The $\alpha_3\beta_3\gamma$ Complex of the F_1-ATPase from Thermophilic *Bacillus* PS3 Containing the $\alpha D_{261}N$ Substitution Fails To Dissociate Inhibitory MgADP from a Catalytic Site When ATP Binds to Noncatalytic Sites†

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ABSTRACT: ATP hydrolyses by the wild-type $\alpha_3\beta_3\gamma$ and mutant $(\alpha D_{261}N)_3\beta_3\gamma$ subcomplexes of the F_1-ATPase from the thermophilic *Bacillus* PS3 have been compared. The wild-type complex hydrolyzes 50 μM ATP in three kinetic phases: a burst decelerates to an intermediate phase, which then gradually accelerates to a final rate. In contrast, the mutant complex hydrolyzes 50 μM or 2 mM ATP in two kinetic phases. The mutation abolishes acceleration from the intermediate phase to a faster final rate. Both the wild-type and mutant complexes hydrolyze ATP with a lag after loading a catalytic site with MgADP. The rate of the MgADP-loaded wild-type complex rapidly accelerates and approaches that observed for the wild-type apo-complex. The MgADP-loaded mutant complex hydrolyzes ATP with a more pronounced lag, and the gradually accelerating rate approaches the slow, final rate observed with the mutant apo-complex. Lauryl dimethylamide oxide (LDAO) stimulates hydrolysis of 2 mM ATP catalyzed by wild-type and mutant complexes 4- and 7.5-fold, respectively. The rate of release of [^{3}H]-ADP from the Mg[^{3}H]ADP-loaded mutant complex during hydrolysis of 40 μM ATP is slower than observed with the wild-type complex. LDAO increases the rate of release of [^{3}H]ADP from the preloaded wild-type and mutant complexes during hydrolysis of 40 μM ATP. Again, release is slower with the mutant complex. When the wild-type and mutant complexes are irradiated in the presence of 2-N_3-[^{3}H]-ADP plus Mg^{2+} or 2-N_3-[^{3}H]ATP plus Mg^{2+} and azide, the same extent of labeling of noncatalytic sites is observed. Whereas ADP and ATP protect noncatalytic sites of the wild-type and mutant complexes about equally from labeling by 2-N_3-[^{3}H]ADP or 2-N_3-[^{3}H]ATP, respectively, AMP-PNP provides little protection of noncatalytic sites of the mutant complex. The results suggest that the substitution does not prevent binding of ADP or ATP to noncatalytic sites, but rather that it affects cross-talk between liganded noncatalytic sites and catalytic sites which is necessary to promote dissociation of inhibitory MgADP.

The F_oF_1-ATP synthases couple ATP synthesis and hydrolysis to proton electrochemical gradients in energy-transducing membranes (Senior, 1990; Pedersen & Amzel, 1993). F_o is an integral membrane protein complex that mediates proton conduction, whereas F_1 is a peripheral membrane protein complex which bears the catalytic sites. When removed from the membrane in soluble form, F_1 is an ATPase. The F_1-ATPases are comprised of five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ and have molecular weights of about 380 000. They contain six nucleotide binding sites, three of which are catalytic (Cross, 1992). The other three, for want of a defined physiological function, are called noncatalytic (Allison et al., 1991). From the recently deduced crystal structure of MF_1[1] (Abrahams et al., 1994), in which the α and β subunits are elongated and arranged alternately like the segments of an orange, it is clear that catalytic and noncatalytic sites are located at different interfaces of α and β subunits. Catalytic sites reside mostly on β subunits, whereas noncatalytic sites are mostly on α subunits.

The overall topologies of the catalytic and noncatalytic nucleotide binding sites are very similar to each other and are strikingly homologous with the nucleotide binding domain of the recA protein (Story & Steitz, 1992; Amano et al., 1994; Abrahams et al., 1994). Among the residues comprising the nucleotide binding sites are two signature sequences common to proteins that bind nucleotides which are known as the Walker A and B motifs (Walker et al., 1982). Motif A, which is also called the P-loop (Saraste et al., 1990), has the consensus sequence GXXXXGKT/S.

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[1] Abbreviations: MF_1, F_1-ATPase from bovine heart mitochondria; TF_1, F_1-ATPase from the thermophilic *Bacillus* PS3; CF_1, F_1-ATPase from spinach chloroplasts; LDAO, lauryl dimethylamine oxide; HPLC, high-performance liquid chromatography; AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate).

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Motif B is comprised of a stretch of hydrophobic residues which is terminated by an aspartate. In the α subunit of TF_1, this sequence is: $_{257}$LVVID$_{261}$.

Steady-state kinetic analyses of the MF_1-, CF_1-, and TF_1-ATPases are complicated by transient entrapment of inhibitory MgADP in a single catalytic site during turnover (Vasilyeva et al., 1982a,b; Zhuo et al., 1988; Paik et al., 1994). Consequently, when nucleotide-depleted MF_1 or TF_1, the latter of which is isolated free of endogenous nucleotides, hydrolyzes low concentrations of ATP in the presence of a regenerating system, three kinetic phases are exhibited (Jault & Allison, 1993; Paik et al., 1994). An initial burst rapidly decelerates to an intermediate rate that gradually accelerates to a final steady-state rate which approaches the initial rate. Transition from the burst phase to the slow, intermediate phase is caused by turnover-dependent entrapment of MgADP in a single catalytic site (Drobinskaya et al., 1985; Zhuo et al., 1988; Guerrero et al., 1990). Slow binding of ATP to noncatalytic sites, which promotes dissociation of MgADP from the affected catalytic site, is responsible for transition from the intermediate phase to the final rate. This latter transition is responsible for the apparent negative cooperativity which is observed when MF_1 and TF_1 hydrolyze 30–3000 μM ATP (Jault & Allison, 1993; Paik et al., 1994).

