Catalytic Activity of the $\alpha_3\beta_3\gamma$ Complex of F₁-ATPase without Noncatalytic Nucleotide Binding Site*

(Received for publication, October 29, 1996, and in revised form, December 12, 1996)

Tadashi Matsui, Eiro Muneyuki, Masahiro Honda, William S. Allison‡, Chao Dou‡, and Masasuke Yoshida§

From the Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226, Japan and the Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093-0601

A mutant $\alpha_3\beta_3\gamma$ complex of F₁-ATPase from thermophilic Bacillus PS3 was generated in which noncatalytic nucleotide binding sites lost their ability to bind nucleotides. It hydrolyzed ATP at an initial rate with cooperative kinetics ($K_{m}(A), 4 \mu \text{M}; K_{m}(P), 135 \mu \text{M}$) similar to the wild-type complex. However, the initial rate decayed rapidly to an inactivated form. Since the inactivated mutant complex contained 1.5 mol of ADP/mol of complex, this inactivation seemed to be caused by entrapping inhibitory MgADP in a catalytic site. Indeed, the mutant complex was nearly completely inactivated by a 10 min prior incubation with equimolar MgADP. Analysis of the progress of inactivation after initiation of ATP hydrolysis as a function of ATP concentration indicated that the inactivation was optimal at ATP concentrations in the range of $K_m(A)$. In the presence of ATP, the wild-type complex dissociated the inhibitory $[^3\text{H}]\text{ADP}$ preloaded onto a catalytic site whereas the mutant complex did not. Lauryl dimethylamino oxide promoted release of preloaded inhibitory $[^3\text{H}]\text{ADP}$ in an ATP-dependent manner and partly restored the activity of the inactivated mutant complex. Addition of ATP promoted single-site hydrolysis of 2',3'-O-(2,4,6-trinitrophenyl)-ATP preloaded at a single catalytic site of the mutant complex. These results indicate that intact noncatalytic sites are essential for continuous catalytic turnover of the F₁-ATPase but are not essential for catalytic cooperativity of F₁-ATPase observed at ATP concentrations below ~300 μM.

F₁-ATPase is the extrinsic membrane sector of H⁺-ATP synthase and comprises five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma$. $\delta\epsilon_1$ (1). According to the crystal structure of bovine heart mitochondrial F₁ (MF₁)$^3$ (2), the $\alpha$ and $\beta$ subunits are arranged alternately to form a hexagonal $\alpha_3\beta_3$ array. The six nucleotide binding sites are located at different interfaces between the $\alpha$ and $\beta$ subunits. The three catalytic sites are mainly on the $\beta$ subunits, whereas the other three sites called noncatalytic nucleotide binding sites are mainly on the $\alpha$ subunits. The overall structural topologies of the catalytic and noncatalytic sites are very similar to each other and both sites contain the two sequences known as the Walker motif A and B, which are commonly found in many nucleotide-binding proteins (3). Motif A, which is also called as P-loop, has the consensus sequence, GXXXGK(T/S), and motif B consists of a stretch of four consecutive hydrophobic residues followed by Asp.

The function of the noncatalytic site is obscure. However, recent studies suggest that the F₁-ATPase is prone to develop turnover-dependent inactivation and the noncatalytic sites play a role in relieving the inactivation (4, 5). When nucleotide-depleted MF₁ or F₁-ATPase from the thermophilic Bacillus PS3 (TF₁) hydrolyzes relatively low concentration of ATP, three kinetic phases are often observed in the presence of an ATP regenerating system. An initial burst rapidly decelerates to an intermediate rate that, in turn, gradually accelerates to a final steady-state rate. It has been postulated that transition from the initial phase to the intermediate phase is caused by turnover-dependent entrapment of inhibitory MgADP in a catalytic site (6–8), and transition from the intermediate phase to the final phase reflects slow binding of ATP to the noncatalytic sites, which promotes dissociation of inhibitory MgADP from the affected catalytic site. After prior loading of a catalytic site of MF₁ (4, 9, 10), TF₁ (5, 11), and chloroplast F₁-ATPase (12) with MgADP, the enzymes hydrolyze ATP with extended lag. The observation that the binding of ATP to noncatalytic sites stimulates ATPase activity was also reported (13–17). All these kinetic features are observed with the $\alpha_3\beta_3\gamma$ complex of TF₁ (18). The $\alpha_3\beta_3\gamma$ complex of TF₁ containing $\alpha$ subunits with a mutation in the Walker motif $\beta$, $\alpha$D261N, dissociates inhibitory MgADP only slowly even in the presence of ATP and the transition from the intermediate phase to the final phase most disappeared, exhibiting a low final rate of ATP hydrolysis, about 30% of that of the wild-type complex (18). Conversely, the $\alpha_3\beta_3\gamma$ complex of TF₁ containing $\beta$ subunits with a mutation in the Walker motif $\alpha$, $\beta$T165S, efficiently dissociates inhibitory MgADP and exhibits a severalfold higher final rate of ATP hydrolysis than that of the wild-type complex (19). These results suggest that F₁-ATPase in the inactivated state with inhibitory MgADP in a catalytic site is reactivated by ATP binding to noncatalytic sites. However, important unanswered questions remain. For instance, does enzyme containing inhibitory MgADP in a single catalytic site have weak residual ATPase activity or is it completely inactive? Is release of inhibitory MgADP totally dependent on ATP binding to noncatalytic sites or is there slow release of inhibitory MgADP that is independent of ATP binding to noncatalytic sites?

