Slow Binding of ATP to Noncatalytic Nucleotide Binding Sites Which Accelerates Catalysis Is Responsible for Apparent Negative Cooperativity Exhibited by the Bovine Mitochondrial $F_1$-ATPase*

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The bovine heart mitochondrial $F_1$-ATPase depleted of nucleotides (nd-MF$_1$) hydrolyzes 50 $\mu$M ATP in three kinetic phases at 30 °C. An initial “burst” rapidly transforms into an intermediate, slower rate, which slowly accelerates to the final, steady-state rate. The intermediate phase disappears progressively as the concentration of ATP in the assay medium is increased and is absent at 2 mM. Activation in the intermediate phase is lost when nd-MF$_1$, is inactivated by 5'-p-fluorosulfonylbenzoyladenosine, which modifies three noncatalytic sites. Correlation of [3H]ATP binding to nd-MF$_1$, after treatment either with 50 $\mu$M Mg[3H]ATP plus a regenerating system or 10 mM free [3H]ATP, with stimulation of the intermediate phase suggests that this phase is abolished when at least two noncatalytic sites are filled with ATP. Prior incubation of nd-MF$_1$, with MgPP, stimulates hydrolysis of 30 $\mu$M to 2 mM ATP and abolishes the intermediate phase. Following incubation with Mg[32P]PP, 3.3 mol of [32P]PP/mol of enzyme are bound, 1 and 0.5 mol of which are released by cold chases with MgATP and MgTP, respectively. Since the cold chases diminish activation only slightly, the stimulatory effect is not caused by PP binding to catalytic sites. A Lineweaver-Burk plot of initial rates of the intermediate phase for hydrolysis of 30 $\mu$M to 2 mM ATP by nd-MF$_1$, is biphasic, extrapolating to apparent $K_d$ values of 120 and 440 $\mu$M. The latter value is the same as the apparent $K_d$ determined from dependence of the rate of activation of the intermediate phase on ATP concentration in the assay medium. After prior incubation of nd-MF$_1$, with MgPP, or free ATP, Lineweaver-Burk plots are linear with the highest $K_m$ disappearing. Thus, this $K_m$ reflects rate acceleration when ATP binds to noncatalytic sites. From these results it is concluded that slow binding of ATP to noncatalytic sites during hydrolysis of low concentrations of substrate, which accelerates catalysis, is responsible for apparent negative cooperativity exhibited by MF$_1$.

The $F_1$-ATPase is the peripheral membrane component of the $F_0$-$F_1$-ATP synthases present in mitochondria, chloroplast thylakoids, and the plasma membranes of bacteria which can be isolated in soluble form. It is composed of five types of subunit with a stoichiometry $\alpha_3\beta_2\gamma\delta\varepsilon$. From the amino acid sequences of its component subunits, the molecular weight of the bovine heart enzyme is 371,000 (1). The $F_1$-ATPases contain six nucleotide binding sites (2–4). For the beef heart enzyme, three of these sites bind and exchange nucleotides readily during catalysis. Thus, each is a potential catalytic site. In contrast, the other three sites, once filled with adenine nucleotides, exchange very slowly during turnover and are therefore called noncatalytic (2, 5).

Chemical modification studies with 2-azido-ATP have provided evidence that Tyr-345 of the $\beta$-subunit is located at catalytic sites of the beef heart enzyme, whereas Tyr-368 is located at noncatalytic sites (6). Selective modification of the catalytic or the noncatalytic site has also been achieved using two other nucleotide analogues, FSBI and FSBA. Maximal inactivation by the former reagent is accompanied by modification of Tyr-345 in a single $\beta$ subunit (7), whereas maximal inactivation by the latter is accompanied by mutually exclusive modification of Tyr-368 or His-427 in all three $\beta$ subunits (8).

That noncatalytic sites are involved in control of catalysis has been suggested on the basis of several experimental criteria for different $F_1$-ATPases. Steady-state kinetic analyses of $F_1$-ATPases from different sources have revealed apparent negative cooperativity when ATP hydrolysis is measured over a wide range of substrate concentration. Two (9–15) or three (16, 17) $K_m$ values have been deduced from steady-state kinetics, depending on the conditions of analysis. The participation of noncatalytic sites in the control of catalysis has also been suggested from the catalytic properties of mutant enzymes with defective ATPase activity (14, 18) and the characteristics of ADP-induced hysteretic inhibition (19–21). Furthermore, it has been shown recently that the ATPase activity of the CF$_1$-ATPase from spinach chloroplasts depends on the binding of ATP to noncatalytic sites (22, 23). We report here that, at 30 °C, saturation of noncatalytic sites with ATP stimulates ATPase activity of the mitochondrial $F_1$-ATPase depleted of endogenous nucleotides (nd-MF$_1$) to an optimal steady-state level.