Related to the turnover-dependent entrapment of inhibitory MgADP in a single catalytic site is the observation that prior loading of a single catalytic site of MF_1 (Drobinskya et al., 1985; Milgrom & Boyer, 1990; Chernyak & Cross, 1992), CF_1 (Feldman & Boyer, 1985), or TF_1 (Yoshida & Allison, 1986) with MgADP severely attenuates the initial rate of ATP hydrolysis in the presence of an ATP regenerating system. A pronounced lag is exhibited, the length of which is dependent on the ATP concentration in the assay medium. In a previous study, it was shown that LDAO stimulates the final phase of ATP hydrolysis catalyzed by TF_1 4-fold and nearly eliminates the intermediate phase (Paik et al., 1994). From these observations, it was proposed that the detergent shortens the intermediate phase either by stimulating binding of ATP to noncatalytic sites, thus promoting dissociation of inhibitory MgADP from a catalytic site, or by directly promoting release of inhibitory MgADP from a catalytic site.

Yohda et al. (1988) reported that the isolated α subunit of TF_1 containing the $D_{261}N$ substitution binds MgADP with a 10-fold lower affinity than the isolated wild-type α subunit. The steady-state catalytic properties of the $\alpha_3\beta_3\gamma$ complexes containing either wild-type α or mutant $\alpha D_{261}N$ subunits were reported to differ significantly. Whereas a Lineweaver–Burk plot for the wild-type complex displays apparent negative cooperativity typical of TF_1, the Lineweaver–Burk plot for the complex containing $\alpha D_{261}N$ subunits is linear. Furthermore, the maximal velocity of the mutant complex is only about 25% that of the wild-type complex. These differences provoked a more thorough characterization of the $(\alpha D_{261}N)_3\beta_3\gamma$ complex to provide new insights on communication between noncatalytic and catalytic sites and on activation of TF_1 by LDAO. Experiments preliminary to this study were performed with $\alpha_3\beta_3\gamma$ complexes which were assembled *in vitro* from wild-type β and γ subunits and either wild-type or mutant α subunits, essentially as described by Yokoyama et al. (1989). Unless noted otherwise, experiments reported here were carried out with mutant and wild-type complexes isolated from lysates after overexpressing a plasmid containing the genes encoding the wild-type β and γ subunits and either the wild-type or mutant α subunit of TF_1 in an *Escherichia coli* strain deleted of the *unc* operon as described by Matsui et al. (1995).

MATERIALS AND METHODS

Materials. Biochemicals used in assays and buffer components were purchased from Sigma. [^{3}H]ADP was purchased from Du Pont–New England Nuclear, and 2-chloro[^{3}H]adenosine used to synthesize 2-N_3-[^{3}H]AT(D)P was purchased from Moravek Biochemicals. [α-^{35}S]dATP used in DNA sequencing was purchased from Amersham. Enzymes and biochemicals used in the coupled assays and buffer components were purchased from Sigma. Coomassie blue was purchased from Pierce. 2-N_3-[^{3}H]ADP and 2-N_3-[^{3}H]ATP were synthesized as described previously (Jault & Allison, 1994b).

The wild-type α, β, and γ subunits were prepared as described by Ohtsubo et al. (1987). *In vitro* reconstitution of the wild-type $\alpha_3\beta_3$ and $\alpha_3\beta_3\gamma$ complexes was accomplished as described by Yokoyama et al. (1989) except that 200 mM Na_2SO_4 was added to the 20 mM Tris·H_2SO_4 buffer and the complexes were separated from free subunits by high-performance gel permeation chromatography on a column of TSK G3000SW_{XL}. The reconstituted complexes were assayed within a few hours after purification in the presence and absence of LDAO in the absence of 200 mM Na_2SO_4. After expression in an *unc*$^-$ strain of *E. coli*, from plasmids encoding the β and γ subunits of TF_1 and either the wild-type or the mutant α subunits, the $\alpha_3\beta_3\gamma$ and $(\alpha D_{261}N)_3\beta_3\gamma$ complexes were purified according to Matsui et al. (1995) and were stored as precipitates in ammonium sulfate at 4 °C. Before use in the studies described, samples of the suspensions were pelleted by centrifugation. After the ammonium sulfate solution was decanted, the pellets were dissolved in 50 mM Tris·HCl, pH 8.0, which contained 0.1 mM EDTA. The protein solutions were then passed through 1 mL centrifuge columns of Sephadex G-50 which were equilibrated with the same buffer (Penefsky, 1977). The complexes that were assembled *in vivo* were stable for at least 1 week when stored in this buffer at 4 °C. However, the complexes were usually used in experiments on the day of preparation. Unless stated otherwise, the complexes used in this study were prepared from lysates after expression in *E. coli*.

Analytical Procedures. Site-directed mutagenesis was performed according to Kunkel et al. (1991). The oligonucleotide 5′-TTCGATAAATCATTAATCACAACCAA-3′ was used to introduce the $\alpha D_{261}N$ substitution. The mutation was confirmed by dideoxynucleotide sequencing (Sanger et al., 1980) using the Amersham Sequenase 2 kit according to the protocol of the manufacturer.