The role of noncatalytic site in the cooperative kinetics of F₁-ATPase also remains to be clarified. F₁-ATPase exhibits negative cooperativity characterized with two or three apparent $K_m$ values which are $1–30 \mu\text{M}$, $100–300 \mu\text{M}$, and above 400
μM (20–24). This apparent negative cooperativity is observed also for the membrane-bound enzyme (25) and proton translocation (26). Slow binding of ATP to noncatalytic sites can explain apparent negative cooperativity at relatively high concentration of ATP represented by the highest $K_a$ value (4). Weber et al. reported a single $K_a$ value for the mutant F$_1$-ATPase from Escherichia coli (EF$_1$) with mutations α-D261N/α-R365W in which nucleotide binding to noncatalytic sites was greatly diminished (27). They stated that the kinetics of this mutant showed no deviation from simple monophasic Michaelis-Menten kinetics. However, they assayed ATPase activity by measuring $P_i$ release, which is not suitable to monitor fluctuation in rate during assay. Therefore, as stated in their paper, they did not scrutinize the kinetic behavior at very low substrate concentrations, which is necessary to detect a $K_a$ at 1–30 μM. Similarly, Yohda et al. reported that α(D261N)βγ complex of TF$_1$ did not exhibit cooperativity, but again they examined kinetics only above 20 μM ATP (28). Therefore, the effect of noncatalytic sites on cooperative kinetics of F$_1$-ATPase remains unsettled.

Since the covalent modification of the noncatalytic sites with 5′-p-fluorosulfonylfenylazidoadenosine inactivates ATPase activity completely (29), it is even possible to argue that noncatalytic sites are essential for the activity of F$_1$-ATPase, although their participation in catalysis is indirect. The mutants reported so far whose noncatalytic sites are impaired, namely EF$_1$ (α-D261N/α-R365W) and α(D261N)βγ complex of TF$_1$, have considerable ATPase activity (18, 27, 28). Especially, the EF$_1$ mutant showed ATPase activity even under the condition where noncatalytic sites were supposed to be empty and Weber et al. concluded that occupancy of the noncatalytic sites by adenine nucleotides was not required for catalysis. However, ambiguity remains because both EF$_1$ (α-D261N/α-R365W) and α(D261N)βγ complex of TF$_1$ have the ability to bind nucleotide to the noncatalytic sites even though the affinity is decreased. To obtain more discriminating data on the role of noncatalytic sites, it is necessary to characterize a mutant F$_1$-ATPase that completely lacks the ability to bind nucleotides to noncatalytic sites. To generate such a mutant, we have replaced four amino acid residues in Walker motif A and B sequences of the TF$_1$-α subunit by Ala residues, and analyzed nucleotide binding properties and ATP hydrolysis catalyzed by the αβγ complex containing the mutated α subunits under a wide range of ATP concentration. Comparison of this mutant αβγ (DN) complex and the wild-type αβγ complex has revealed the essential role of noncatalytic sites in steady-state catalytic turnover of F$_1$-ATPases.

EXPERIMENTAL PROCEDURES

**E. coli Strains and Plasmids—** E. coli strains used were JM109 (30) for preparation of plasmids, CJ236 (31) for generating uracil-containing single-stranded plasmids for site-directed mutagenesis, and JM103ΔuncB-uncD (32) for expression of the wild-type and ΔNC αβγ complexes of TF$_1$. Plasmids pTABG1 and pKABG1 (33), which carried genes for the α, β, and γ subunits of TF$_1$, were used for mutagenesis and for gene expression, respectively. Terrific broth (34) was used as a culture media and supplemented with ampicillin (50–100 μg/ml). Helper phage M13K07 was obtained from Pharmacia, Japan. The expression plasmid for the ΔNC complex, the noncatalytic sites of which are incapable of binding of adenine nucleotides, was constructed as follows. The four mutations, α-Lys-175 → Ala, α-Thr-176 → Ala, α-Glu-261 → Ala, and α-Glu-262 → Ala, were introduced into pTABG1 by using two synthetic oligonucleotides: 5′-ATTGGCCAGGCCTACGCG-CCGCCCGTTGTG-3′ and 5′-TGCTGCAGATGCCTGCGATCAACG-3′ (changed bases are underlined) (31). The EcorRI-BglII fragment from the resultant plasmid was ligated into the EcoRI-BglII site of pKABG1 to produce the expression plasmid, pKABG1-αK175A/αT176A/αD261A/αD262A. Recombinant DNA procedures were performed as described in the manual (34).

