catalysis but completely abolished the well-known stimulatory effect of oxyanions on the MgATPase activity of the enzyme. The results are thus consistent with observations on the TF_1 enzyme indicating that the catch, like the bearing, does not play an essential role in rotational catalysis driven by ATP-hydrolysis. On the other hand, the results have provided evidence for a role of γ C-terminal residues in the process of oxyanion activation.

B. Materials and Methods

1. Materials

RF_1 α-his6 (six histidine tag at the C-terminus), RF_1 β, and CF_1 γ were expressed in insoluble inclusion bodies as described (Du and Gromet-Elhanan 1999). DEAE cellulose, antibiotics (ampicillin, tetracycline, and chromophenicol), and Ni-NTA resin were purchased from Sigma. Tryptone and yeast extract were from DIFCO. ATP (grade II) and urea (ultra pure) were purchased from Fluka. 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.

2. Generation of Gamma Subunit Mutants

Mutant subunits were constructed by enzymatic amplification of the expression plasmid pET8c-gamma.BB1 (Sokolov, Lu et al. 1999; Samra, Gao et al. 2006). Primers (obtained from IDTDNA, Iowa) 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 pET8cgamma bb1 plasmid (Tucker, Du et al. 2001). PCR products were purified using the QIAquick gel extraction kit (Qiagen, Valencia CA). The purified DNA was circularized by incubating 100-200 ng of the DNA with 3 U of T4 DNA ligase (Promega) in T4 DNA ligase buffer

overnight at room temperature.

3. Transformation

Competent *E.coli* XL1-blue and BL-21 cells were grown in 25 ml of Luria-Bertani (LB) broth medium at 37 ºC to an optical cell density (light absorbance of 0.4 at 600 nm) and cooled rapidly on ice. Cells were collected by centrifugation for 10 min at 4,500 x g at 4ºC. Cell pellets were resuspended in 8 ml of 0.1 M cold $CaCl_2$ and 15% (v/v) glycerol and placed on dry ice to rapidly freeze. Frozen cells were stored at -80ºC in 100 µl aliquots. Plasmids from PCR reactions were added to 100 µl of competent *E.coli* XL1-blue cells and the mixture was placed on ice for 30 min. The mixture was heat-shocked at 42ºC for 45 sec and immediately placed on ice. 0.8 ml of warm LB broth was then added to cells and the resulting mixture was incubated at 37ºC for 1 hr. The cells were harvested by centrifugation at 13,000 x g for 1 min and pellets resuspended in 0.6 ml of LB broth. The cell suspension was poured onto an LB plate (LB medium with 1% agar) that contained 100 µg/ml ampicillin and 12.5 µg/ml tetracycline to ensure that only transformed cells could grow. After incubation at 37ºC for 16 hr, cells in single colonies were taken from the plate for sub-culturing. Cloned plasmid was isolated from XL1-blue cells using QIAprep Spin Miniprep kit (Qiagen, Valencia CA) and transformed into the expression host *E.coli* BL21(DE3)/pLysS. The entire sequence of each mutant gene was confirmed by

the Iowa State DNA Sequencing Facility.

4. Over-expression of Subunit Proteins

Transformed *E.coli* BL21(DE3)/pLysS cells were grown in 50 ml of LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloamphenicol at 37ºC overnight and transferred into 950 ml of same medium. The resulting culture was incubated at 37ºC for 2 hr or until the optical cell density was reached. The broth was added 0.1 mM over-expression inducing agent isothiopropylgalactosedase (IPTG) for 5 hr. Cells were harvested by centrifugation at 3,000 x g for 10 min at 4ºC. The pellet was washed once with TE buffer (50 mM Tris-HCl, pH 8.0, and 2 mM EDTA) and centrifuged again. The cell pellet was chilled at -80ºC overnight to facilitate lysis. Cells were thawed slowly on ice and resuspended in 30 ml of TE buffer with 10 mM $MgCl_2$ and 10 mg DNase I (Fisher). After incubation on ice for 20 min the cells were sheared by sonication with a Branson 250 sonifier three times for 15 sec at an output of 4 and a duty cycle of 10. The resulting mixture was washed in 30 ml of TE buffer and centrifuged at 3,000 x g for 10 min at 4ºC. The same washing and centrifugation steps were repeated three times and the white inclusion bodies (pellets) resuspended in 5 ml of TE buffer containing small amounts of protease inhibitors (Sigma). Inclusion bodies were stored at -20 ºC in small aliquots.

5. Reconstitution and Purification of Hybrid Assemblies

Hybrid proteins containing $RF_1\alpha\beta$ and $CF_1\gamma$ were reconstituted according to (Du and Gromet-Elhanan 1999). Inclusion bodies of subunit proteins ($RF_1\alpha$his6, $RF_1\beta$, and $CF_1\gamma$) were slowly thawed on ice and slurries 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 one hour and centrifuged at 20,000 x g at 4ºC to remove any insoluble material. Solubilized α, β and γ subunits were mixed at concentrations of 50, 50, and 30 μg/ml, respectively, and dialyzed overnight at 4 ºC against 8 volumes of dialysis buffer (50 mM Tricine–NaOH, pH 7.8, 20% (v/v) glycerol). Insoluble material was removed by centrifugation (20,000 x g, 4ºC). The supernatant was diluted into two volumes of TEA buffer that contained 20 mM Tricine–NaOH (pH 7.8), 2 mM EDTA, and 1 mM ATP. The protein mixture was loaded onto a column packed with 6 cm^3 of DEAE anion exchange resin equilibrated with the same TEA buffer. The bound protein was eluted with 15 ml of TEA with 0.4 M NaCl and the resulting solution applied to a Ni–NTA affinity column (packed with 1 ml of resin slurry) 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 (BioRad) 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 -80ºC.

6. 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 to Figures 8 & 10) for 2 minutes at 37ºC. 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 to Figures 9 & 11). The ATP hydrolysis reaction was stopped by addition of 1 ml of 0.5 M trichloroacetic acid (TCA) and the concentration of inorganic phosphate was measured as previously described (Taussky and Shorr 1953). Protein concentrations were determined by the Bradford method (Bradford 1976). Kinetics constants were obtained using the built–in nonlinear regression tools in SigmaPlot 8.0.

C. Results

1. 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, nickel affinity chromatography and gel filtration as described in the Materials and Methods. A sodium dodecylsulfate polyacrylamide gel showing individual subunit proteins purified from inclusion bodies and used for assembly, and a trace diagram of the FPLC purification profile after reconstitution of the assembly are presented in Figure 7. The catalytic properties of this RF_1/CF_1 hybrid assembly were described in detail elsewhere (Du, Tucker et al. 2001) and the assembly was shown to be capable of CaATP-dependent γ rotation (Tucker, Schwarz et al. 2004).

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 (Abrahams, Leslie et al. 1994). The residues γArg302, 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 (Abrahams, Leslie et al. 1994). The loop residues are also highly conserved. The MF_1 residue

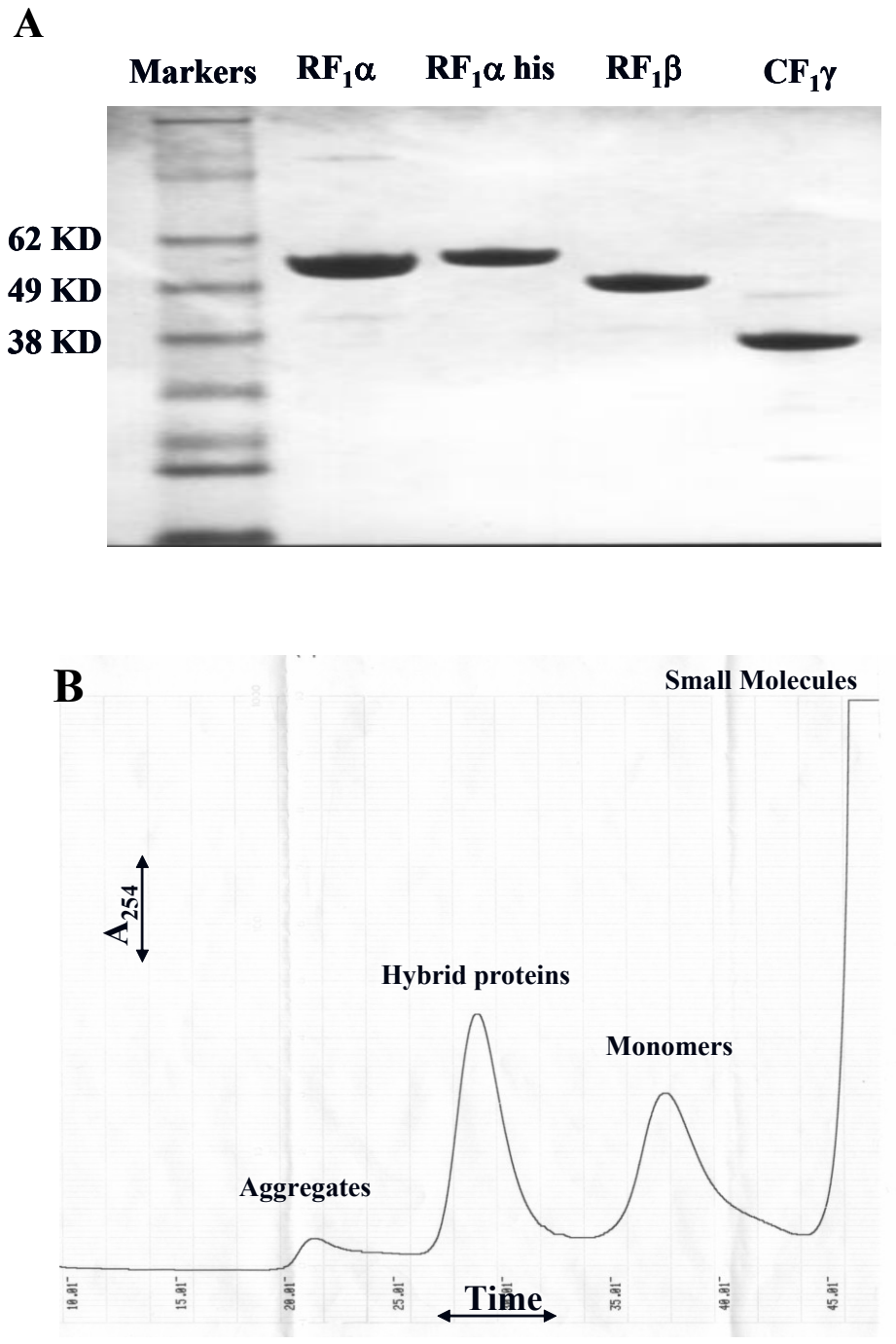

Figure 7. Over-expression and purification of F_1 subunits. **(A)** solubilized inclusion bodies of the RF_1 α, RF_1 β, and CF_1 γ subunits were subjected to SDS gel electrophoresis on an SDS-Page gradient gel (4 – 12%, Invitrogen). **(B)** FPLC separation of reconstituted hybrid proteins on a superdex 200 column with a buffer containing 50 mM Tricine-NaOH (pH 7.8) and 50 mM NaCl at a flow rate of 0.5 ml / min.

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 (see Figure 12) between the α 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 (Abrahams, Leslie et al. 1994). 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 above mutations 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.

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 I). 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 8. Remarkably, the γR302L mutation, while having little effect on the calcium-dependent activity, caused a nearly three-fold increase in the rate of

Table I. Assembly of CF_1 γ mutants with *R.rubrum* α and β subunits.

Mutant CF_1 γ subunits[1]	Assembly competence[2]
γ wild type	+
γ R302L	+
γ R304L	+
γ Q305A	+
γ K307A	-
γ R302L;R304L	+
γ R304A;Q305A	+
γ R302A;R304A;Q305A	+

[1] CF_1 γ mutants within the C-terminal region were reconstituted with RF_1 $\alpha_3\beta_3$ subunits as described in the *Materials and Methods*.

[2] Assembly competence was judged by the yield of purified assembly relative to wild type: +, normal yield; - , insufficient yield to test enzymatic activity.

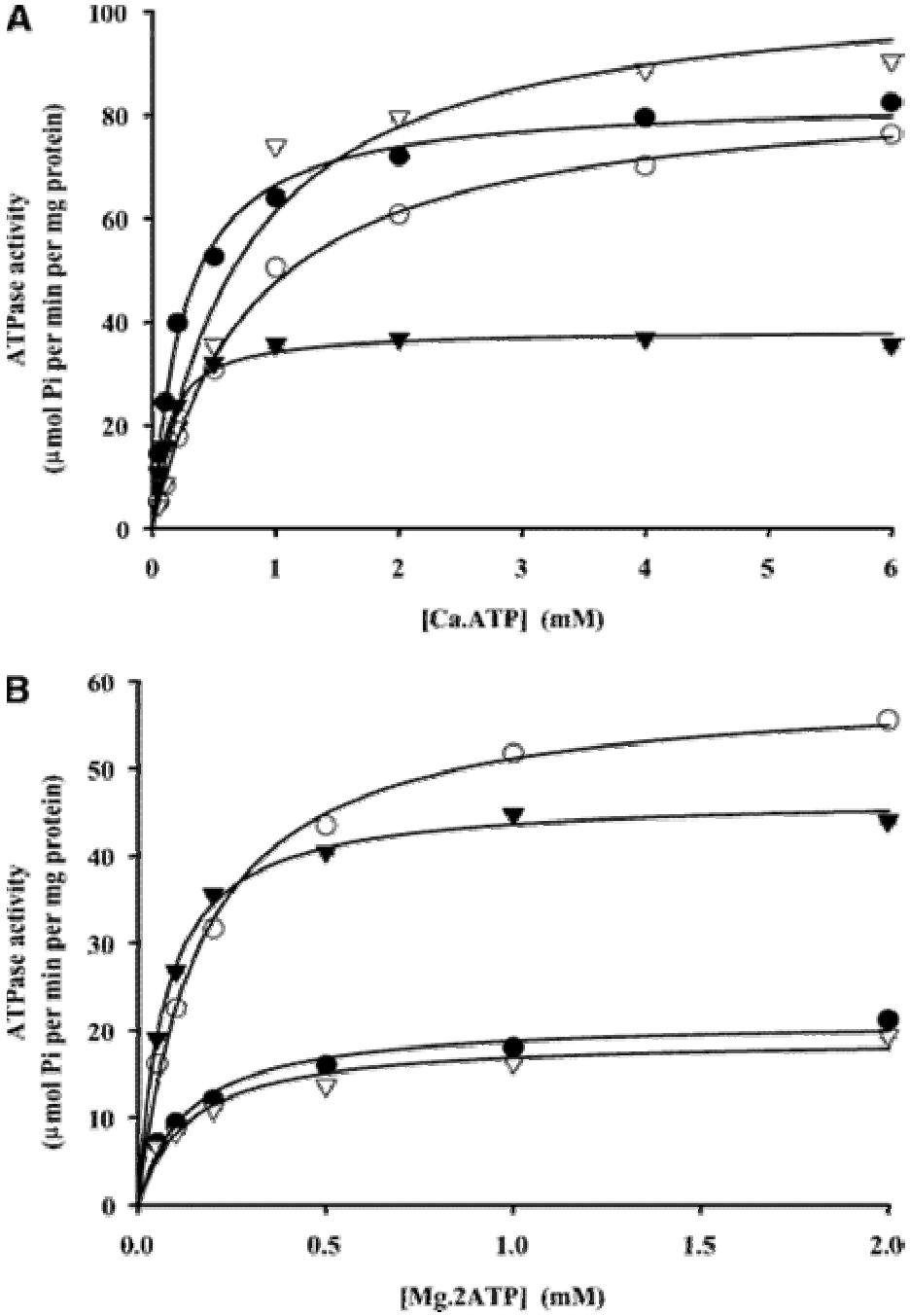

Figure 8. Substrate titrations of hybrid F_1 complexes containing single γ C-terminal mutations. The ATP hydrolysis activities of purified enzyme assemblies were determined as described in the *Materials and Methods* section. **(A)** The CaATP concentration was varied as indicated while maintaining a constant molar ratio of $CaCl_2$ to ATP of 1:1. **(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.

MgATP hydrolysis. The γR304L mutation had a similar effect on MgATPase activity but reduced the CaATPase activity to about 50% that of the wild type assembly. In contrast, the γQ305A mutation had no apparent effect on MgATP ase activity and a small stimulatory effect on CaATPase activity. This result was surprising since the analogous mutation reduced the ATPase activity of the EcF1 by 99% (Greene and Frasch 2003).

The magnesium-dependent activity of the hybrid was shown previously to increase significantly (>20-fold) in the presence of stimulatory oxyanions such as sulfite (Tucker, Du et al. 2001). The stilmulatory 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 (Du and Boyer 1990; Murataliev and Boyer 1994). Oxyanions are required for high rates of magnesium-dependent catalytic turnover in both of these systems but not by EcF_1 which exhibits very high rates of magnesium-dependent ATPase activity in the absence of oxyanions, whereas oxyanions are inhibitory (Richter, unpublished data).

The responses to added sulfite of the MgATPase activities of the single mutants are shown in Figure 9. The wild type assembly exhibited a nearly four-fold stimulation at optimal sulfite concentration (25 mM). The stimulatory effect was reduced at higher concentrations due to a non-specific inhibitory effect of ionic strength (He, data not shown). Both the γR302L and γR304L mutants

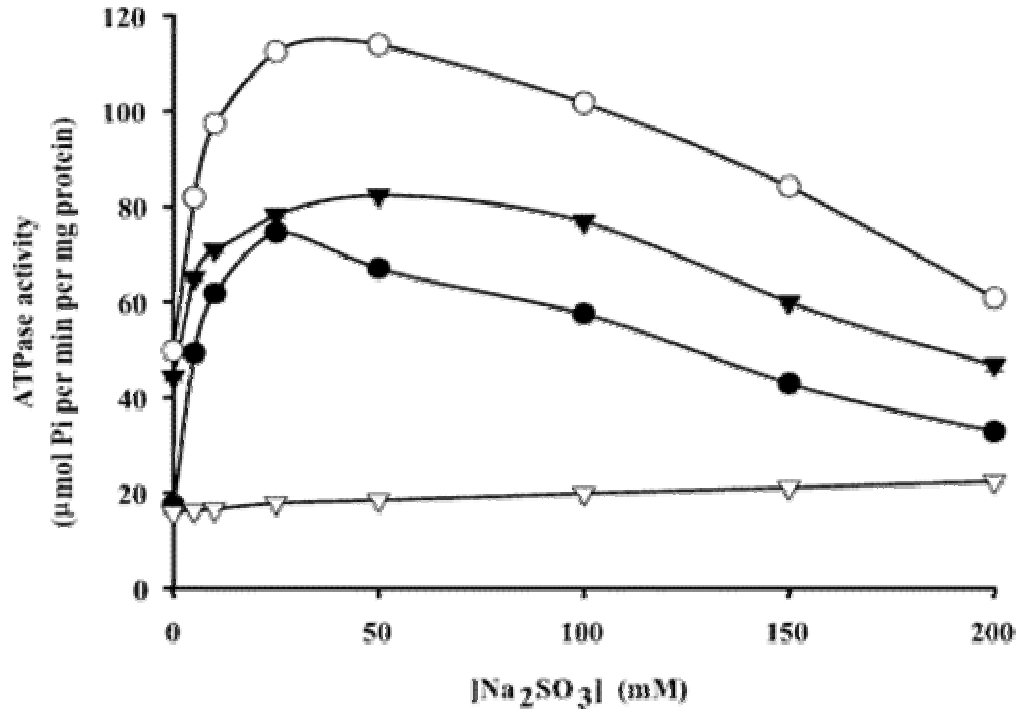

Figure 9. Sulfite effects on hybrid F_1 complexes containing chloroplast γ subunits with single mutations in the C-terminus. The ATP hydrolysis activities of purified enzyme assemblies were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$ and the indicated concentrations of sodium sulfite as described in the *Materials and Methods* section: (●) $\alpha_3\beta_3\gamma$wild-type, (○) $\alpha_3\beta_3\gamma$R302L, (▼) $\alpha_3\beta_3\gamma_C$R304L, and (▽) $\alpha_3\beta_3\gamma$Q305A.

also showed further stimulation by sulfite, pushing the turnover rates to values similar to or exceeding the maximum turnover values observed with CaATP as substrate (Figure 8, Table II). The k_{cat} of the γR302L mutant 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 9).