**EXPERIMENTAL PROCEDURES**

$F_1$ was prepared from bovine heart mitochondria with a previously described (24) modification of the method of Knowles and Penefsky (25) and was depleted of nucleotides by gel permeation chromatography in the presence of 50% glycerol (v/v) as described by Garrett and Penefsky (26). After chromatography, protein frac-

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1 The abbreviations used are: FSBI, 5'-p-fluorosulfonylbenzoyladenosine; FSBA, 5'-p-fluorosulfonylbenzoyl-8-azidoadenosine; MF$_1$, bovine heart mitochondrial $F_1$-ATPase; nd-MF$_1$, MF$_1$-ATPase depleted of nucleotides; CF$_1$, $F_1$-ATPase from spinach chloroplasts; TF$_1$, $F_1$-ATPase from the thermophilic bacterium PS3.
tions with an $A_{280}/A_{260}$ ratio of $>1.95$ were 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 analyses by HPLC as previously described (21), the nd-MF$_1$ used in this study contained about 0.2 mol of ATP/mol of enzyme and essentially no bound ADP. The nd-MF$_1$ hydrolyzed 190–120 nmol of ATP/mg of protein at 30 °C. ATPase activity was determined spectrophotometrically at 30 °C with an assay medium containing 50 mM Hepes/KOH, pH 8.0, containing 4 mM EDTA and 50% glycerol.

An Activation Phase Is Exhibited When nd-MF$_1$ Hydrolyzes Low, but Not High Concentrations of ATP—Hydrolysis of 50 µM ATP by nd-MF$_1$ proceeds in three distinct phases as shown in Fig. 1 (trace $a$). In the first two phases, an initial "burst," lasting less than 20 s, rapidly transforms to a slow, interna-

diate phase of hydrolysis. This phenomenon has been described in detail by several laboratories (31–35). The slow intermediate phase gradually accelerates to a final steady-state rate. The latter transition is an activation process which has not been previously described for MF$_1$. Whereas the initial burst is not affected, 10 mM Na$_2$SO$_4$ in the assay medium nearly completely abolishes activation of the intermediate phase as shown by trace $b$. Sulfate introduced with pyruvate kinase and lactate dehydrogenase is sufficient to affect the intermediate phase. Therefore, these enzymes were added as solutions in glycerol to provide strict exclusion of sulfate from the assay medium. As shown by traces $a$, $c$, and $d$, which are recordings of assays initiated by addition of 2.1, 1.4, and 0.7 µg of nd-MF$_1$, respectively, the magnitude of the burst phase decreases with decreasing quantities of enzyme in the assay medium and is barely discernible when 0.7 µg of enzyme are assayed. In contrast, activation of the intermediate phase remains prominent when less than 1 µg/ml enzyme is assayed. In order to eliminate interference from the initial burst, the remainder of the enzyme assays reported in this study were carried out with less than 1 µg of nd-MF$_1$/ml. Recordings of hydrolysis were initiated 20 s after addition of enzyme to the spectrophotometer cell. Therefore, "initial rate" in these experiments refers to the rate in the 20–60-s interval after initiating hydrolysis by addition of enzyme.

Fig. 2, in which trace $a$ is a control, illustrates conditions which affect activation of the intermediate phase when nd-

MF$_1$ hydrolyzes 50 µM ATP. As the ATP concentration in
the assay medium is increased, the intermediate phase becomes progressively shorter and is entirely absent when assays are conducted at 2 mM ATP as shown by Fig. 2B (trace a). Prior incubation of nd-MF1 with either 10 mM ATP (Fig. 2A, trace b) or 50 μM ATP in the presence of Mg2+ and an ATP regenerating system (not shown) also eliminates the intermediate phase. These prior incubations have no effect on the linear, initial rate of hydrolysis of 2 mM ATP (Fig. 2B, trace b). Prior incubation of nd-MF1, with 2 mM PPi, in the presence of Mg2+ not only abolishes the intermediate phase observed during hydrolysis of 50 μM ATP, but also stimulates ATPase activity during assay at low and high ATP concentrations as shown by comparison of traces c with traces a in Fig. 2 (A and B). Addition of 20 mM bicarbonate to the assay medium does not eliminate the intermediate phase observed during hydrolysis of 50 μM ATP but does stimulate the final steady-state rate and the rate of hydrolysis of 2 mM ATP (Fig. 2, panels A and B, traces d).