ATPase activity was determined spectrophotometrically using a coupled enzyme assay as described previously (Jault & Allison, 1994a). Protein concentrations were determined by the method of Bradford (1976) using the Coomassie Plus protein assay reagent from Pierce. Assessment of nucleotide binding to catalytic and noncatalytic sites of the complexes by photoaffinity labeling by 2-N_3-[^{3}H]ADP and 2-N_3-[^{3}H]-ATP was performed by HPLC as described in detail previously for TF_1 (Jault et al., 1994a).

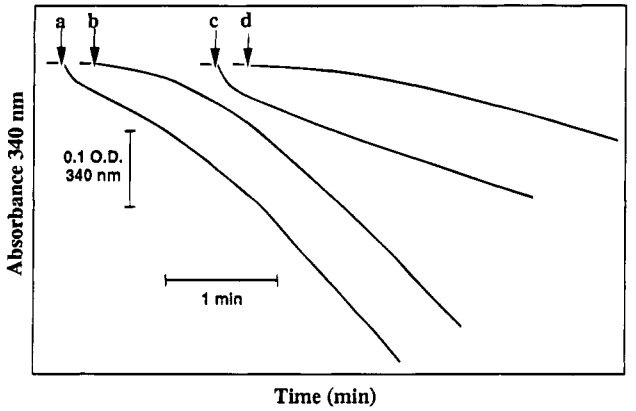

FIGURE 1: Comparison of hydrolysis of 50 μM ATP by the wild-type $\alpha_3\beta_3\gamma$ and mutant $(\alpha D_{261}N)_3\beta_3\gamma$ complexes. Solutions of the wild-type or mutant complexes, at 1 mg/mL, were incubated at 23 °C for 15 min in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA and 1 mM Mg^{2+} in the presence of 3.6 μM ADP (b, d) or absence of ADP (a, c). Then 4.0 μL of wild-type (a, b) or mutant complex (c, d) was withdrawn and injected into 1 mL of assay medium containing 50 μM ATP plus 1.05 mM Mg^{2+}.

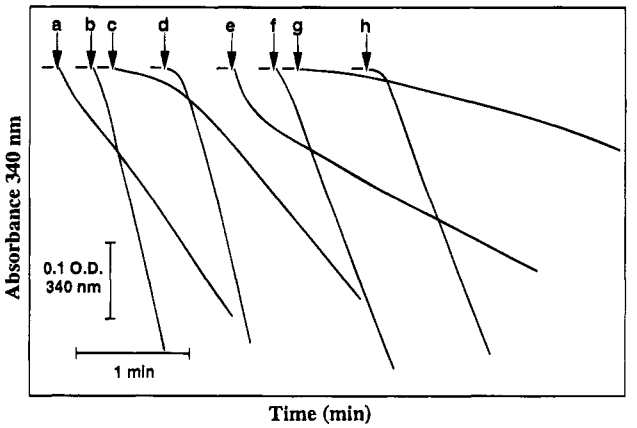

FIGURE 2: Comparison of hydrolysis of 2 mM ATP by the wild-type $\alpha_3\beta_3\gamma$ and the mutant $(\alpha D_{261}N)_3\beta_3\gamma$ complexes under various conditions. Solutions of the wild-type or mutant complexes, at 1 mg/mL, were incubated at 23 °C for 15 min in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA and 1 mM Mg^{2+} in the presence of 3.6 μM ADP (c, d, g, h) or absence of ADP (a, b, e, f). Samples of the wild-type (a–d) or mutant complex (e–h) were withdrawn and injected into 1.0 mL of assay medium containing 2 mM ATP and 3 mM Mg^{2+}, in the presence (b, d, f, h) or absence (a, c, e, g) of 0.06% LDAO. For assays conducted in the presence of LDAO, 1.5 μL samples were used. For those conducted in the absence of LDAO, 2.0 μL samples were used.

RESULTS

Comparison of the Hydrolytic Properties of the Wild-Type $\alpha_3\beta_3\gamma$ and the Mutant $(\alpha D_{261}N)_3\beta_3\gamma$ Complexes. Figure 1 compares hydrolysis of 50 μM ATP by the wild-type and mutant complexes in the presence of a regenerating system before and after loading a catalytic site with MgADP. Trace a shows that the wild-type complex, initially free of bound nucleotides, hydrolyzes 50 μM ATP in three distinct kinetic phases: an initial burst decelerates to an intermediate phase, which, in turn, accelerates to a final rate (6.2 μmol of ATP hydrolyzed min^{-1} mg^{-1}) that approaches the initial rate. In contrast, trace c illustrates that only two phases are observed when the mutant complex, initially free of bound nucleotides, hydrolyzes 50 μM ATP. A burst decelerates to a slow, constant rate (2.2 μmol of ATP hydrolyzed min^{-1} mg^{-1}) which is equivalent to the intermediate phase observed for the wild-type complex (trace a). It is clear from comparison of traces a and c that the major effect of the mutation is to abolish acceleration from the intermediate rate to the final rate. Traces b and d illustrate hydrolysis of 50 μM ATP by the wild-type and mutant complexes, respectively, after loading a single catalytic site of each with MgADP. In both cases, the initial rate of hydrolysis is severely attenuated. However, the rate of ATP hydrolysis catalyzed by the wild-type enzyme accelerates much more rapidly than observed with the mutant complex.