**Materials—** 2-N$_3$-[3H]ATP and 2-N$_3$-[3H]ADP were synthesized and purified as described elsewhere (35). [3H]ADP was purchased from DuPont NEN. Laurdylidimethylamineoxyde (LDAO) was purchased from Calbiochem. Synthesis and purification of 2,3′-O-2,4,6-trinitropheno-lyl-ATP (TPN-ATP) were carried out as described previously (36). The wild-type and ΔNC αβγ complexes were purified as described before (33). Purified complexes did not contain detectable amount of endogenously bound adenine nucleotide (<0.05 mol/mol of complex).

**Analytical Methods—** Protein concentrations of TF$_1$ and αβγ complexes were determined by measurement of absorbance at 280 nm using the factor 0.45 of absorbance for 1 mg/ml of protein. ATPase activity was measured at 25 °C in the presence of an ATP regenerating system. An assay mixture contained 50 mM Tris-Cl (pH 8.0), 100 mM KCl, indicated concentrations of ATP, 2.5 mM phosphoenolpyruvate, 50 μg/ml pyruvate kinase (rabbit muscle), 50 μg/ml lactate dehydrogenase (pig muscle), and 0.2 mM NADH. Pyruvate kinase and lactate dehydrogenase (Boehringer Japan, Tokyo) were diluted from solution in glycerol. These amounts of auxiliary enzymes were confirmed to be sufficient for the rapid ATP hydrolysis in the initial burst phase of catalysis. MgCl$_2$ concentration was maintained at 2 mM excess over that of ATP in the assay mixture. Typically, the reaction was initiated by addition of the enzyme to 2 ml of the assay mixture and the rate of ATP hydrolysis was monitored as the rate of oxidation of NADH determined by the absorbance decrease at 340 nm. The data were stored in an on-line computer for subsequent analyses. We obtained a device on the spectrophotometer that enabled us to start the reaction by injecting the enzyme solution without opening it. The spectrophotometer was equipped with a small stirrer to ensure rapid mixing. We confirmed that the maximum dead time of measurement was below 4 s after the start of the reaction and the data from 5 to 20 s were usually used for analysis. The initial rates were obtained from exponential extrapolation of the experimental data between 5 and 20 s to time zero. One unit of activity was defined as the activity that hydrolyzed 1 μmol of ATP/min. Assessment of nucleotide binding to catalytic and noncatalytic sites of the complexes by photoaffinity labeling with 2-N$_3$-[3H]AT(D)P was carried out as described previously (37). Briefly, the solution (100 μl) containing 1.5 mg of the wild-type or ΔNC αβγ complex, 150 μl 2-N$_3$-[3H]AT(D)P (2700 cpm/nmol), MgCl$_2$ 100 mM, and 50 mM Tris-Cl, was irradiated for 40 min at room temperature with a Minilam, and digested with 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin after denaturation of the proteins and removal of unbound nucleotides. An aliquot of the digested solution was injected into a C4 reversed-phase HPLC column and developed with a gradient of CH$_3$CN in 0.1% HCl as follows: 0–10 min, 0%; 10–100 min, 0–24%; 100–115 min, 24–48%; 115–120 min, 48–80%. Fractions, 1 ml each, were collected and radioactivity of each fraction was measured. Difference spectra induced by the interaction between TNP-ATP and the proteins and single-site hydrolysis of TNP-ATP were measured according to previous papers (38–40). The bound nucleotide content of the enzyme complex was determined after separating free nucleotide from enzyme-bound nucleotides by centrifuge elution using a 1-ml column of Sephadex G-50, packed with perchloric acid, and analyzed by HPLC (38). Release of [3H]ADP from the complexes was monitored according to the methods described by Jault et al. (18).

**RESULTS**

**Generation of the Stable ΔNC αβγ Complex—** The crystal structure of MF$_1$ shows that, in the noncatalytic nucleotide binding site, Lys and Thr in the Walker motif A and Asp in the motif B of the α subunit lie close to the terminal phosphate and Mg$^{2+}$ of bound Mg-AMP-P(NH)$_2$P, a substrate analogue (2). In addition, the conserved Asp just adjacent to the Asp of the catalytic nucleotide binding site, Lys and Thr in the Walker motif A and Asp in the motif B sequence of the α subunit are conservatively replaced by Ala residues. Four mutations, α-Lys-175 → Ala, α-Thr-176 → Ala, α-Glu-261 → Ala, and α-Glu-262 → Ala, were introduced into pTABG1 by using two synthetic oligonucleotides: 5′-ATTGGCCAGGCCTACGCG-CCGCCCGTTGTG-3′ and 5′-TGCTGCAGATGCCTGCGATCAACG-3′ (changed bases are underlined) (31). The EcoRI-BglII fragment from the resultant plasmid was ligated into the EcoRI-BglII site of pKABG1 to produce the expression plasmid, pKABG1-αK175A/αT176A/αD261A/αD262A. Recombinant DNA procedures were performed as described in the manual (34).
The Role of Noncatalytic Sites in F\textsubscript{1}-ATP\textsubscript{ase}

used for the purification of the wild-type complex including the incubation at 60 °C for 30 min.

