ADP−Fluoroaluminate Complexes Are Formed Cooperatively at Two Catalytic Sites of Wild-Type and Mutant $\alpha_3\beta_3\gamma$ Subcomplexes of the F_1-ATPase from the Thermophilic *Bacillus* PS3[†]

Chao Dou,[‡] Neil B. Grodsky,[‡] Tadashi Matsui,[§] Masasuke Yoshida,[§] and William S. Allison*,[‡]

Department of Chemistry & Biochemistry, School of Medicine, University of California at San Diego, La Jolla, California 92093, and Research Laboratory of Resource Utilization, R-1, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226, Japan

Received September 17, 1996; Revised Manuscript Received December 20, 1996[⊗]

ABSTRACT: Addition of Al^{3+} and F^- to the $\alpha_3\beta_3\gamma$ subcomplex of the TF_1-ATPase containing MgADP in one catalytic site causes slow, complete inactivation as the ADP−fluoroaluminate complex is formed. This conflicts with the "bisite" stochastic model suggested earlier (Issartel, J. P., Dupuis, A., Lunardi, J. & Vignais, P. V. (1991) *Biochemistry 30,* 4726−4733] on the finding that complete inactivation of the bovine mitochondrial F_1-ATPase by Al^{3+}, F^-, Mg^{2+}, and excess ADP occurs as ADP−fluoroaluminate complexes form in two catalytic sites. When Al^{3+} and F^- were added to $\alpha_3\beta_3\gamma$ containing MgADP in two catalytic sites, inactivation accelerated 8-fold, indicating catalytic to catalytic site cooperativity. When added to $\alpha_3\beta_3\gamma$ containing MgADP bound to one or two catalytic sites prior to addition of Al^{3+} and F^-, phosphate inhibits formation of the ADP−fluoroaluminate complex. When introduced after adding 200 μM ADP plus Mg^{2+} to $\alpha_3\beta_3\gamma$, but before adding Al^{3+} and F^-, phosphate accelerated formation of the ADP−fluoroaluminate complex 3-fold. Sulfite accelerated formation of the ADP−fluoroaluminate complex 9-fold when 200 μM ADP plus Mg^{2+} was added to $\alpha_3\beta_3\gamma$ before adding Al^{3+} and F^-. The accelerations induced by phosphate or sulfite in the presence of excess ADP and Mg^{2+} suggest noncatalytic to catalytic site cooperativity. When Al^{3+} and F^- were added to the $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplex containing MgADP in a single catalytic site, the ADP−fluoroaluminate complex formed at least 10-fold more slowly than observed with wild-type under the same conditions. Therefore, the catalytic site containing MgADP recognizes the $\alpha D_{261}N$ substitution when noncatalytic sites are empty. Cross-linking α to γ or β to γ by oxidizing the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes, respectively, abolishes cooperative formation of ADP−fluoroaluminate complexes in two catalytic sites. ADP−fluoroaluminate complex formation is restricted to a single catalytic site in the oxidized double mutants. The $\alpha_3\beta_3\delta$ subcomplex does not form an inhibitory ADP−fluoroaluminate complex under any of the conditions examined for the $\alpha_3\beta_3\gamma$ subcomplexes.

The F_1-ATPase is the peripheral membrane component of the F_oF_1-ATP synthases. It is comprised of five different subunits which are present in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. Isolated F_1 functions only as an ATPase (Pedersen & Amzel, 1993). The $\alpha_3\beta_3\gamma$ subcomplex of the TF_1-ATPase[1] from the thermophilic *Bacillus* PS3 has essentially the same catalytic characteristics as the complete enzyme (Yokoyama et al., 1989; Paik et al., 1993; Jault et al., 1995, 1996). A plasmid bearing the genes encoding the α, β, and γ subunits of TF_1 can be overexpressed in an *unc*$^-$ strain of *E. coli* to produce the assembled $\alpha_3\beta_3\gamma$ complex. This allows purification of the expressed wild-type and mutant subcomplexes in very high yield from host cell lysates (Matsui & Yoshida, 1995). Comparison of the catalytic properties of the wild-type and mutant $\alpha_3\beta_3\gamma$ subcomplexes has proved to be a useful method for examining structure−function relationships in the F_1-ATPase (Jault et al., 1995, 1996).

The F_1-ATPases contain six nucleotide binding sites. Three are catalytic sites which are predominantly on β subunits, whereas the others are noncatalytic sites which are predominantly on α subunits. The overall topologies of the catalytic and noncatalytic sites are very similar in the crystal structure of MF_1 (Abrahams et al., 1994). Among the residues comprising the nucleotide binding sites are two signature sequences known as the Walker A and B motifs (Walker et al., 1982) which are common to proteins that hydrolyze nucleoside triphosphates (Saraste et al., 1990; Amano et al., 1994). In common with the G proteins (Sternweis & Gilman, 1982), the F_1-ATPases form stable fluoroaluminum and fluoroberyllium complexes with ADP when incubated with ADP rather than GDP in the presence of Mg^{2+}, Al^{3+} or Be^{2+}, and F^- (Lunardi et al., 1988). The ADP−fluorometal complexes bound to F_1 are inactive as ATPases. Issartel et al. (1991) reported that full inactivation of MF_1 is accompanied by formation of 2 mol of ADP−fluorometal complex per mole of enzyme. From this

[†] This study was supported by Grant GM-16974 from the U.S. Public Health Service to W.S.A.

[‡] University of California at San Diego.

[§] Tokyo Institute of Technology.

[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1997.

[1] Abbreviations: TF_1, MF_1, and CF_1, F_1-ATPases from the thermophilic *Bacillus* PS3, bovine heart mitochondria, and spinach chloroplasts, respectively; nd-MF_1, MF_1 depleted of endogenous nucleotides; BSA, bovine serum albumin; CDTA, *trans*-1.2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; HPLC, high-performance liquid chromatography; LDAO, lauryldimethylamine oxide; SDS, sodium dodecyl sulfate; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

S0006-2960(96)02353-7 CCC: $14.00

observation, they concluded that the enzyme hydrolyzes ATP by a stochastic process in which the catalytic sites participate cooperatively as interactive pairs. In contrast, modification of a single catalytic site of F_1-ATPases with Nbf-Cl (Ferguson et al., 1975; Andrews et al., 1984), FSBI (Bullough et al., 1986), or 2-N_3-ADP (Milgrom & Boyer, 1990; Chernyak & Cross, 1992) is sufficient to abolish ATPase activity completely. Furthermore, after loading a single catalytic site of MF_1 (Drobinskaya et al., 1985; Jault & Allison, 1993) or TF_1 (Jault et al., 1995) with MgADP, the enzymes are initially inactive when injected into assay medium containing ATP and then slowly reactivate as ATP binds to noncatalytic sites. To provide an explanation for the apparent discrepancy between the reported observation of Issartel et al. (1991) that complete inactivation of MF_1 occurs on formation of ADP−fluoroaluminate complexes in two catalytic sites, whereas other inactivations proceed with modification of a single catalytic site, the rates of inactivation of the wild-type and mutant $\alpha_3\beta_3\gamma$ subcomplexes of TF_1 have been examined in the presence of ADP, Mg^{2+}, Al^{3+}, and F^- under a variety of conditions. The results of this study clearly show that slow, but complete, inactivation is observed when Al^{3+}, and F^- are added to wild-type and mutant $\alpha_3\beta_3\gamma$ subcomplexes containing MgADP bound to a single catalytic site. However, when two catalytic sites are filled with MgADP, the enzyme subcomplexes are inactivated rapidly, forming ADP−fluoroaluminate complexes in two catalytic sites.

EXPERIMENTAL PROCEDURES

Materials. Biochemicals used in the assay and buffers were purchased from Sigma. The [^{3}H]ADP was supplied by Du Pont New England Nuclear. The 2-chloro[^{3}H]-adenosine used to synthesize 2-N_3-[^{3}H]AD(T)P was from Moravek Biochemicals. Synthesis of 2-N_3-[^{3}H]ADP was carried out as previously described (Jault & Allison, 1994). Sodium fluoride was obtained from Aldrich, and solutions of it were prepared and stored in plastic containers. $AlCl_3$ was obtained from Fisher Scientific. The oligonucleotides used for mutagenesis were purchased from Gibco BRL.