Modification of Noncatalytic Sites with FSBA Abolishes the Lag Phase Observed during Hydrolysis of 50 or 100 μM ATP by nd-MF1—Recordings for hydrolysis of 50 μM ATP are progressively transformed from curved to linear as nd-MF1 is inactivated with FSBA. Trace e of Fig. 2A shows a recording of the hydrolysis of 50 μM ATP catalyzed by nd-MF1, which had been inactivated by 81% with FSBA. In contrast, the lag phase remains after inactivation of nd-MF1, with FSBI. Trace f of Fig. 2A illustrates hydrolysis of 50 μM ATP by nd-MF1, inactivated by 55% with FSBI. The lag phase remains even after inactivation of nd-MF1 with FSBI reaches 95% (not shown). Recordings of activity remained linear when residual ATPase activity was determined with 2 mM ATP during inactivation of nd-MF1, with either FSBA or FSBI as illustrated by Fig. 2B (traces e and f).

Recordings for hydrolysis of 50 μM ATP by samples of nd-MF1 undergoing inactivation by FSBA become more linear as noncatalytic sites are increasingly modified. This suggests that the progressive increase in activity observed during the lag phase exhibited by unmodified enzyme is caused by slow binding of ATP to noncatalytic sites. Therefore, it was of interest to compare the rate of inactivation of nd-MF1, by FSBA, determined by monitoring the initial, slow rate, on the one hand, and the final, fast rate, on the other, during hydrolysis of low concentrations of ATP. As illustrated in Fig. 3A, the initial rate of hydrolysis of 100 μM ATP is much less sensitive to inactivation by FSBA than is the final steady-state rate. The half-times observed when inactivation is measured by monitoring the initial, slow rate and final, fast rate of ATP hydrolysis are, respectively, 42 and 15 min. When inactivation by FSBA is monitored by assaying samples with 2 mM ATP, the rate of inactivation is close to that determined by monitoring the final steady-state rate during hydrolysis of 100 μM ATP (Fig. 3A). After prior incubation of nd-MF1 with 10 mM ATP, the rate of inactivation of the enzyme by FSBA is the same when residual activity is monitored by assay with either 100 μM or 2 mM ATP as illustrated in Fig. 3B. It should be noted that the FSBA concentration is higher in this experiment in order to compensate for the protective effect of ATP.

Also shown in Fig. 3B, prior incubation of nd-MF1 with 2 mM PPi, plus Mg2+ leads to the same rate of inactivation of the enzyme when residual activity is monitored by assaying samples at 100 μM or 2 mM ATP. Comparison of the rate of inactivation of nd-MF1, monitored with 2 mM ATP in the assay medium in the presence (Fig. 3B) and absence (Fig. 3A) of prior incubation with PPi, clearly shows that binding of PPi to the enzyme accelerates inactivation by FSBA. Pyrophosphate has also been observed to accelerate inactivation of MF, by 8-N3-FSBA in the dark (36).

Binding of [3H]ATP to Noncatalytic Sites Correlates with Disappearance of the Lag Phase Observed during Hydrolysis of 50 μM ATP by nd-MF1—The amount of bound [3H]ATP, measured by gel permeation chromatography on Sephadex G-50 centrifuge columns, increases from 1.3 to about 3 mol/mol of enzyme when nd-MF1 is incubated with 0.5–10.0 mM [3H]ATP in the absence of Mg2+ as shown in Fig. 4A (upper curve). When ATP and Mg2+ are added to final concentrations of 75 and 10 mM, respectively, in a cold chase prior to applying samples to centrifuge columns, the amount of [3H]-labeled nucleotide bound decreases by about 1 mol/mol of enzyme.
ATP Binding to Noncatalytic Sites Promotes ATP Hydrolysis

I. Appearance of the lag phase observed during hydrolysis of 50 pM ATP with the amount of [3H]ATP bound during prior incubation of nd-MF1 with [3H]ATP, with and without a cold chase, is shown in Fig. 4B. The results obtained after the cold chase indicate that maximal stimulation occurs when two noncatalytic sites are saturated with [3H]ATP.

Fig. 5A shows that prior incubation of nd-MF1 with 50 pM ATP plus 1 mM Mg$^{2+}$ in the presence of an ATP regenerating system leads to a time-dependent disappearance of the lag phase when the activity is subsequently monitored by assay with 50 pM ATP. Measurements of the binding of [3H]ATP to the enzyme under these conditions (Fig. 5B) show that