**Nucleotide Binding Properties of the ΔNC αβγ Complex**—To assess binding of adenine nucleotides to the noncatalytic sites, tryptic digests from the wild-type and ΔNC αβγ complexes which were photolabeled with 2-N3-[3H]ATP in the presence of Mg\textsuperscript{2+} were analyzed. The profiles of tryptic peptides resolved by reversed-phase HPLC are shown in Fig. 1. Elutions were carried out under the same conditions reported previously in which assignment of radioactive peaks was established (42). A radioactive peak eluted at around 78 min contains the tryptic peptide with β-Tyr-364 derivatized, which is a part of the noncatalytic site, and peaks eluted between 90 and 100 min contain the tryptic peptides with β-Tyr-341 derivatized, which is a part of the catalytic site (43). It has been shown that the tryptic peptides from the catalytic site are often eluted as two (or more) peaks as shown in Fig. 1 because of the heterogeneity arising from hydrolysis of ATP tethered to the catalytic subunit, whereas a trough at 395 nm and a peak at around 420 nm were observed for the α subunit (data not shown). These results show that 2-N3-adenine nucleotides cannot bind to the catalytic sites of the ΔNC complex.

The binding of nucleotide was further examined by TNP-ATP-induced difference spectra (Fig. 2). The difference absorption spectra induced by binding of TNP-ATP/DP to the isolated wild-type α or β subunit are significantly different from each other. A trough at 450 nm and a peak at 510 nm were observed for the α subunit whereas a trough at 395 nm and a peak at around 420 nm were observed for the β subunit (Fig. 2). The ΔNC complex showed spectra typical for the β subunit even at a 6:1 TNP-ATP/molar ratio (Fig. 2). In contrast, the ΔNC complex showed spectra typical for the β subunit even at a 6:1 TNP-ATP/molar ratio (Fig. 2). This indicates that TNP-ATP binds exclusively to the catalytic sites on the β subunits of the ΔNC complex and that noncatalytic sites on the α subunit are unable to bind TNP-ATP. Based on these results, we conclude that the noncatalytic sites of the ΔNC complex do not bind adenine nucleotides.

**ATPase Activity of the ΔNC αβγ Complex**—Fig. 3 shows time courses of ATP hydrolysis by the wild-type and ΔNC αβγ complexes in the presence of an ATP regenerating system. The wild-type complex hydrolyzed 20 μM ATP in three phases (trace b), an initial burst decelerated to an intermediate phase that then accelerated to a final state (18). At 2 mM ATP, the tran-
**Fig. 3.** ATP hydrolysis by the wild-type or ΔNC α₃β₃γ complexes. The wild-type and ΔNC complexes (5 μM) were incubated at 25 °C for 10 min in the presence (traces a, c, e, and h) or absence (traces b, d, f, and i) of 6 μM MgADP. Then, 1 μl of the wild-type (a–d) or 10 μl of the ΔNC (e, f, h, and i) complexes were removed and injected into 2 ml of an ATP assay mixture. ATP concentration in the assay mixture was 20 μM (a, b, c, f, and g) or 2 μM (c, d, h, i, and j). ATP hydrolysis was monitored at 25 °C in the presence of an ATP regenerating system. For comparison, the kinetic traces by the wild-type complex with the same amount (10 μl of 5 μM) that was used for the assay of the ΔNC complex are illustrated by dotted lines (g and j). Other experimental conditions are described under “Experimental Procedures.”

A transition from the intermediate phase to the final phase was not seen and it appeared to proceed in two phases, an initial burst phase and a following decelerated constant phase (Fig. 3). The wild-type and ΔNC complexes also hydrolyzed 20 μM and 2 mM ATP with an initial burst (traces f and i). The rates of the initial bursts by ΔNC complex at both concentrations were very similar to those observed for the wild-type complex which are shown by overlaid dotted lines (traces g and j). However, the initial burst of the ΔNC complex rapidly decelerated and hydrolysis stopped in a short period. We analyzed the kinetics of initial rates of the burst phase of the ΔNC complex at a wide range of ATP concentrations (1–2000 μM) and compared them to those of the wild-type complex (Fig. 4). Although there were some differences at high ATP concentrations, the ΔNC complex hydrolyzed ATP in a similar manner to the wild-type complex. As shown in the inset of Fig. 4, the Eadie-Hofstee plots of the initial rates of ATP hydrolysis by the wild-type and ΔNC complexes are concave downward, indicating that ATP hydrolysis by the ΔNC complex, like the wild-type complex, exhibits negative cooperativity. Apparent kinetic parameters are calculated by a nonlinear regression curve-fitting (24) and summarized in Table I.