The wild-type $\alpha_3\beta_3\gamma$ subcomplex and the mutant $(\alpha D_{261}N)_3\beta_3\gamma$, $\alpha_3(\beta T_{165}S)_3\gamma$, $\alpha_3(\beta E_{190}Q)_3\gamma$, $a_3(\beta Y_{341}W)_3\gamma$, $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$, and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes were prepared after expression of the wild-type and mutant plasmids in an unc$^-$ strain of *E. coli* according to Matsui and Yoshida (1995). The $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ mutant subcomplex was prepared as described by Matsui et al. (1996). The purified proteins were stored as suspensions in 70% saturated ammonium sulfate at 4 °C. The composition and purity of the subcomplexes were assessed by SDS−PAGE. Stock solutions of the subcomplexes were prepared by removing them from the ammonium sulfate suspensions by centrifugation and dissolving the pellets in 50 mM Tris-HCl, pH 8.0, containing 1 mM CDTA. After incubating at least 30 min at room temperature to allow chelation of Mg^{2+}, the solutions were passed through 1 mL centrifuge columns of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8.0. After treatment with CDTA, the enzyme preparations were essentially free of endogenous nucleotides as assessed by HPLC.

Nucleotide-depleted MF_1 was prepared by gel permeation chromatography in the presence of 50% glycerol (v/v) as described by Garrett and Penefsky (1975). After chromatography, protein fractions with an A_{280}/A_{260} greater than 1.95 were combined and stored at room temperature in 100 mM Tris−SO_4, pH 8.0, containing 4 mM EDTA and 50% glycerol (v/v). When submitted to nucleotide analysis by HPLC as previously described (Bullough et al., 1988), the nd-MF_1 used in this study was free of bound ADP. Before use, the 4 mg/mL stock solution of nd-MF_1 in 50% glycerol was diluted 8 times with deionized water.

Methods. Site-directed mutagenesis was performed as described by Kunkel et al. (1991). The $\alpha D_{261}N$ and $\beta T_{165}S$ mutants were prepared as described previously (Jault et al., 1995, 1996). The oligonucleotides used to introduce the $\beta E_{190}Q$ and $\beta Y_{341}W$ substitutions were, respectively: 5′-TC-GCG-TGT-CCG-TTG-GCC-AAC-GCC-AGC-AAA-G-3′ which contained a new site for *Bal*I; and 5′-AG-CGG-GTC-AAC-TGC-AGG-CCA-AAT-CCC-CAT-CT-3′ which contained a new site for *Pst*I. The restriction sites incorporated into the oligonucleotides allowed facile screening of the mutants. Restriction mapping showed that the constructed plasmids contained the desired substitutions. This was confirmed by DNA sequencing (Prober et al., 1987).

Construction of $\alpha A_{396}C/\gamma A_{22}C$ and $\beta D_{390}C/\gamma S_{90}C$ double mutants involved the following. The oligonucleotides for the $\alpha A_{396}C$, $\gamma A_{22}C$, βD390C, and γS90C substitutions were, respectively: 5′-GCC-GAA-TTG-GCA-GAA-GGC-CTC-GAG-CTC-ACG-3′ containing a new site for *Stu*I; 5′-GAC-CAT-TTC-CAT-GCA-TTT-TGT-AAT-TTG-G-3′ containing a new site for *Nsi*I, 5′-GTC TTC-ATC-CGA-GAG-CTC-ACA-CAT-CCC-CAA-G-3′ containing a new site for *Sac*I; and 5′-TTG-GTA-CAC-GAG-TCT-TAA-GAC-GTT-GCA-GTT-GTA-CGC-3′ containing a new site for *Afl*II. The mutated $\alpha A_{396}C$ gene fragment, $\gamma A_{22}C$ gene fragment, $\beta D_{390}C$ gene fragment, and $\gamma S_{90}C$ gene fragment were removed with restriction enzymes from the mutated pTD-$\alpha\gamma\beta$ plasmids and were then ligated to the pKK-$\alpha\gamma\beta$ plasmid from which the wild-type α and γ or the wild-type β and γ gene fragments were deleted. Restriction mapping showed that the resulting plasmids contained the appropriate substitutions. This was confirmed for each mutation by DNA sequencing (Prober et al., 1987).

Protein concentrations were determined by the method of Bradford (1976). Binding of [^{3}H]ADP and [^{3}H]ADP·AlF_4 to the wild-type $\alpha_3\beta_3\gamma$ and mutant complexes was carried out with the use of 1 mL centrifuge columns of Sephadex G-50 (Penefsky, 1977) as previously described (Jault & Allison, 1993). Radioactivity was detected with a Packard 1600TR counter using Ecoscint from National Diagnostics. Assignment of nucleotide binding to catalytic or noncatalytic sites was made by submitting tryptic digests of the enzyme complexes photolabeled by 2-N_3-ADP·AlF_4 to HPLC as described previously for TF_1 (Jault et al., 1994).

The rates of conversion of the reversibly inhibited subcomplexes containing MgADP in catalytic sites to irreversibly inhibited subcomplexes with ADP−fluoroaluminate complexes bound to catalytic sites were determined as follows. The enzyme samples were assayed at pH 8.0 and 30 °C with 2 mM ATP in the regeneration system previously described (Jault & Allison, 1994) that couples NADH oxidation monitored spectrophotometrically to ATP hydrolysis. When a control sample containing bound MgADP in one or more catalytic sites, but in the absence of Al^{3+} and F^-, is submitted to this assay, the spectrophotometer trace shows a short lag which accelerates to a final, constant rate within 1 min (Jault et al., 1995, 1996). The final rate of the

control represents v_0. At any time after adding Al^{3+} and F^- to the samples containing MgADP in catalytic sites, the final rate v, is directly proportional to the amount of reversibly inhibited enzyme remaining. The rate constants for inactivation were calculated from semilogarithmic plots of v/v_0 vs time by the method of Guggenheim (1926).

The oxidized forms of the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes were prepared and assayed in the following manner. To initiate oxidation, *o*-iodosobenzoate was added to 100 μL of the CDTA-treated $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ mutant subcomplex at 1 mg/mL in 50 mM Tris-HCl, pH 8.0, to a final concentration of 1 mM. The resulting solution was incubated for 30 min at 23 °C, at which time it was passed through a 1 mL centrifuge column of Sephadex G50 equilibrated with 50 mM Tris-HCl, pH 8.0. Oxidation of the $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ mutant subcomplex was initiated by adding $CuCl_2$ to a final concentration of 100 μM to100 μL of the subcomplex at 1 mg/mL in 50 mM Tris-HCl, pH 8.0. After incubating for 16 h at 4 °C, the reaction mixture was passed through a 1 mL centrifuge column of Sephadex G50 which was equilibrated with 50 mM Tris-HCl, pH 8.0. After oxidation, the ATPase activity of both double mutants was inactivated by greater than 98%.

RESULTS

Formation of the ADP−Fluoroaluminate Complex at a Single Catalytic Site Is Sufficient for Complete Inactivation of both the Wild-Type $\alpha_3\beta_3\gamma$ Subcomplex of TF_1 and Nucleotide-Depleted MF_1. When MF_1, TF_1, and the wild-type TF_1 $\alpha_3\beta_3\gamma$ subcomplex containing MgADP in a single catalytic site are assayed with low concentrations of ATP in the presence of a regenerating system, they are initially inactive and then slowly activate as ATP binds to noncatalytic sites (Drobinskaya et al., 1985; Jault & Allison, 1993; Jault et al., 1995). Therefore, it was of interest to determine if the reversibly inhibited wild-type $\alpha_3\beta_3\gamma$ subcomplex containing MgADP in a single catalytic site is converted to the irreversibly inhibited ADP−fluoroaluminate complex when $AlCl_3$ and NaF are added to it. Figure 1A shows that after incubating 3 μM wild-type TF_1 subcomplex with 3 μM [^{3}H]ADP and 2 mM $MgCl_2$ for 1 h followed by addition of 200 μM $AlCl_3$ and 5 mM NaF, the ATPase activity was inactivated by more than 90% in 6 h ($k_{inact} = 7.0 \times 10^{-3}$ min^{-1}) when assays were conducted with 2 mM ATP. This was accompanied by incorporation of 0.9 mol of [^{3}H]ADP per mole of subcomplex which was presumably present as the ADP−fluoroaluminate complex. From these results, it is concluded that formation of the ADP−fluoroaluminate complex in a single catalytic site of F_1 is sufficient for complete inactivation of the enzyme. The same results were obtained when $AlCl_3$ and NaF were added to MF_1 containing MgADP in a single catalytic site as illustrated in Figure 1B. Comparison of Figure 1A and 1B shows that MF_1 containing MgADP in a single catalytic site is inactivated 4 times more rapidly ($k_{inact} = 2.9 \times 10^{-2}$ min^{-1}) than the TF_1 $\alpha_3\beta_3\gamma$ subcomplex containing MgADP in a single catalytic site when $AlCl_3$ and NaF are added to the preloaded enzymes. Figure 1 also shows that incubation of the wild-type $\alpha_3\beta_3\gamma$ subcomplex of TF_1 or nd-MF_1 with 200 μM ADP and 2 mM Mg^{2+} before adding 200 μM $AlCl_3$ and 5 mM NaF led to a greatly enhanced rate of inactivation (closed triangles). The first-order rate constants for the inactivations under these conditions were as follows: wild-type $\alpha_3\beta_3\gamma$, 5.8×10^{-2} min^{-1}; nd-MF_1, 1.4×10^{-1} min^{-1}.