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**Fig. 4.** Correlation of binding [3H]ATP to noncatalytic sites with loss of the lag phase observed when nd-MF1 hydrolyzes 50 pM ATP. A, solutions of 0.7 mg/ml nd-MF1 in 90 mM Tris-SO$_4$, pH 8.0, containing 24 mM EDTA, 10% (w/v) glycerol, and increasing concentrations of [3H]ATP were prepared at 23°C. After 5 min, unbound nucleotide was removed by passing the enzyme solutions through centrifuge columns equilibrated in 50 mM Tris-SO$_4$, pH 8.0, containing 5% glycerol, 1 mM MgCl$_2$, and 1 mg/ml bovine serum albumin, with (●) or without (○) a subsequent 20-s cold-chase with ATP and MgCl$_2$ added to final concentrations of 75 and 10 mM, respectively. B, the reaction mixtures the same as in A except that [3H]ATP was replaced by cold ATP. After incubating the reaction mixtures for 5 min at 23°C, 1-μl sample of each was assayed with 50 pM ATP with a regeneration system. Relative activation represents the ratio of the initial rate (measured 20–60 s after adding enzyme) to the final rate (measured 4.0–4.5 min after adding enzyme) divided by the same ratio determined for nd-MF1, in the absence of prior incubation with ATP. The concentrations of ATP in the assay mixtures were adjusted for the amount of ATP injected concomitantly with enzyme and kept constant at 50 pM. Moles of [3H]ATP/mol of MF1 were determined from the values obtained in A.

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**Fig. 5.** Correlation of binding of [3H]ATP in the presence of Mg$^{2+}$ plus a regenerating system to noncatalytic sites with loss of the lag phase observed when nd-MF1 hydrolyzes 50 pM ATP. A, a solution of 0.07 mg/ml nd-MF1, was prepared in 50 mM Hepes/KOH, pH 8.0, which contained 10% glycerol with 50 pM ATP in a medium containing 1.1 mM MgCl$_2$, 10 mM phosphoenolpyruvate (tris(cyclohexylammonium) salt), 3.3 mg/ml pyruvate kinase, and 0.5 mM [3H]ATP. At the times indicated, 5 μl were assayed with 50 pM ATP plus a regenerating system. Relative activation is defined in the legend of Fig. 4A. The reaction mixture was the same as in A except that [3H]ATP was used. At the times indicated, samples were passed through centrifuge columns of Sephadex G-50 equilibrated with 50 mM Hepes/KOH, pH 8.0, containing 5% glycerol, 1 mM MgCl$_2$, and 1 mg/ml bovine serum albumin to remove unbound [3H]ATP with (●) or without (○) a 20-s cold chase by addition of ATP and MgCl$_2$ to final concentrations of 2 mM each.
about 4.5 mol of nucleotide/mol of enzyme are bound during
the time required to abolish the lag phase (upper curve).
However, after a cold chase with 2 mM ATP plus 2 mM Mg²⁺
to unload catalytic sites, about 3 mol of nucleotide remain
bound to the enzyme (Fig. 5B, lower curve). Approximately
1.5 mol of nucleotide/mol of MF, is removed in the cold chase,
irrespective of the time of initial incubation of enzyme with
[^32P]PPi, suggesting that only noncatalytic sites are involved
in eliminating the lag phase. Fig. 5A (inset) shows that
activation of nd-MF, by ATP plus Mg²⁺ in the presence of the
regeneration system occurs with saturation of the second
and third noncatalytic sites with ATP. Taking into account
differences in experimental conditions, this observation is not
necessarily inconsistent with the results illustrated in Fig. 4,
which show that prior incubation of nd-MF, with free ATP
eliminates the lag phase with apparent filling of the first two
noncatalytic sites. When loading of noncatalytic sites is ac-
complished with free ATP, the high affinity noncatalytic site,
which is only seen in the presence of Mg²⁺, might fill rapidly
on introducing the enzyme to the assay medium.

Correlation of the Binding of Pyrophosphate to nd-MF, with
Stimulation of ATPase Activity—As shown in Table I, incu-
bation of nd-MF, with [³²P]PP, at concentrations that stim-
ulate the hydrolytic activity 2-fold (see Fig. 2) is accompa-
nied by binding of about 3 mol of [³²P]PP/mol of enzyme as
detected by gel permeation chromatography on centrifuge
columns of Sephadex G-50. When a cold chase with ATP
or ITP preceded gel permeation chromatography, the amount
of [³²P]PP bound decreases by ~1 and ~0.5 mol/mol of enzyme,
respectively. Interestingly, the enzyme remains nearly
completely activated after a cold chase with ATP or ITP, indicat-
ing that at least part, if not all of the binding sites for PPi
are occupied by ATP or ITP, independent of the noncatalytic
sites with ATP. Taking into account
values cannot be

![Fig. 6. Lineweaver-Burk plots for ATP hydrolysis catalyzed by nd-MF, in the presence and absence of effectors. A, the stock solution of 0.7 mg/ml nd-MF, was prepared in 90 mM Tris-
SO₄, pH 8.0, containing 0.4 mM EDTA and 10% (w/v) glycerol. Rates
were recorded using 0.7 mg of nd-MF/assay for the hydrolysis of
30-2000 μM ATP. The initial rate of ATP hydrolysis was measured
20-40 s after adding enzyme (●). The final rate was measured after
a cold steady-state rate was attained (○). B, variations in conditions
over those shown in A are as follows. ■, the assay medium also
contained 20 mM HCO₃⁻; ▲, the enzyme stock solution contained
10 mM ATP, and the concentration of EDTA was 2.4 mM; ▲, the enzyme
stock solution contained 2 mM PPi + 2.5 mM Mg²⁺. The concentration
of ATP in the assay medium was adjusted for the ATP added
with enzyme for the rate measurements conducted after prior incubation
of the enzyme with 10 mM ATP.