2. 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 catalytic turnover (Figure 10, Table II). Both the CaATPase and MgATPase activities of the γR302L,R304L double mutant were reduced to less than half that of the wild type assembly. Similarly, the CaATPase activity of the γR304A, Q305A double mutant was about 50% that of wild type, whereas the MgATPase activity of this mutant was about 18% that of 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 about 25% of wild type CaATPase activity but only about 10% of wild type MgATPase activity. The effects of the combined mutations on the sulfite-stimulated MgATPase activity were even more pronounced (Figure 11), the

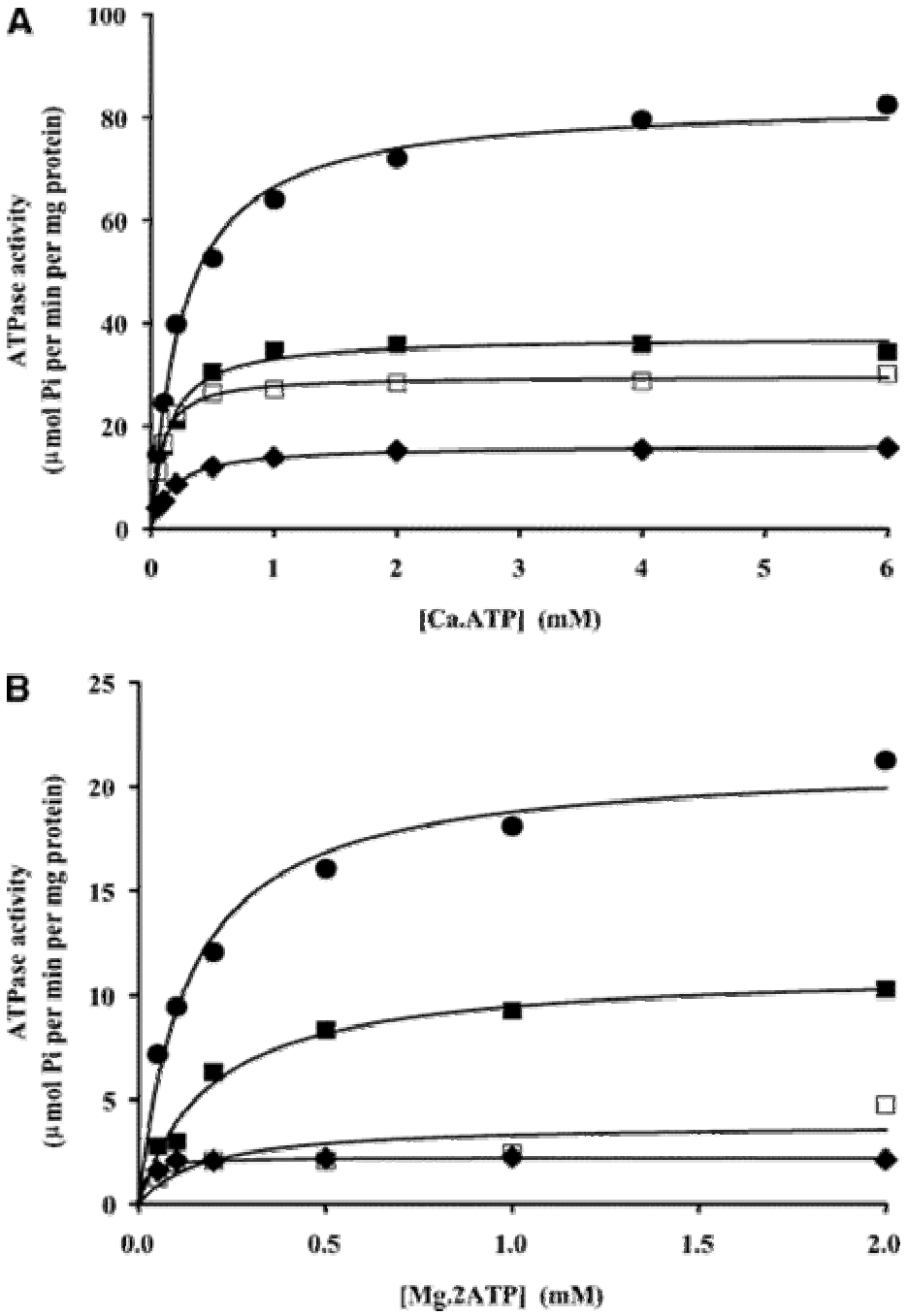

Figure 10. Substrate titrations of hybrid F_1 complexes containing chloroplast γ subunits with multiple mutations in the C-terminus. The ATP hydrolysis activities of purified enzyme assemblies were determined as described in the *Materials and Methods* section. **(A)** The CaATP concentration was varied as indicated while maintaining a constant molar ratio of $CaCl_2$ to ATP of 1:1. **(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.

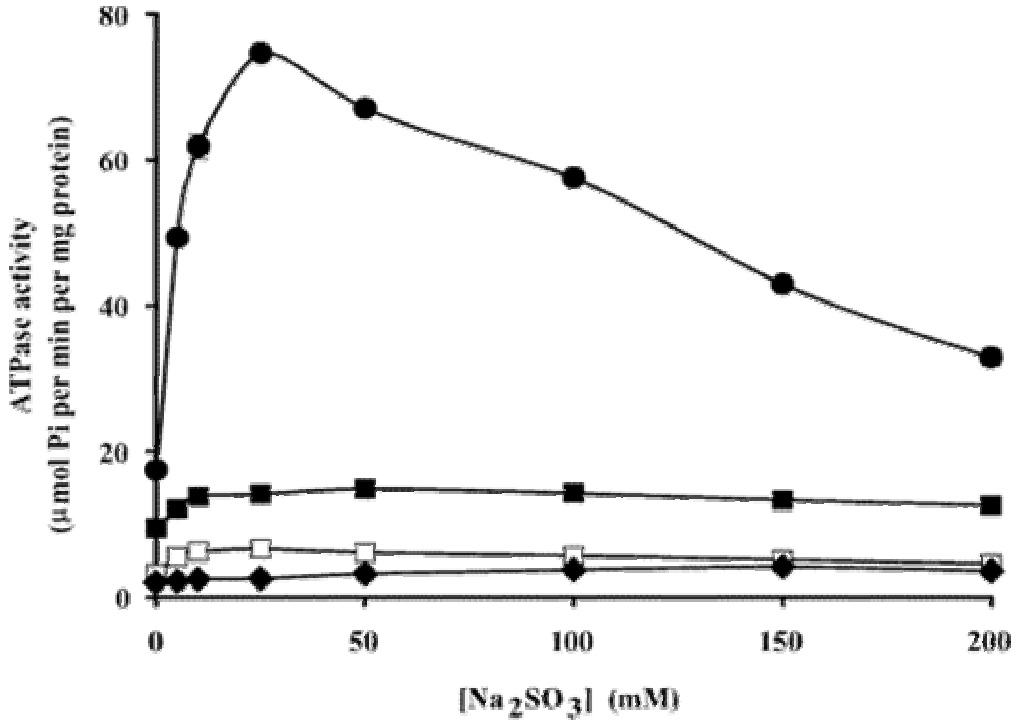

Figure 11. Sulfite effects on double and triple hybrid F_1 mutants. The ATP hydrolysis activities of purified enzyme assemblies were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$ and the indicated concentrations of sodium sulfite as described in the *Materials and Methods* section: (●) $\alpha_3\beta_3\gamma$wild-type, (■) $\alpha_3\beta_3\gamma$R302L;R304L, (□) $\alpha_3\beta_3\gamma$R304A;Q305A, and (♦) $\alpha_3\beta_3\gamma$R302A;R304A;Q305A.

Table II. Summary of kinetic rate constants for hybrid F_1 mutant assemblies

Hybrid F_1 assemblies[1]	K_{cat} (S^{-1}) [2]	
	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

[1] Hybrid F_1 mutants were assembled, purified and assayed as described in the *Materials and Methods*.

[2] Catalytic rate constants (K_{cat}) were calculated using the *nonlinear regression* tools in *Sigma Plot 8.0*. Errors are expressed as standard deviations with n = 3.

MgATPase rate of the triple mutant being less than 3% that of the wild type assembly.

D. Discussion

The hybrid enzyme used in this study has provided the means to genetically engineer F_1 subunits for structure/function analysis and rotation studies of F_1 enzymes derived from photosynthetic and eukaryotic sources. The k_{cat} for CaATP hydrolysis by the hybrid $\alpha_R\beta_R\gamma_C$ enzyme is substantially higher than those of the native RF_1 and CF_1 enzymes (Tucker, Du et al. 2001). The k_{cat} for MgATP hydrolysis by the hybrid in the absence of stimulatory oxyanions is similar to that of RF_1 but substantially higher than that of CF_1 whereas the maximum sulfite-stimulated k_{cat} is similar in all three enzymes. The differences in catalytic rates between the hybrid and the 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 (Tucker, Schwarz et al. 2004) and coupled ATP synthesis (He, McCarty et al. 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 escapement mechanism for nucleotide-driven γ rotation (Greene and Frasch 2003), then substitution of CF_1 γR304 and γQ305 with leucine or alanine would be expected to reduce 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 catalytic turnover (Greene and Frasch 2003). In stark contrast, however, the γR304L mutant hybrid greatly stimulated MgATPase activity while retaining significant rates of CaATPase activity. Similarly, substitution of the nearby arginine residue at position 302 led to a significant stimulation in 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 catalysis they did not eliminate it. In the triple mutant, the stretch of residues between 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 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 α or β subunits, this could explain why the 304/305 mutations in the EcF_1 were so effective in blocking catalysis. Unfortunately, attempts to substitute γLys307 for alanine in this study were unsuccessful so we were 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 (Mueller 1989; Murataliev and Boyer 1994; Sokolov, Lu et al. 1999; Malyan and Vitseva 2001; Muller, Panke et al. 2002; Greene and Frasch 2003; Hossain, Furuike et al. 2006). 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 between the enzyme surface and the nucleotide phosphates (Abrahams, Leslie et al. 1994). This residue has been mutated to serine in several respiratory F_1 ATPases (Mueller 1989; Omote, Maeda et al. 1992; Jault, Dou et al. 1996), in *chlamydomonas reinhardtii* (Hu, Strotmann et al. 1998) and in the hybrid photosynthetic F_1 used in this study (Du, Tucker et al. 2001), in each case resulting in elevated MgATPase activity and reduced oxyanion stimulation.

Recent studies with CF_1 (Malyan 2002; Malyan 2003), however, have indicated that oxyanions act by blocking binding of ADP or ATP to non-catalytic sites. The extent of inhibition of nucleotide binding observed with different oxyanions closely mirrored the extent of stimulation of MgATPase activity (Malyan 2003). This apparent contradiction may be resolved by taking into account some earlier studies with the yeast MF_1 (Mueller 1989) 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 one acting on a different part of the catalytic process. Magnesium ions may, in fact, stabilize binding of inhibitory ADP to both catalytic and non-catalytic sites.

The structural organization of the αβ pair that is associated with the catch residues in the bovine MF_1 is shown in Figure 12. On the upper panel of the figure, 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 non-catalytic 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 non-catalytic interface. On the bottom panel of Figure 12, two of the three catalytic site configurations of the bovine MF_1 enzyme, the empty site on β_E and the site with ADP bound on β_D, are shown. In β_D, the anionic loop

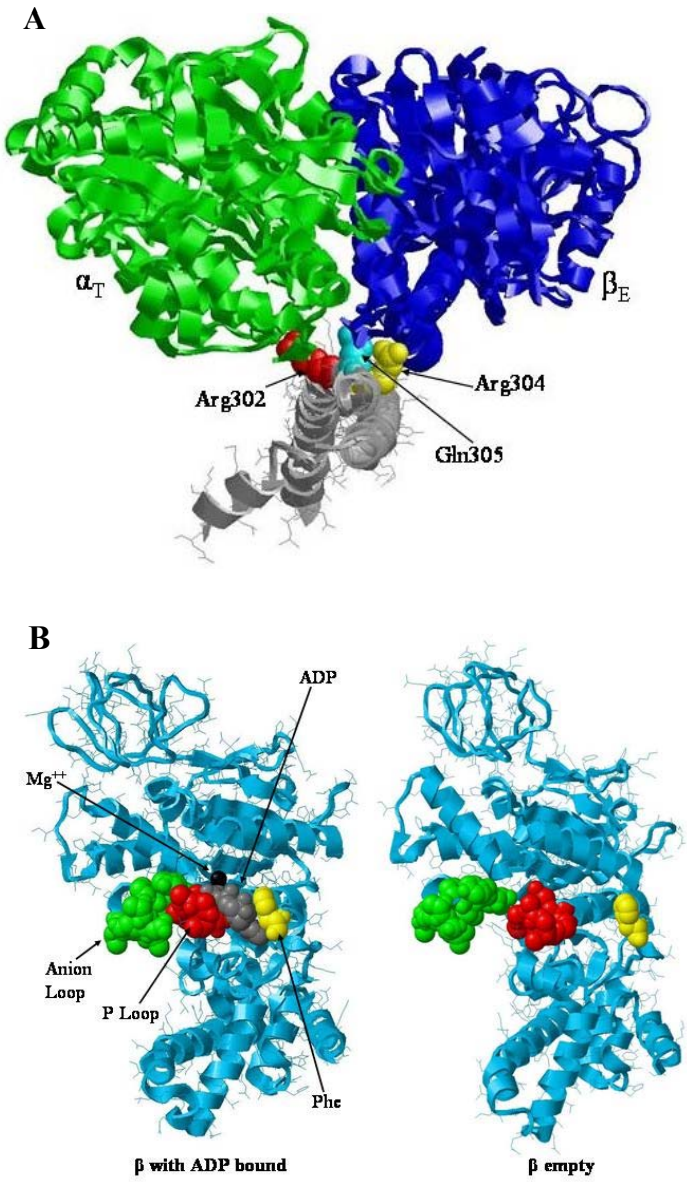

Figure 12. Modulation of catalytic sites by the catch interaction. (A) Top view of the proposed catch region extending across the non-catalytic interface formed between the β_E and α_T subunits in CF_1. The model (courtesy of Dr. Engelbrecht) indicates that γArg304 (yellow) and γGln305 (cyan) contact an anionic loop on β_E in the same manner as in the MF_1 structure (Abrahams, Leslie et al. 1994). 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 (grey) and Phe424 (yellow) on the β_D and β_E subunits.

is closely associated with the p-loop that binds the nucleotide phosphates which, together with the adjacent phenylalanine residue, forms a compact arrangement and very tight nucleotide binding. 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 (Abrahams, Leslie et al. 1994) and which forms a separate catch with residues further along from the C-terminus in the γ subunit, has also pulled away from the p-loop. Presumably, 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 the 302-305 catch residues on catalysis by the hybrid enzyme. When isolated after ammonium sulfate precipitation, the chloroplast F_1 contains two very tightly bound ADP molecules, one in a catalytic site and the other in a non-catalytic site (Malyan 2006). Tight binding of ADP to one of the three non-catalytic 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 (Gao, Lipscomb et al. 1995), it is a reasonable assumption that the non-catalytic 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 non-catalytic sites with MgATP (Murataliev and Boyer 1992), replacement of the tightly bound non-catalytic 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 non-catalytic interface. The stimulatory effect of oxyanions such as sulfite would result from destabilization of non-catalytic ADP allowing it to be rapidly exchanged with MgATP.

In this model, mutations in the γ subunit that weaken the catch interaction (γR302L, γR305A) would be expected to result in increased activation of the MgATPase activity and a decreased 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 non-catalytic site. This explanation, although remaining hypothetical, is consistent with observations by Malyan's group (Malyan 2002; Malyan 2003; Malyan 2005; Malyan 2006) and is strongly reinforced by an earlier observation that the presence of sulfite dramatically increases the rate of exchange of tightly bound non-catalytic ADP for MgATP in CF_1 (Hu and Richter unpublished experiments).

In summary, the results of this study have shown that catch residues identified in the MF_1 structure to interact with the anionic loops on the β subunits

are neither universally nor obligatorily essential for catalytic turnover in F_1 enzymes, but are involved in modulating catalysis. The arginine residue at position 302 has also been shown to have a modulatory effect on catalysis, possibly by forming a bridge between α and β subunits at a non-catalytic interface. The results have also provided insight into how oxyanions activate MgATP hydrolysis by the mitochondrial and chloroplast enzymes and the role of non-catalytic sites in the catalytic process. The fact that the transmembrane proton gradient replaces oxyanions in activating the MgATPase activity of membrane-bound CF_1 (Du and Boyer 1990; Zhang, Letham et al. 1993) underscores its relevance to regulation of the ATP synthase under physiological conditions.

Chapter Three

C-terminal mutations in the chloroplast ATP synthase gamma subunit impair ATP synthesis and stimulate ATP hydrolysis

A. 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 transporter. 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 α and β subunits alternate to form a hexameric ring with three catalytic sites located one on each of the three β subunits at αβ interfaces, and three non-catalytic sites located one on each of the three α subunits, at the alternate αβ interfaces (Girault, Berger et al. 1988; Abrahams, Leslie et al. 1994).

The three catalytic sites are structurally asymmetric, the result of differential interactions between each of the three αβ pairs and the single-copy γ subunit (Boyer 1993; Abrahams, Leslie et al. 1994; Gao, Lipscomb et al. 1995). During ATP hydrolysis, cooperative ATP binding, hydrolysis and ADP release

result in directional rotation of the γ subunit (Boyer 1993; Abrahams, Leslie et al. 1994; Boyer 1997; Cross 2000). 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 (Boyer 1993; Cross 2000; Milgrom and Cross 2005). 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 (Abrahams, Leslie et al. 1994). 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 Ångstroms (Abrahams, Leslie et al. 1994). This observation implies that formation of the catch helps to reduce the affinity of the nucleotide at this site (He, Samra et al. 2007), and forming and breaking the catch is considered to constitute an 'escapement

mechanism' that is a critical step in the cooperative binding change process (Greene and Frasch 2003). 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 (Greene and Frasch 2003).