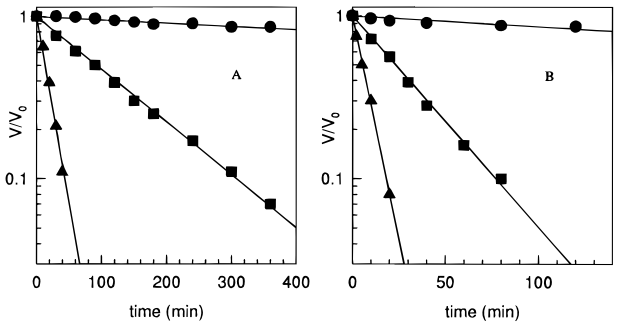


FIGURE 1: Addition of $AlCl_3$ and NaF to F_1 containing MgADP in a single catalytic site of the wild-type $\alpha_3\beta_3\gamma$ subcomplex or nd-MF_1 leads to complete inactivation. (A) The wild-type $\alpha_3\beta_3\gamma$ subcomplex at 1.0 mg/mL in 100 μL of 50 mM Tris-HCl, pH 8.0, was incubated with 3.0 μM [^{3}H]ADP plus 2 mM $MgCl_2$ for 1 h at room temperature. Then NaF and $AlCl_3$ were added to final concentrations of 5 mM and 200 μM, respectively. The reaction mixture was incubated at 30 °C. At the times indicated, 5 μL samples were withdrawn and assayed. The remaining solution was passed through a 1 mL centrifuge column of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8.0, containing 2 mM $MgCl_2$. The moles of bound [^{3}H]ADP per mole of subcomplex was 0.92. The control (●) was treated identically except that [^{3}H]ADP was not included in the first incubation. Another inactivation mixture containing excess ADP was prepared by incubating the subcomplex with 200 μM ADP and 2 mM Mg^{2+} for 30 min at 30 °C before adding $AlCl_3$ and NaF to final concentrations of 5 mM and 200 μM, respectively. The reaction mixture was then incubated at 30 °C. At the times indicated (closed triangles), 5 μL samples were removed and assayed. (B) A stock solution of nd-MF_1 in 100 mM Tris-HCl, pH 8.0, containing 4 mM EDTA and 50% glycerol was diluted 8 times with deionized water. Then $MgCl_2$ and [^{3}H]-ADP were added to final concentrations of 2.5 mM and 0.7 μM, respectively, bringing the ratio of [^{3}H]ADP to nd-MF_1 to 1:1. This solution (stoichiometric reaction mixture) was then incubated for 1 h at room temperature before adding NaF and $AlCl_3$ to final concentrations of 5 mM and 200 μM, respectively. Another inactivation mixture containing excess ADP was prepared by incubating nd-MF_1 with 200 μM ADP and 2 mM Mg^{2+} for 30 min at 30 °C before adding $AlCl_3$ and NaF to final concentrations of 5 mM and 200 μM, respectively. The inactivation mixtures were incubated at 30 °C. At the times indicated, 2 μl samples were withdrawn and assayed with 2 mM ATP. The symbols represent: (■), stochiometric ADP; (▲), 200 μM ADP; and (●), a control reaction mixture treated identically except that NaF was not added.

Table 1: Comparison of the Rates of ATP Hydrolysis and Rates of Formation of the ADP−Fluoroaluminate Complexes Catalyzed by the Wild-Type and Mutant $\alpha_3\beta_3\gamma$ Subcomplexes[a]

subcomplex	specific activity (μmol of ATP min^{-1} mg^{-1})	k_{inact}(MgADP) (min^{-1})
wild-type	18	5.8×10^{-2}
$\alpha D_{261}N$	6	4.6×10^{-3}
$\beta T_{165}S$	80	7.0×10^{-2}
$\beta Y_{341}W$	27	1.4×10^{-1}

[a] The pseudo-first-order rate constants were determined by the method of Guggenheim (1926) from the inactivation of the mutant and wild-type TF_1 subcomplexes with 200 μM ADP, 2 mM Mg^{2+}, 200 μM $AlCl_3$, and 5 mM NaF essentially as described in the legend of Figure 2.

Comparison of the Rates of Formation of the Inhibitory Fluoroaluminate Complex by the Wild-Type $\alpha_3\beta_3\gamma$ Subcomplex and the $\alpha D_{261}N$, $\beta T_{165}S$, and $\beta Y_{341}W$ Mutant Subcomplexes in the Presence of 200 μM ADP. Table 1 compares the first-order rate constants obtained for inactivations initiated by addition of 200 μM $AlCl_3$ and 5 mM NaF to the wild-type and mutant subcomplexes after incubation with 200 μM ADP and 2 mM Mg^{2+} for 30 min. Compared to wild-type, each mutant has altered capacity to entrap

inhibitory MgADP in a catalytic site during turnover when noncatalytic sites are not saturated with ATP. This is reflected by differences in specific activity. The $\alpha D_{261}N$ mutant, which does not release inhibitory MgADP from a catalytic site when it binds ATP to noncatalytic sites (Jault et al., 1995), has the lowest specific activity (6 μmol of ATP hydrolyzed per mg per min) and also exhibits the slowest rate of formation of the inhibitory ADP−fluoroaluminate complex. The $\beta T_{165}S$ mutant, which does not entrap inhibitory MgADP in a catalytic site during ATP hydrolysis, has a specific activity of 80 μmol of ATP hydrolyzed mg^{-1} min^{-1} (Jault et al., 1996) and forms the ADP−fluoroaluminate complex faster than the wild-type complex. Surprisingly, the $\beta Y_{341}W$ mutant, which corresponds to the $\beta Y_{331}W$ mutant of *E. coli* F_1 examined extensively by Weber et al. (1993, 1994; Weber & Senior, 1996a), which has a specific activity of 27 μmol of ATP hydrolyzed mg^{-1} min^{-1} formed the ADP−fluoroaluminate complex with the highest rate under all conditions examined. The increased specific activity of the $\beta Y_{341}W$ mutant subcomplex over that of the wild-type subcomplex (18 μmol of ATP hydrolyzed mg^{-1} min^{-1}) reflects decreased propensity of the mutant subcomplex to entrap inhibitory MgADP in a catalytic site during turnover, but to a substantially lesser extent than observed for the $\beta T_{165}S$ mutant (C. Dou, unpublished experiments). Clearly, the rates of formation of the fluoroaluminate complexes in the mutant and wild-type complexes do not correlate well with their capacities to entrap inhibitory MgADP in a catalytic site.

Issartel et al. (1991) reported that complete inactivation of MF_1 with Al^{3+} and F^- in the presence of excess ADP plus Mg^{2+} correlates with formation of fluoroaluminate complexes in two catalytic sites. To examine the stoichiometry of incorporation of [^{3}H]ADP during inactivation with excess [^{3}H]ADP present, the wild-type and $\alpha D_{261}N$, $\beta T_{165}S$, and $\beta Y_{341}W$ mutant subcomplexes were incubated with 100 μM [^{3}H]ADP for 30 min in the presence of 2 mM Mg^{2+}, at which time 200 μM $AlCl_3$ and 5 mM NaF were added to initiate inactivations. When inactivated by at least 95%, the enzyme subcomplexes were passed through two successive 1 mL centrifuge columns of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA to remove unbound reagents. The moles of [^{3}H]ADP bound per mole of the wild-type and mutant subcomplexes after gel filtration were the following: wild-type, 2.0; $\alpha D_{261}N$, 2.0; $\beta T_{165}S$, 1.8; and $\beta Y_{341}W$, 1.8. The $\beta E_{190}Q$ mutant subcomplex, which is inactive as an ATPase (Ohtsubo et al., 1987), also incorporated 2.0 mol of [^{3}H]ADP per mole when it was treated identically to the wild-type complex. E_{190} of TF_1 is equivalent to E_{188} of MF_1, which, in the crystal structure, appears to be the general base at the catalytic site that activates the attacking water molecule during ATP hydrolysis (Abrahams et al., 1994).