Prior incubation of nd-MF, with 10 mM ATP or 2 mM PPi,
plus Mg²⁺ abolishes apparent negative cooperativity. The kinetic parameters estimated from Lineweaver-Burk plots
constructed from rate measurements obtained following
incubation with ATP or PP, (Fig. 5B) are, respectively: Kₘ =
108 μM, and Vₘₐₓ = 759 s⁻¹ (115 units/mg); Kₘ = 120 μM, and
Vₘₐₓ = 1353 s⁻¹ (205 units/mg). When assayed at low ATP
central concentrations in the presence of 20 mM bicarbonate, a lag
phase remains as shown in Fig. 2 (trace d). Lineweaver-Burk plots
of initial rates obtained in the presence of bicarbonate exhibit
apparent negative cooperativity (not shown). In con-
trast, a Lineweaver-Burk plot of the final steady-state rates
obtained in the presence of bicarbonate (Fig. 6B) is linear,
from which a Kₘ of 155 μM and a Vₘₐₓ of 1188 s⁻¹ (180 units/
mg) are estimated.

| Conditions | No chase | ATPMg chase | ITPMg chase |
|------------|---------|-------------|-------------|
| mol PP/mol nd-MF, | 3.33 ± 0.08 | 2.38 ± 0.04 | 2.63 ± 0.12 |
Semilogarithmic plots of the time-dependent activation observed during hydrolysis of 30–300 μM ATP are linear, from which first order rate constants, \( k' \), for the activation process are obtained as shown in Fig. 7A. As illustrated in Fig. 7B, a double-reciprocal plot of the activation constants, \( k' \), against ATP concentration is linear. Extrapolation of the line in Fig. 7B to the abscissa and ordinate intercepts reveals a maximal activation constant of \( 6.6 \times 10^{-3} \text{ s}^{-1} \) and an apparent \( K_d \) of 430 μM for the activation process. The latter value is the same as \( K_{sc} \) estimated by extrapolation of the steep phase of the upper Lineweaver-Burk plot shown in Fig. 6A. The pseudobimolecular rate constant calculated from the ratio, \( k'/K_d \), is \( 1.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1} \).

**DISCUSSION**

The results presented clearly show that nd-MF1 hydrolyzes low concentrations of ATP in three kinetic phases. An initial burst phase decelerates rapidly to a slow intermediate phase, which, in turn, gradually accelerates to a final steady-state rate. Binding of ATP to two noncatalytic sites during prior treatment of nd-MF, with high free ATP concentrations or low MgATP concentrations in the presence of a regenerating system suggests that activation is caused by slow binding of MgATP to the second and third noncatalytic sites, whereas rapid binding of MgATP to the first site has no effect on the intermediate phase to the final steady-state rate. Activation of the intermediate phase appears to occur when only two noncatalytic sites are loaded by prior incubation of nd-MF, with free ATP. However, it is probable that MgATP binds rapidly to the open noncatalytic site when enzyme preloaded in this manner is introduced into the assay medium.

From the preloading experiments, it appears that a major component of the final steady-state rate depends on binding of ATP to noncatalytic sites, whereas the initial rate of the intermediate phase is a basal rate which is observed in the absence of apparent regulatory site to catalytic site cooperativity. This contention is supported by the observation that the initial rate of inactivation of nd-MF1 by FSBA, which modifies noncatalytic sites, is considerably faster when loss of activity is monitored by assaying the final steady-state rate than when monitored by assay of the initial rate of the intermediate phase.

Comparison of ATP binding to noncatalytic sites when nd-MF1 is incubated with high concentrations of free ATP or low concentrations of MgATP in the presence of a regenerating system suggests heterogeneity of noncatalytic sites. One noncatalytic site binds MgATP rapidly, but when filled alone, does not accelerate rate in the intermediate phase. Whether this site must be filled to observe the initial burst of ATPase activity and/or subsequent activation of rate in the intermediate phase when ATP binds to the second and third noncatalytic sites remains an open question. What relationship this site has to the noncatalytic site that binds MgADP with low affinity described by Kironde and Cross (37) is not obvious.