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 (Abrahams, Leslie et al. 1994) 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 (Oster and Wang 2000; Cui, Li et al. 2004; Gao, Yang et al. 2005). 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 six and twenty amino acids deleted from the γ C-terminus (Sokolov, Lu et al. 1999). 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 (Muller, Panke et al. 2002) and TF_1 (Hossain, Furuike et al. 2006) γ subunits are likewise dispensable for ATP hydrolysis and γ rotation. Deletion of residues beyond the last 14 in CF_1 γ, down to and including the putative catch residues Arg304 and Gln305, however, 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 (Hu, Mills et al. 1993; Sokolov and Gromet-Elhanan 1996). 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 twenty-one residues from the γ C-terminus, although reducing the ATPase rate to less than 1% that of wild type, failed to abolish γ rotation indicating that multisite catalytic cooperativity was retained (Hossain, Furuike et al. 2006).

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 (Sokolov, Lu et al. 1999). Removing twenty residues from the C-terminus abolished oxyanion (sulfite) stimulation altogether (Sokolov, Lu et al. 1999). Free magnesium ions or MgADP strongly inhibit the ATP

hydrolysis activities of F_1-ATPases by forming a stable MgADP-enzyme complex (Moyle and Mitchell 1975; Zhou, Xue et al. 1988; Du and Boyer 1990). 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 (Du and Boyer 1990; Du, Tucker et al. 2001). 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 (Du and Boyer 1990; Sokolov, Lu et al. 1999; Du, Tucker et al. 2001). The loss of oxyanion stimulation in the deletion mutants provided the first evidence for an involvement of the γ C-terminus in oxyanion activation (Sokolov, Lu et al. 1999).

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 the F_1 of the photosynthetic bacterium *Rhodospirillum rubrum* (RrF_1) (Tucker, Du et al. 2000; Du, Tucker et al. 2001; Tucker, Du et al. 2001; Tucker, Du et al. 2001). 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 (Tucker,

Schwarz et al. 2004). 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 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 non-catalytic sites (He, Samra et al. 2007).

In this study the involvement of catch residues in proton-coupled processes in the photosynthetic enzyme was examined 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 (Gao, Lipscomb et al. 1995). 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}, 500 s^{-1} compared to 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 multi-site cooperative binding change process nor, therefore, in ATP-dependent generation of gamma subunit rotation.

B. Materials and Methods

1. Materials

Fresh spinach leaves were obtained from the supermarket. DEAE cellulose, antibiotics (ampicillin, tetracycline, and chloramphenicol), Sephadex G50 resin, and Tentoxin were purchased from Sigma. Hydroxyapatite HTP gel was from BioRad. Tryptone and yeast extract were obtained from DIFCO. ATP (grade II) and urea (ultra pure) were purchased from Fluka. Dialysis tubing (8,000 M.W. cut-off) was obtained from Biodesign Inc. (New York). Cheesecloth was from Fisher and Miracloth from Calbiochem Inc. All other chemicals were of the highest quality reagent grade available.

2. Preparation of CF_1

Intact CF_1 and CF_1 lacking δ and ε subunits were isolated from fresh market spinach as described in (McCarty and Racker 1968; Richter, Snyder et al. 1985). Spinach leaves, about 2 kg in weight, were deveined, cleaned and chilled at 4°C overnight. Leaves were divided into 150 g batches and blended in a Waring Blender for 25 sec, chilled on ice before use, where 200 ml of cold STN buffer containing 0.4 M sucrose, 20 mM Tricine-NaOH (pH 8.0), and 10 mM NaCl was added to each batch. The homogenate was filtered through three layers of cheesecloth, then twelve layers of cheesecloth. The filtrate was centrifuged at 11,000 x g for 15 min at 4°C and the pellet was kept and resuspended in 10 mM NaCl (2 L for 2 kg of leaves). The resuspension was centrifuged at the same speed for 25, 35 and 45 min with supernatant discarded and pellet resuspended in 10 mM NaCl in between. The pellet resulting from the last centrifugation was resuspended carefully in 0.75 mM EDTA (pH 8.0) and homogenized to obtain thylakoid pellets that were then diluted with 40 L of 0.75 mM EDTA (pH 8.0). The mixture was incubated at room temperature with constant stirring for 30 min before adding DEAE-cellulose resin (1.5 L wet) equilibrated in TEA buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1 mM ATP). The resulting mixture was incubated at room temperature for another 30 min and filter through a single layer of Miracloth (Calbiochem Inc.) The collected DEAE resin was washed with 500 ml of MEA buffer (20 mM

MES-NaOH (pH 6.5), 2 mM EDTA, and 1 mM ATP), then 1 L of MEA that contained 0.1 M NaCl followed by 1.5 L of MEA that contained 0.15 M NaCl. Resin was scraped off the Miracloth, equilibrated in 200 ml of MEA with 0.15 M NaCl, and packed onto a fast-flow column (12 cm in diameter). The bound protein was eluted with MEA plus 0.4 M NaCl. The eluate was contained in a 500 ml fraction and the protein was precipitated by addition of $(NH_4)_2SO_4$ at 31 g/100 ml and stored at 4°C. The precipitated protein was collected by centrifugation at 9,000 x g for 15 min at 10 °C. The pellet was dissolved in 200 ml of TEA buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA and 4 mM ATP. Insoluble green material was removed by an additional centrifugation step (9,000 x g, 15 min, and 10 °C). The supernatant was diluted to 500 ml with TEA buffer and loaded onto a column packed with 50 ml of wet DEAE-cellulose resin which was equilibrated in TEA buffer. The column was washed with 400 ml of TEA buffer that contained 85 mM $(NH_4)_2SO_4$, then 200 ml of TEA with 105 mM $(NH_4)_2SO_4$, and finally 150 ml of TEA plus 200 mM $(NH_4)_2SO_4$ to elute the CF_1. Purified CF_1 was stored in an ammonium sulfate precipitated form at 4°C.

3. Removal of Delta and Epsilon Subunits

CF_1 was precipitated by centrifugation at 9,000 x g for 15 min at 10 °C. The pellet was dissolved in 100 ml of a buffer that contained 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 50 mM DTT and 10 mM ATP. The solution was incubated at

37°C for 5 hr and diluted with 300 ml of TE buffer (25 mM Tris-HCl (pH 8.0) and 1 mM EDTA). The solution was passed through a column packed with 3 ml of wet DEAE equilibrated in TE buffer containing 1 mM ATP. The resin was washed with 100 ml of an ice cold buffer containing 25 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM DTT, 5 mM ATP, 35 mM NaH_2PO_4, 20% (v/v) ethanol and 30% (v/v) glycerol. The flow-through containing approximately equal amounts of the native ε and δ subunits was collected and stored for short periods (1-3 days) at 4^oC or for longer periods at -20^oC (Younis, Winget et al. 1977; Richter, Patrie et al. 1984; Richter, Snyder et al. 1985). The CF_1 (-εδ) was eluted with 50 ml of TE buffer plus 0.4 M NaCl and stored as an ammonium sulfate precipitate at 4 °C.

4. Preparation of Alfa-Beta Hexamer

20 mg of CF_1 (-εδ) were dissolved in 4 ml of 30 mM K_2HPO_4 (pH 7.0) and passed through a desalting Sephadex G50 column (30 ml of bed size). Desalted protein was diluted with 15 ml of the same buffer supplemented with 5.5 mM ATP and 5 mM $MgCl_2$. The mixture was added to 10 M ice cold LiCl to a final concentration of 2 M and incubated at 4°C for 2 hr with slow stirring. The protein solution was loaded on a column packed with 20 ml of wet HTP resin equilibrated with a running buffer that contained 30 mM K_2HPO_4 (pH 7.0), 0.3 M LiCl, 1 mM ATP, and 1 mM $MgCl_2$. CF_1 ($\alpha_3\beta_3$) protein was collected in the flow-through while the γ subunit bound to HTP resin. The resulting hexamer

was dialyzed against a dialysis buffer (20 mM Hepes-NaOH (pH 7.0), 1 mM ATP, 1 mM $MgCl_2$, 2 mM DTT, and 10% (v/v) glycerol) at 4°C overnight. Dialyzed protein was collected and applied to a 3 ml DEAE column equilibrated with HEA buffer (20 mM Hepes-NaOH (pH 7.0), 1 mM ATP and 1 mM EDTA). Free β subunit was eluted with HEA buffer that contained 0.12 M NaCl, and the $\alpha_3\beta_3$ eluted with HEA plus 0.17 M NaCl. Purified $\alpha_3\beta_3$ was dialyzed overnight at 4°C against ten volumes of 20 mM Hepes-NaOH (pH 7.0), 1 mM ATP, 1 mM $MgCl_2$, and 20% (v/v) glycerol.

5. Assembly and Purification of Recombinant CF_1 Three-Subunit Complexes

CF_1 γ inclusion bodies were solubilized to about 20 mg/ml in 8 M urea on ice and rapidly diluted to 0.4 mg/ml with 4 M urea, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA and 5 mM DTT. Any insoluble material was removed by centrifugation at 15,000 x g for 15 min at room temperature.

Folded γ subunits were added, drop-wise, to a mixture containing $\alpha_3\beta_3$ hexamers, 20 mM Hepes-NaOH (pH 7.0), 1 mM ATP, and 1 mM $MgCl_2$. The protein mixture was loaded onto a DEAE column and protein forms other than reconstituted CF_1 were washed from the column with the Hepes buffer containing 0.25 M NaCl. The recombinant complexes were eluted with Hepes buffer plus 0.4 M NaCl and 20% (v/v) glycerol.

6. ATP Hydrolysis Assays

The Mg^{2+}- and Ca^{2+}-dependent ATP hydrolysis activities were measured by incubating 2 μg of enzyme for 5 minutes at 37ºC in 1 ml of assay mixture containing 25 mM Tris-HCl (pH 8.0) and ATP, $MgCl_2$ or $CaCl_2$ at the concentrations indicated in the figure and table legends. An equal volume of 0.5 M TCA was added to stop the reaction and the concentration of inorganic phosphate was measured as described elsewhere (Taussky and Shorr 1953). Protein concentrations were determined using the Bradford method (Bradford 1976). Kinetics constants were obtained using the SigmaPlot software.

7. Reconstitution of CF_1 Assemblies with Thylakoid Membranes Deficient in CF_1

Spinach chloroplast thylakoids were prepared and treated with NaBr to generate CF_1-deficient membranes (Cruz, Harfe et al. 1995; Cruz, Radkowski et al. 1997). Purified reconstituted $\alpha_3\beta_3\gamma$ assemblies were incubated with the ε and δ subunits at a saturating molar ratio (15 μg δε per 10 μg $\alpha_3\beta_3\gamma$) at room temperature in 40 mM Tris-HCl (pH 8.0) for one hour. The assembly was passed through a Sephadex G50 centrifuge column to remove unbound ε and δ subunits. 100 μg of the purified assembly were 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

(BSA) in a final volume of 0.5 ml on ice for 10 min (Andreo, Patrie et al. 1982; Patrie and McCarty 1984).

8. Proton Permeability Measurements

Reconstituted thylakoid membranes equivalent to 20 μg of chlorophyll were assayed for pH gradient formation, shown in Figure 13, using fluorescence quenching of 9-Amino-6-chloro-2-methoxyacridine (ACMA) in an assay buffer of 1 ml total volume containing 40 mM Tricine – NaOH (pH 8.0), 50 mM NaCl, 50 μM phenazine methosulfate, 2 μM ACMA, and 2.5 mM ascorbate (PH 6.8). Fluorescence quenching of ACMA (excitation at 410 nm and emission at 450 nm) was measured as described in (Cruz, Harfe et al. 1995). The quenching signal was reported as percentage of the ratio of ΔF/F of mutant assemblies relative to wild type CF_1, where ΔF was the difference in fluorescence and F the steady state fluorescence (Evron and McCarty 2000).

9. ATP Synthesis Measurements

ATP synthesis was measured 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

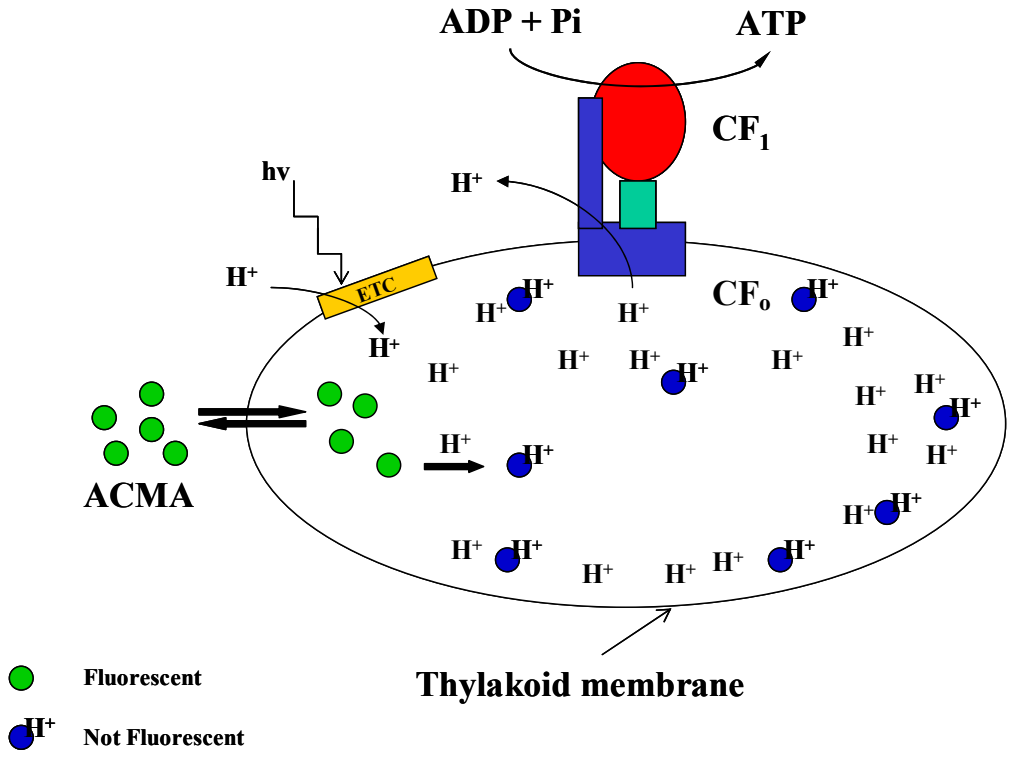

Figure 13. Measurement of ΔpH. Thylakoid particles were prepared and treated with NaBr to strip off the entire CF_1. Electron Transport Chain (ETC) is labeled in yellow. CF_1 wild type and assembly proteins were reconstituted with the membrane and the membranes were washed to remove unbound protein. ACMA is a pH sensitive dye that can penetrate through thylakoid membrane freely. Once protonated, ACMA becomes non-fluorescent and unable to penetrate the membrane (McCarty 1982). When the light is switched on, protons are translocated through ETC and the pH decreases inside the thylakoid membrane. The binding of CF_1 to the membrane helps block the leakage of protons through CF_o and the proton gradient builds.

chlorophyll. The reaction was carried out under a light intensity of 2.5 $\times 10^6$ ergs/cm^2/s for 2 min at 22ºC (Andreo, Patrie et al. 1982). Trichloroacetic acid was added to the reaction after light was switched off to a final concentration of 0.5% (v/v) and the mixture centrifuged at 30,000 x g for 5 min at room temperature. 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 sec after injection. The same reaction mix was used to determine concentrations of ATP standards (10^{-11} M to 10^{-5} M in 10 – fold increments) dissolved in 25 mM Tris-acetate (pH 7.75).

C. Results

1. 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 (Gao, Lipscomb et al. 1995; Sokolov, Lu et al. 1999). The reconstituted assemblies were purified by gel filtration (Tucker, Du et al. 2001) and exhibited

electrophoretic profiles on sodium dodecylsulfate gels that were indistinguishable from that of the native CF_1-δ,ε complex (not shown). The purified $\alpha_3\beta_3$ hexamer displayed specific activities for MgATP and CaATP hydrolysis of approximately 0.1 μmol/min/mg 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/mg and a MgATPase activity of 2.5 μmol/min/mg 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-δ,ε (Richter, Patrie et al. 1984; Richter, Snyder et al. 1985).

2. 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 CF_1 γ were identified in the crystal structure of the 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 (Abrahams, Leslie et al. 1994). A residue equivalent to Arg302 in CF_1 γ is sufficiently close to an anionic loop on an α subunit in the bovine MF_1 to form a salt link (Abrahams, Leslie et al. 1994). 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 (He, Samra et al. 2007).

Arg302 and Arg304 were initially substituted with leucine to negate the positive charge while maintaining a side chain of similar 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 pre-incubation 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 (Richter, Snyder et al. 1985). The ATPase activities of the F_1 assemblies containing the mutant γ subunits are shown in Figure 14 and the k_{cat} values are summarized in Table III. 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 14B), the greatest increase (~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 (~50%) also being observed with the γR304L mutant (Figure 15 and Table III).

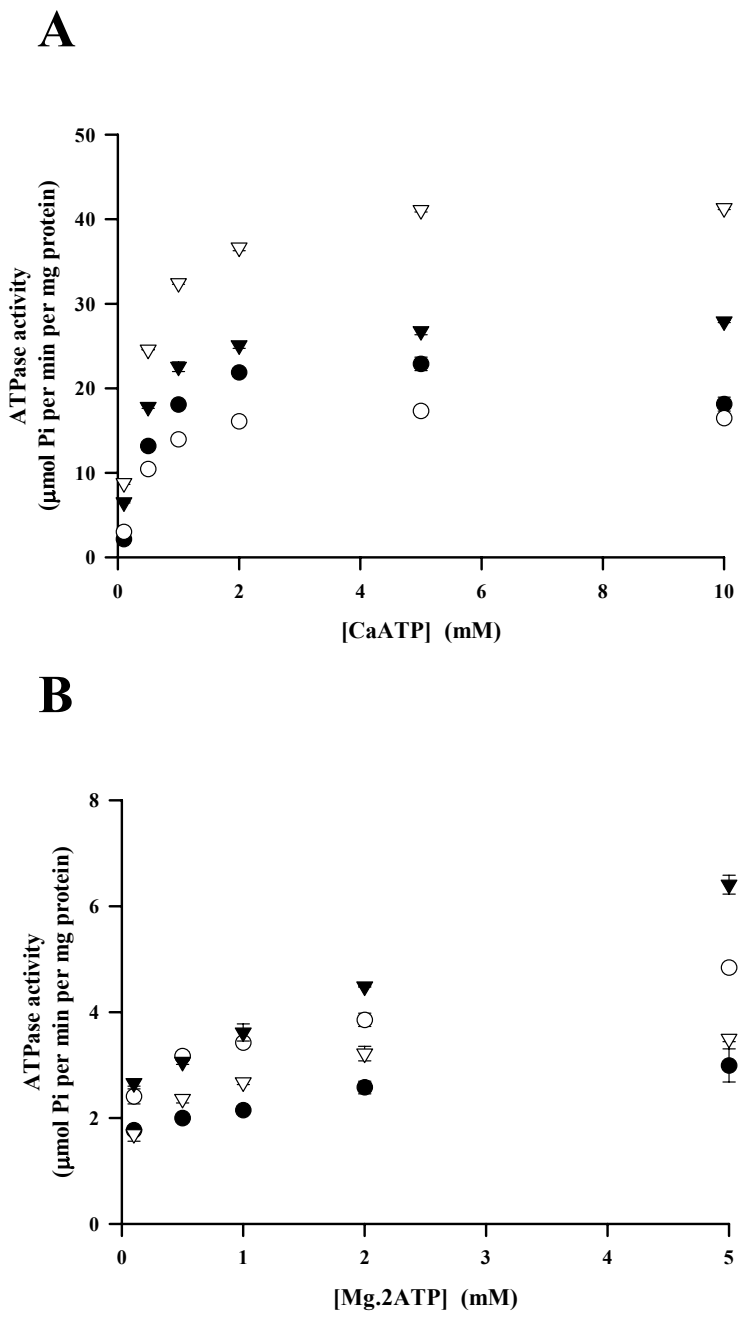

Figure 14. ATP hydrolysis by CF1 assemblies containing single C-terminal γ mutations. ATP hydrolysis assay mixtures contained Ca^{2+}:ATP, 1:1 (A) or Mg^{2+}:ATP, 0.5:1 (B) at the concentrations indicated. ●, $CF_1\gamma_{wild\text{-}type}$; ○, $CF_1\gamma_{R302L}$; ▼, $CF_1\gamma_{R304L}$; ▽, $CF_1\gamma_{Q305A}$. Error bars represent standard deviations with n = 3.