To test whether the 2 mol of ADP incorporated per mole of F_1 subcomplex in these experiments is indeed bound to catalytic sites, the enzyme subcomplexes were incubated with 100 μM 2-N_3-[^{3}H]ADP and 2 mM $MgCl_2$ for 1 h in the dark before adding 200 μM $AlCl_3$ and 5 mM NaF to initiate inactivation which also proceeded in the dark. When inactivated by greater than 95%, the samples were passed through two successive 1 mL centrifuge columns of Sephadex G-50 before irradiating them for 90 min to induce covalent labeling. After irradiation, the moles of [^{3}H]ADP bound per mole of the different subcomplexes were as follows: wild-type, 1.8; $\alpha D_{261}N$, 1.6; $\beta T_{165}S$, 1.5; and $\beta Y_{341}W$, 2.1. The irradiated enzyme subcomplexes were digested with trypsin and submitted to HPLC on a C_4 reversed-phase column under the conditions described by Jault et al. (1995). With the exception of the digest prepared from the labeled $\beta Y_{341}W$ mutant subcomplex, the majority of the radioactivity eluted from the column between 90 and 100 min, and negligible radioactivity eluted between 70 and 80 min, indicating that catalytic sites were derivatized at βTyr_{341} and noncatalytic sites were not derivatized at βTyr_{364}. In the case of the labeled $\beta Y_{341}W$ subcomplex, the majority of the radioactivity eluted from the column between 90 and 100 min, but a detectable amount also eluted between 70 and 80 min, indicating that a noncatalytic site was occupied by 2-N_3-[^{3}H]ADP to a limited extent.

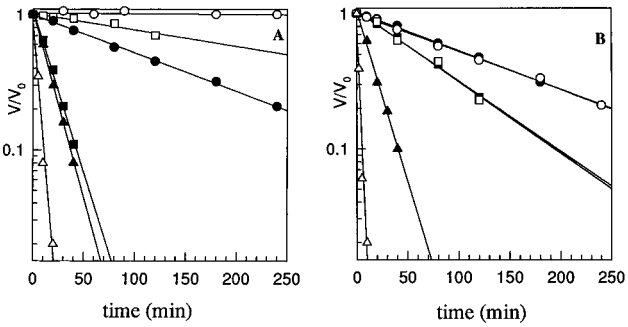


FIGURE 2: Effects of P_i and sulfite on the rate of formation of ADP−fluoroaluminate complexes at the catalytic sites of the wild-type $\alpha_3\beta_3\gamma$ subcomplex under different conditions. (A) Effects of P_i. Wild-type subcomplex containing MgADP in a single catalytic site: A 100 μL solution of 1 mg/mL CDTA-treated wild-type subcomplex in 50 mM Tris-HCl, pH 8.0, containing 3 μM ADP and 2 mM $MgCl_2$ was incubated at 30 °C for 30 min, at which time P_i was added to a final concentration of 2 mM. After 3 min, NaF and $AlCl_3$ were added to final concentrations of 5 mM and 200 μM, respectively. At the times indicated, 5 μL samples were withdrawn and assayed with 2 mM ATP (○). A control (●) was treated identically, except P_i was not included. *Effect of 2 mM P_i on the inactivation of wild-type complex containing MgADP in two catalytic sites:* A 400 μL solution containing 1.0 mg/mL CDTA-treated wild-type subcomplex in 50 mM Tris-HCl, pH 8.0 was incubated with 50 μM [^{3}H]ADP and 2 mM $MgCl_2$ for 60 min, at which time it was passed through a 5 mL centrifuge column of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8.0. The gel-filtered enzyme subcomplex contained 1.9 mol of [^{3}H]ADP per mole. P_i was added to half of the gel filtrate to a final concentration of 2 mM (□) and then incubated for 3 min before adding NaF and $AlCl_3$ to final concentrations of 5 mM and 200 μM to initiate inactivation. At the times indicated, 5 μL samples were withdrawn and assayed with 2 mM ATP. The other half of the gel filtrate used as a control (■) was prepared in the same manner except P_i was not added. *Effect of 2 mM P_i on the inactivation of wild-type complex in the presence of 200 μM ADP*: A 100 μL sample containing 1.0 mg/mL of CDTA-treated wild-type subcomplex was incubated with 2 mM $MgCl_2$ and 200 μM ADP for 30 min at 23 °C, at which time P_i was added to a final concentration of 2 mM. After an additional 3 min, NaF and $AlCl_3$ were added to final concentrations of 5 mM and 200 μM, respectively, to initiate inactivation. At the times indicated, 5 μL samples were withdrawn and assayed with 2 mM ATP (open triangles). A control sample was treated identically except that P_i was not added (closed triangles). (B) Effects of sulfite. The reaction conditions were the same as those described under (A) for P_i except that 10 mM Na_2SO_3 replaced 2 mM P_i where indicated. Also, 1.5 mol of ADP was bound per mole of F_1 in the experiments designated by (□, ■) in (B) rather than 1.9 mol of ADP per mole of F_1 designated by the same symbols in (A).

Effects of P_i and Sulfite on the Rate of Inactivation of the TF_1 Subcomplexes by Al^{3+} and F^- in the Presence of ADP and Mg^{2+} under Different Conditions. Figure 2A clearly

Table 2: Effect of P_i on the Binding of Mg[^{3}H]ADP and to a Single Catalytic Site of the Wild-Type and Mutant $\alpha_3\beta_3\gamma$ Complexes[a]

additions to $\alpha_3\beta_3\gamma$ subcomplexes	mol of [^{3}H]ADP/mol of wild-type	mol of [^{3}H]ADP/mol of $\alpha D_{261}N$	mol of [^{3}H]ADP/mol of $\beta T_{165}S$
Mg[^{3}H]ADP	1.0	0.85	0.84
Mg[^{3}H]ADP, then P_i	0.91	0.84	0.80

[a] To load a single catalytic site, 100 μL of the CDTA-treated subcomplexes, 0.5 mg/mL in 50 mM Tris-HCl containing 2 mM $MgCl_2$, pH 8.0, was incubated with 2 μM [^{3}H]ADP for 45 min. Then P_i was added to a final concentration of 2 mM, and the resulting solution was incubated an additional 30 min, at which time the enzyme solutions were passed through 1 mL columns of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8.0. Samples of the effluents were removed to determine protein concentration and radioactivity. Controls were prepared and analyzed in the same manner except P_i was not added.

shows that addition of Al^{3+} and F^- to the wild-type enzyme with MgADP bound to two catalytic sites (closed squares) was inactivated severalfold more rapidly ($k_{inact} = 5.3 \times 10^{-2}$ min^{-1}) than when they were added to the 1:1 F_1·MgADP complex (closed circles) ($k_{inact} = 7.0 \times 10^{-3}$ min^{-1}). Essentially the same rate of inactivation was observed when Al^{3+} and F^- were added to the wild-type complex after prior incubation with 200 μM ADP plus Mg^{2+} (closed triangles) ($k_{inact} = 5.8 \times 10^{-2}$ min^{-1}) which suggests that occupancy of two catalytic sites with MgADP is optimal for formation of ADP−fluoroaluminate complexes.

The following background is pertinent to results obtained on the effects of P_i on the rate of formation of the ADP−fluoroaluminate complexes at the catalytic sites of the TF_1 subcomplexes. The F_1-ATPases contain both high- and low-affinity binding sites for P_i (Penefsky, 1977; Penefsky & Grubmeyer, 1984). Drobinskaya et al. (1985) observed that [^{14}C]ADP remains bound to MF_1 after adding 2.5 mM P_i to the enzyme containing Mg[^{14}C]ADP bound to a single catalytic site. Kozlov and Vulfson (1985) reported that ADP must be bound to a catalytic site of MF_1 in order to observe binding of [^{32}P]P_i, indicating that P_i binds to catalytic sites containing bound ADP. The crystal structure of transducin containing the GDP−fluoroaluminate complex bound at the catalytic site has been determined at 1.7 Å resolution (Sondek et al., 1994). The liganding of Al^{3+} in the deduced crystal structure is thought to resemble that of pentacovalent phosphate in the transition state that develops when transducin hydrolyzes GTP. Therefore, if the ADP−fluoroaluminate complex bound to catalytic sites of F_1 were to have a similar structure, the binding of P_i to catalytic sites containing MgADP would be expected to inhibit formation of the ADP-fluoroaluminate complex. Figure 2A shows that this is indeed the case. Compared to a control reaction mixture not containing P_i (closed circles), formation of the the ADP−fluoroaluminate complex was nearly completely inhibited when the wild-type subcomplex containing MgADP at a single catalytic site was incubated with 2 mM P_i for 3 min before adding $AlCl_3$ and NaF (open circles). The addition of P_i to the subcomplexes containing MgADP in a catalytic site does not cause dissociation of ADP as shown by the results presented in Table 2. Similar results have been reported for MF_1 by Drobinskaya et al. (1985).