The initial burst observed during hydrolysis of low concentrations of ATP was first reported for native MF1 containing endogenous nucleotides and for submitochondrial particles by Vasilyeva et al. (31). Subsequently Drobinskaia et al. (32) reported that a lag phase replaces the initial burst phase after treating MF1 containing endogenous nucleotides with Mg2+ prior to assay with 100 μM ATP. The rate of the lag phase induced by treatment with Mg2+ slowly accelerates to the rate of the slow phase observed in the absence of treatment with Mg2+ (the intermediate phase described in this study). They also found that prior treatment of nd-MF1 with Mg2+ did not alter the initial burst phase, but reported that treatment of nd-MF1 with an equivalent amount of ADP in the presence of Mg2+ also leads to replacement of the initial burst phase with a lag phase which slowly accelerates to a rate equivalent to the slow phase observed in the absence of pretreatment. From these observations, Drobinskaia et al. (32) proposed that deceleration in the initial burst phase in the absence of pretreatment is caused by abortive binding of MgADP formed during catalysis to a catalytic site. That this might be the case is supported by recent affinity labeling studies with 2-N3-ADP carried out independently by Milgrom and Boyer (34) using nd-MF1 and Chernyak and Cross (35) using MF1, with two or three noncatalytic sites filled with adenine nucleotides.

**FIG. 7. Dependence of activation observed in the intermediate phase on ATP concentration in the assay medium.**

A, the recorder traces from the assays with increasing ATP concentrations conducted to obtain the results presented in Fig. 6A were analyzed in the following manner. \( V_b \), the final rate, was taken as the steady-state rate after maximal activation was reached. The transient rates, \( V_t \), were estimated from tangents of the curved traces at the times indicated. The concentrations of ATP in the assay medium represented by the lines were: ●, 30 mM; ○, 50 mM; ▲, 100 mM; ▲, 150 mM, ■, 300 mM. To ensure clarity, results are presented for activation observed at a limited number of ATP concentrations B, the reciprocals of the activation constants, \( k' \), determined from the slopes of the straight lines in A, were plotted against the reciprocal of the concentration of ATP in the assay medium.
Both laboratories reported that prior incubation of MF₁ with stoichiometric 2-N₃-[³²P]ADP in the presence of Mg²⁺ elicits inhibition similar to that caused by MgADP binding. Subsequent irradiation of the resulting complexes is accompanied by derivatization of Tyr-β345, which is present in the catalytic site. Therefore, the transition from the initial burst phase to the intermediate phase described in this and other studies (31–35) appears to be associated with retention of MgADP at a catalytic site.

The slow rate acceleration observed in this study during transition of the intermediate phase to the final steady-state rate when nd-MF₁ hydrolyzes of 50 or 100 μM ATP was not noted in the studies cited above (31–35). In all but one of these studies, the rate of hydrolysis of 1–100 μM ATP was recorded for only short time intervals after initiating catalysis, thus precluding observation of the activation phase. The presence of 1 mM sulfate in the assay medium used by Cher- nyak and Cross (35) may have precluded appearance of the activation phase in the longer time interval examined in their study. As shown in this study, sulfate inhibits the transition from the intermediate phase to the faster, final steady-state phase during hydrolysis of 50 μM ATP by nd-MF₁, presumably by interfering with binding of ATP to noncatalytic sites.

Milgrom et al. (22) have reported direct evidence that sulfate lowers the affinity of noncatalytic sites of CF₁ for ATP. It is interesting that the CF₁ preparation which they examined contained about 2 mol of ADP/mol, one bound to a catalytic site and the other to a noncatalytic site. This enzyme preparation exhibits a lag when hydrolyzing 20 μM ATP. The lag is significantly extended in the presence of 5 mM sulfate. After prior incubation of the enzyme with ATP plus a regeneration system to load noncatalytic sites, the CF₁ preparation hydrolyzes 20 μM ATP without a lag in the presence or absence of sulfate. An initial burst phase is not observed when the CF₁ preparation hydrolyzes low concentrations of ATP, possibly because inhibitory ADP is bound initially at a catalytic site. The TF₁-ATPase, which is isolated free of nucleotides (38), hydrolyzes ATP without exhibiting an initial burst. Yoshida and Allison (39) showed that the lag is extended after binding MgADP to a single catalytic site. Yohda et al. (14) reported that a mutant form of the α subunit of TF₁, with the D⁹⁵⁵N substitution has a 10-fold diminished affinity for ADP. Whereas the Lineweaver-Burk plot for ATP hydrolysis catalyzed by the reconstituted α₁β₂γ complex of TF₁ containing wild-type α subunit is biphasic and hydrolyses ATP with a pronounced lag, the Lineweaver-Burk plot for the reconstituted complex containing the mutant αD⁹⁵⁵N subunit is linear and the mutant complex hydrolyzes ATP without a lag. These observations suggest that the apparent negative cooperativity exhibited by the α₁β₂γ complex of TF₁ and the lag observed in ATPase activity catalyzed by the complex are associated with binding of ATP to noncatalytic sites.