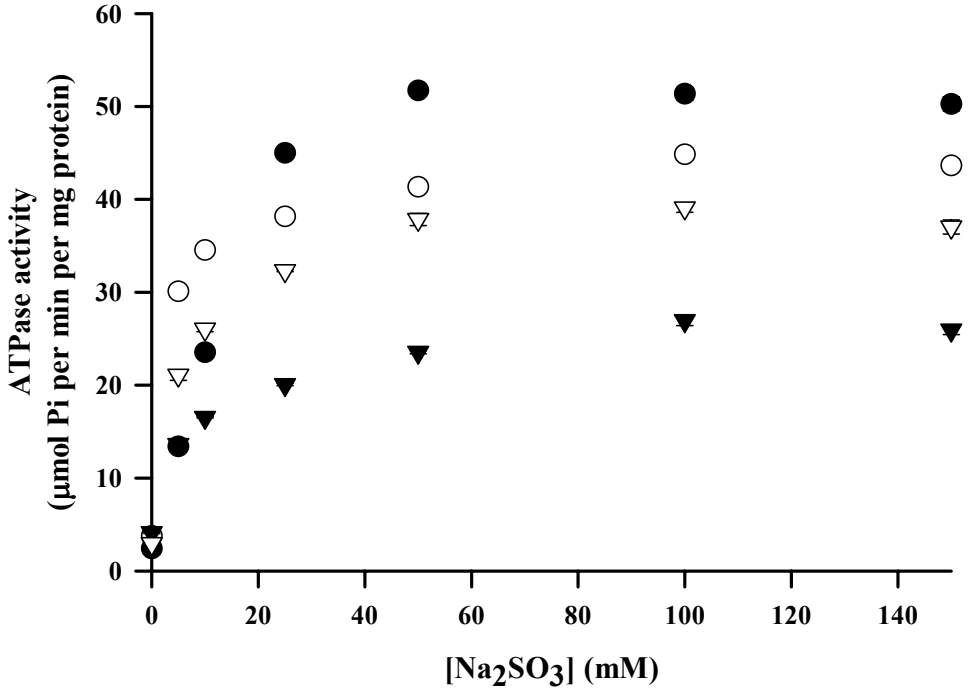

Figure 15. Sulfite activation of CF_1 assemblies that contain chloroplast γ subunits with single C-terminal mutations. The ATP hydrolysis activities were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$ and the indicated concentrations of sodium sulfite. ●, $CF_1\gamma_{wild\text{-}type}$; ○, $CF_1\gamma_{R302L}$; ▼, $CF_1\gamma_{R304L}$; ▽, $CF_1\gamma_{Q305A}$. Error bars represent standard deviations with n = 3.

The effects of simultaneous substitution of γArg302 and γR304, γR304 and γQ305 or of all three residues together, on the ATPase activities are shown in Figure 16 and the k_{cat} values are summarized in Table III. 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 three-fold higher than the activity of the wild type enzyme. The MgATPase activities of the double and triple mutants were also elevated three to four-fold over the wild type activity. In contrast, the sulfite-activated catalytic rates were significantly reduced in all of the mutants (Figure 17, Table III).

3. Loss of sensitivity to tentoxin

The fungal phytotoxin tentoxin is a potent inhibitor of cooperative multisite catalysis by CF_1 (Hu, Mills et al. 1993). 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 (Pinet, Gomis et al. 1996; Mochimaru and Sakurai 1997; Santolini, Haraux et al. 1999). The results of titration of the wild type and mutant assemblies with tentoxin are shown in Figures 18 and 19. The data are representative titrations that were performed twice with the same outcome.

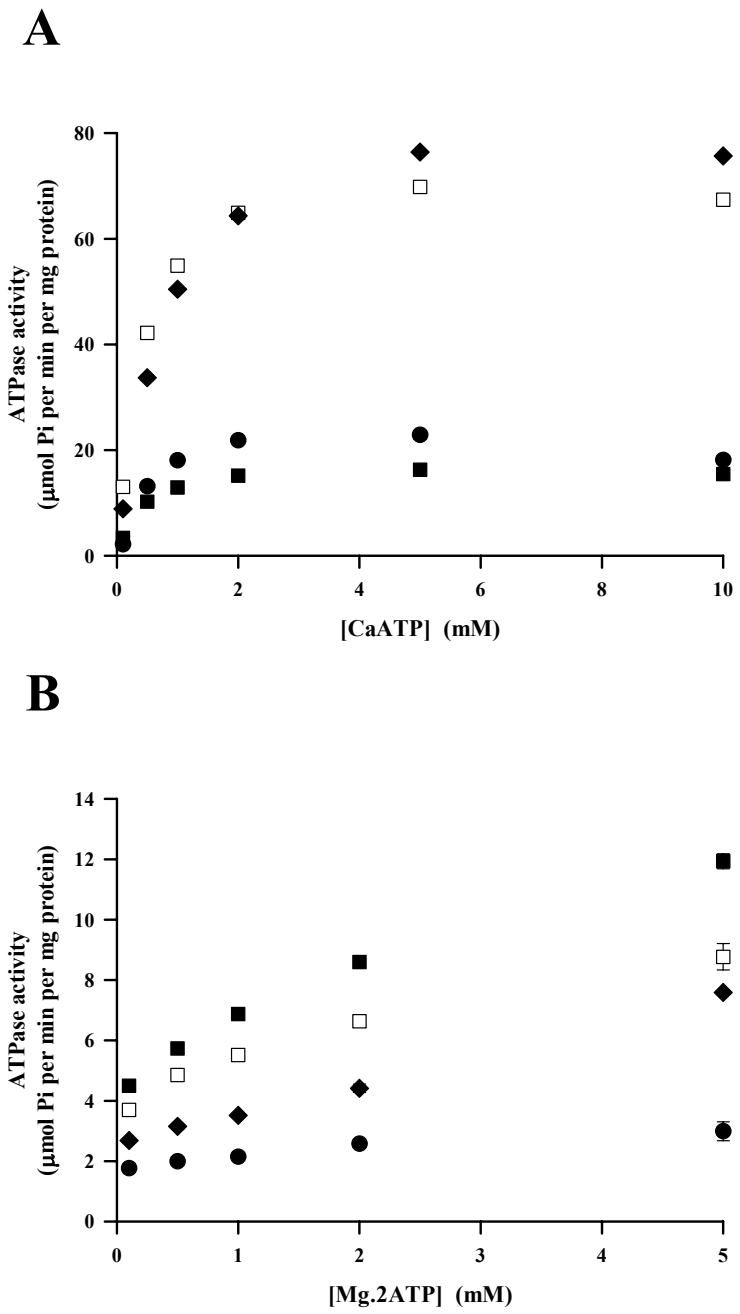

Figure 16. ATP hydrolysis by CF_1 assemblies containing multiple C-terminal γ mutations. ATP hydrolysis assay mixtures contained either Ca^{2+}:ATP, 1:1 (A) or Mg^{2+}:ATP, 0.5:1 (B) at the concentrations indicated. ●, $CF_1\gamma_{wild\text{-}type}$; ■, $CF_1\gamma_{R302L;R304L}$; □, $CF_1\gamma_{R304A;Q305A}$; ♦, $CF_1\gamma_{R302A;R304A;Q305A}$. Error bars represent standard deviations with n = 3.

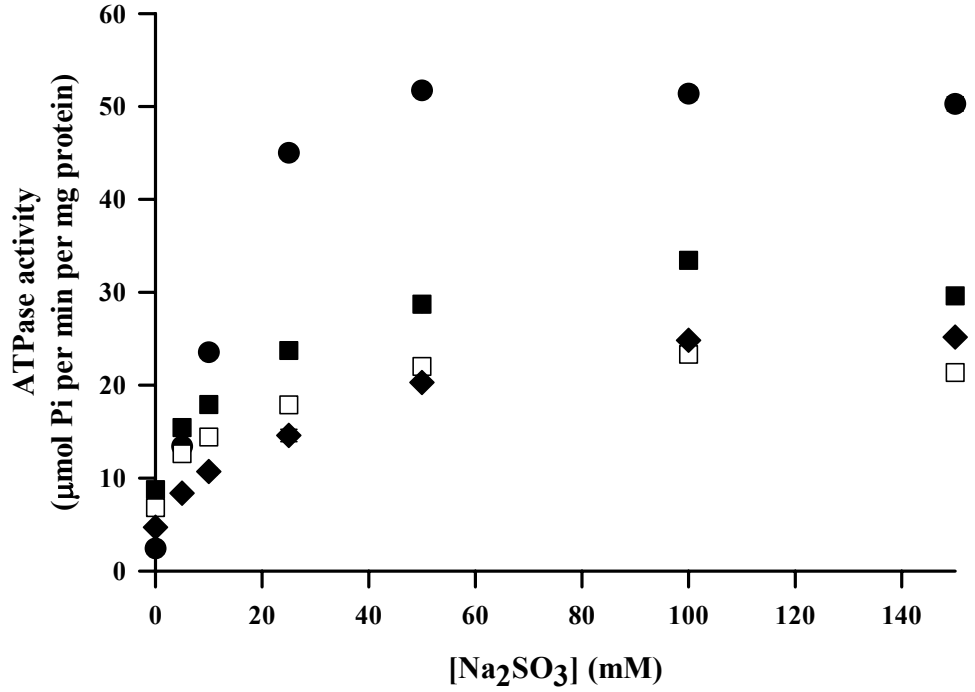

Figure 17. Sulfite activation of CF_1 assemblies with double and triple C-terminal γ mutations. The ATP hydrolysis activities of purified enzyme assemblies were determined in the presence of 4 mM ATP, 2 mM $MgCl_2$ and the indicated concentrations of sodium sulfite. ●, $CF_1\gamma_{wild\text{-}type}$; ■, $CF_1\gamma_{R302L;R304L}$; □, $CF_1\gamma_{R304A;Q305A}$; ♦, $CF_1\gamma_{R302A;R304A;Q305A}$. Error bars represent standard deviations with n = 3.

Table III. Summary of k_{cat} values for γ mutant F_1 assemblies

Gamma mutants	k_{cat} (s^{-1}) [1]			
	Ca^{2+}	Mg^{2+}	Mg^{2+} + Sulfite[2]	R[3]
$CF_1(\alpha\beta)_3\gamma$ *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

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

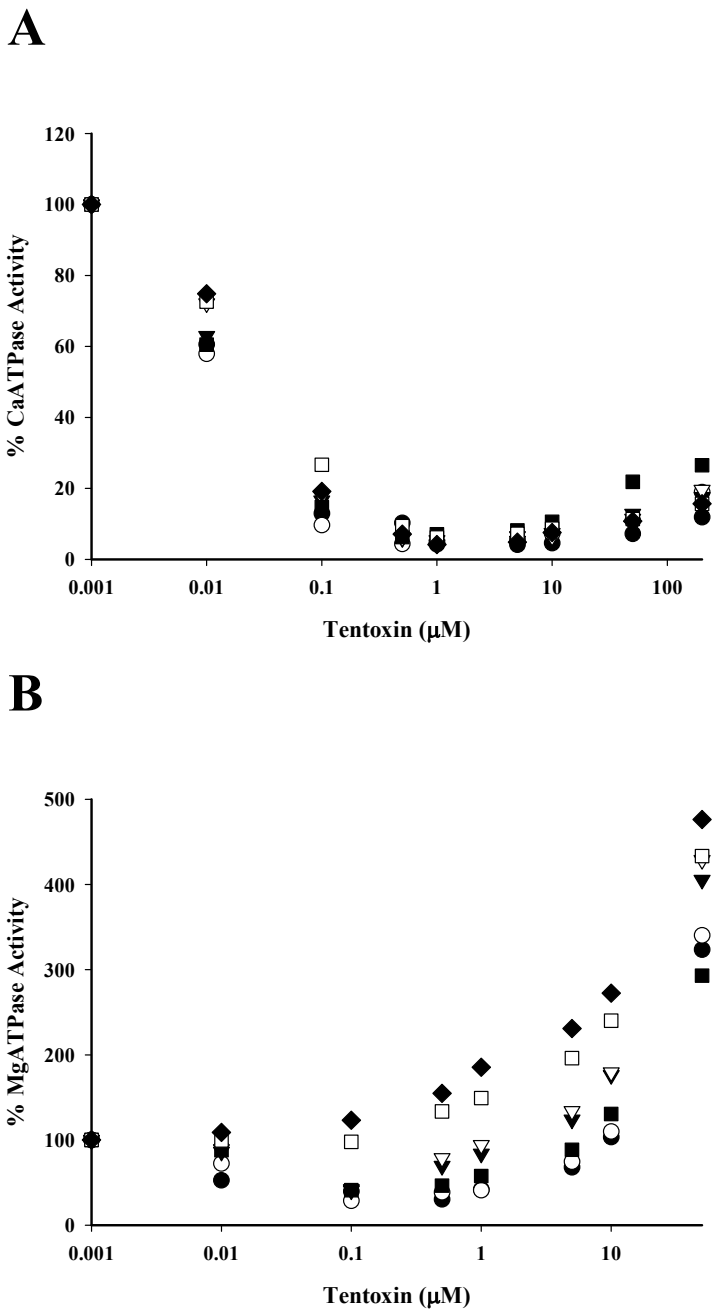

Figure 18. Titration of gamma mutants with tentoxin. ATP hydrolysis assay mixtures contained either 5 mM ATP and 5 mM $CaCl_2$ (A) or 4 mM ATP and 2 mM $MgCl_2$ (B). Samples were preincubated with tentoxin at the concentrations indicated for five minutes prior to initiation of the assay. ●, $CF_1\gamma_{wild\text{-}type}$; ○, $CF_1\gamma_{R302L}$; ▼, $CF_1\gamma_{R304L}$; ▽, $CF_1\gamma_{Q305A}$; ■, $CF_1\gamma_{R302L;R304L}$; □, $CF_1\gamma_{R304A;Q305A}$; ♦, $CF_1\gamma_{R302A;R304A;Q305A}$. Error bars represent standard errors with n = 2.

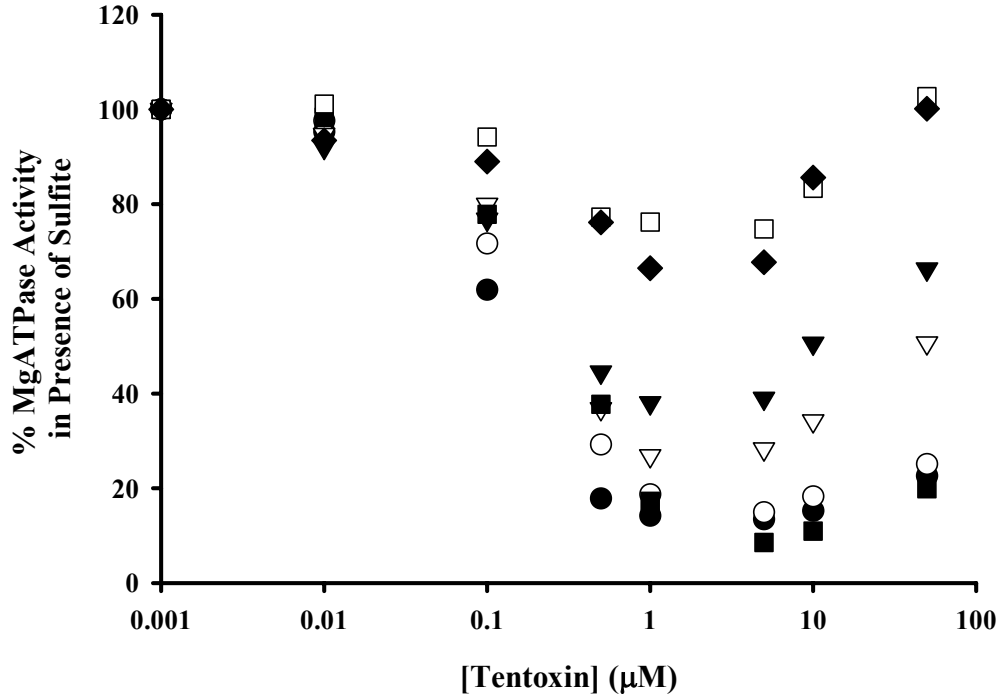

Figure 19. Tentoxin inhibition of sulfite-stimulated MgATP hydroysis. ATP hydrolysis assay mixtures contained 4 mM ATP, 2 mM $MgCl_2$ and 25 mM Na_2SO_3. Samples were preincubated with tentoxin at the concentrations indicated for five minutes prior to initiation of the assay. ●, $CF_1\gamma_{wild\text{-}type}$; ○, $CF_1\gamma_{R302L}$; ▼, $CF_1\gamma_{R304L}$; ▽, $CF_1\gamma_{Q305A}$; ■, $CF_1\gamma_{R302L;R304L}$; □, $CF_1\gamma_{R304A;Q305A}$; ♦, $CF_1\gamma_{R302A;R304A;Q305A}$. Error bars represent standard errors with n = 2.

The Ca^{2+}-dependent ATPase activities of wild type and mutants show full sensitivity to inhibition by tentoxin (Figure 18A). In contrast, the MgATPase activities of the mutants (Figure 18B) show 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 19. 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 showed the typical reactivation/stimulation of both MgATPase and CaATPase activities that occurs at higher concentrations of tentoxin indicating that only inhibition of MgATPase activity was affected.

4. 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 then reconstituted with CF_1-deficient thylakoid membranes (Patrie and McCarty 1984; Richter, Patrie et al. 1984). In the absence of CF_1, protons flow freely through CF_O and the thylakoid membranes are unable to maintain an 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 (McCarty and Racker 1968; Kamienietzky and Nelson 1975; Richter, Patrie et al. 1984).