**TABLE I**

| Complex      | $K_m^{(1)}$ | $V_{max}^{(1)}$ | $K_m^{(2)}$ | $V_{max}^{(2)}$ |
|--------------|-------------|----------------|-------------|----------------|
| Wild-type α₃β₃γ | 3.7         | 7.4            | 187         | 31             |
| ΔNC α₃β₃γ    | 4.0         | 8.8            | 135         | 22             |

The Role of Noncatalytic Sites in $F_1\text{-ATPase}$

**Fig. 4.** Initial rate of ATP hydrolysis by the wild-type and ΔNC α₃β₃γ complexes during the initial burst phase. The wild-type (WT) and mutant complexes (each 50 pmol) were injected into 2 ml of an ATPase assay mixture containing various concentrations of ATP in the presence of an ATP regenerating system. Inset, Eadie-Hofstee plots. Lines are drawn by a curve fitting program based on kinetic parameters listed in Table I. Open circle, wild-type complex; closed circle, ΔNC complex. Other experimental conditions were described under “Experimental Procedures.”

...almost the same as $K_m^{(1)}$ and $V_{max}^{(1)}$ of the wild-type complex. $K_m^{(2)}$ and $V_{max}^{(2)}$ of the ΔNC complex are also close (about 70%) to those of the wild-type complex. Thus, the α₃β₃γ complex without functional noncatalytic sites exhibits cooperative kinetics which are very similar to those of the wild-type complex with intact noncatalytic sites.

Inactivation of the ΔNC α₃β₃γ Complex—Although the initial rate of ATP hydrolysis by the ΔNC complex obeys similar kinetics to those of the wild-type complex, it is rapidly inactivated during catalytic turnover and hydrolysis stops completely in a short period as described above (Fig. 3, traces f and i). The dependence of the rate of inactivation on ATP concentration is shown in Fig. 5 (A and B). The time course of inactivation was exponential curve, and the first order rate constants of inactivation were obtained at various ATP concentrations. As shown in Fig. 5C, the rate constants of inactivation exhibit monophasic dependence on ATP concentration. The ATP concentration that gave a half-maximal rate of inactivation was 5 μM. This value agrees well with the $K_m^{(1)}$ (4 μM) obtained from analysis of the rate in the initial burst phase. The maximal rate of inactivation was 0.33 s⁻¹. Combining the rate of inactivation and the rate of ATP hydrolysis, we can calculate the average number of catalytic turnovers required for the inactivation at each ATP concentration. For example, at 0.53, 1.1, 1.4, 4.4, 11, 22, and 33 μM ATP, 17, 31, 63, 80, 97, and 105 turnovers are required for the inactivation, respectively. Therefore, inactivation is not simply proportional to turnover number. When ATP concentration is low, the enzyme is inactivated in relatively few turnovers. More turnovers are required for inactivation as ATP concentrations increased. Accordingly, the size of initial burst is smaller when...
showed attenuated initial rates of hydrolysis of 20 mM MgADP by a prior incubation with equimolar MgADP for 5 min investigated after incubating the complex with MgADP. As re-
of the bound ADP in the inactivated complex was further in-
D complex, while a control
As shown in Fig. 6, the extent of inactivation of the
inactivated state. Analysis of the bound nucleotides revealed that the
have ATPase activity, ensuring that it was still in an inacti-
fuge column. The complex recovered in the effluent did not
were removed by passing through the Sephadex G-50 centri-
cannot bind MgADP, we conclude that the ΔNC complex is
was observed. Since noncatalytic sites in the ΔNC complex
is only to amplify the conformational signal generated by the

Effect of LDAO—It has been shown in previous studies that
the ATPase activities of TF \( \alpha \) and the wild-type \( \alpha \)Δβ\( \gamma \) complex are stimulated significantly by the neutral detergent LDAO (5, 18, 44). The stimulation of ATPase activity by LDAO is thought
to promote release of inhibitory MgADP from a catalytic site in
the presence of ATP (19). When LDAO was present in the
reaction mixture, the wild-type complex hydrolyzed 2 mM ATP at
a constant rate from the beginning. The constant rate in the
presence of LDAO was very close to the rate in the initial burst
in the absence of LDAO (Fig. 7A). It appears that LDAO does
not affect the rate of ATP hydrolysis in the initial burst, but
rather allows the initial burst to continue linearly without
deceleration. Similar to the wild-type complex, LDAO had little
effect on the initial burst of the ΔNC complex (Fig. 7B).
However, different from the wild-type complex, the initial burst
decelerated even in the presence of LDAO and reached a slow,
final rate. When LDAO was added to the ΔNC complex, which
was previously inactivated by aging in the assay mixture or by
prior incubation with MgADP, the same slow rate was restored
(Fig. 7C). The above experiments were performed at 2 mM ATP.
However, a similar effect of LDAO on the ΔNC complex was
observed when it hydrolyzed 20 mM ATP (data not shown). The
stimulating effect of LDAO on the final steady-state hydrolysis
was saturated at 0.04% LDAO for the wild-type complex
and was nearly (but not completely) saturated at 0.15% LDAO for
the ΔNC complex (Fig. 7D). If the mechanism of action of LDAO
is only to amplify the conformational signal generated by the
ATP binding to noncatalytic sites that causes release of inhib-
itory MgADP, then LDAO would not have an effect on the
ΔNC complex. Probably, LDAO has a direct effect on the catalytic
site occupied by inhibitory ADP. This effect might be small
because the extent of activity restored by LDAO in the case of
the ΔNC complex is much smaller than that of the wild-type
complex as described above.