The observation that prior incubation of nd-MF₁ with PP, abolishes the intermediate phase and accelerates the final steady-state rate when the enzyme hydrolyzes 50 μM ATP is consistent with the results of Kalashnikova et al. (33). They reported that the initial rate of hydrolysis of 100 μM ATP is unaffected by the presence of 2 mM PP, in the assay medium, but deceleration of the initial burst phase is markedly decreased resulting in a stimulated final rate. It was also shown that the inactive complex formed on incubating nd-MF₁ with equimolar ADP in the presence of Mg²⁺ rapidly reactivates when treated with 1 mM PP. From the observation that PP, increases the affinity of P₁, presumably bound to a catalytic site, Kalashnikova et al. (33) concluded that PP, binds to noncatalytic sites. In contrast, Peinnequin et al. (40) recently reported that PP, binds to three sites on MF₁, irrespective of occupancy of catalytic and noncatalytic sites with ligands, but that the total number of sites occupied with nucleotides and PP, never exceeds 6 sites/mol of MF₁. From these results, they suggested that the binding sites for PP, are unique and interact with both catalytic and noncatalytic sites. The observation reported here that prior incubation of nd-MF₁ with PP, in the presence of Mg²⁺ mimicks prior incubation of the enzyme with ATP or MgATP in that the intermediate phase disappears, suggests that PP, binds to noncatalytic sites. However, the observation that PP, accelerates inactivation of the enzyme by FSBA contradicts this suggestion, unless simultaneous binding of PP, and FSBA to noncatalytic sites accelerates derivatization.

It is interesting that the higher Kₐ of 440 μM determined for the hydrolysis of 30–2000 μM ATP by nd-MF₁ is very close to the apparent Kₐ of 430 μM determined from the dependence of activation of the intermediate phase on the ATP concentration in the assay medium. Prior binding of ATP or MgATP to noncatalytic sites of nd-MF₁ eliminates the intermediate phase and modification of noncatalytic sites with FSBA freezes the enzyme in the intermediate phase when low concentrations of ATP are hydrolyzed. Therefore, the apparent Kₐ value represents binding of ATP to noncatalytic sites. Given these observations, we propose that the apparent negative cooperativity observed when the initial rate of the intermediate phase is plotted according to the method of Line- weaver and Burk is caused by slow binding of ATP to non- catalytic sites of nd-MF₁. At sufficiently low substrate concentrations, very little ATP is bound to noncatalytic sites during the interval of the initial rate measurement. However, at higher concentrations of ATP in the assay medium, the noncatalytic sites begin to saturate within the time frame of the rate measurement leading to stimulation of the rate of ATP hydrolysis. Therefore, the apparent Kₐ of 440 μM observed on Lineweaver-Burk plots for hydrolysis of ATP by nd-MF₁ does not reflect binding of ATP to catalytic sites, but rather represents binding of ATP to noncatalytic sites which accelerates catalysis, presumably by promoting release of inhibitory MgADP from a catalytic site. Supporting this contention is the observation that Lineweaver-Burk plots are linear when ATP hydrolysis is catalyzed by nd-MF₁, after loading noncatalytic sites with ATP. Lineweaver-Burk plots are also linear after treating nd-MF₁ with PP, plus Mg²⁺, which, according to Kalashnikova et al. (33), promotes dissolution of inhibitory MgADP from a catalytic site. The Kₐ values of 108 and 120 μM observed with enzyme activated with ATP and Mg²⁺ plus PP₁, respectively, are equivalent to the lower Kₐ of 120 μM observed for the hydrolysis of ATP by nd-MF₁, in the absence of pretreatment.

The proposal that slow saturation of noncatalytic sites with ATP promotes dissociation of inhibitory MgADP from the affected catalytic site during hydrolysis of low concentrations of substrate by nd-MF₁ is consistent with the results of a study reported by Milgrom and Murataliev (41) on interaction of MgADP with nd-MF₁. After loading a catalytic site of nd-MF₁ with Mg [+³¹P]ADP, addition of ATP promoted dissociation of the complex with an apparent pseudo-bimolecular constant of 1.1 × 10⁸ M⁻¹ s⁻¹. This is very close to the apparent pseudo-bimolecular rate constant of 1.6 × 10⁸ M⁻¹ s⁻¹ determined in this study from the dependence of the rate of activation of the intermediate phase during catalysis on ATP concentration.