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 (Evron, Johnson et al. 2000; Evron and McCarty 2000). Thylakoid membranes reconstituted with CF_1 assembled using the recombinant wild type γ subunit showed a maximum light-dependent quenching of ACMA fluorescence of about 60%, the same as that observed in membranes reconstituted with native CF_1 (Figure 20). In contrast, the capacities of the assemblies containing the γ mutants to block proton flow through CF_O were significantly reduced (Table IV). This was not likely to be due to reduced 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 gamma subunit of CF_1 (e.g. reduction and specific cleavage by trypsin) do not affect the binding of CF_1 to CF_O (McCarty 2005), and third, generation of a measurable transmembrane pH gradient requires that 90% or more of the CF_O is complexed with CF_1 (Weiss and McCarty 1977). Thus the assemblies containing the γ mutants appeared to be proton-leaky.

The results shown in Table IV further indicate a loss of ATP synthesis capacity by the mutant enzymes. The triple mutant was most affected, retaining

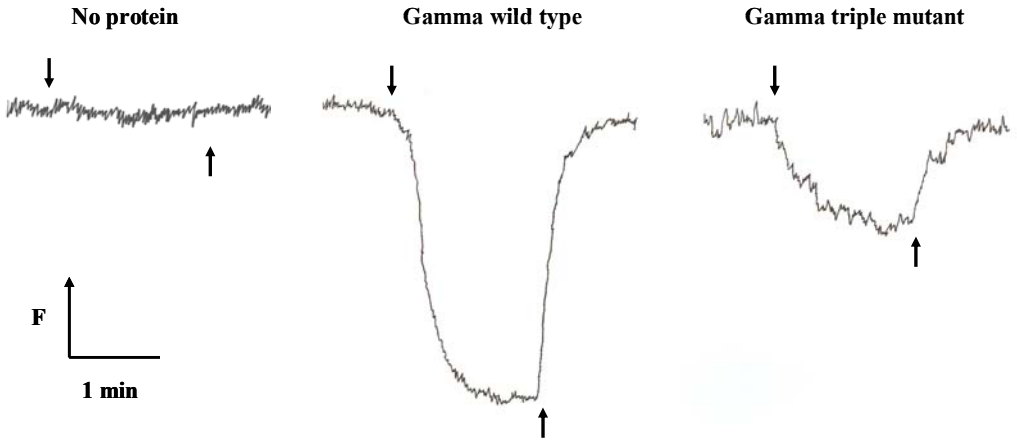

Figure 20. Comparison of proton gradient formation by thylakoid membranes reconstituted with the wild type and triple mutant CF_1 assemblies. Proton gradient formation in thylakoid membrane preparations reconstituted with No CF_1 (left trace), $CF_1\gamma_{wild\ type}$ (middle trace) or $CF_1\gamma_{R302A;R304A;Q305A}$ (right trace) was determined by measuring ACMA fluorescence quenching as described in the *Materials and Methods*. The arrows indicate light on (↓) and off (↑).

Table IV. Proton gradient formation and ATP synthesis by mutant γ F_1 assemblies

Gamma mutants	Relative ΔF/F (%)[1]	ATP Synthesis (μmol P_i /h/mg chl)	(%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)

[1] ACMA quenching and ATP synthesis by purified F_1 assemblies were determined as described in the *Materials and Methods*. Errors are expressed as standard deviations with n = 4.

only about 13% of wild type ATP synthesis. Interestingly, the reduced ATP synthesis capacity of the mutants paralleled the reduction in sulfite-stimulated MgATPase activity (Table III) which in turn paralleled the increase in CaATPase activity (Table IV).

D. DISCUSSION

1. Catch residues are not essential for cooperative multi-site 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 MgATP hydrolysis to a greater extent than the R304L mutant whereas the R304L mutant increased CaATP hydrolysis to a greater extent than the 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 the 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 catalytic turnover (Greene and Frasch 2003). Remarkably, the double

γR304A,Q305A and triple γR302A,R304A,Q305A mutants showed a greater than three-fold increase in CaATPase activity compared to the wild type enzyme. The elevated CaATP hydrolysis was fully sensitive to tentoxin indicating that it resulted from normal cooperative multi-site nucleotide interactions (Hu, Mills et al. 1993). 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 multi-site 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 (Hossain, Furuike et al. 2006).

2. 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 sulfite-activation of ATP hydrolysis (Table III). 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* (He, Samra et al. 2007). In that case, replacement of Gln305 with alanine alone or in combination with mutations of Arg302 and Arg304 completely eliminated the sulfite-induced stimulation of MgATP hydrolysis.

It was noted previously (He, Samra et al. 2007) that residues equivalent to Arg302, Arg304 and Gln305 in the bovine MF_1 structure (Arg252, Arg254 and Gln255) form a bridge across a non-catalytic αβ interface, linking the adjacent α and β subunits together. Freshly isolated CF_1 contains two very tightly bound molecules of ADP (Shapiro, Huber et al. 1991), one in a catalytic site and one in a non-catalytic site (Digel and McCarty 1995; Digel, Kishinevsky et al. 1996; Malyan 2006). The enzyme is latent with respect to MgATP hydrolysis due to the presence of MgADP bound tightly in a non-catalytic site on the enzyme (Digel and McCarty 1995; Digel, Kishinevsky et al. 1996). Pre-treatment of CF_1 with MgATP results in activation of the MgATPase activity by filling one or more non-catalytic site with MgATP (Milgrom, Ehler et al. 1991). 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 (Murataliev and Boyer 1992). Oxyanions such as sulfite decrease the binding affinity of nucleotides at non-catalytic sites and increase nucleotide exchange at catalytic sites (Malyan and Vitseva 2001). 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 to the hypothesis that the activating effect of oxyanions results from stimulation of exchange of MgATP for MgADP in a non-catalytic site and that occupancy of the non-catalytic site by MgATP is communicated to the catalytic site via the catch residues (He, Samra et al. 2007). 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 non-catalytic interface that occurs with the γ triple mutant results in 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 (Du and Boyer 1990).

Tentoxin is known to block the cooperative exchange of nucleotides among the three catalytic sites that leads to high rates of multi-site catalysis (Hu, Mills et al. 1993). 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 reduced. If this is indeed the case, the residual tentoxin-insensitive activity must then result from single-site or bi-site catalysis at the remaining site or sites. A thorough analysis

of exchange of tightly bound ADP from catalytic and non-catalytic sites in the triple mutant is presented in the next chapter to determine if the non-exchangeable ADP is located in a catalytic or a non-catalytic site.

The observation that cooperative multi-site 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 (Du, Tucker et al. 2001). 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 multi-site ATP hydrolysis and can drive γ subunit rotation (Tucker, Schwarz et al. 2004), 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 the previous study in which the mutants were assembled with the hybrid enzyme (He, Samra et al. 2007). 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}~640. s^{-1}), the double and triple mutants partially inhibited CaATPase activity (50 to 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 activites 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 (Sokolov, Lu et al. 1999). 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.

3. 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 IV) paralleled the loss of sulfite-stimulated MgATP hydrolysis (Table III). The triple mutation in the γ subunit caused CF_OF_1 to become proton-leaky and reduced the ATP synthesis capacity to ~13% of wild type. The ratio of sulfite-stimulated to unstimulated MgATPase activity was similarly reduced by ~85% in the triple mutant compared to 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 non-catalytic sites which is non-exchangeable with medium nucleotides, to a form in which the tightly bound nucleotides can be rapidly exchanged with medium nucleotides (Zhou, Xue et al. 1988; Malyan 2005; Malyan 2006). 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 (Carmeli and Lifshitz 1972).

Activation of the latent ATPase activity of membrane-bound CF_OF_1 is a two-step process. Formation of a transmembrane 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 (Ketcham, Davenport et al. 1984). Disulfide reduction has been shown to result in a shift in the conformation of the inhibitory ε subunit, releasing its inhibitory effect (Richter and McCarty 1987; Soteropoulos, Ong et al. 1994). 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 (Richter, Snyder et al. 1985; Soteropoulos, Ong et al. 1994; McCarty 2005). The MgATPase activity of the enzyme, however, 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 (McCarty 2005). 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 non-catalytic site and communication of this exchange to catalytic sites. Mutation of the catch residues in the hybrid enzyme resulted in complete loss of the oxyanion stimulation of catalysis (He, Samra et al. 2007). In CF_1, however, mutating the catch residues reduced, 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 non-catalytic sites in CF_1 (Tucker, Schwarz et al. 2004), they only affect non-catalytic 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 (Tucker, Du et al. 2001).

In the crystal structure of bovine MF_1 the β_D (with ADP bound) and β_T (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 N-terminus of the γ subunit from Met23 to Val26 and Leu77 to Lys87, and from Arg228 to 230 on the γ subunit C-terminus (Abrahams, Leslie et al. 1994). 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 (Abrahams, Leslie et al. 1994). Interestingly, an earlier study with the EcF_1 (Al-Shawi and Nakamoto 1997) indicated that mutation of one of the catch residues, Met23, to lysine strongly affected proton coupling and ATP synthesis activities but had a much smaller effect on ATPases activity. In a more recent study in TF_1 (Yoshida and Anraku 2000) the entire DELSEED loop was substituted with alanine residues. The substitutions were expected to eliminate the γ-β 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 on 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 gamma 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 αβ 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 non-catalytic 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.

Chapter Four

Nucleotide Exchange of Catalytic and Non-catalytic Site(s) of the Chloroplast F_1 ATP Synthase

A. Introduction

The CF_1 segment of the ATP synthase has six nucleotide binding sites, heterogeneous in their properties, located within the $\alpha_3\beta_3$ ring. Three sites reside primarily on the β subunits and are catalytic while the other three are primarily on the α subunits and are considered to be non-catalytic (Xue, Miller et al. 1987; Xue, Melese et al. 1988; Boyer 1993). It was suggested that the three non-catalytic sites play important regulatory roles. Although all three non-catalytic sites are considered to be tight nucleotide binding sites, (i.e. K_d values for ADP or ATP of less than 1 μM), one of the non-catalytic sites can bind nucleotide very quickly (fills within 2 minutes) and the other two fill more slowly (up to 2 hours) (Xue, Miller et al. 1987). It was shown that the binding of MgATP to non-catalytic sites was required to activate CF_1 as an ATPase (Milgrom, Ehler et al. 1990).

The *Binding Change* mechanism states that during catalysis, three catalytic sites rotate through three different conformational states: open, loose, and tight during a single catalytic cycle (Boyer 1989; Boyer 1993). In a bi-site model, on the other hand, four nucleotide binding sites or two pairs participate (Shapiro and

McCarty 1988; Boyer 2002). The nucleotide occupying properties of CF_1 have been studied in detail by McCarty's group, who characterized the six nucleotide binding sites of CF_1 individually and labeled them site 1 through site 6. Each site has been studied thoroughly using fluorescent probes.

Site 1 binds all adenine nucleotides tightly and independently of metal ions. The bound nucleotides exchange rapidly with ADP and slowly with ATP in the absence of Mg^{2+} ions (Shapiro, Huber et al. 1991). In the presence of Mg^{2+} ions the exchange can be accelerated. Site 2 binds ATP with high affinity only in the presence of Mg^{2+} ions and does not bind ADP or MgADP (Feldman and Sigman 1982). The bound ATP is stabilized by Mg^{2+}, making it not exchangeable. However precipitation of CF_1 by ammonium sulfate can dissociate any nucleotide from this site (Shapiro, Huber et al. 1991). This site was thought to be non-catalytic (Feldman and Sigman 1983). Site 3 binds ATP or ADP loosely with a K_d in the μM range with and without Mg^{2+} ions (Bruist and Hammes 1981). Nucleotide binding to this site is freely reversible. This site is hypothesized to be a catalytic site and can switch properties with site 1 in the presence of MgATP (Carlier and Hammes 1979; Shapiro and McCarty 1988; Shapiro and McCarty 1990).

Site 4 has properties similar to site 1 (Shapiro, Gibson et al. 1991). Bound nucleotides exchange rapidly with ADP and ATP in solution in the presence of Mg^{2+} (Shapiro, Gibson et al. 1991). This site has been shown

capable of slow ATP hydrolysis when site 1 was occupied by ADP (Shapiro, Huber et al. 1991).

Site 5 binds to nucleotides in the same way that site 2 does. Mg^{2+} ions facilitate tight ATP binding but not ADP binding to this site (Shapiro, Huber et al. 1991). Site 5 can not hydrolyze bound ATP in the absence of medium nucleotides (Shapiro, Huber et al. 1991). Site 6 was poorly characterized but assumed to have site 3-like properties, that is, it binds nucleotides with low affinities in the absence of metal ions and has catalytic function (Girault, Berger et al. 1988).

It was proposed by the McCarty group that four of the six nucleotide binding sites are arranged in two pairs with site 1 and site 3 in one pair and site 4 and site 6 in the other. Two sites in each pair switch properties during catalysis and do so in a cooperative manner, while the remaining two sites, site 2 and site 5, play no catalytic role but have structural functions to maintain the integrity of the $\alpha_3\beta_3$ ring (Shapiro, Huber et al. 1991). However the functional properties of the six nucleotide binding sites remain debatable. In addition, the proposal that two pairs of sites alternate to hydrolyze ATP is highly controversial and is incompatible with the rotational catalysis model.

When isolated from chloroplasts, CF_1 retains four tightly bound nucleotides even after dialysis and gel filtration (Xue, Miller et al. 1987; Xue, Melese et al. 1988). When treated with ammonium sulfate CF_1 looses two of the

four bound nucleotides, leaving only two tightly bound nucleotides behind, both of which are ADP (Gao, Lipscomb et al. 1995). One of these two sites was thought to be site 1, which is a catalytic site and able to exchange its contents for nucleotides in the medium (Bruist and Hammes 1981). The second site may be analogous to McCarty's site 5 and is likely to be non-catalytic (Malyan 2002; Malyan 2006).

To examine the properties of the two sites on CF_1 that retain tightly bound ADP, and to examine the effects of the γ C-terminal mutants described in Chapters Two and Three on nucleotide exchange at these two sites, the ADP bound to isolated CF_1 was exchanged with the fluorescent ADP analog TNP-ADP. The exchange of bound TNP-ADP with medium nucleotides was monitored under hydrolysis conditions using ATP as well as nonhydrolyzable AMP-PNP. Exchange of ADP from one of the two sites correlated closely with activation of catalysis while exchange of the other one did not. The γR302A,R304A,Q305A mutant was also tested for nucleotide exchange and the results showed that one of the two sites in the mutant protein failed to exchange for medium nucleotides under all catalytic conditions. This result suggests that the catch interaction between the C-terminus of γ and the $\alpha_3\beta_3$ hexamer plays an important role in cooperative nucleotide exchange in CF_1.

B. Materials and Methods

1. Materials

Fresh spinach leaves were obtained from the supermarket. Sephadex G50 resin, AMP-PNP, TNP-ADP, Nicotinamide Adenine Dinucleotide (NADH), pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, and trypsin were purchased from Sigma. ATP (grade II) and ADP (grade II) were from Fluka. All other chemicals were of the highest quality reagent grade available.

2. Preparation of CF_1

CF_1 was prepared from fresh spinach leaves as described previously (Sokolov, Lu et al. 1999). The ε and δ subunits were removed and the remaining $\alpha_3\beta_3\gamma$ complex was desalted using sephadex G50 resin (Richter, Patrie et al. 1984). The $\alpha_3\beta_3\gamma$ protein containing the triple mutant, γR302A,R304A,Q305A, was reconstituted as described in Chapter Three (He, Samra et al. 2008).

3. Nulceotide Binding to CF_1

Ammonium sulfate precipitated CF_1 was dissolved to ~ 1 mg / ml in 50 mM Tricine-NaOH buffer (pH 8.0) containing 50 mM NaCl at room temperature. Dithiothreitol was added to the mixture to a final concentration of 20 mM and the mixture was incubated for 30 min to reduce the disulfite bond in the γ subunit. In other experiments, 50 μM $CuCl_2$ was added to the enzyme in place of DTT for 30 min to fully oxidize the disulfide bond. The reduced or oxidized protein was

passed consecutively through two Sephadex G50 centrifuge columns equilibrated with 50 mM Tricine-NaOH (pH 8.0) / 50 mM NaCl buffer at room temperature. A sample containing 100 μg of desalted CF_1 was mixed with 50 mM Tricine-NaOH (pH 8.0) buffer containing 50 mM NaCl, and 1 mM TNP-ADP. The protein mixture was incubated at room temperature for 1 min to load TNP-ADP into one binding site and for 2 hr to load TNP-ADP into two binding sites. The mixture was then passed consecutively through three Sephadex G50 centrifuge columns equilibrated with 50 mM Tricine-NaOH (pH 8.0) / 50 mM NaCl buffer. The amount of TNP-ADP bound was determined by measuring absorbance at 416 nm using a Varian Cary 100 spectrophotometer. Protein concentration was obtained using the Bradford method (Bradford 1976). The molecular ratio of TNP-ADP to CF_1 (mr) was calculated using $mr = (A_{416} / \varepsilon) / [CF_1]_{Molar}$ where ε is the extinction coefficient (26,000 M^{-1}) for TNP-ADP and the molecular weight of CF_1 is 360,000 g / mol.

4. Nucleotide Release from CF_1

Free TNP-ADP released from the protein has less than 20% of the fluorescence amplitude than that of the bound form. The TNP-ADP labeled CF_1 was diluted with 50 mM Tricine-NaOH (pH 8.0) and 50 mM NaCl. Calcium - dependent release was carried out in the presence of 100 μg CF_1, 4 mM $CaCl_2$, and 4 mM nucleotide (ADP, ATP or AMP-PNP) while the magnesium dependent

release was carried out with 100 μg CF_1, 2 mM $MgCl_2$, 4 mM nucleotide (ADP, ATP or AMP-PNP), and in some cases 50 mM Na_2SO_3. The total volume of each release experiment was 400 μl contained in a micro cuvette. The release was triggered by the addition of the exchanging nucleotide and the fluorescence emission was measured at 550 nm with the excitation at 415 nm in a Varian Eclipse fluorometer. The signal was saved by averaging continuous fluorescence over 5 second intervals. In all release experiments, 10 μg of solid trypsin, which digests CF_1 and causes it to release all nucleotides, was added to the sample after 60 minutes to determine the baseline signal or minimum fluorescence by free TNP-ADP. Fluorescent plots were obtained by subtracting the baseline signal using the Sigma Plot software.