Figure 2 compares hydrolysis of 2 mM ATP by the wild-type and mutant complexes under various conditions in the presence of an ATP regenerating system. In the absence of effectors, the wild-type complex (trace a) hydrolyzes 2 mM ATP with a slight initial deceleration which is followed by a slight acceleration to a final rate of 18.9 μmol of ATP hydrolyzed mg^{-1} min^{-1}. This behavior is reminiscent of the pronounced triphasic behavior illustrated in Figure 1 (trace a) when the wild-type complex hydrolyzes 50 μM ATP under the same conditions. In contrast, in the absence of effectors, the mutant complex (trace e) hydrolyzes 2 mM ATP with a prominent burst that rapidly decelerates to a slow, constant rate of 6.6 μmol of ATP hydrolyzed min^{-1} mg^{-1}. Traces b and f illustrate hydrolysis by the wild-type and mutant complexes, respectively, in the presence of 0.06% LDAO. After a short lag, hydrolysis of ATP by both the wild-type and mutant complexes is accelerated considerably in the presence of LDAO to final rates of 73 and 42 μmol of ATP hydrolyzed mg^{-1} min^{-1}, respectively. Traces c and g compare the rates of ATP hydrolysis observed after loading a single catalytic site of the wild-type and mutant complexes, respectively, with MgADP. In the case of the preloaded, wild-type complex, acceleration from a very slow initial rate to a final steady-state rate occurs within 1 min. In contrast, hydrolysis of ATP by the preloaded, mutant complex accelerates much more slowly from a severely attenuated initial rate. Even after 3 min, the rate of the preloaded mutant complex does not begin to approach the final rate of the mutant complex hydrolyzing 2 mM ATP in the absence of effectors. Traces d and h illustrate that the presence of 0.06% LDAO in the assay medium overcomes the inhibition induced by preloading a catalytic site of either the wild-type (trace d) or the mutant complex (trace h) with MgADP.

Comparison of the Effects of LDAO and Rhodamine 6G on the Activity of the Wild-Type $\alpha_3\beta_3\gamma$ and Mutant $(\alpha D_{261}N)_3\beta_3\gamma$ Complexes. It has been shown that LDAO stimulates the ATPase activity of *E. coli* F_1 (Lötscher et al., 1984; Bragg & Hou, 1986; Dunn et al., 1990) and TF_1 (Paik et al., 1993, 1994). Figure 3A, which illustrates final rates, shows that LDAO-induced stimulation of hydrolysis of 2 mM ATP catalyzed by the mutant complex is nearly twice that observed for the wild-type complex. A previous study showed that low concentrations of rhodamine 6G stimulate the ATPase activity of TF_1 and certain subcomplexes assembled from the subunits of TF_1, whereas higher concentrations inhibit (Paik et al., 1993). Figure 3B shows that the final rate of ATP hydrolysis catalyzed by the mutant complex is stimulated to a greater extent by low concentrations of rhodamine 6G than hydrolysis catalyzed by the wild-type complex. Stimulation of the final rates of the mutant and wild-type complexes shows the same dependence on the concentration of both activators. Therefore, the much greater

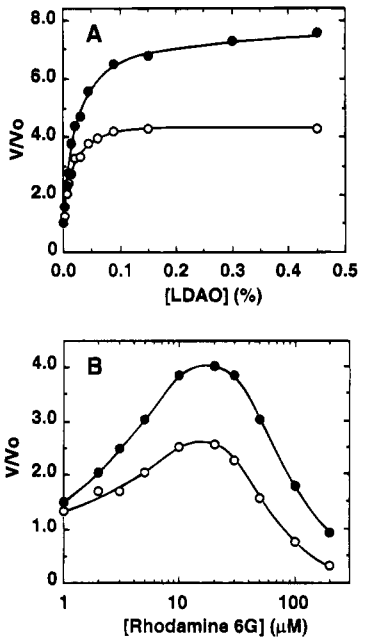

FIGURE 3: Effects of LDAO and rhodamine 6G on the ATPase activity of the wild-type $\alpha_3\beta_3\gamma$ and mutant $(\alpha D_{261}N)_3\beta_3\gamma$ complexes. The experimental conditions were the same as for Figure 2 except that 1.1 mg/mL stock solutions of complexes were used. Samples, 1 μL each, of the wild-type (O) or 2 μL of the mutant complex (●) were withdrawn and injected into assay medium containing 2 mM ATP plus 3 mM Mg^{2+} and the concentrations of LDAO (A) or rhodamine 6G (B) specified. The specific activities of the wild-type and mutant complexes were 18 and 6 μmol of ATP hydrolyzed mg^{-1} min^{-1}, respectively.

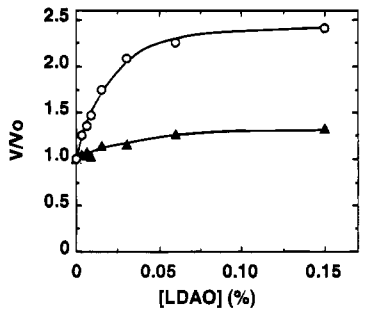

FIGURE 4: Comparison of the effects of LDAO on the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3$ subcomplexes of TF_1. The $\alpha_3\beta_3$ (▲) and $\alpha_3\beta_3\gamma$ (O) complexes were reconstituted from individual subunits as described under Materials and Methods. Then 2 μL of each solution was injected into an ATP assay medium containing 2 mM ATP plus 3 mM Mg^{2+} in the absence of Na_2SO_4 and the concentrations of LDAO specified. Under these conditions, the initial rates of the $\alpha_3\beta_3$ and $\alpha_3\beta_3\gamma$ complexes yielded specific activities of 3.5 and 11 μmol of ATP hydrolyzed mg^{-1} min^{-1}, respectively.

stimulations observed for the mutant complex do not reflect greater affinities of this complex for LDAO or rhodamine 6G.