Table II compares the two Kₐ and associated Vₘₐₓ values determined in this study with the multiple Kₐ and Vₘₐₓ values previously reported by others for the hydrolysis of ATP by
bovine heart MF₁. In the studies reported by Gresser et al. (12), Wong et al. (16), and Roveri and Calceterra (17), rate measurements were determined at lower concentrations of ATP than used in this study and in the study reported by Cross et al. (15), thus revealing Kₘ values in the 1 μM range. Neglecting the Kₘ values in the 1 μM range, there is considerable variation in the higher Kₘ values reported by the different laboratories. This may be due, in part, to variations in the assay media used. For instance, the assay mixtures of Cross et al. (15) contained both 1 mM phosphate, an activating anion (9), and at least 0.5 mM sulfate, an inhibiting anion (11). The assay mixtures of Wong et al. (16) contained at least 1.0 mM sulfate. Given the triphasic kinetic profile observed in this study (burst, intermediate, and final phases) when MF₁ hydrolyzes 50 or 100 μM ATP, the time interval in which the rates are measured has great bearing on the number and magnitude of Kₘ values observed in the high micromolar range of ATP concentrations. For instance, extrapolation of the Lineweaver-Burk plot constructed from initial rates of the intermediate phase (Fig. 6A) clearly reveals two Kₘ values of 120 and 440 μM. In contrast, the Lineweaver-Burk plot constructed from the final steady-state rates of hydrolysis of ATP is curved throughout. It is interesting, however, that tangents to the initial and final portions of the curve extrapolate to arbitrary Kₘ values of 33 and 500 μM, which are very close to the Kₘ values reported by Cross et al. (15) for the hydrolysis of 10–2000 μM ATP by MF₁ containing endogenous nucleotides. It is curious that Gresser et al. (12) reported Kₘ values of 1.7 and 250 μM for the hydrolysis of 0.05–2000 μM ATP by MF₁ containing endogenous nucleotides, whereas Wong et al. (16) reported three Kₘ values of 150, 1000, and 5000 μM for the hydrolysis of 1–5000 μM ATP by MF₁ containing endogenous nucleotides and two Kₘ values of 1 and 260 μM by nd-MF₁. Roveri and Calceterra (17) reported three Kₘ and associated Vₘₐₓ values shown in Table II for hydrolysis of 3–2000 μM ATP by MF₁ containing endogenous nucleotides which was stored in buffer containing 10 mM sulfate and 2 mM EDTA. After removing sulfate and EDTA by dialysis, the same enzyme preparation exhibited two Kₘ values of 10 and 145 μM. Since the time intervals of the rate measurements in the studies of others summarized in Table II were not reported, it is not possible to comment further on the differences or similarities among the Kₘ values.

The multiple Kₘ and associated Vₘₐₓ values determined for MF₁ have been variously interpreted in terms of the number of catalytic sites participating in catalysis at given concentrations of ATP. For instance, Cross et al. (15) have suggested that hydrolysis of MgATP at a single site of MF₁ occurs with such high affinity and slow product release that it cannot be detected by steady-state measurements. They also proposed that the Kₘ values of 30 and 150 μM determined from steady-state measurements represent catalysis with two (bisite) and three (trisite) catalytic sites filled with substrate. An opposing interpretation was put forward by Harris (42), who observed that MF₁ exhibits a single Kₘ of about 1 μM in the presence of 1 mM azide, which is equivalent to the low Kₘ value reported by Gresser et al. (12), Wong et al. (16), and Roveri and Calceterra (17). Harris (42) suggested that the Kₘ of about 1 μM represents ATP hydrolysis at a single catalytic site and that all three catalytic sites are operative during ATP hydrolysis in the physiological range. The results of this study are not easily accommodated by either interpretation. As described in detail earlier, it is our view that the Kₘ of 150 μM and associated Vₘₐₓ of 600 s⁻¹ determined by Cross et al. (15), represent augmentation of ATP hydrolysis when ATP binds to noncatalytic sites. If the Vₘₐₓ value of 726 s⁻¹ reported in this study represents cooperative hydrolysis of ATP with three catalytic sites operating maximally, then the Vₘₐₓ value of 284 s⁻¹ should represent ATP hydrolysis with two catalytic sites operating cooperatively and the third occupied with inhibitory MgADP. If this is the case, then the Kₘ value of about 1 μM should represent bisite catalysis.

It has been suggested that prior dissociation of product Pᵢ from a catalytic site is responsible for the transition in rate that is observed when the initial burst phase decelerates to the intermediate phase (32, 35). If this is indeed the case, then the binding of ATP to noncatalytic sites must be necessary to ensure optimal dissociation of MgADP from catalytic sites, thus preventing entrapment of inhibitory MgADP at a catalytic site. Vasilyeva et al. (33) reported that MF₁ containing endogenous nucleotides exhibits a burst phase which decelerates to a slower phase equivalent to the intermediate phase described in this study. Given that MF₁ is usually isolated with 2 mol of adenine nucleotides bound to noncatalytic sites and exhibits apparent negative cooperativity (9, 43), it appears that binding of ATP to the third noncatalytic site to be filled promotes dissociation of inhibitory MgADP from a catalytic site in this case as well.
ATP Binding to Noncatalytic Sites Promotes ATP Hydrolysis

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