5. ATPase Activity Measurements

Wild type or mutant CF_1 with TNP-ADP bound was diluted with 50 mM Tricine-NaOH (pH 8.0) and 50 mM NaCl to a 0.5 ml final volume and transferred to a plastic cuvette. ATP hydrolysis was triggered by addition of 0.5 ml of reaction mixture that contained 50 mM Tricine-NaOH (pH 8.0), 10 mM KCl, 5 mM phosphoenolpyruvate, 4 mM ATP, 2 mM $MgCl_2$, 0.3 mM NADH, 20 units / ml of pyruvate kinase, and 50 units / ml of lactate dehydrogenase at room temperature (Richter, Gromet-Elhanan et al. 1986). The change in light absorbance at 340 nm was monitored in a Varian Cary 100 spectrophotometer at

2.5 second intervals. The ATPase activity was calculated based on the disappearance of absorption assuming a molar ratio of Pi over NADH of 1:1

C. Results

1. TNP-ADP Binding to CF_1

CF_1 (-δε) isolated from spinach leaves was prepared using several steps of gel filtration and ammonium sulfate precipitation, leaving only two ADP bound in the nucleotide binding sites (Shapiro, Gibson et al. 1991; Gao, Lipscomb et al. 1995). The CF_1 wild type enzyme was incubated with TNP-ADP in the presence or absence of the oxyanion sulfite for different time periods and the results are shown in Figure 21. Under experimental conditions in which a high concentration of TNP-ADP was present, one nucleotide binding site exchanged rapidly with the ADP analog within 2 minutes. A second site exchanged with TNP-ADP in a much slower process, taking more than 90 minutes for complete exchange. The oxyanion sulfite partially stimulated the binding of TNP-ADP (shown in Figure 21). In both cases, with and without sulfite, a maximum of 2 moles of TNP-ADP bound per mole of enzyme even after 4 hr of incubation (data not shown). Hereafter, the two sites will be designated the “first site” and the “second site” in the order in which they fill with TNP-ADP.

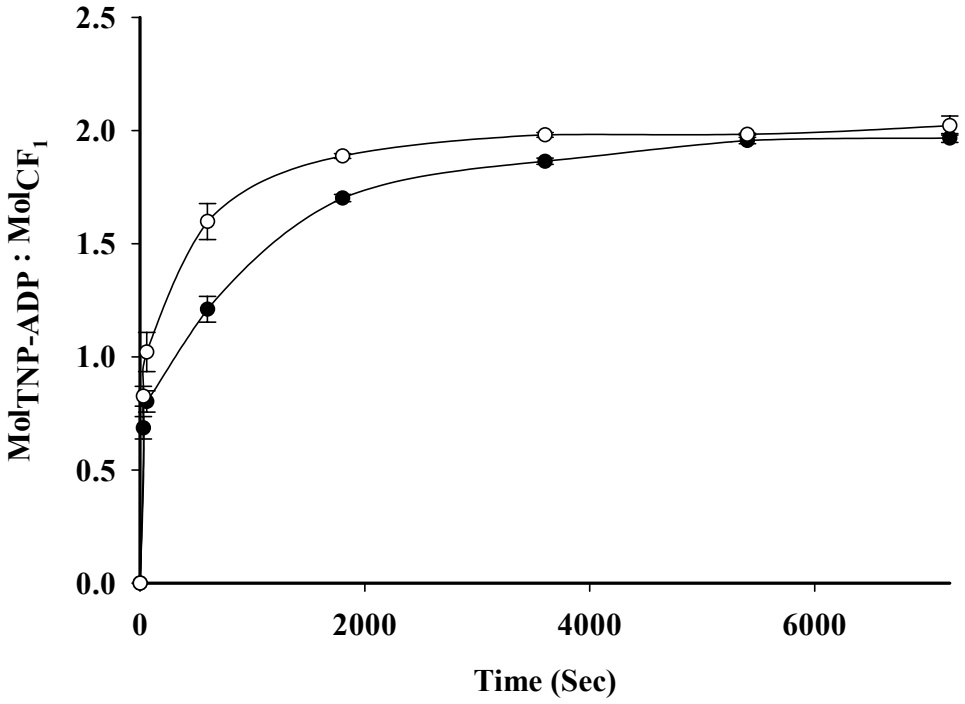

Figure 21. Titrations of TNP-ADP binding to CF_1. CF_1 $\alpha_3\beta_3\gamma$ protein was isolated and desalted as described in *Materials and Methods*. The enzyme was incubated with 1 mM TNP-ADP with (○) and without (●) 50 mM Na_2SO_3 for different time periods from 30 sec up to 2 hr. The labeled protein was passed consecutively through three Sephadex G50 centrifuge columns to eliminate any unbound nucleotides. The number of TNP-ADP bound on each CF_1 was determined as indicated in the *Materials and Methods*. The values were obtained by averaging results from two independent experiments (n=2).

2. TNP-ADP Exchange with Adenosine Nucleotides

When bound to CF_1 TNP-ADP shows an increased fluorescence due to the highly nonpolar environment in the nucleotide binding pocket(s) (Mills and Richter 1991). The bound fluorescent nucleotide analog can be exchanged with medium ADP (Leckband and Hammes 1988; Shapiro, Gibson et al. 1991). Release of TNP-ADP can be readily monitored by following the decrease in the fluorescence intensity as TNP-ADP dissociates (Mills and Richter 1991). In this study, trypsin was employed to treat the CF_1 after 30 minutes of incubation with non-fluorescent nucleotide(s) to completely release the remaining bound TNP-ADP. The residual fluorescence signal was then used as a baseline that was subtracted from the raw fluorescence data. The resulting fluorescence traces were then normalized to yield plots of percentages of TNP-ADP bound as a function of time upon introduction of non-fluorescent nucleotide into the solution.

ADP-induced TNP-ADP release is illustrated in Figure 22. TNP-ADP stayed bound indefinitely when no nucleotide was present in the solution. Metal ions, such as Ca^{2+} and Mg^{2+}, facilitated the exchange of medium ADP for bound TNP-ADP whereas sulfite, in addition to Mg^{2+} ions, accelerated the exchange process notably. Less than 5% of the TNP-ADP was retained in CF_1 after 30 min of incubation in the presence of sulfite. Both ATP and the ATP analog, AMP-PNP, were used to exchange for the bound TNP-ADP in the presence of Mg^{2+} ions or Ca^{2+} ions (Figure 23). AMP-PNP, which is structurally similar to

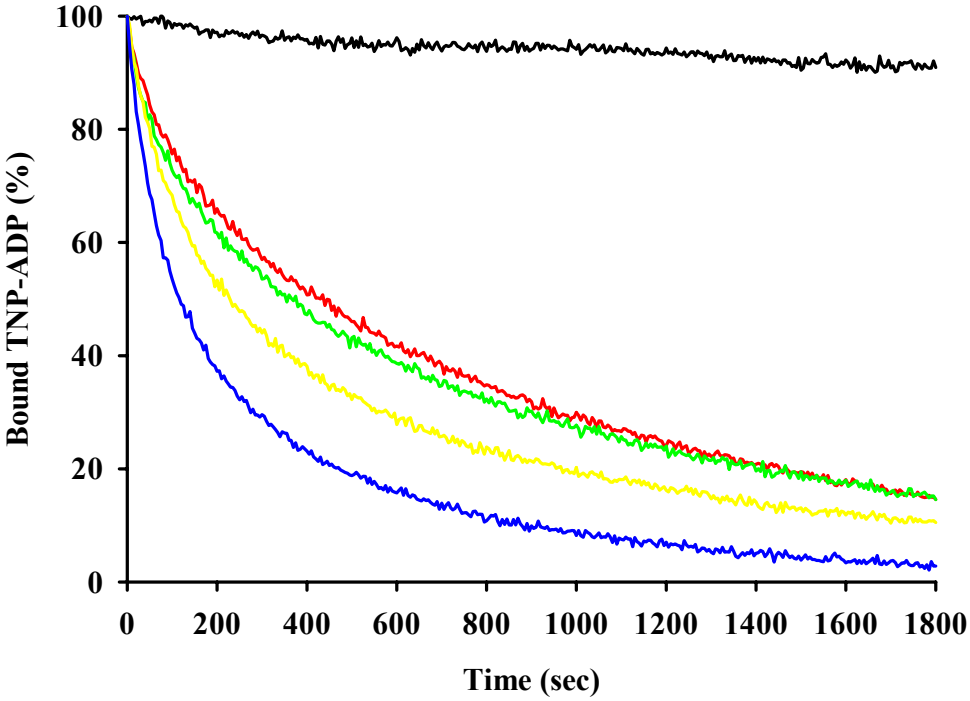

Figure 22. ADP induced release of TNP-ADP from CF_1. Wild type CF_1 was fully reduced by treatment with 20 mM DTT for 30 min at room temperature. Desalted CF_1 wild type was incubated with 1 mM TNP-ADP for 2 hr resulting in two molecules of TNP-ADP per molecule of CF_1. ADP was added to exchange the bound TNP-ADP under the following conditions: (black) no ADP; (red) 4 mM ADP; (green) 4 mM $CaCl_2$ and 4 mM ADP; (yellow) 2 mM $MgCl_2$ and 4 mM ADP; and (blue) 2 mM $MgCl_2$, 4 mM ADP and 50 mM Na_2SO_3. TNP-ADP release was monitored at room temperature by monitoring the decrease in fluorescence with excitation at 415 nm and emission at 550 nm as described in the *Materials and Methods*.

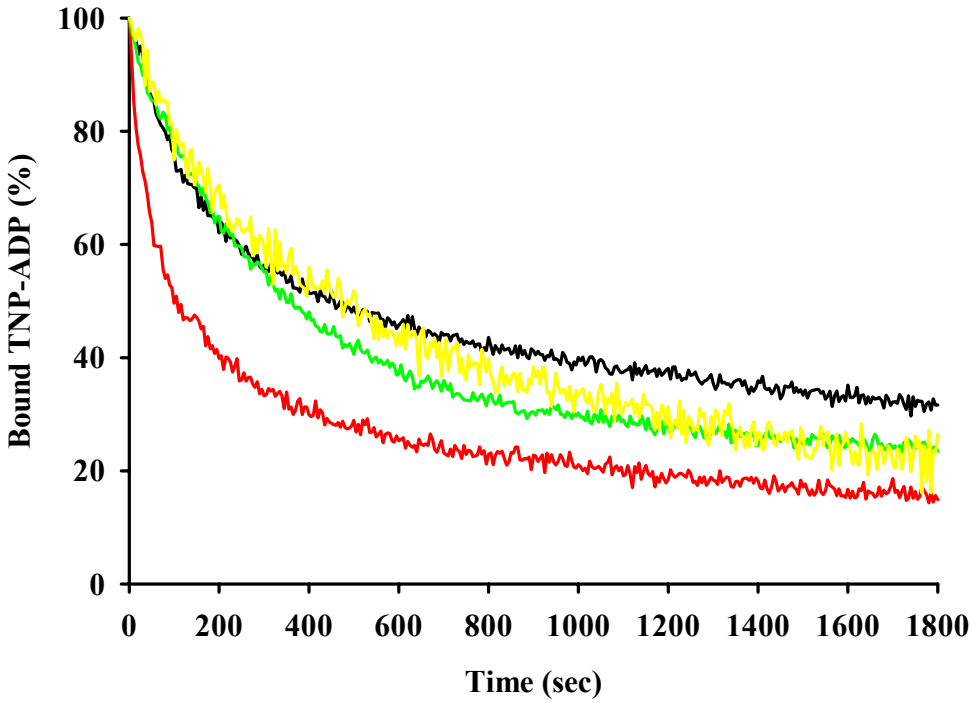

Figure 23. ATP and AMP-PNP induced release of TNP-ADP from CF_1. Wild type CF_1 was fully reduced by treatment with 20 mM DTT for 30 min at room temperature. Desalted CF_1 wild type was incubated with 1 mM TNP-ADP for 2 hr resulting in two molecules of TNP-ADP per molecule of CF_1. ADP was added to exchange the bound TNP-ADP under the following conditions: (black) 4 mM ATP; (red) 4 mM $CaCl_2$ and 4 mM ATP; (green) 2 mM $MgCl_2$ and 4 mM ATP; and (yellow) 2 mM $MgCl_2$, 4 mM AMP-PNP. TNP-ADP release was monitored at room temperature by monitoring the decrease in fluorescence with excitation at 415 nm and emission at 550 nm as described in the *Materials and Methods*.

ATP but not hydrolyzed by CF_1 to an appreciable extent, was able to exchange with TNP-ADP bound on CF_1 at a slower rate than ATP.

The time required for 50% of TNP-ADP exchange (T_{50}) was determined and summarized in Table V for all three types of non-fluorescent nucleotides. In the absence of metal ions, ADP and ATP were able to exchange TNP-ADP at a similar rate, which was significantly faster than that of the AMP-PNP induced exchange. Calcium ions were able to facilitate the TNP-ADP exchange with both ATP and AMP-PNP but not with ADP. Sulfite ions greatly stimulated nucleotide exchange in all cases. Higher TNP-ADP exchange rates were observed in the presence of ATP compared to those in the presence of AMP-PNP, indicating that the exchange is dependent upon catalytic turnover.

3. The Redox State of the γ Dithiol Affects TNP-ADP Exchange

The dithiol found in the γ subunit is closely involved in regulating CF_1 function (Richter, Hein et al. 2000). Fully oxidized and reduced enzymes were compared for nucleotide exchange as shown in Figure 24. Neither form of the enzyme could retain TNP-ADP when medium nucleotides were available. The reduced enzyme released bound nucleotides at a faster rate than its oxidized counterpart, correlating with the difference in the rates of hydrolysis activity between the reduced and oxidized enzymes. The T_{50} values of reduced and oxidized enzyme were 108 and 275 seconds, respectively, when CaATP was

Table V. TNP-ADP exchange of the wild type CF_1 ATP synthase[1]

Metal ion[2]	T_{50} (sec) of TNP-ADP exchange[3]		
	ADP	ATP	AMP-PNP
None	433 ± 18	465 ± 14	900 ± 28
Ca^{2+}	350 ± 21	108 ± 4	243 ± 21
Mg^{2+}	218 ± 11	318 ± 18	415 ± 28
$Mg^{2+} + SO_3^{2-}$	108 ± 11	40 ± 14	83 ± 11

[1] Wild type CF_1 was fully reduced by treatment with 20 mM DTT for 30 min at room temperature. Desalted CF_1 was incubated with 1 mM TNP-ADP for two hours resulting in two molecules of TNP-ADP per molecule of CF_1.

[2] Metal ion and nucleotide concentrations were: 4 mM nucleotide; 4 mM $CaCl_2$ and 4 mM nucleotide; 2 mM $MgCl_2$ and 4 mM nucleotide; and 2 mM $MgCl_2$, 4 mM nucleotide and 50 mM Na_2SO_3.

[3] T_{50} (in seconds) represents the time required to exchange 50% of the TNP-ADP from CF_1. All T_{50} numbers were determined from duplicating fluorescence traces (indicated by standard deviations) from which baselines were subtracted using the remaining fluorescence after the complete release of TNP-ADP triggered by trypsin treatment.

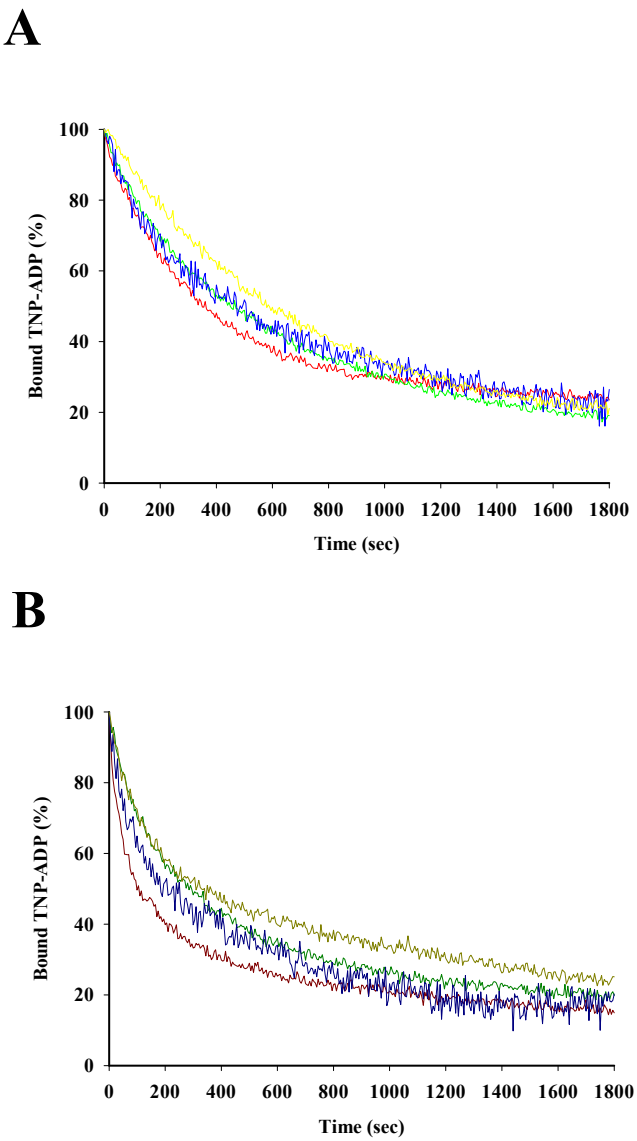

Figure 24. Comparison of TNP-ADP release from oxidized and reduced wild type CF_1. CF_1 was prepared with two molecules of labeled nucleotides per CF_1 as described in the *Materials and Methids*. Prior to the nucleotide loading, the disulfide bond within the γ subunit was oxidized by incubation with 50 μM $CuCl_2$ for 30 min at room temperature or reduced by addition of 20 mM DTT for 30 min at room temperature. **(A)** Mg^{2+} ion (2 mM) induced TNP-ADP release: (red) reduced CF_1 with 4 mM ATP, (green) oxidized CF_1 with 4 mM ATP, (blue) reduced CF_1 with 4 mM AMP-PNP, and (yellow) oxidized CF_1 with 4 mM AMP-PNP. **(B)** Ca^{2+} ion (4 mM) induced TNP-ADP release: (dark red) reduced CF_1 with 4 mM ATP, (dark green) oxidized CF_1 with 4 mM ATP, (dark blue) reduced CF_1 with 4 mM AMP-PNP, and (dark yellow) oxidized CF_1 with 4 mM AMP-PNP. TNP-ADP release was monitored at room temperature by changes in fluorescence with excitation at 415 nm and emission at 550 nm as described in the *Materials and Methods*.

present, and, 318 and 440 seconds in the case of MgATP.

4. TNP-ADP Release from Individual Sites on Wild Type CF_1

CF_1 was incubated with fluorescent TNP-ADP for one minute to selectively load the first binding site. The exchange of TNP-ADP under these conditions was compared to that of CF_1 that had been incubated for two hours resulting in loading of both sites. To estimate the exchange properties of the second, slower filling site, the fluorescence amplitude corresponding to this site was calculated using the data from the first (fast filling) site and those from two sites together. In this approach, assuming independence between the exchange of the two sites, normalized spectra from two sites were multiplied by a factor of two and the resulting relative fluorescence traces were subtracted with those from the first site to yield the normalized fluorescence. The normalized fluorescence amplitude changes should indicate the rate of TNP-ADP release from the second site.