P_i also inhibited the rate of inactivation of the wild-type subcomplex when $AlCl_3$ and NaF were added to enzyme containing MgADP bound to two catalytic sites as illustrated in Figure 2A. In the absence of P_i, the rate constant for inactivation of the subcomplex containing MgADP in two catalytic sites was 5.3×10^{-2} min^{-1} (closed squares), whereas after incubation of the enzyme with P_i for 3 min before adding $AlCl_3$ and NaF, the rate constant reduced to 3.0×10^{-3} min^{-1} (open squares). Figure 2A also illustrates the unexpected result encountered when the wild-type complex was incubated with 200 μM ADP and 2 mM Mg^{2+} for 30 min and then P_i for 3 min before initiating inactivation by adding $AlCl_3$ and NaF. This protocol led to a 3-fold acceleration in the rate of inactivation of the wild-type enzyme (open triangles) over that observed for a control in which P_i was not introduced subsequent to incubation with ADP plus Mg^{2+} (closed triangles). To ensure that these are common phenomena, mutant enzyme subcomplexes were examined in the same manner. The effects of P_i on the rates of inactivation initiated on addition of $AlCl_3$ and NaF to the wild-type and mutant subcomplexes containing MgADP bound to one catalytic site or in the presence of 200 μM ADP plus Mg^{2+} are summarized in Table 3. Addition of P_i to the mutant enzymes containing MgADP in a single catalytic site prior to initiating inactivation clearly inhibited the rate of inactivation of the $\beta T_{165}S$ and $\beta Y_{341}W$ mutants, but not nearly to the extent observed for the wild-type complex. Since the $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplex containing MgADP in a single catalytic site was inactivated too slowly with or without added P_i to allow determination of first-order rate constants with accuracy, it is not included in the comparison. The rates of inactivation observed when Al^{3+} and F^- were added to the mutant complexes after prior incubation with 200 μM ADP plus Mg^{2+} were 5−10-fold greater than observed when Al^{3+} and F^- were added to the enzymes containing MgADP in a single catalytic site.

Table 3: Effect of P_i on the Rate of Formation of the ADP−Fluoroaluminate Complexes with and without Excess ADP[a]

	k_{inact} (min^{-1})			
$\alpha_3\beta_3\gamma$ subcomplex	stoichiometric ADP	stoichiometric ADP + 2 mM P_i	200 μM ADP	200 μM ADP + 2 mM P_i
wild-type	7.0×10^{-3}	$<10^{-3}$	5.8×10^{-2}	1.7×10^{-1}
$\alpha D_{261}N$	too slow[b]	too slow[b]	4.6×10^{-3}	1.7×10^{-2}
$\beta T_{165}S$	1.3×10^{-2}	6.7×10^{-3}	7.0×10^{-2}	3.5×10^{-1}
$\beta Y_{341}W$	1.3×10^{-2}	4.0×10^{-3}	1.4×10^{-1}	4.6×10^{-1}

[a] The pseudo-first-order rate constants were determined from Guggenheim plots (1926) from data obtained under the conditions described in Figure 2. [b] Inactivation of the $\alpha D_{261}N$ mutant was too slow under these conditions to estimate a first-order rate constant.

Issartel et al. (1991) reported that sulfite stimulates the rate of formation of the ADP−fluoroberyllium complex of MF_1. Figure 2B shows that sulfite also stimulated the rate of inactivation of the wild-type $\alpha_3\beta_3\gamma$ subcomplex when added to the enzyme after incubation with 200 μM ADP and Mg^{2+} but prior to the addition of $AlCl_3$ and NaF. In contrast to what is observed with P_i, sulfite has no effect on the rate of inactivation when it was added to the wild-type subcomplex containing MgADP bound to 1.0 (●, ○) or 1.5 (■, □) catalytic sites prior to addition of NaF and $AlCl_3$ as shown in Figure 2B. However, when sulfite was added to the subcomplex containing 200 μM ADP plus Mg^{2+} before adding $AlCl_3$ and NaF, the rate of irreversible inactivation was accelerated 9-fold (open triangles) ($k_{inact} = 5.0 \times 10^{-1}$ min^{-1}) compared to a control not containing sulfite (closed triangles) ($k_{inact} = 5.8 \times 10^{-2}$ min^{-1}).

The stimulatory effects of P_i and sulfite may be related to results reported by Larson et al. (1989), who observed that

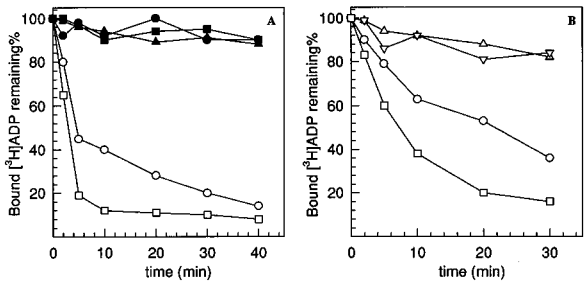


FIGURE 3: Synergistic effect of ADP with P_i or sulfite in promoting dissociation of Mg[^{3}H]ADP from a single catalytic site of the wild-type, $(\alpha D_{261}N)_3\beta_3\gamma$, and $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ subcomplexes. The wild-type and the $(\alpha D_{261}N)_3\beta_3\gamma$ and $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ subcomplexes were incubated at 1.0 mg/mL with 2 mM $MgCl_2$ and 30 μM [^{3}H]ADP in 100 μL of 50 mM Tris-HCl, pH 8.0, for 30 min, at which time the samples were passed through two 1 mL centrifuge columns of Sephadex G-50 equilibrated with the same buffer. The moles of [^{3}H]ADP bound per mole of enzyme subcomplex were determined to be 1.0 ± 0.1 for the three subcomplexes. The gel filtrate of each subcomplex was diluted 20-fold into five separate solutions containing 2 mM Mg^{2+} and either 1 mM ADP; 2 mM P_i; 10 mM Na_2SO_3; 1 mM ADP plus 2 mM P_i; or 1 mM ADP plus 10 mM Na_2SO_3 in 50 mM Tris-HCl. Samples, 100 μL each, of the 2.0 mL dilutions were removed at the times indicated and passed through 1 mL centrifuge columns of Sephadex G-50 which were equilibrated and eluted with 50 mM Tris-HCl, pH 8.0, containing 2 mM $MgCl_2$ and 1 mg/mL BSA. The elutions were submitted to liquid scintillation counting. (A) Results obtained for the wild-type subcomplex diluted in 1 mM ADP (▲); 2 mM P_i, (●); 10 mM Na_2SO_3, (■); 1 mM ADP plus 2 mM P_i, (○); and 1 mM ADP plus 10 mM Na_2SO_3 (□). (B) Results obtained for the $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplex diluted in 1 mM ADP plus 2 mM P_i (○) and 1 mM ADP plus 10 mM Na_2SO_3 (□); and for the $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ subcomplex diluted in 1 mM ADP plus 2 mM P_i (△) and 1 mM ADP plus 10 mM Na_2SO_3 (▽). The controls for the two mutant subcomplexes, which were essentially the same as those for the wild-type complex, are not included for the sake of clarity.

a combination of ADP and sulfite promotes release of [^{3}H]-ADP bound to a catalytic site of CF_oF_1. To test this possibility, the effects of P_i and sulfite, each alone or in combination with ADP, on promotion of release of Mg[^{3}H]-ADP from a single catalytic site of the wild-type, $(\alpha D_{261}N)_3\beta_3\gamma$, and $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ subcomplexes have been compared. The $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ subcomplex does not bind ATP or ADP to noncatalytic sites in the presence of Mg^{2+} (Matsui et al., 1996). Figure 3A illustrates that 1 mM ADP in combination with either 2 mM P_i (open circles) or 10 mM sulfite (open squares) promotes dissociation of Mg[^{3}H]ADP from a single catalytic site of the wild-type subcomplex, whereas P_i (closed circles), sulfite (closed squares), or ADP (closed triangles) by themselves do not. The effects of ADP in combination with P_i or sulfite on the release of [^{3}H]ADP from a single catalytic site of the $(\alpha D_{261}N)_3\beta_3\gamma$ and $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ mutant subcomplexes are illustrated in Figure 3B. Whereas P_i (open circles) or sulfite (open squares) in combination with ADP promotes dissociation of Mg[^{3}H]ADP from a single catalytic site of the $(\alpha D_{261}N)_3\beta_3\gamma$ mutant, albeit at a slower rate than observed for wild-type, P_i (open triangles) or sulfite (open inverted triangles) in combination with ADP has little or no effect on dissociation of Mg[^{3}H]-ADP from a single catalytic site of the $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ mutant.