Release of Inhibitory MgADP—The effect of ATP and LDAO
on the release of preloaded, inhibitory \(^{3} \text{H}\)ADP from the
wild-type and ΔNC complexes was examined (Fig. 8). For the
wild-type complex, release of preloaded \(^{3} \text{H}\)ADP was promoted by
ATP but not by LDAO (Fig. 8A). However, when both ATP and
LDAO were present in the solution, the release was greatly
enhanced and most \(^{3} \text{H}\)ADP was released in 30 s. For the ΔNC
complex, neither ATP nor LDAO, when added alone, promoted
release of \(^{3} \text{H}\)ADP. A moderate promotion of the release was

---

**Fig. 5.** Rate of generation of the inactivated ΔNC \( \alpha \)β\( \gamma \) complex plotted as a function of ATP concentration. The ΔNC complex (150 pmol) was injected at the time indicated by the arrows into 1.2 ml of the ATPase assay mixture containing various concentrations of ATP. Typical kinetic traces at 0.53 mM ATP (A) and 33 mM ATP (B) are illustrated. It was confirmed that the initial rapid drop of absorbance observed in A was caused by dilution. Theoretical lines of time courses were calculated assuming an exponential decrease of the absorbance and overlaid in A and B by solid lines. In B, the line completely overlapped the experimental data. C, the rate of inactivation obtained from the fitted time course was plotted as a function of ATP concentration. The solid line in C is a theoretical one calculated from kinetic parameters described in the text. Other experimental conditions were described under “Experimental Procedures.”
The Role of Noncatalytic Sites in $F_1$-ATPase

Fig. 7. Effect of LDAO on the ATP hydrolysis by the wild-type and $\Delta NC\ alpha betagamma$ complexes. A, hydrolysis of 2 mM ATP by the wild-type complex in the absence or presence of 0.1% LDAO. B, hydrolysis of 2 mM ATP by the $\Delta NC$ complex in the absence or presence of 0.1% LDAO. C, effect of addition of 0.1% LDAO to the $\Delta NC$ complex inactivated by a prior incubation with MgADP or by aging in the ATP assay solution. D, effect of LDAO concentration on the steady-state ATPase activities. Open circle, wild-type (WT) $\alpha beta gamma$ complex; closed circle, $\Delta NC$ complex. Other experimental conditions were described under “Experimental Procedures.”

Fig. 8. Release of preloaded $[^3H]ADP$ from a catalytic site of the wild-type and $\Delta NC\ alpha beta gamma$ complexes. A and B, the wild-type (WT) and $\Delta NC$ complex (B) (each 2.8 M) were incubated at 25 °C for 30 min with a solution containing 4 $\mu$m $[^3H]ADP$, 1 mM MgCl2, 100 $\mu$m EDTA, and 50 mM Tris-Cl (pH 8.0). Then the solution was diluted 20-fold with 50 mM Tris-Cl buffer (pH 8.0) containing 2 mM MgCl2, and 40 $\mu$m ATP and/or 0.1% LDAO as indicated. The diluted solution was incubated at 25 °C. At the times indicated, an aliquot was taken out and subjected to a Sephadex-G50 centrifuge column to remove unbound $[^3H]ADP$. The amount of $[^3H]ADP$ bound to the complex contained in the effluent was obtained. Other experimental conditions were described under “Experimental Procedures.”

Fig. 9. Hydrolysis of substoichiometric amount of TNP-ATP by the wild-type and $\Delta NC\ alpha beta gamma$ complexes. The reaction mixture containing 0.3 mM TNP-ATP, 2 mM MgCl2, and 1 $\mu$m of the wild-type (A, WT) or $\Delta NC$ complex (B) was incubated at 25 °C. At indicated time, the reaction was terminated by addition of perchloric acid (~ATP chase) or was chased by addition of 3.3 mM ATP (+ATP chase). The reaction was terminated 5 s after chase addition of ATP by the addition of perchloric acid. The amounts of produced TNP-ADP and remaining TNP-ATP were measured by HPLC. Other experimental conditions were described under “Experimental Procedures.”

MgADP when LDAO is present in the assay mixture and, as a consequence, almost all of the enzyme is in an active state, showing uninhibited ATPase activity. For the $\Delta NC$ complex, in contrast, the presence of ATP does not promote release of inhibitory MgADP. Once inhibitory MgADP is entrapped, it fails to dissociate keeping the complex in an inactivated state. When LDAO and ATP are present, inhibitory MgADP is released slowly and an equilibrium is established with a small fraction of the complex free of inhibitory MgADP resulting in partial restoration of activity.