Exchange of TNP-ADP from the first site followed single-exponential decay kinetics indicating a relatively homogeneous process. The T_{50} rate of TNP-ADP exchange in the presence of ATP alone was 575 seconds. A significant acceleration was observed by adding divalent metal ions and a very large increase in the MgATP exchange rate was detected upon addition of the oxyanion sulfite (Figure 25 A). The differences in the initial rates of TNP-ADP

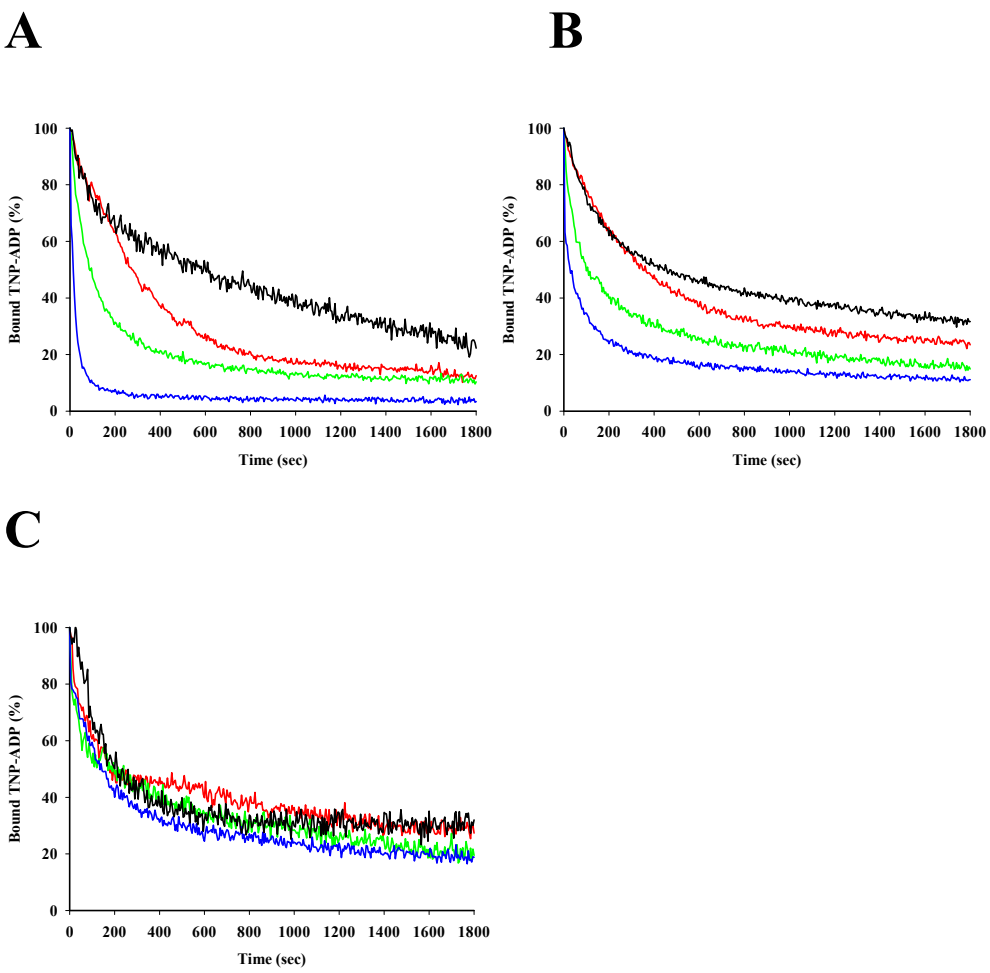

Figure 25. TNP-ADP release from wild type CF_1 induced by ATP nucleotides. Wild type CF_1 was fully reduced by 20 mM DTT for 30 min at room temperature. Desalted CF_1 was loaded with TNP-ADP in **(A)** one site or **(B)** two sites as described in the *Materials and Methods*. Nucleotide exchange in the second site, shown in **(C)**, was calculated by subtracting the exchange of the first site from that of the two sites together. Exchange was triggered by the addition of 4 mM ATP plus: (black) no metal ion, (red) 2 mM $MgCl_2$, (green) 4 mM $CaCl_2$, and (blue) 2 mM $MgCl_2$ and 50 mM Na_2SO_3. TNP-ADP release was monitored at room temperature by changes in fluorescence with excitation at 415 nm and emission at 550 nm as described in the *Materials and Methods*.

exchange mirrored the relative rates of ATP hydrolysis obtained with CF_1 in the presence of different metal ions and sulfite observed in Chapter Three. In contrast, the calculated exchange rates for the second site (Figure 25 C) showed very little variation for the different conditions. Similar results were obtained when AMP-PNP was the medium nucleotide instead of ATP. The second site displayed essentially complete exchange of TNP-ADP with or without Mg^{2+} ions while there was a marked stimulatory effect by the metal ion of TNP-ADP exchange by the first site (Figure 26).

5. ATPase Activities of TNP-ADP – loaded Wild Type CF_1

A coupled assay was carried out to measure ATP hydrolysis by the enzymes in the presence of Mg^{2+} ions. The assay included an ATP–regenerating system to eliminate inhibition of the reaction by the product ADP. The enzyme without TNP-ADP pre–loaded exhibited a classical steady–state behavior following a short lag of approximately 100 seconds (Figure 27) that is thought to be due to exchange of the ADP for ATP in the non-catalytic site (Du and Boyer 1990; Boyer 2002).

Pre-loading the enzyme with TNP-ADP at the first, rapidly exchanging site increased the lag to about 250 seconds. It is known that TNP-ADP binds to CF_1 with a higher affinity than ADP and so a slower rate of exchange under catalytic conditions is expected (Mills and Richter 1991). The steady-state rate of ATP

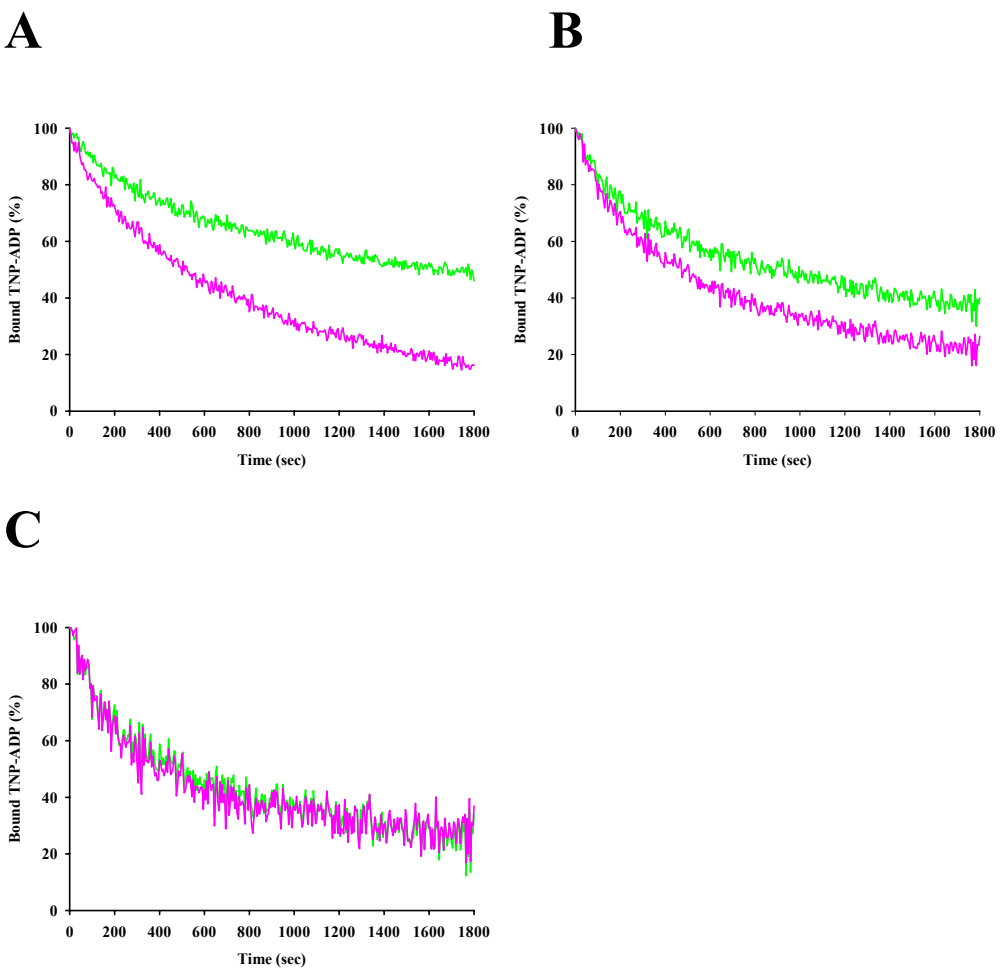

Figure 26. Mg^{2+} ion dependency of TNP-ADP exchange for nonhydrolsable AMP-PNP nucleotide. Wild type CF_1 was fully reduced by 20 mM DTT for 30 min at room temperature. Desalted CF_1 was loaded with TNP-ADP in **(A)** one site or **(B)** two sites as described in the *Materials and Methods*. Nucleotide exchange in the second site, shown in **(C)**, was calculated by subtracting the exchange of the first site from that of the two sites together. Nucleotide exchange was triggered by (green) 4 mM AMP-PNP only or (pink) 2 mM $MgCl_2$ and 4 mM AMP-PNP. TNP-ADP release was monitored at room temperature by changes in fluorescence with excitation at 415 nm and emission at 550 nm as described in the *Materials and Methods*.

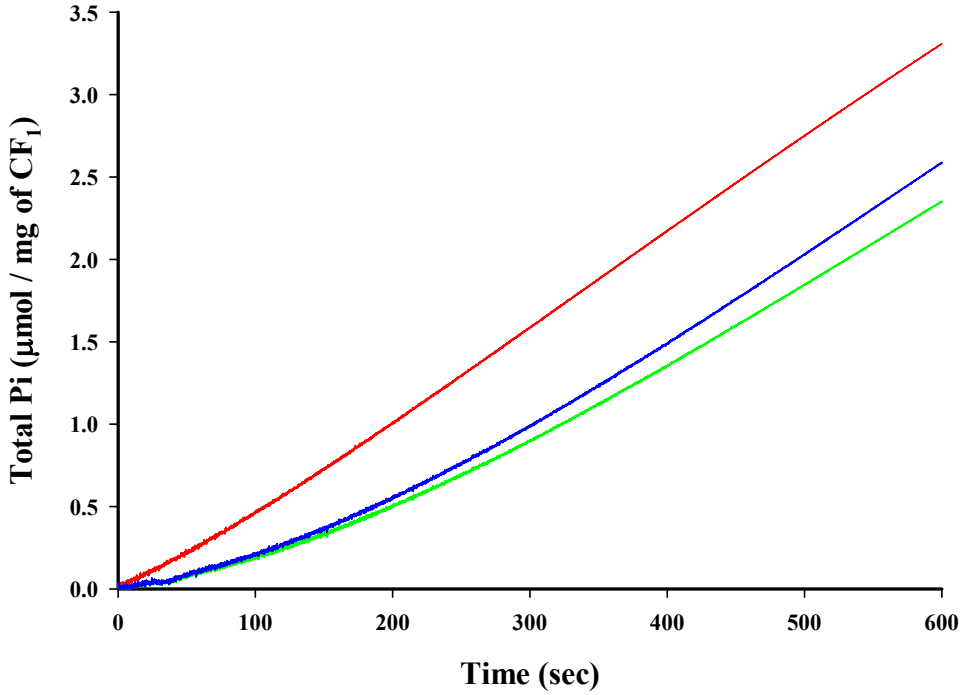

Figure 27. MgATPase activities of CF_1. Wild type CF_1 was fully reduced by 20 mM DTT for 30 min at room temperature. The enzyme was desalted through Sephadex G50 and loaded with TNP-ADP as described in the *Materials and Methods*: (red) no TNP-ADP control, (blue) one molecule of TNP-ADP bound per CF_1, and (green) two molecules of TNP-ADP bound per CF_1. The Mg^{2+} dependent ATP hydrolysis by 100 µg of CF_1 was carried out in a cuvette with a total volume of 1 ml. The assay was coupled with NAD^+ reduction and ATP regeneration as described in the *Materials and Methods*. The total amount of Pi produced was calculated based on change in absorbance of NAD^+ measured at 340 nm.

hydrolysis reached following the lag period was the same as the control. Pre-loading the enzyme with two molecules of TNP-ADP per enzyme did not increase the lag significantly but did slightly decrease (~ 10%) the steady–state rate of hydrolysis (Figure 27).

6. Nucleotide Exchange in the γ Triple Mutant is Altered

The enzyme assembly containing the γR302A,R304A,Q305A triple mutation expressed higher MgATPase activities and showed a major impairment in sulfite stimulation as well as ATP synthesis (chapter three). The nucleotide exchange properties of the triple mutant were examined. As for the wild type enzyme, the mutant was loaded with one molecule of TNP-ADP following a thirty second labeling and two molecules of TNP-ADP after two hours of incubation. The TNP-ADP binding properties were essentially identical to those of wild type CF_1 (not shown).

TNP-ADP release curves shown in Figure 28 indicated a significant difference between the wild type and mutant enzymes. The TNP-ADP exchange from the first site was notably faster than that of the wild type enzyme without addition of sulfite ions. Sulfite significantly increased the exchange in the presence of MgATP. In contrast to the wild type enzyme, however, the exchange of TNP-ADP in the second site was much slower. Only 20% of the TNP-ADP was released after thirty minutes in the presence of calcium

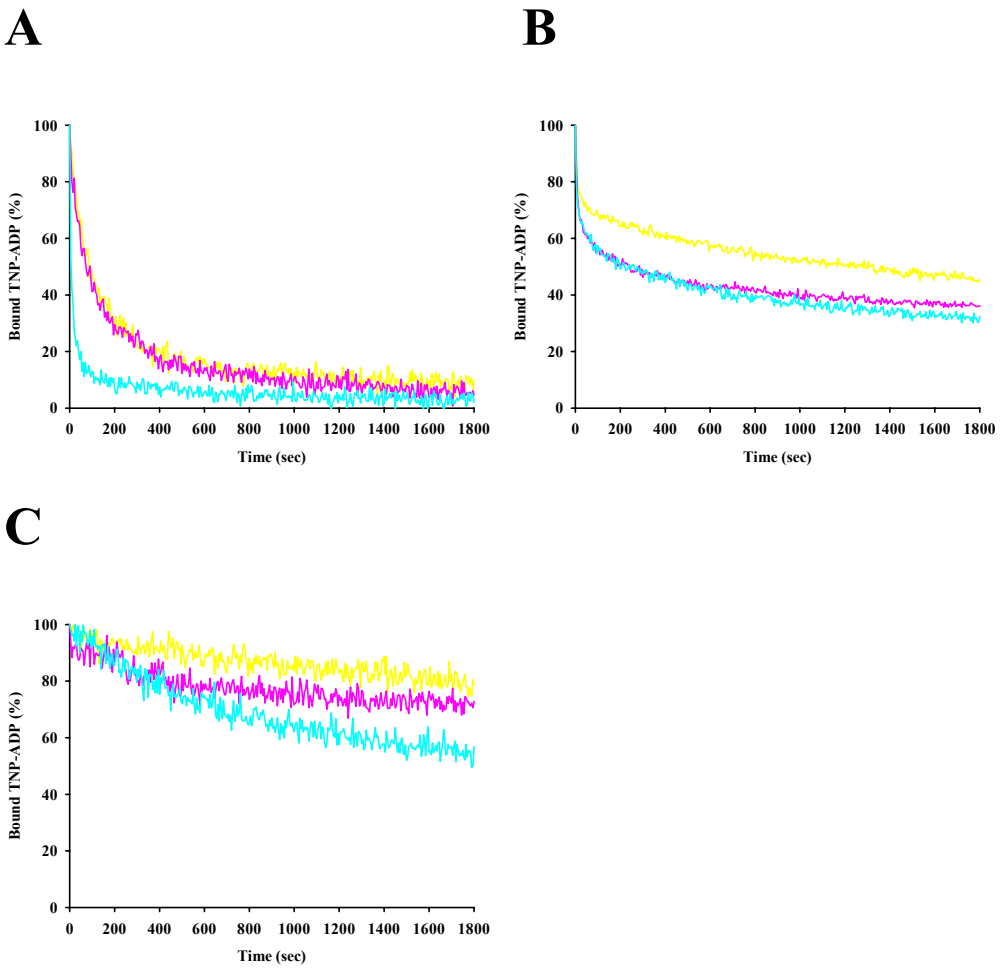

Figure 28. TNP-ADP release from the gamma triple mutant induced by ATP nucleotides. CF_1 containing the γR302A,R304A,Q305A mutation was fully reduced by 20 mM DTT for 30 min at room temperature. The enzyme was desalted and loaded with TNP-ADP in **(A)** one site or **(B)** two sites as described in the *Materials and Methods*. Nucleotide exchange in the second site, shown in **(C)**, was calculated by subtracting the exchange of the first site from that of the two sites together. Exchange was triggered by the addition of 4 mM ATP plus: (yellow) 2 mM $MgCl_2$, (pink) 4 mM $CaCl_2$, and (cyan) 2 mM $MgCl_2$ and 50 mM Na_2SO_3. TNP-ADP release was monitored at room temperature by changes in the fluorescence with excitation at 415 nm and emission at 550 nm as described in the *Materials and Methods*.

or magnesium and ATP (Figure 28 C). Even in the presence of sulfite, which induced 80% of the TNP-ADP to be exchanged in the second site of the wild type enzyme, there was still 60% of the TNP-ADP bound in the mutant after 30 minutes of incubation. The values of T50 in each of the two sites were determined for both wild type and mutant enzymes and are listed in Table VI.

The MgATPase activities of the triple mutant before and after loading with TNP-ADP are shown in Figure 29. The triple mutant displayed faster turnover rates compared to the wild type enzyme in the presence of the ATP regenerating system. When loaded with one molecule of TNP-ADP, the catalysis of the triple mutant showed a very short lag that was similar to the wild type enzyme. In striking contrast, however, the rate of ATP hydrolysis by the triple mutant enzyme with two TNP-ADP molecules bound was approximately 60% lower than that of the enzyme without TNP-ADP bound or with only the first site filled with TNP-ADP (indicated by the slopes of the curves in Figure 29).

D. Discussion

McCarty's and Hammes' research groups have characterized five of six nucleotide binding sites on CF_1 enzyme using fluorescent nucleotide analogs (Carlier and Hammes 1979; Bruist and Hammes 1981; Leckband and Hammes 1988; Shapiro and McCarty 1988; Shapiro and McCarty 1990). Four sites were

Table VI. TNP-ADP exchange in the wild type and the γ triple mutant enzymes[1]

	Wild type T_{50}[3]		Triple mutant T_{50}[3]	
Nucleotide[2]	**First site**	**Second site**	**First site**	**Second site**
CaATP	**90 ± 7**	**193 ± 32**	**78 ± 4**	**> 1800**
MgATP	**290 ± 21**	**168 ± 11**	**80 ± 7**	**> 1800**
MgATP + SO_3^{2-}	**18 ± 4**	**133 ± 18**	**10 ± 7**	**> 1800**

[1] Wild type and the γ 302A,304A,305A mutant CF_1 were fully reduced by treatment with 20 mM DTT for 30 min at room temperature. Desalted CF_1 was incubated with 1 mM TNP-ADP for one minute or two hours resulting in one or two molecules of TNP-ADP bound per molecule of CF_1, respectively.

[2] Metal ion and nucleotide concentrations were: 4 mM $CaCl_2$ and 4 mM ATP; 2 mM $MgCl_2$ and 4 mM ATP; and 2 mM $MgCl_2$, 4 mM ATP and 50 mM Na_2SO_3.