Cross-Linking of α to γ or β to γ Abolishes Cooperative Formation of ADP−Fluoroaluminate Complexes in Two Catalytic Sites. It has been demonstrated that addition of oxidizing agents to mutant *E. coli* F_1-ATPases containing

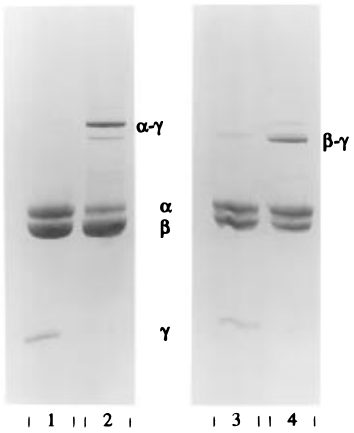


FIGURE 4: Comparison of the polypeptides resolved by SDS-PAGE before and after oxidation of the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes. The reduced subcomplexes prepared in 50 mM Tris-HCl, pH 8.0 were incubated at 2 mg/mL with 1 mM dithiothreitol at 23 °C for 30 min prior to application to the gel. The oxidized subcomplexes were prepared at 2 mg/mL as described under Experimental Procedures. To dissociate the complexes, 5 μL samples of the reduced and oxidized subcomplexes were added to 20 μL of 0.5 mM Tris-HCl, pH 6.8, containing 10% (w/v) SDS, 0.5% bromthymol blue, and 10% glycerol. The diluted samples were heated at 95 °C for 4 min prior to application to the 12% gel which was prepared in the absence of thiols. Lane 1, reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex; lane 2, oxidized $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex; lane 3, reduced $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplex; and lane 4, oxidized $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplex.

introduced cysteines in the DELSEED loop in the C-terminal domain of the β subunit leads to disulfide bond formation with an endogenous cysteine in the γ subunit (Aggeler et al., 1995; Duncan et al., 1995). Inactivation of ATP hydrolysis accompanies $\beta-\gamma$ cross-linking of *E. coli* F_1 which is completely reversed by the addition of dithiothreitol. To examine the effects of cross-linking the α or β subunit to the γ subunit on formation of the ADP−fluoroaluminate complex, the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ mutant subcomplexes were prepared. Assuming that the structures of TF_1 and MF_1 are homologous, the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ mutant contains an introduced cysteine in the major loop in the α-helical domain in the C-terminal region of the α subunit and an introduced cysteine in the N-terminal strand of the coiled-coil of the γ subunit that is located in the central cavity of the crystal structure of MF_1. Again, assuming homologous structures, the $\alpha_3(\beta D_{390}C)_3\beta_3(\gamma S_{90}C)$ mutant contains an introduced cysteine in the DELSEED segment of the β subunit and an introduced cysteine in the α-helical spur that appends the coiled-coil of the γ subunit. The two introduced cysteines in each of the double mutants are adjacent at one contact point of the asymmetrically arranged γ subunit with the α or β subunit (Abrahams et al., 1994). When the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ mutant was treated with *o*-iodosobenzoate or the $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ mutant was treated with $CuCl_2$, the ATPase activity was inactivated by greater than 98%. Figure 4 shows that oxidation of the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes was accompanied by nearly quantitative formation of $\alpha-\gamma$ and $\beta-\gamma$ cross-links, respectively. ATPase activity was completely recovered when each cross-linked subcomplex was assayed in the presence of 10 mM dithiothreitol, indicating that a disulfide bond can be formed and then broken between the α and γ or β and γ subunits without irreversible loss of catalytic activity. However, in the case of the oxidized $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ mutant, a much longer

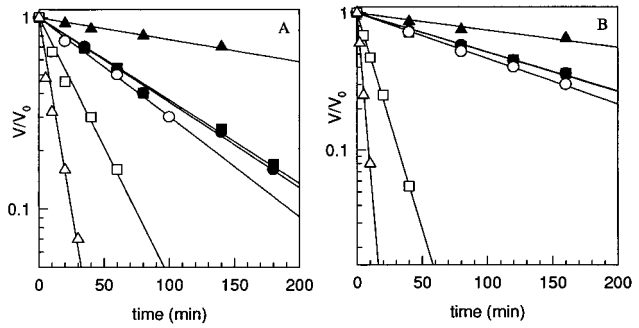


FIGURE 5: Effects of cross-linking the α to γ and β to γ subunits of the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes, respectively, on the formation of the ADP−fluoroaluminate complex under various conditions. The oxidized and reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes were prepared as described under Experimental Procedures. (A) Oxidized and reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex: To 1 mg/mL solutions of the oxidized and reduced subcomplex were added stoichiometric ADP plus $MgCl_2$ to a final concentration of 2 mM or ADP plus $MgCl_2$ to final concentrations of 200 μM and 2 mM, respectively. The samples were incubated for 30 min at 23 °C, at which time NaF and $AlCl_3$ were added to final concentrations of 5 mM and 200 μM, respectively. In another set, P_i was added to a final concentration of 2 mM 30 min after incubating the oxidized and reduced samples with 200 μM ADP plus 2 mM $MgCl_2$. After addition of P_i, the samples were incubated an additional 3 min before adding NaF and $AlCl_3$ to final concentrations of 5 mM and 200 μM, respectively. At the times indicated, 5 μL samples of the reaction mixtures were withdrawn and assayed with 2 mM ATP in media containing 10 mM dithiothreitol. The symbols represent: (○) reduced subcomplex with stoichiometric ADP; (□) reduced subcomplex with 200 μM ADP; (△), reduced subcomplex with 200 μM ADP plus 2 mM P_i; (●), oxidized complex with stoichiometric ADP; (■) oxidized complex with 200 μM ADP; (▲), oxidized subcomplex with 200 μM ADP plus 2 mM P_i. (B) Oxidized and reduced $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplex: The samples were prepared as described under (A) for the oxidized and reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex. The symbols are the same as described for the reaction mixtures under (A).

lag preceded attainment of a final steady-state rate corresponding to that of the reduced complex. The longer lag might represent slow cleavage of the disulfide bond or slow conversion of an inactive conformation to an active conformation subsequent to cleavage of the disulfide bond. Figure 5A compares the rates of formation of the ADP−fluoroaluminate complex observed when the reduced and oxidized $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplexes were treated with $AlCl_3$, NaF, Mg^{2+}, and ADP under various conditions. Approximately the same rate of inactivation ($k_{inact} \cong 1.1 \times 10^{-2}$ min^{-1}) was observed when $AlCl_3$ and NaF were added to the oxidized or reduced subcomplex containing stoichiometric MgADP in a single catalytic site (solid or open circles) or when added to the oxidized subcomplex after exposure to 200 μM ADP and 2 mM Mg^{2+} for 30 min (solid squares) before adding 200 μM $AlCl_3$ and 5 mM NaF. In contrast, when $AlCl_3$ and NaF were added to the reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex after incubation with 200 μM ADP and 2 mM Mg^{2+}, the rate of inactivation was considerably faster ($k_{inact} = 2.9 \times 10^{-2}$ min^{-1}) (open squares) than observed when $AlCl_3$ and NaF were added to the reduced mutant subcomplex containing MgADP in a single catalytic site (open circles). When the reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex was incubated with 200 μM ADP, 2 mM Mg^{2+}, and 2 mM P_i before adding $AlCl_3$ and NaF, the rate of inactivation was accelerated further ($k_{inact} = 8.3 \times 10^{-2}$ min^{-1}) (open triangles). On the other hand, when 2 mM P_i was added to the oxidized complex with 200 μM ADP plus 2 mM Mg^{2+} before adding $AlCl_3$ and NaF, the rate of inactivation was slowed considerably ($k_{inact} = 2.7 \times 10^{-3}$ min^{-1}) (solid triangles). When the oxidized and reduced forms of the the $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ mutant were submitted to the same series of experiments described for the oxidized and reduced forms of the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex, essentially the same responses were observed as illustrated in Figure 5B.

It is clear from the results illustrated in Figure 5 that the cross-linked $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes do not exhibit cooperative formation of the ADP−fluoroaluminate complexes in two catalytic sites. To determine whether the ADP−fluoroaluminate complex forms at one or two catalytic sites when $AlCl_3$ and NaF were added to the oxidized $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes after incubating them with 200 μM ADP and 2 mM Mg^{2+}, the following test was performed. The oxidized, mutant subcomplexes and the wild-type subcomplex were incubated with 200 μM [^{3}H]ADP plus 2 mM Mg^{2+} for 30 min, at which time they were passed through 1 mL centrifuge columns of Sephadex G-50. At this interval, the moles of [^{3}H]ADP bound per mole of each subcomplex were as follows: $\alpha_3\beta_3\gamma$, 2.1; $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$, 2.2; and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$, 2.3. $AlCl_3$ and NaF were added to each sample to initiate inactivation. After the samples were inactivated by greater than 98%, they were passed through 1 mL centrifuge columns of Sephadex G-50. Then CDTA, which has a high affinity for Mg^{2+}, was added to the effluents to a final concentration of 5 mM to promote dissociation of the MgADP complex bound to catalytic sites while leaving the bound MgADP·AlF_4^- complex intact. After incubating for 2 h with CDTA, the samples were passed through 1 mL centrifuge columns of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA. The moles of [^{3}H]ADP remaining bound to each subcomplex after the last centrifuge column were the following: $\alpha_3\beta_3\gamma$, 1.9; $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$, 1.05 ± 0.05; and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$, 1.01 ± 0.05. From these results, it is concluded that the oxidized, mutant subcomplexes only form an ADP−fluoroaluminate complex in a single catalytic site even when excess ADP is available. This means that cooperative conversion of MgADP bound to two catalytic sites to ADP−fluoroaluminate complexes requires movement of the γ subunit which is restricted by cross-linking α to γ or β to γ, respectively, in the $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes.