In a previous study it was shown that the ATPase activity of subcomplexes lacking the γ subunit is not stimulated by low concentrations of rhodamine 6G (Paik et al., 1993). This prompted an examination of the effect of LDAO on the ATPase activity of the $\alpha_3\beta_3$ complex. Figure 4 compares the effects of LDAO on the hydrolysis of 2 mM ATP by the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3$ complexes reconstituted *in vitro*. Whereas LDAO stimulates the $\alpha_3\beta_3\gamma$ complex nearly 2.5-fold under these conditions, the $\alpha_3\beta_3$ complex is stimulated by only 30%. Since Hirada et al. (1994) have reported that the $\alpha_3\beta_3$ complex undergoes nucleotide-induced dissociation, the

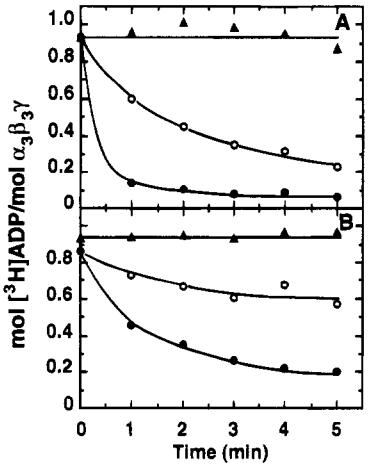

FIGURE 5: Effect of $\alpha D_{261}N$ mutation and LDAO on the release of inhibitory ADP from a catalytic site. Solutions of 1 mg/mL (2.8 μM) wild-type (A) and mutant (B) complexes were incubated at 23 °C for 30 min in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA, 1 mM Mg^{2+}, and 4 μM [^{3}H]ADP. Samples were then diluted 20 times with 50 mM Tris-HCl, pH 8.0, containing 2 mM Mg^{2+} and 40 μM ATP, in the presence (●) or absence (O) of 0.06% LDAO. At the times indicated, the samples were passed through 1 mL centrifuge columns of Sephadex G50 which were equilibrated with 50 mM Tris-HCl, pH 8.0, containing 2 mM Mg^{2+} and 1 mg/mL BSA. Bound [^{3}H]ADP was determined by scintillation counting. Control experiments (▲) were performed in the presence of LDAO but in the absence of ATP.

stimulation afforded by LDAO may only reflect stabilization of the complex during assay. That LDAO might stabilize the $\alpha_3\beta_3$ complex is supported by the observation that low concentrations of rhodamine 6G slightly stimulate the $\alpha_3\beta_3$ complex, whereas they inhibit the $\alpha_3\beta_3\delta$ complex (Paik et al., 1993), which is not subject to nucleotide-induced dissociation (Yokoyama et al., 1989). From these observations, we conclude that the relatively weak stimulation of the ATPase activity of the $\alpha_3\beta_3$ complex by LDAO may not be related to the strong stimulation of ATPase activity observed when TF_1 and the subcomplexes containing the γ subunit are assayed in the presence of LDAO. The lower activation that LDAO confers to the $\alpha_3\beta_3\gamma$ complex reconstituted *in vitro* shown in Figure 4 compared to that conferred to the $\alpha_3\beta_3\gamma$ complex assembled *in vivo* shown in Figure 3A probably reflects incomplete assembly of the complex during *in vitro* reconstitution.

Effects of the $\alpha D_{261}N$ Substitution on Release of Inhibitory [^{3}H]ADP from a Catalytic Site under Turnover Conditions. Figures 5A and 5B compare the rate of dissociation of [^{3}H]-ADP from the mutant and wild-type complexes which is observed when each complex, loaded with Mg[^{3}H]ADP at a catalytic site, is then submitted to turnover conditions in the presence or absence of LDAO. In the absence of LDAO, hydrolysis of 40 μM ATP promotes dissociation of 75% of the bound [^{3}H]ADP from the wild-type complex in 5 min (Figure 5A, open circles), whereas in the presence of LDAO nearly complete dissociation is observed within 1 min (Figure 5A, closed circles). In contrast, dissociation of preloaded [^{3}H]ADP from the mutant complex during hydrolysis of 40 μM ATP is much slower. Only one-third of the preloaded [^{3}H]ADP dissociates from the preloaded mutant complex in 5 min (Figure 5B, open circles). Although LDAO increases the rate and extent of dissociation of [^{3}H]ADP which occurs during hydrolysis of 40 μM ATP by the mutant complex

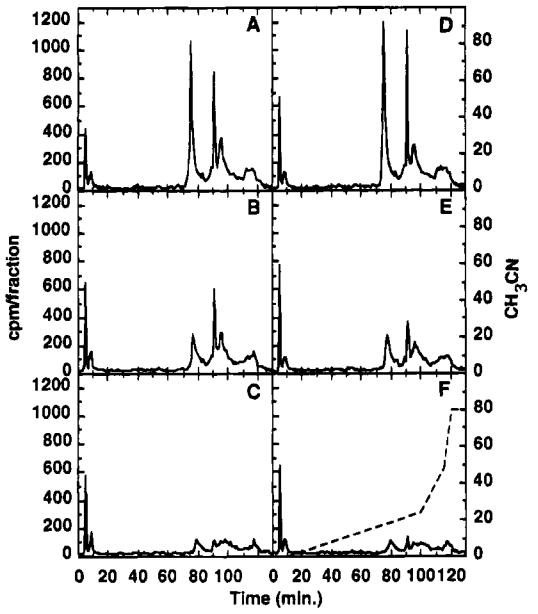

FIGURE 6: ADP protects against binding of 2-N_3-[^{3}H]ADP to both catalytic and noncatalytic sites of the wild-type $\alpha_3\beta_3\gamma$ and $(\alpha D_{261}N)_3\beta_3\gamma$ mutant complexes. Solutions, 200 μL each, containing 3 mg/mL of the wild-type complex (A−C) or the mutant complex (D−F), were incubated at 23 °C for 15 min in 16 mM Tris-HCl, pH 8.0, containing 0.03 mM EDTA and 1 mM Mg^{2+} in the absence (A, D) or presence of 98 μM (B, E) or 490 μM ADP (C, F). Then 2-N_3-[^{3}H]ADP was added to a final concentration of 147 μM. After 15 min, the samples were irradiated and digested with trypsin as described previously (Jault et al., 1994a). Radioactive peptides in the tryptic digest were resolved on a C-4 reversed-phase column which was equilibrated with 0.1% HCl and eluted with the gradient of acetonitrile illustrated. The collected 1 mL fractions were submitted to liquid scintillation counting.