Single-site TNP-ATP Hydrolysis and Chase-promotion—$\mathrm{TF}_1$ (38) and the $\alpha beta gamma$ complex (40) hydrolyze TNP-ATP slowly when TNP-ATP is added to the enzyme in a substoichiometric molar ratio. Slow hydrolysis of TNP-ATP is greatly accelerated by chase-promotion with ATP. It has been suggested that the ATP binding site responsible for chase-promotion is the second catalytic site to be filled (39, 45). Fig. 9 illustrates time courses of TNP-ATP hydrolysis by the wild-type and $\Delta NC$ complexes. Similar to the wild-type complex, the $\Delta NC$ complex slowly hydrolyzed substoichiometric TNP-ATP and chase-promotion with ATP accelerated the hydrolysis of the TNP-ATP. From this result we conclude that participation of noncatalytic sites is not necessary for cooperativity between two catalytic sites.

DISCUSSION

Noncatalytic Sites Are Essential for Continuous Catalytic Turnover—This work provides solid support for the view that entrapping inhibitory MgADP at a catalytic site, either during incubation with MgADP or during turnover under assay conditions, causes inactivation of $F_1$-ATPase. In addition, it is now clear that enzyme that retains inhibitory MgADP at a single catalytic site is completely inactive in ATP hydrolysis (Figs. 3 and 6). Differing from the wild-type enzyme, inhibitory MgADP is not released from the $\Delta NC$ complex even in the presence of ATP (Fig. 8). Since catalytic sites in the $\Delta NC$ complex are as intact and available for ATP binding as those of the wild-type complex, the failure of ATP to promote release of inhibitory MgADP from a catalytic site can only be attributed to the lack of ability of noncatalytic sites to bind ATP. Thus, it is concluded that ATP binding to noncatalytic sites is essential for continuous catalytic turnover. This may have physiological importance since Richard et al. suggested that ATP synthesis by H$^+$-ATP synthase from thermophilic Bacillus PS3 is stimulated when the noncatalytic sites were occupied by ATP (46).

Noncatalytic Sites Are Not Essential for Cooperative Kinetics

observed only in the presence of both ATP and LDAO (Fig. 8B). Based on these results, an explanation can be given for the observations shown in Figs. 3 and 7. For the wild-type complex, both entrapping and release of inhibitory MgADP occur during turnover in the presence of ATP. A fraction of the complex is always in an inactive state during steady-state catalysis in the absence of LDAO. However, LDAO promotes release of the inhibitory MgADP from the affected catalytic site and this converts nearly all of the enzyme to an active state. This equilibrium was driven in the direction of release of inhibitory

DISCUSSION

Noncatalytic Sites Are Essential for Continuous Catalytic Turnover—This work provides solid support for the view that entrapping inhibitory MgADP at a catalytic site, either during incubation with MgADP or during turnover under assay conditions, causes inactivation of $F_1$-ATPase. In addition, it is now clear that enzyme that retains inhibitory MgADP at a single catalytic site is completely inactive in ATP hydrolysis (Figs. 3 and 6). Differing from the wild-type enzyme, inhibitory MgADP is not released from the $\Delta NC$ complex even in the presence of ATP (Fig. 8). Since catalytic sites in the $\Delta NC$ complex are as intact and available for ATP binding as those of the wild-type complex, the failure of ATP to promote release of inhibitory MgADP from a catalytic site can only be attributed to the lack of ability of noncatalytic sites to bind ATP. Thus, it is concluded that ATP binding to noncatalytic sites is essential for continuous catalytic turnover. This may have physiological importance since Richard et al. suggested that ATP synthesis by H$^+$-ATP synthase from thermophilic Bacillus PS3 is stimulated when the noncatalytic sites were occupied by ATP (46).

Noncatalytic Sites Are Not Essential for Cooperative Kinetics

observed only in the presence of both ATP and LDAO (Fig. 8B). Based on these results, an explanation can be given for the observations shown in Figs. 3 and 7. For the wild-type complex, both entrapping and release of inhibitory MgADP occur during turnover in the presence of ATP. A fraction of the complex is always in an inactive state during steady-state catalysis in the absence of LDAO. However, LDAO promotes release of the inhibitory MgADP from the affected catalytic site and this converts nearly all of the enzyme to an active state. This equilibrium was driven in the direction of release of inhibitory

DISCUSSION

Noncatalytic Sites Are Essential for Continuous Catalytic Turnover—This work provides solid support for the view that entrapping inhibitory MgADP at a catalytic site, either during incubation with MgADP or during turnover under assay conditions, causes inactivation of $F_1$-ATPase. In addition, it is now clear that enzyme that retains inhibitory MgADP at a single catalytic site is completely inactive in ATP hydrolysis (Figs. 3 and 6). Differing from the wild-type enzyme, inhibitory MgADP is not released from the $\Delta NC$ complex even in the presence of ATP (Fig. 8). Since catalytic sites in the $\Delta NC$ complex are as intact and available for ATP binding as those of the wild-type complex, the failure of ATP to promote release of inhibitory MgADP from a catalytic site can only be attributed to the lack of ability of noncatalytic sites to bind ATP. Thus, it is concluded that ATP binding to noncatalytic sites is essential for continuous catalytic turnover. This may have physiological importance since Richard et al. suggested that ATP synthesis by H$^+$-ATP synthase from thermophilic Bacillus PS3 is stimulated when the noncatalytic sites were occupied by ATP (46).