Given the different responses of the oxidized and reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ subcomplexes in the experiments described, it was of interest to examine the rate of formation of the ADP−fluoroaluminate complex using the $\alpha_3\beta_3\delta$ subcomplex of TF_1. To this end, the $\alpha_3\beta_3\delta$ subcomplex was prepared from the isolated wild-type subunits and purified as described by Yokoyama et al. (1989). Surprisingly, the $\alpha_3\beta_3\delta$ subcomplex was not inactivated in the presence of Mg^{2+}, ADP, Al^{3+}, and F^- under any of the conditions leading to inactivation of the wild-type $\alpha_3\beta_3\gamma$ subcomplex. This suggests that the γ subunit must be present to observe isomerizations involved in forming inactive ADP−fluoroaluminate complexes at catalytic sites.

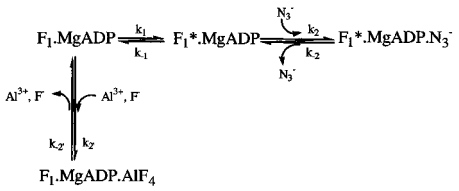
DISCUSSION

The results presented clearly illustrate that catalytic site to catalytic site cooperativity is exhibited during formation

of inactive ADP−fluoroaluminate complexes at catalytic sites of the wild-type and mutant $\alpha_3\beta_3\gamma$ subcomplexes of TF_1. The rate of reaction of Al^{3+} and F^- with the enzyme subcomplexes containing MgADP bound to a single catalytic site is slow, whereas when MgADP is bound to two catalytic sites, the rate of reaction is accelerated considerably. The same acceleration is observed when the enzyme subcomplexes are incubated in the presence of 200 μM ADP plus Mg^{2+} for 30 min before adding Al^{3+} and F^-. Therefore, the ADP−fluoroaluminate complexes form optimally when MgADP is bound to two catalytic sites. However, this does not mean that complete inactivation requires formation of ADP−fluoroaluminate complexes in two catalytic sites as suggested by Issartel et al. (1991). The subcomplexes are slowly, but completely inactivated when Al^{3+} and F^- are added to enzyme containing MgADP bound to a single catalytic site. Therefore, formation of an ADP−fluoroaluminate complex in the single site is sufficient for complete inactivation. This is consistent with the demonstrated "one-third-the-sites" reactivity of reagents that derivatize catalytic sites (Ferguson et al., 1975; Andrews & Allison, 1984; Bullough et al., 1986; Milgrom & Boyer, 1990; Chernyak & Cross, 1992) which has been taken as evidence for sequential participation of catalytic sites (Boyer, 1993). These results are not consistent with the stochastic model proposed by Issartel et al. (1991), which postulates that the three catalytic sites function cooperatively as interactive pairs, but are compatible with models which propose sequential participation of catalytic sites (Boyer, 1993; Allison et al., 1995; Weber & Senior, 1996).

As previously suggested by Lunardi et al. (1988), the rate of formation of the inactive ADP−fluoroaluminate complexes probably reflects isomerization of the catalytic site to accommodate the MgADP·AlF_4^- transition state analog as it assembles. According to the crystal structure of MF_1, the equivalent of βT_{165} of TF_1 is liganded to the Mg^{2+} ion chelated to ADP in the catalytic site designated β_{DP} by Abrahams et al. (1994), whereas the equivalent of βY_{341} of TF_1 is adjacent to the adenine of ADP bound to β_{DP}. Although the $\alpha_3(\beta T_{165}S)_3\gamma$ and $\alpha_3(\beta Y_{341}W)_3\gamma$ subcomplexes have increased specific activities compared to the wild-type subcomplex and form inhibitory ADP−fluoroaluminate complexes more rapidly than the wild-type subcomplex, other catalytic characteristics of the two mutant F_1-ATPases are very different. In contrast to wild-type, the $\alpha_3(\beta T_{165}S)_3\gamma$ subcomplex does not entrap significant inhibitory MgADP in a catalytic site during turnover (Jault et al., 1996). The $\alpha_3(\beta Y_{341}W)_3\gamma$ subcomplex entraps inhibitory MgADP in a catalytic site during turnover, but to a lesser extent than wild-type (C. Dou, unpublished experiments, 1996). For instance, the ATPase activity of the $\alpha_3(\beta Y_{341}W)_3\gamma$ mutant is inhibited by increasing concentrations of Mg^{2+} in the assay medium, activated when LDAO is present during assay, and undergoes abrupt turnover-dependent inhibition in the presence of azide. None of these characteristics is displayed when the $\alpha_3(\beta T_{165}S)_3\gamma$ subcomplex hydrolyzes ATP (Jault et al., 1996). This suggests that both the adenine and pyrophosphoryl moieties of ADP shift positions with respect to residues that abut them in the catalytic site during the enzyme isomerization that accompanies the conversion of the reversibly inhibited F_1·MgADP complex to the irreversibly inhibited F_1·MgADP·AlF_4^- complex. The amino acid substitutions in both the $\alpha_3(\beta T_{165}S)_3\gamma$ and $\alpha_3(\beta Y_{341}W)_3\gamma$ mutant F_1-ATPases lessen constraints hindering isomerization with the $\beta Y_{341}W$ substitution easing formation of the transition state analogue to a greater extent.

Scheme 1



The results presented show that the $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplex forms the inactive ADP−fluoroaluminate complex at least 10-fold more slowly than the wild-type subcomplex under all conditions examined. This suggests that noncatalytic site to catalytic site cooperativity also influences the rate of formation of the MgADP·AlF_4^- transition state analogue. The finding that the $(\alpha D_{261}N)_3\beta_3\gamma$ mutant enzyme containing MgADP in a single catalytic site is inactivated much more slowly on addition of Al^{3+} and F^- than wild-type under the same conditions was unexpected. This means that the single catalytic site of the mutant subcomplex which is occupied by MgADP recognizes the $\alpha D_{261}N$ substitution even when noncatalytic sites are empty. In the crystal structure of MF_1, the equivalent of αAsp_{261} is liganded to the Mg^{2+} ion chelated to AMP-PNP bound to noncatalytic sites.

The different rates of formation of the ADP−fluoroaluminate complex observed when MgADP is bound to a single catalytic site of the wild-type, $\alpha D_{261}N$, or $\beta T_{165}S$ $\alpha_3\beta_3\gamma$ subcomplexes are consistent with the equilibria illustrated in Scheme 1. F_1·MgADP represents enzyme in an active conformation with MgADP bound to a single catalytic site, whereas F_1*·MgADP represents enzyme in an inactive conformation with MgADP bound to a catalytic site (Vasilyeva et al., 1982). It has been demonstrated that isomerization of F_1·MgADP to F_1*.MgADP is slow (Milgrom & Murataleiv, 1986) and does not occur in the $\alpha_3\beta_3$ or $\alpha_3\beta_3\delta$ (Jault et al., 1995) subcomplexes, indicating that the γ subunit is required to form the inactive conformation. Evidence also suggests that, in the case of the $\alpha_3(\beta T_{165}S)_3\gamma$ subcomplex, the equilibrium between F_1·MgADP and F_1*·MgADP favors F_1·MgADP, whereas, in the case of the $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplex, the equilibrium is in favor of F_1*·MgADP (Jault et al., 1995, 1996). Given that the order of reactivity of the wild-type and mutant $\alpha D_{261}N$ and $\beta T_{165}S$ subcomplexes with MgADP bound to a single catalytic site is $\alpha_3(\beta T_{165}S)_3\gamma > \alpha_3\beta_3\gamma > (\alpha D_{261}N)_3\beta_3\gamma$, it appears that the ADP−fluoroaluminate complex is formed from F_1·MgADP rather than from F_1*·MgADP. Supporting this contention is the observation that the rate of inactivation of the wild-type complex was 3-fold slower when it was incubated with 200 μM ADP, 2 mM Mg^{2+}, and 0.2 mM NaN_3 before adding Al^{3+} and F^-, than in the absence of NaN_3 during prior incubation with ADP and Mg^{2+} (data not shown). Azide stabilizes the F_1*·MgADP complex (Vasilyeva et al., 1982; Hyndman et al., 1994).