[3] T_{50} (in seconds) represents the time required to exchange 50% of the TNP-ADP from CF_1. All T_{50} numbers were determined from duplicate fluorescence traces (indicated by standard deviations) from which baselines were subtracted using the remaining fluorescence after the complete release of TNP-ADP triggered by trypsin treatment. The fluorescence decrease corresponding to the second, slow filling site was derived from the normalized two-site signal after subtracting the portion contributed by the first site. T_{50} numbers of the exchange in the second site were calculated using the resulting percentage curves.

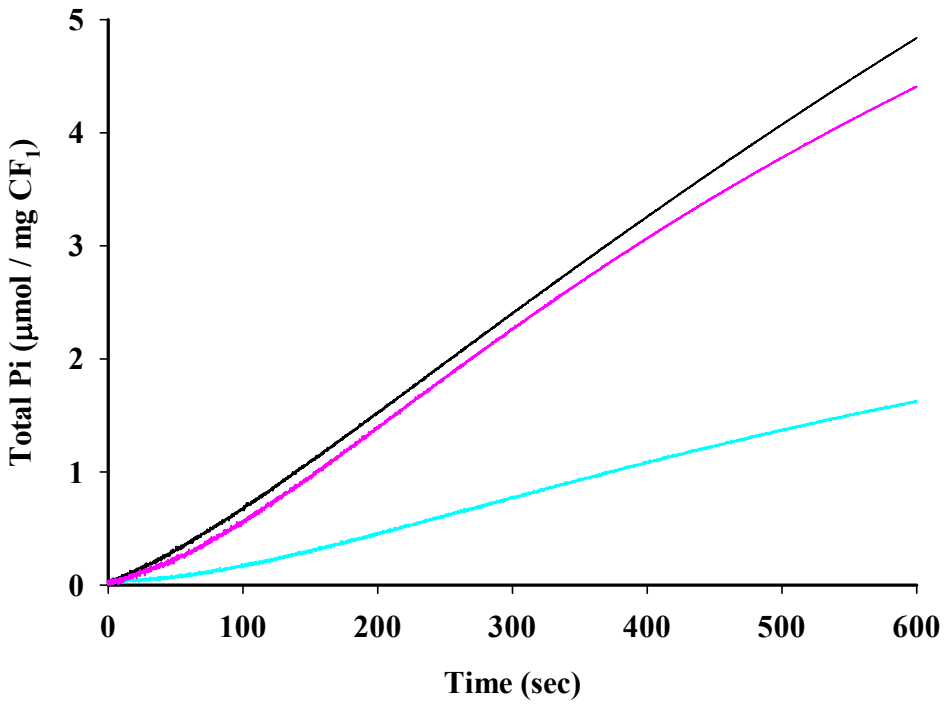

Figure 29. MgATPase activities of the CF_1 gamma triple mutant. CF_1 containing the γR302A,R304A,Q305A triple mutation was fully reduced by 20 mM DTT for 30 min at room temperature. The enzyme was desalted and loaded with TNP-ADP as described in the *Materials and Methods*: (black) no TNP-ADP control, (pink) one molecule of TNP-ADP per CF_1 in the first site, and (cyan) two molecules of TNP-ADP per CF_1. The Mg^{2+} dependent ATP hydrolysis by 100 µg of CF_1 was carried out in a cuvette with a total volume of 1 ml. The assay was coupled with NAD^+ reduction and ATP regeneration as described in the *Materials and Methods*. The total amount of Pi produced was calculated based on change in absorbance of NAD^+ measured at 340 nm.

identified to bind nucleotides tightly when CF_1 was isolated (Bruist and Hammes 1981). Two sites would lose their bound nucleotides once the enzyme was treated with ammonium sulfate and EDTA (Bruist and Hammes 1981; Gao, Lipscomb et al. 1995). The remaining two sites on CF_1 retain tightly bound ADP which can be exchanged with medium ADP in a process that can take more than two hours (Leckband and Hammes 1988; Shapiro, Gibson et al. 1991). It was also suggested that three of the tight binding sites were non-catalytic (Xue, Miller et al. 1987). In this study, isolated CF_1 was shown to bind the fluorescent ADP analog, TNP-ADP, at millimolar concentrations in exchange for ADP at each of the two sites (Figure 21). One of the two sites binds TNP-ADP much faster than the other site under experimental conditions, offering an opportunity to characterize the two sites separately. Oxyanions, such as sulfite, have been observed to stimulate the MgATPase activity of the chloroplast ATP synthase by competing with the enzyme binding MgADP in the catalytic site and facilitating the release of product ADP (Du and Boyer 1990; Malyan 2003). Sulfite was also found to have a stimulatory effect on nucleotide exchange in both catalytic and non-catalytic sites (Malyan and Vitseva 2001).

In the presence of sulfite, ADP was replaced with TNP-ADP at a slightly faster rate than in the absence of sulfite (Figure 21). Up to two nucleotide binding sites were able to bind the fluorescent nucleotide, the same result as seen previously for radioactively labeled ADP (Shapiro and McCarty 1990). Freshly

isolated CF_1 contains between 1.1 and 2.0 molecules of ADP per molecule of enzyme that is associated with one catalytic and one non-catalytic site (Shapiro, Huber et al. 1991; Hu, Mills et al. 1993; Malyan 2006). The ADP is very tightly bound and resists dissociation following multiple desalting steps (Hu, Mills et al. 1993). It is likely that one site is partially emptied following the purification of CF_1 and fills with nucleotide analog very rapidly, which corresponds to the very rapid labeling (less than one minute). The longer loading time is presumably due to the slow exchange between bound ADP and medium TNP-ADP, which has a significantly higher affinity than ADP (Mills and Richter 1991). Both sites which retained ADP after CF_1 isolation were shown to release their nucleotide contents and bind solution nucleotides *in vitro* under certain conditions (Bruist and Hammes 1981). Studies in this chapter show that high concentrations of medium nucleotides are able to exchange with TNP-ADP molecules that are bound on CF_1.

To investigate the nucleotide binding properties of CF_1, the wild type enzyme was loaded with two molecules of TNP-ADP and three types of medium nucleotides were employed in this study: ATP, ADP, and AMP-PNP. Both ADP and AMP-PNP are considered poorly hydrolysable by CF_1. The ADP induced release of TNP-ADP, shown in Figure 22, was accelerated by Ca^{2+} ions and sulfite, which resulted in more than 90% of the dissociation of the bound nucleotide. Ca^{2+} ions are known to bind nucleotides to CF_1 with a lower affinity than Mg^{2+} does (Hochman, Gong et al. 2000). Moreover, MgADP has been found to bind

to both catalytic and non-catalytic sites tightly (Du and Boyer 1990; Malyan 2006) which suggests that the different rates of exchange in the presence of Ca^{2+} or Mg^{2+} ions might be caused by the difference in energy required to dissociate the bound TNP-ADP from the nucleotide binding site(s). Sulfite ions are known to compete with amino acid side chains within the binding sites on CF_1 for ligand binding, resulting in acceleration of the nucleotide release (Malyan 2003). The comparison between ATP and AMP-PNP showed a clear pattern. ATP exchanged with TNP-ADP much faster than AMP-PNP under all experimental conditions (Table V). This indicates that the nucleotide exchange, when two sites on CF_1 are labeled, correlates to the ATP hydrolysis function.

When different labeling conditions were applied, CF_1 could be loaded with either one or two molecules of TNP-ADP depending on the incubation time. It is assumed that the biphasic time dependence for TNP-ADP binding reflects the different properties of two ADP-binding sites on the enzyme. ATP induced nucleotide exchange was monitored in both cases and the results were numerically analyzed to gain information on the behavior of the individual sites (Figure 25). The first site exchanged rapidly when divalent metal ions were present and the exchange was strongly stimulated by sulfite ions (Figure 25 A). The exchange in the second site, however, did not depend on metal ions (Figure 25 C and Table VI). It has been well established that nucleotide exchange in a non-catalytic site does not depend on magnesium while that in a catalytic site is stimulated in the

presence of Mg^{2+} (Cunningham and Cross 1988; Shapiro and McCarty 1988; Milgrom and Boyer 1990; Milgrom, Ehler et al. 1990; Shapiro and McCarty 1990; Shapiro, Gibson et al. 1991; Shapiro, Huber et al. 1991; Murataliev and Boyer 1992). The metal ion is required in this case as a co-substrate for ATP hydrolysis. The TNP-ADP exchange measurements with ATP with and without Mg^{2+} are shown in Figure 26. The results revealed that the two sites might have different identities. The first site appears to have catalytic properties while the second site does not, according to the magnesium dependency.

Wild type CF_1 labeled in both ways (one site and two sites) were examined for MgATPase activity and compared to the enzyme unoccupied by TNP-ADP, shown in Figure 27. Both labeled enzymes showed a lag at the beginning of the catalysis, directly reflecting an activation process observed by Boyer's group (Zhou, Xue et al. 1988; Du and Boyer 1990). The lag was almost identical in both labeling scenarios and was clearly related to the nucleotide exchange in the first site, which is assumed catalytic. Since exchanges in both sites happen simultaneously it is not possible to analyze the sole contribution from the second site to the lag. The ATP hydrolysis rate in unlabeled CF_1 displayed linear, apparent steady-state kinetics. The enzymes with both one and two molecules of TNP-ADP were able to reach the same steady state after the lag, presumably, during which the fluorescent nucleotide at the fast exchange site was released. Calcium dependent ATP hydrolysis could not be examined using the same assay

system which required Mg^{2+} to regenerate ATP.

The γR302A,R304A,Q305A mutation in the CF_1 enzyme resulted in more than three-fold increases in Ca^{2+} and Mg^{2+} dependent ATP hydrolysis activities and a marked impairment in sulfite sensitivity (Table III). Furthermore, CF_1 containing the triple mutation only retained 13% of the ATP synthesis activity of the wild type enzyme (Table IV). These results indicate that the contact is not required for ATP hydrolysis but is important for sulfite-activation and for ATP synthesis. The triple mutant exhibited similar labeling properties to those of wild type CF_1 when exposed to TNP-ADP and could be labeled in one or two sites depending on the incubation time (not shown). The release of bound nucleotides from the CF_1 triple mutant is demonstrated in Figure 28 and T_{50} numbers summarized in Table VI. The nucleotide exchange in the first site, although manifesting a similar single exponential phase, was much more rapid and more complete compared to the wild type enzyme. This further suggests that the first site might be a catalytic site. Surprisingly, the ability of the second site to exchange its nucleotide content was greatly impaired (Figure 28 C). The results suggested that more than 50% of the TNP-ADP was retained in the second site after 30 min of incubation with a high concentration of ATP even when sulfite was present. The CF_1 triple mutant with zero, one or two molecules of TNP-ADP was subjected to MgATPase activity measurement and the results shown in Figure 29. The unlabeled triple mutant displayed higher ATP hydrolysis activity than

the wild type enzyme, agreeing with the results included in Chapter Three. The ATPase activity of the mutant enzyme with one TNP-ADP bound showed a shorter lag time (~ 100 seconds) before it reached the steady state rate. This is probably due to the more rapid exchange in the first site of CF_1. When two sites were occupied with fluorescent nucleotide, the mutant manifested a similar lag but could never reach the same steady state hydrolysis rate under experimental conditions. It has been observed previously that ADP bound to a non-catalytic site is inhibitory to ATP hydrolysis by F_1 enzymes (Melese, Xue et al. 1988; Hisabori and Mochizuki 1993; Malyan 2006). Therefore, the second site is likely to be non-catalytic. The results obtained in this study confirmed that complete activation of ATP hydrolysis by CF_1 requires ATP binding at both catalytic and non-catalytic sites, a property reported previously (Milgrom, Ehler et al. 1990). Further investigations including photo-affinity labeling and mass spectroscopy can be used to directly reveal the catalytic roles of these individual sites.

The γ C-terminal interaction with the anionic loop on β subunit is permanently disrupted in the γR302A,R304A,Q305A mutant. The site directed mutagenesis studies suggested that the contact, though not required for ATP hydrolysis, is essential for ATP synthesis (Chapter Three). According to those results the contact probably plays an important role in proton-driven activation of CF_1. The nucleotide exchange results presented in this chapter further suggest

that the contact is critical for the communication between catalytic and non-catalytic sites. Studies showed that CF_1 undergoes conformational changes among nucleotide binding sites that allow a site to switch properties between tight and loose (Strotmann, Bickel-Sandkotter et al. 1979; Shapiro and McCarty 1990; Weber, Wilke-Mounts et al. 1993). The disruption of the contact between the C terminus of the γ subunit and the anionic loops on α and β subunits may affect the disability of the enzyme to communicate between the non-catalytic site(s) and the catalytic site(s). The establishment of the contact functions as a bridge to propagate the conformational changes in the nucleotide binding sites. In the direction of ATP hydrolysis, the triple mutant retains TNP-ADP, which has very high binding affinity to CF_1, in a non-catalytic site, partially inhibiting the cooperative catalysis. In the case of ATP synthesis, it has been suggested that in the presence of the proton gradient, the catalytic sites have to switch properties in order for bound MgADP not to escape (Boyer 1993; Weber and Senior 2000). It is also know that the non-catalytic sites have to exchange their nucleotide contents during MgADP recruitment, that is, releasing the inhibitory ADP and binding ATP (McCarty 2005). The results shown in this chapter suggest that the contact is important for the nucleotide exchange of the non-catalytic sites upon activation of the latent form of the enzyme. Since all the nucleotide exchange experiments were performed in the absence of the proton gradient, further studies with the membrane-associated form of CF_1 are required to reveal the true physiological

role(s) of the contact.

Chapter Five

Conclusions

This study has focused on the C-terminal region of the γ subunit of the ATP synthase that has been identified to form a "catch" interaction with an anionic loop on one of the three β subunits within the αβ hexamer in the crystal structure of the mitochondrial F_1 enzyme (Abrahams, Leslie et al. 1994). There are two highly conserved amino acid residues in this region, an arginine and a glutamine (Arg304 and Gln 305 in CF_1). Homology modeling studies also suggested that Arg302 of the CF_1 γ subunit makes a close contact with the anionic loop on an adjacent α subunit (Figure 12). These three residues were substituted both one at a time and simultaneously to leucines or alanines using site-directed mutagenesis. The resulting CF_1 γ mutants were assembled with α and β subunits from *Rhodospirillum rubrum* F_1 into a hybrid photosynthetic F_1 that was shown to carry out both MgATPase and CaATPase activities and ATP-dependent γ rotation (Tucker, Schwarz et al. 2004).

All single mutations in the γ subunit increased both the Mg^{2+}- and Ca^{2+}-dependent ATP hydrolysis activities with the exception of Arg304Leu that had only about 50% of the wild type CaATPase activity, while the double and triple mutations greatly impaired ATP hydrolysis (Table II). In addition, any mutants that had a substitution at Gln305 abolished the well known sulfite stimulatory

effect. These results indicate that the catch differentially affects the Mg^{2+} and Ca^{2+}-dependent processes and that it is not essential for F_1 ATPase activities. The hybrid enzymes displayed higher catalytic turnover rates than wild type homogeneous CF_1, probably caused by small difference in energy requirements as a result of the inhomogeneous interactions between the γ and the αβ hexamer during rotational catalysis. The interactions were further reduced by single mutations within the C-terminal catch region on the γ subunit, increasing ATPase activities even more. Combinations of mutations, however, affected the interactions such that catalysis became significantly inhibited.

To examine the effects of the mutations on ATP synthesis, the γ mutants were assembled with the native CF_1 α and β subunits. For comparison, the ATP hydrolysis studies described for the hybrid enzyme were repeated with the homogeneous enzyme. A general trend was observed between the two systems in that the mutants tended to show similar or increased CaATPase activities as well as stimulated MgATPase activities (Table III). The sulfite stimulation, on the other hand, was significantly reduced in all mutant assemblies. In combination with the hybrid enzyme studies, these results clearly show that the catch is not required for ATP hydrolysis *in vitro*. The catch is, however, necessary for the activation of the enzyme by oxyanions as suggested by the MgATPase assays in the presence of sulfite. Further evidence was collected from studies using tentoxin, a potent phytotoxic inhibitor that specifically affects

the cooperativity of CF_1. The tentoxin inhibition was not affected by the mutations in the case of Ca^{2+}-depedent ATP hydrolysis but was noticeably impaired in the presence of MgATP with or without sulfite (Figures 18 and 19). The simultaneous substitution of Arg304 and Gln305 completely abolished the tentoxin effect in the absence of sulfite ions. One possible interpretation of this effect is that the exchange of a tightly bound ADP is either blocked or greatly reduced by the mutations which results in a loss of cooperativity (He, Samra et al. 2008).

The assembled enzymes containing γ mutants were also reconstituted with the native ε and δ subunits isolated from CF_1, and then the reconstituted complex was reconstituted with thylakoid membranes from which the native CF_1 had been stripped. All membranes reconstituted with the mutant enzymes showed a decrease in their ability to maintain a light-induced transmembrane proton gradient and the amount of ATP synthesis was consequently decreased. The effect was greatest in the triple mutant which exhibited a ~80% decrease in photophosphorylation. These effects suggest a critical role of the γ catch residues in photophosphorylation.

TNP-ADP was used to study the nucleotide binding and exchange of CF_1. The results in Figure 21 clearly suggest two binding sites on CF_1, a fast filling site (~ 1 min) and a slow filling site (~ 2 hr). Wild type CF_1 with either one or two molecules of TNP-ADP bound was incubated with non-fluorescent nucleotides

and the nucleotide exchange within individual sites was determined. The exchange within the fast filling site was stimulated in the presence of Mg^{2+} ions while that in the slow filling site was not affected, suggesting that the two sites have different properties and the fast filling site is probably catalytic and the slow filling site non-catalytic (Cunningham and Cross 1988; Milgrom and Boyer 1990; Milgrom, Ehler et al. 1990). The triple mutant containing substitutions at all three locations (Arg302, Arg304 and Gln305) was loaded with one or two molecules of TNP-ADP using the same method and a comparison of the T_{50} values between wild type and mutant CF_1 is summarized in Table VI. The mutant displayed significantly faster TNP-ADP exchange from the catalytic site, in agreement with the higher ATP hydrolysis rates compared to those of the wild type. In marked contrast, the slow filling site on mutant CF_1 retained more than 60% of the TNP-ADP after 30 minutes even in the presence of sulfite ions. In parallel with the dramatically reduced exchange at this site, TNP-ADP present in the slow filling site also reduced the MgATPase activity to about 40% that of the control without TNP-ADP bound. This could be attributed to the inability of achieving full activation which requires the binding of ATP to the non-catalytic sites (Milgrom, Ehler et al. 1990).

In summary, the mutational studies within the C-terminal region of the CF_1 γ subunit show that the catch between the γ and $\alpha\beta$ ring is not essential for ATP hydrolysis in both hybrid and native systems. Although rotational experiments

with the hybrid enzymes containing γ mutants were not included, recent studies with the thermophilic bacterial F_1 have shown that the catch is not required for ATP-driven rotation (Hossain, Furuike et al. 2006). The proton coupling and ATP synthesis results indicate that the catch is, instead, important for photophosphorylation by CF_1. The nucleotide exchange results further suggest that the catch is probably involved in activation of the enzyme from its latent state , as well as in the recruitment of MgADP (i.e. the very tight binding of MgADP) in the presence of an overwhelming concentration of ATP.

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