(Figure 5B, closed circles), dissociation is much slower than observed with the wild-type complex under the same conditions.

Dilution of the complexes preloaded with Mg[^{3}H]ADP into buffer containing LDAO in the absence of ATP does not promote dissociation of [^{3}H]ADP (triangles). As previously reported for TF_1 (Yoshida & Allison, 1986; Hisabori et al., 1994), the same results are observed when the complexes, preloaded with Mg[^{3}H]ADP, are diluted into buffer alone.

$\alpha D_{261}N$ Substitution Does Not Affect Binding of 2-N_3-[^{3}H]-AT(D)P, ATP, or ADP to Noncatalytic Sites of the Mutant $(\alpha D_{261}N)_3\beta_3\gamma$ Complex, but Severely Decreases the Affinity of Noncatalytic Sites of the Mutant Complex for AMP-PNP. To assess the effect of the $\alpha D_{261}N$ substitution on binding of adenine nucleotides to catalytic and noncatalytic sites of the $\alpha_3\beta_3\gamma$ mutant complex, the capacity of ADP, ATP, and AMP-PNP to protect the mutant and wild-type complexes against photolabeling with 2-N_3-[^{3}H]ADP or 2-N_3-[^{3}H]ATP has been examined. After photolabeling the wild-type and mutant complexes with and without prior incubation with nucleotides, tryptic digests were prepared and submitted to reversed-phase HPLC to resolve derivatized peptides arising from noncatalytic and catalytic sites. Panels A and D of Figure 6 illustrate elution profiles obtained on submitting tryptic digests of the wild-type and mutant complexes, photolabeled with 147 μM 2-N_3-[^{3}H]ADP plus Mg^{2+} in the absence of prior binding of nucleotides, to reversed-phase HPLC. The peak of radioactivity eluting at 78 min contains the tryptic peptide with β-Tyr_{364} derivatized (Jault et al., 1994a), a residue that is part of the noncatalytic site. The

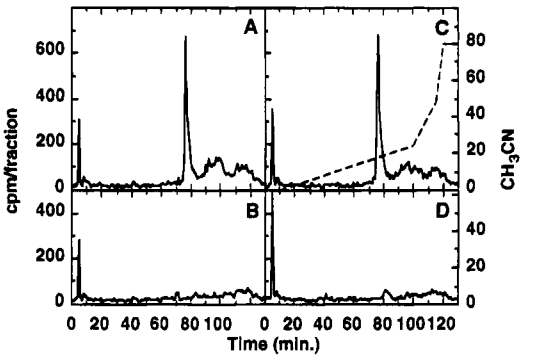

FIGURE 7: ATP protects against binding of 2-N_3-[^{3}H]ATP to the noncatalytic sites of both the wild-type $\alpha_3\beta_3\gamma$ and mutant $(\alpha D_{261}N)_3\beta_3\gamma$ complexes. Solutions, 200 μL each, containing 2 mg/mL of the wild-type (A, B) or mutant (C, D) complex, were incubated at 23 °C for 5 min in 25 mM Tris-HCl, pH 8.0, containing 0.05 mM EDTA, 1 mM Mg^{2+}, and 5 mM NaN_3 in the absence (A, C) or presence of 100 μM ATP (B, D). Then 2-N_3-[^{3}H]ATP was then added to a final concentration of 190 μM. Five minutes later the samples were irradiated and were then treated as described in the legend to Figure 6.

two peaks eluting between 90 and 100 min contain two forms of the tryptic peptide with β-Tyr_{341} derivatized, a residue that is part of the catalytic site. From results of previous studies in which TF_1 was labeled with 2-N_3-[β,γ-^{32}P]ATP in the presence of Mg^{2+} (Jault et al., 1994a) or MF_1 was labeled with 2-N_3-[^{3}H]ADP in the presence or absence of Mg^{2+} (Jault & Allison, 1994b), it is probable that the radioactive peak eluting at about 95 min represents the tryptic peptide containing βTyr_{341} with tethered [^{3}H]ADP. The radioactive peak eluting at about 90 min probably represents the tryptic peptide containing βTyr_{341} with tethered [^{3}H]AMP formed by hydrolysis of tethered [^{3}H]ADP (Jault & Allison, 1994b). Panels B and E of Figure 6 illustrate the protection afforded by 98 μM ADP against labeling of the wild-type and mutant complexes by 2-N_3-[^{3}H]ADP, respectively. Panels C and F of Figure 6 show that prior incubation of both the wild-type and mutant complexes with 490 μM ADP nearly eliminates photolabeling with 147 μM 2-N_3-[^{3}H]ADP. These results indicate that the $\alpha D_{261}N$ substitution does not significantly affect binding of 2-N_3-[^{3}H]ADP or ADP to either catalytic or noncatalytic sites.