The observation that ADP−fluoroaluminate complexes are formed more rapidly when P_i or sulfite is added to the enzyme in the presence of excess ADP may reflect noncatalytic site to catalytic site cooperativity. P_i effectively blocks formation of the ADP−fluoroaluminate complex when added to enzyme containing MgADP bound to one or two catalytic sites prior to the addition of Al^{3+} and F^-. This demonstrates

that the stimulation observed in the presence of excess ADP and P_i is not caused by binding of P_i to catalytic sites containing bound ADP. Sulfite also stimulates formation of the ADP−fluoroaluminate complexes only when excess ADP is present. The synergistic stimulation of formation of the ADP−fluoroaluminate complexes observed in the presence of excess ADP plus P_i or sulfite might reflect (a) that ADP and the activating anions bind to noncatalytic sites simultaneously, which promotes isomerization of catalytic sites, or (b) that P_i or sulfite binds to a site which is part of the pathway for transmitting conformational signals to catalytic sites when noncatalytic sites bind ADP. The following observations support the first possibility. In the presence of Mg^{2+}, ADP plus phosphate or sulfite (1) promotes rapid dissociation of inhibitory Mg[^{3}H]ADP from a single catalytic site of the wild-type $\alpha_3\beta_3\gamma$ subcomplex; (2) promotes slow dissociation of inhibitory Mg[^{3}H]ADP from a single catalytic site of the $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplex; and (3) does not promote dissociation of inhibitory Mg[^{3}H]-ADP from a single catalytic site of the $(\alpha K_{175}A/T_{176}A/D_{261}N/D_{262}A)_3\beta_3\gamma$ subcomplex which does not bind adenine nucleotides to noncatalytic sites (Matsui et al., 1996).

It has been demonstrated that the reduced $(\alpha A_{396}C)_3\beta_3$-$(\gamma A_{22}C)$ and $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ mutant subcomplexes exhibit cooperative formation of the ADP−fluoroaluminate complexes in two catalytic sites, whereas the corresponding oxidized complexes containing an $\alpha-\gamma$ or $\beta-\gamma$ cross-link, respectively, do not. Therefore, it appears that positional interchange of the γ subunit with respect to an α subunit or a β subunit is required for cooperative formation of ADP−fluoroaluminate complexes in two catalytic sites. The same rate of formation of the ADP−fluoroaluminate complex is observed when Al_3^+ and F^- are added to the oxidized or reduced $(\alpha A_{396}C)_3\beta_3(\gamma A_{22}C)$ subcomplex containing MgADP in a single catalytic site. Similarly, the same rate of formation of the complex is observed when Al_3^+ and F^- are added to the oxidized or reduced $\alpha_3(\beta D_{390}C)_3(\gamma S_{90}C)$ containing MgADP in a single catalytic site. These observations suggest that little or no positional interchange of the γ subunit occurs when the ADP−fluoroaluminate complex is formed in a single catalytic site. However, the observation that the $\alpha_3\beta_3\delta$ complex does not form the ADP−fluoroaluminate complex under any condition, nevertheless, indicates that formation of the ADP−fluoroaluminate complex in a single catalytic site requires the presence of the γ subunit.

REFERENCES

Abrahams, J. P., Leslie, A. G., Lutter, R., & Walker, J. E. (1994) *Nature 370*, 621−628.

Allison, W. S., Jault, J. M., Grodsky, N. B., & Dou, C.(1995) *Biochem. Soc. Trans. 23*, 752−756.

Amano, T., Yoshida, M., Matsuo, Y., & Nishikawa, K. (1994) *FEBS Lett. 351*, 1−5.

Andrews, W. W., Hill, F. C., & Allison, W. S. (1984) *J. Biol. Chem. 259*, 8219−8225.

Boyer, P. D. (1993) *Biochim. Biophys. Acta 1140*, 215−250.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Bullough, D. A., & Allison, W. S. (1986) *J. Biol. Chem. 261*, 14171−14177.

Bullough, D. A., Brown, E. L., Saario, J. D., & Allison, W. S. (1988) *J. Biol. Chem. 263*, 14053−14060.

Chernyak, B. V., & Cross, R. L. (1992) *Arch. Biochem. Biophys. 295*, 247−252.

Drobinskaya, I. Y., Kozlov, I. A., Murataliev, M. B., & Vulfson, E. N. (1985) *FEBS Lett. 182*, 419−424.

Ferguson, S. J., Lloyd, W. J., Lyons, M. H., & Radda, G. K. (1975)- *Eur. J. Biochem. 54*, 117−126.

Garrett, N. E., & Penefsky, H. S. (1975) *J. Biol. Chem. 250*, 6640−6647.

Guggenheim, E. A. (1926) *Philos. Mag. 2*, 538−543.

Hyndman, D. J., Milgrom, Y. M., Bramhall, E. A., & Cross, R. L. (1994) *J. Biol. Chem. 269*, 28871−28877.

Issartel, J.-P., Dupuis, A., Lunardi, J., & Vignais, P. V. (1991) *Biochemistry 30*, 4726−4733.

Jault, J.-M., & Allison, W. S. (1993) *J. Biol. Chem. 268*, 1558−1566.

Jault, J.-M., & Allison, W. S. (1994) *J. Biol. Chem. 269*, 319−325.

Jault, J.-M., Matsui, T., Jault, F. M., Kaibara, C., Muneyuki, E., Yoshida, M., Kagawa, Y., & Allison, W. S. (1995) *Biochemistry 34*, 16412−16418.

Jault, J.-M., Dou, C., Grodsky, N. B., Matsui, T., Yoshida, M., & Allison, W. S. (1996) *J. Biol. Chem. 271*, 28818−28824.

Kozlov, I. A., & Vulfson, E. N. (1985) *FEBS Lett. 182*, 425−428.

Kunkel, T. A., Benebek, K., & McClary, J. (1991) *Methods Enzymol. 204*, 125−139.

Larson, E. M., Umbach, A., & Jagendorf, A. T. (1989) *Biochim. Biophys. Acta 973*, 78−85.

Lunardi, J., Dupuis, A., Garin, J., Issartel, J.-P., Michael, L., Chabre, M., & Vignais, P. V. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8958−8962.

Matsui, T., & Yoshida, M. (1995) *Biochim. Biophys. Acta 1231*, 139−146.

Matsui, T., Muneyuki, E., Honda, M., Dou, C., Allison, W. S., & Yoshida, M. (1996) *J. Biol. Chem.* (in press).

Milgrom, Y. M., & Murataliev, M. B. (1989) *Biochim. Biophys. Acta 975*, 50−58.

Milgrom, Y. M., & Boyer, P. D. (1990) *Biochim. Biophys. Acta 1020*, 43−48.

Ohtsubo, M., Yoshida, M., Ohta, S., Kagawa, Y., Yohda, M., & Date, T. (1987) *Biochem. Biophys. Res. Commun. 142*, 705−710.

Paik, S. R., Yokoyama, K., Yoshida, M., Ohta, T., Kagawa, Y., & Allison, W. S. (1993) *J. Bioenerg. Biomembr. 25*, 679−684.

Pedersen, P. L., & Amzel, L. M. (1993) *J. Biol. Chem. 268*, 9937−9940.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.

Penefsky, H. S., & Grubmeyer, C. (1984) in *H^+-ATPase (ATP Synthase): Structure, Function, Biogenesis, the F_oF_1 Complex of Coupling Membranes* (Papa, S., Altendorf, K., Ernster, L., & Packer, L., Eds.) pp 195−204, ICSU Press, Adriatica Edritrice, Bari, Italy.

Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A., & Baumeister, K. (1987) *Science 238*, 336−341.

Saraste, M., Sibbald, P. R., & Wittinghofer, A. (1990) *Trends Biochem. Sci. 15*, 430−434.

Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., & Sigler, P. B. (1994) *Nature 372*, 276−279.

Sternweis, P. C., & Golman, A. G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4888 −4891.

Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., & Vinogradov, A. D. (1982) *Biochem. J. 202*, 15−23.

Walker, J. E., Saraste, M., Runswick, M. J., & Gay, N. J. (1982) *EMBO J. 1*, 945−951.

Weber, J., & Senior, A. E. (1996) *Biochim. Biophys. Acta 1275*, 101−104.

Weber, J., Wilke-Mounts, S., Lee, R. S.-F., Grell, E., & Senior, A. E. (1993) *J. Biol. Chem. 271*, 20126−20133.

Weber, J., Wilke-Mounts, S., & Senior, A. E. (1994) *J. Biol. Chem. 269*, 20462−20467.

Yokoyama, K., Hisabori, T., & Yoshida, M. (1989) *J. Biol. Chem. 264*, 21837−21841.

Yoshida, M., & Allison, W. S. (1983) *J. Biol. Chem. 258*, 14407−14412.

Yoshida, M., & Allison, W. S. (1986) *J. Biol. Chem. 261*, 5714−5721.

BI962353+