Noncatalytic Sites Are Not Essential for Cooperative Kinetics

observed only in the presence of both ATP and LDAO (Fig. 8B). Based on these results, an explanation can be given for the observations shown in Figs. 3 and 7. For the wild-type complex, both entrapping and release of inhibitory MgADP occur during turnover in the presence of ATP. A fraction of the complex is always in an inactive state during steady-state catalysis in the absence of LDAO. However, LDAO promotes release of the inhibitory MgADP from the affected catalytic site and this converts nearly all of the enzyme to an active state. This equilibrium was driven in the direction of release of inhibitory

DISCUSSION

Noncatalytic Sites Are Essential for Continuous Catalytic Turnover—This work provides solid support for the view that entrapping inhibitory MgADP at a catalytic site, either during incubation with MgADP or during turnover under assay conditions, causes inactivation of $F_1$-ATPase. In addition, it is now clear that enzyme that retains inhibitory MgADP at a single catalytic site is completely inactive in ATP hydrolysis (Figs. 3 and 6). Differing from the wild-type enzyme, inhibitory MgADP is not released from the $\Delta NC$ complex even in the presence of ATP (Fig. 8). Since catalytic sites in the $\Delta NC$ complex are as intact and available for ATP binding as those of the wild-type complex, the failure of ATP to promote release of inhibitory MgADP from a catalytic site can only be attributed to the lack of ability of noncatalytic sites to bind ATP. Thus, it is concluded that ATP binding to noncatalytic sites is essential for continuous catalytic turnover. This may have physiological importance since Richard et al. suggested that ATP synthesis by H$^+$-ATP synthase from thermophilic Bacillus PS3 is stimulated when the noncatalytic sites were occupied by ATP (46).
of the F$_1$-ATPase—Comparison of the rates of ATP hydrolysis in the initial burst by the wild-type and ΔNC complexes revealed that both enzymes obey very similar cooperative kinetics (Fig. 4, Table I). Both the $K_m$ and $V_{max}$ values of the ΔNC complex are almost the same or close to the corresponding values of the wild-type complex. In addition, the ΔNC complex can catalyze single-site hydrolysis and chase-promotion using TNP-ATP as a substrate (Fig. 9). The remarkably similar kinetic of the ΔNC complex and the wild-type complex strongly indicates that the catalytic sites of the ΔNC complex are intact and behave in a similar manner to the wild-type complex. In other words, noncatalytic sites contribute little, if anything, to cooperation of the $\alpha_2\beta_2\gamma$ complex. Thus, cooperative features of ATP hydrolysis by F$_1$-ATPase characterized by two sets of parameters, $K_{m(1)} = 1$–30 μM and $K_{m(2)} = 100$–300 μM, reflect catalytic site to catalytic site cooperativity. The apparent $K_{m(2)}$ of 140 μM observed here agrees well with the $K_m$ for proton translocation which was membrane potential independent (47). Cooperativity observed at high ATP concentration (>400 μM) at steady-state catalysis is a phenomenon attributed to slow nucleotide binding to noncatalytic sites (4).

Simultaneous Occupation of Two Catalytic Sites Promotes Entrapment of Inhibitory MgADP at a Catalytic Site—Interestingly, the rate of the progressive inactivation of the ΔNC complex during turnover shows a hyperbolic dependence on ATP concentration and exhibits an apparent $K_m$ of 5 μM (Fig. 5). This $K_m$ value corresponds to $K_{m(1)}$ (4 μM) obtained from initial rate analysis. This $K_m$ is thought to reflect a catalytic cycle operating when two catalytic sites are occupied, so-called bi-site catalysis (22). Owing to the very low $K_d$ and $K_{m(1)}$ values for ATP hydrolysis when only one catalytic site is occupied, single site catalysis is not amenable to steady-state kinetic analysis. This means that occupancy of two catalytic sites promotes transition from an active to an inactive enzyme. On the other hand, we observed that loading a catalytic site with exogenous MgADP is sufficient to inactivate the ΔNC complex completely (Fig. 6). This apparent contradiction can be accommodated as follows. The formation of inactivated MgADP-TP$_1$ from TF$_1$ and MgADP is a slow process (2 m$^{-1}$ s$^{-1}$) (11) and this is also the case for the $\alpha_2\beta_2\gamma$ complex. The rate-limiting step is most likely to be the isomerization from a transient, active MgADP-enzyme complex into the stable, inactive MgADP-enzyme complex (6, 19). If ATP hydrolysis operating with two catalytic sites can facilitate the isomerization, ATP hydrolysis characterized by $K_{m(1)}$ becomes an apparently responsible step for the generation of the inactive complex. After isomerization, MgADP at one of the two catalytic sites might be released during gel filtration procedures to analyze bound nucleotide. In this mechanism, cooperative interaction between two catalytic sites is assumed to accelerate not only catalysis but also generation of inhibitory MgADP-ΔNC complex (7, 19). Azide inhibition and inactivation of the ΔNC complex during turnover probably operate by a similar mechanism.