The substitution also does not appear to prevent binding of 2-N_3-[^{3}H]ATP to noncatalytic sites of the mutant complex. Panels A and C of Figure 7 show that the noncatalytic sites of the wild-type and mutant complexes are labeled to the same extent after irradiation in the presence of 190 μM 2-N_3-[^{3}H]ATP plus Mg^{2+} and 5 mM sodium azide. Curiously, very little labeling of the catalytic site is observed under these conditions. This appears to be caused by azide, which was added to prevent appreciable hydrolysis of 2-N_3-[^{3}H]ATP. When the same experiment was performed in the absence of azide, the labeling pattern obtained was nearly the same as that observed when photolabeling was performed with 2-N_3-[^{3}H]ADP illustrated in Figure 6. Panels B and D of Figure 7 illustrate that prior addition of 100 μM ATP to the wild-type and mutant complexes, respectively, virtually eliminates photolabeling of noncatalytic sites by 2-N_3-[^{3}H]-ATP.

Although the protection experiments indicate that the $\alpha D_{261}N$ substitution does not significantly impair binding of ADP or ATP to noncatalytic sites of the mutant complex, the results illustrated in Figure 8 show that this substitution

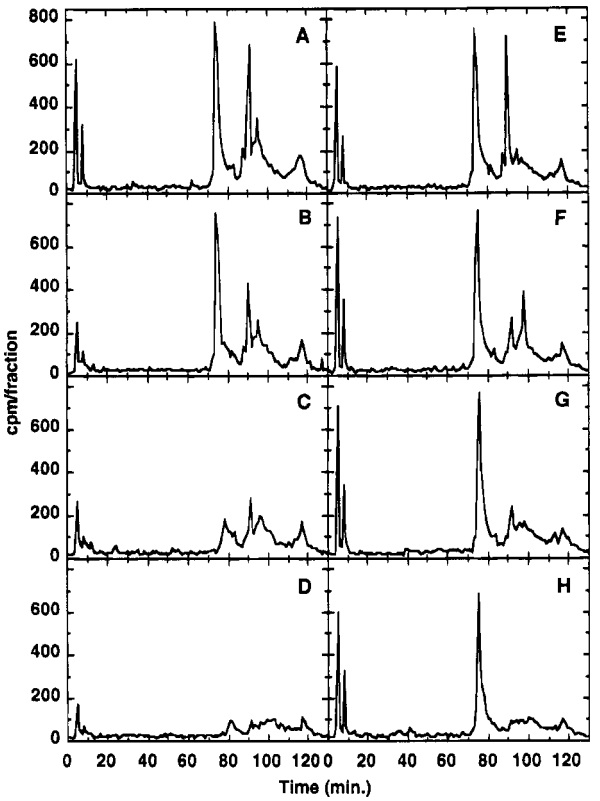

FIGURE 8: Comparison of AMP-PNP-induced protection of catalytic and noncatalytic sites of the wild-type $\alpha_3\beta_3\gamma$ and mutant $(\alpha D_{261}N)_3\beta_3\gamma$ complexes against binding of 2-N_3-[^{3}H]ADP. Solutions, 200 μL each, containing 2 mg/mL of the wild-type (A−D) or mutant (E−H) complex, were incubated at 23 °C for 15 min in 10 mM Tris-HCl, pH 8.0, containing 0.02 mM EDTA and 1 mM Mg^{2+} in the absence (A, E) or presence of 10 μM (B, F), 40 μM (C, G), or 500 μM AMP-PNP (D, H). Then 2-N_3-[^{3}H]ADP was added to a final concentration of 184 μM. After 15 min, the samples were irradiated and then treated as described in Figure 6. The gradient of acetonitrile is the same as shown in Figure 6.

has a prominent effect on the apparent affinity of noncatalytic sites for AMP-PNP, whereas it does not affect binding of AMP-PNP to catalytic sites. Panels A−D of Figure 8 show the labeling patterns obtained from HPLC of tryptic digests of the wild-type complex which had been irradiated in the presence of 184 μM 2-N_3-[^{3}H]ADP and Mg^{2+} after prior incubation with 0, 10, 40, and 500 μM AMP-PNP, respectively. Panels E−H of Figure 8 show the labeling patterns obtained from HPLC of tryptic digests of the mutant complex which had been irradiated in the presence of 2-N_3-[^{3}H]ADP after prior incubation with the same increasing concentrations of AMP-PNP. Whereas nearly complete protection of both catalytic and noncatalytic sites of the wild-type enzyme and catalytic sites of the mutant complex is afforded by 40 μM AMP-PNP, no protection of the mutant complex against photolabeling is observed after prior incubation with 10 and 40 μM AMP-PNP, and only slight, if any, protection is observed after prior incubation with 500 μM AMP-PNP.

DISCUSSION

The results presented clearly show that the $\alpha D_{261}N$ substitution severely attenuates ATP-induced dissociation of inhibitory MgADP from a catalytic site which either is entrapped in the mutant $\alpha_3\beta_3\gamma$ complex during hydrolysis of ATP or is loaded there by prior incubation of the complex with MgADP. The crystal structure of MF_1 indicates that the corresponding aspartate is near the Mg^{2+} ion that is chelated to phosphoryl oxygens of AMP-PNP bound to noncatalytic sites (Abrahams et al., 1994). When this structural information was considered with the observation of Yohda et al. (1988) that the isolated $\alpha D_{261}N$ subunit binds ADP with 10-fold lower affinity than the wild-type α subunit, it was anticipated that the $(\alpha D_{261}N)_3\beta_3\gamma$ complex would not bind adenine nucleotides to noncatalytic sites. Clearly, this is not the case. Noncatalytic sites of the mutant and wild-type complexes are photolabeled to the same extent by 150 μM 2-N_3-[^{3}H]ADP or 190 μM 2-N_3-[^{3}H]ATP in the presence of Mg^{2+}. Furthermore, prior incubation with 100 μM ATP in the presence of azide and Mg^{2+} affords complete protection against photolabeling of noncatalytic sites in both the mutant and wild-type complexes by 3-N_3-[^{3}H]ATP. Under the conditions of the experiments, the inhibitory $Mg{\cdot}ADP{\cdot}N_3^-$ complex should be entrapped in a catalytic site, which would prevent substantial hydrolysis of ATP or 2-N_3-[^{3}H]ATP. Therefore, the very slow rate of hydrolysis of 2 mM ATP observed when the mutant complex containing a catalytic site is preloaded with MgADP is not caused by lack of binding of ATP to noncatalytic sites. From these results, we conclude that the $\alpha D_{261}N$ substitution affects cross-talk between liganded noncatalytic sites and liganded catalytic sites. This interaction is necessary to promote dissociation of inhibitory MgADP from a catalytic site when ATP binds to noncatalytic sites.

The kinetic properties of the $(\alpha D_{261}N)_3\beta_3\gamma$ complex reported here are consistent with the steady-state kinetic properties of the mutant complex reported by Yohda et al. (1988). It has been shown that the apparent negative cooperativity exhibited during hydrolysis of 30−3000 μM ATP by MF_1 is caused by slow binding of ATP to noncatalytic sites which promotes dissociation of inhibitory MgADP from a catalytic site (Jault & Allison, 1993). Therefore, the observation that the $(\alpha D_{261}N)_3\beta_3\gamma$ complex entraps inhibitory MgADP in a catalytic site during turnover, which is not released when ATP binds to noncatalytic sites, is consistent with the linear Lineweaver−Burk plot reported by Yohda et al. (1988) for the mutant complex. The V_{max} estimated from the Lineweaver−Burk plot reported by Yohda et al. (1988) is about 25% that of the wild-type complex. The results reported here show that the $(\alpha D_{261}N)_3\beta_3\gamma$ complex hydrolyzes 2 mM ATP at about 35% the rate exhibited by the wild-type complex.

The triphasic behavior observed when MF_1, TF_1, and the wild-type $\alpha_3\beta_3\gamma$ complex hydrolyze 50 μM ATP has been partly explained (Jault & Allison, 1993; Paik et al., 1994). Transition from the burst to the intermediate phase is associated with entrapment of inhibitory MgADP in a catalytic site, and acceleration from the intermediate phase to the final rate is caused by slow binding of ATP or, more likely, MgATP to noncatalytic sites. However, what is responsible for entrapment of inhibitory MgADP in a catalytic site during turnover when noncatalytic sites are not saturated with ATP is not known. Several lines of evidence suggest that the γ subunit might be involved in this process. Yokoyama et al. (1989) reported that, unlike TF_1 and the $\alpha_3\beta_3\gamma$ complex, the $\alpha_3\beta_3\delta$ complex does not bind ADP tightly to a catalytic site in the presence of Mg^{2+}. They also reported that the $\alpha_3\beta_3\delta$ complex is much more active hydrolyzing low concentrations of ATP than TF_1 or the $\alpha_3\beta_3\gamma$ complex, suggesting that MgADP is not entrapped in a

catalytic site of this complex during turnover. Miwa and Yoshida (1989) showed that the ATPase activity of the $\alpha_3\beta_3$ complex is insensitive to inhibition by azide which acts by increasing retention of inhibitory MgADP in a catalytic site (Vasilyeva et al., 1982b; Murataliev et al., 1991; Divita et al., 1992). These observations, taken together with the finding reported here that the $\alpha_3\beta_3$ complex is relatively insensitive to stimulation by LDAO compared to the $\alpha_3\beta_3\gamma$ complex, suggest that the γ subunit must be present for retention of inhibitory MgADP in a catalytic site.

Evidence has accumulated from several laboratories which suggests that the inactive complex containing inhibitory MgADP in a single catalytic site of F_1-ATPases is formed in a two-step process (Vasilyeva et al., 1982b; Murataliev & Milgrom, 1989; Murataliev, 1992; Hyndman et al., 1994). It has been postulated that a complex, designated F_1•ADP•-Mg, which either is active (Vasilyeva et al., 1982a,b; Bulygin & Vinogradov, 1991) or is easily reactivated in the presence of MgATP (Milgrom & Murataliev, 1989; Murataliev, 1992; Murataliev et al., 1991; Hyndman et al., 1994), is formed initially which slowly isomerizes to a stable, inactive complex, designated F_1*•ADP•Mg. The complexes are proposed to be in an equilibrium which is shifted in the direction of the F_1•ADP•Mg complex by activating anions such as bisulfate or bicarbonate which bind to unknown sites (Vasilyeva et al., 1982a) or binding ATP, or PP_i in the case of MF_1, to noncatalytic sites (Jault & Allison, 1993; Kalashnikova et al., 1988; Jault et al., 1994b). In the presence of azide, the F_1*•ADP•Mg complex is converted to the more stable F_1*•ADP•Mg•N_3^- complex which effectively decreases the concentration of the F_1•ADP•Mg complex (Vasilyeva et al., 1982b; Murataliev, 1992; Hyndman et al., 1994). Slow isomerization of the F_1•ADP•Mg complex to the F_1*•ADP•Mg complex might represent movement of the γ subunit to an abortive position, either in a random event during turnover or after loading a single catalytic site with MgADP when noncatalytic sites are not saturated with ATP.

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