Nucleotides Binding Sites on Beef Heart Mitochondrial F₁-ATPase

COOPERATIVE INTERACTIONS BETWEEN SITES AND SPECIFICITY OF NONCATALYTIC SITES*

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We have studied the properties of beef heart mitochondrial F₁, having inhibitory MgADP bound at one of the three catalytic sites and various levels of occupancy of the three noncatalytic nucleotide sites including zero, two, or three ADP/ATPs or two ADP/ATP plus one GTP. The properties examined include the rate of MgATP-dependent reactivation and the rate of increase in the fraction of F₁ containing transiently bound intermediates. For each form of the enzyme tested, the rate of reactivation closely paralleled the rate of increase in the level of bound intermediates, indicating that when one catalytic site on F₁ is blocked by inhibitory MgADP, the remaining two sites are incapable of residual uni- or bi-site activity. It was also found that the stability of the MgADP-inhibited complex decreases with full occupancy of the noncatalytic sites. This demonstrates that the noncatalytic sites modulate the properties of catalytic sites. Finally, it was found that the noncatalytic sites on mitochondrial F₁ do not, as has long been believed, bind adenine nucleotides exclusively. Evidence is presented that both GTP and PPᵢ bind tightly at noncatalytic sites.

F₁-ATPase, the catalytic component of proton-translocating ATP-synthase complexes from eubacteria, mitochondria, and chloroplasts, has a subunit structure of α₃β₃γ₆ε₇ (for reviews see Senior, 1988; Boyer, 1989; Futai et al., 1989; Penefsky and Cross, 1991; Issartel et al., 1992) and possesses six nucleotide binding sites (Cross and Nalin, 1982; Wise et al., 1983; Girault et al., 1988). Three of the sites exchange nucleotide rapidly during catalytic turnover and are considered to be potential catalytic sites. The remaining three sites do not exchange rapidly and are referred to as noncatalytic sites. Nucleotide sites on F₁ appear to be positioned at or near α/β subunit interfaces (Schafer and Dose, 1984; Zhuo et al., 1992), and catalytic and noncatalytic sites may be in close proximity and oriented like the ATP and AMP sites on adenylate kinase (Vogel and Cross, 1991).

Catalytic sites show strong catalytic cooperativity (Kayalar et al., 1977), as indicated by the ability of substrate binding at one site to accelerate the rate of product release from a second site by up to 5 orders of magnitude (Cross et al., 1982; O’Nea and Boyer, 1984; Duncan and Senior, 1985; Cun-
ningham and Cross, 1988). In addition, complete or near complete inactivation of F₁ can be obtained following the covalent modification of, or the binding of MgADP to, a single catalytic site. However, it remains uncertain whether blocking a single catalytic site completely prevents the capacity for catalysis by the two remaining sites (Cross, 1988, 1992).

The role of noncatalytic sites is unknown, and the results of studies of their specificity and interactions with catalytic sites are controversial. Affinity labeling of noncatalytic sites has been reported to inhibit F₁ activity (Bullough and Allison, 1986; Cross et al., 1987; Melese et al., 1988), suggesting interactions between noncatalytic and catalytic sites. However, it is possible that the effect is caused by nonphysiological structural changes induced by the chemical modification reactions. Binding of ADP to noncatalytic sites was reported to inhibit hydrolytic activity of F₁ from beef and pig heart mitochondria (Harris et al., 1978; DiPietro et al., 1980) and from chloroplasts (Milgrom et al., 1991). In the case of CF₁, inhibition could also be induced by GTP binding to noncatalytic sites. In contrast, it was reported that mitochondrial and Escherichia coli F₁ do not bind guanine nucleotides at noncatalytic sites (Harris, 1978; Perlin et al., 1984).

In the present studies, we address several questions regarding the interactions and properties of catalytic and noncatalytic nucleotide binding sites on F₁ from beef heart mitochondria. The results show that when inhibitory MgADP is bound at one catalytic site, the two remaining unoccupied catalytic sites are incapable of either uni- or bi-site catalysis. We also show that noncatalytic sites on mitochondrial F₁ can bind GTP and PPᵢ, and that the occupancy of noncatalytic sites affects the interaction of catalytic sites with inhibitory MgADP.

**EXPERIMENTAL PROCEDURES**

**MATERIALS—**ATP, GTP, phosphoenolpyruvate, Mops, Tris, bovine serum albumin, NADH, Sephadex G-50-80, and lyophilized pyruvate kinase were obtained from Sigma. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Mannheim in glycerol-containing buffers. [³H]ATP (45 Ci/mmol) and [³H]GTP (9 Ci/mmol) were obtained from Amersham, and [³H]ADP (30 Ci/mmol) was from Du Pont-New England Nuclear.

Native enzyme (F₁[2,1]) was prepared from beef heart mitochon-

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1 The abbreviations used are: CF₁, soluble ATPase portion of spinach chloroplast ATP synthase; Mops, 4-morpholinepropanesulfonic acid; ANP, ADP and/or ATP; F₁[2A+1G,1A], soluble ATPase portion of beef heart mitochondrial ATP synthase having X mol of ANP bound at noncatalytic sites and Y mol of ANP bound at catalytic sites per mol of enzyme (X and Y are averaged rounded off to whole numbers); F₁[2A+1G,1A], having two ANP and one GTP bound at noncatalytic sites plus one ANP at a catalytic site: GNP, GDP and/or GTP; MTSEM, 20 mM Mops/Tris (pH 8.0), 150 mM sucrose, 0.2 mM EDTA, and 2 mM Mg(CH₃COO)₂; MTSEMP, MTSEM buffer containing 1 mM Pi; NTP, nucleoside triphosphate; HPLC, high performance liquid chromatography.
dria and stored according to Knowles and Penevsky (1972). A aliquot
of the stock ammonium sulfate suspension of F1 was centrifuged, the
dissolved in buffer containing 150 mM sucrose, 20 mM Mops/Tris
(pH 8.0), and 2 mM EDTA, and desalted on a Sephadex centrifug-
fuge column (Penevsky, 1977) equilibrated with the same buffer. An
equal volume of glycerol was added to the effluent, diluted F1 to approximately 50 μM, and the enzyme was stored at −15 °C for up to
3 months. Anion-exchange HPLC analysis of extracted nucleotides
showed that this form of the enzyme contained 2.1 mol of ADP and
1.0 mol of ATP/mole NTP-depleted enzrme (F1[0,0]) was
prepared according to Garrett and Penevsky (1975), and fractions having a A260/A280 ratio greater than 1.96 were pooled. Analysis of extracted
nucleotides showed that this form of the enzyme retained 0.07 mol of
ADP/mole ATP. MgADP-bound F1[2,1] (10 μM) incubating
F1[2,1] at 5.5 μM with 5 mM MgATP for 2 min in MTSEM buffer (20
mM Mops/Tris (pH 8.0), 150 mM sucrose, 0.2 mM EDTA, and 2 mM
Mg(CH2COO)2) containing 1 mM P; (MTSEM buffer). Unbound
nucleotides were removed by passage through a centrifuge column
equilibrated with MTSEM buffer. The eluate is eluted from a
second column equilibrated with MTSEM buffer and, following an additional 10-min incubation, passed through a third column. F1 having two ANP occupancies (F1[3,1]) was obtained by preincubating F1[2,1] at 6 μM with 5 mM MgATP for 30
s in MTSEM buffer. After removing unbound nucleotide on a
centrifuge column equilibrated with MTSEM buffer, the enzyme
was incubated for 1 min with 6 mM MgATP. Unbound nucleotides
were removed by passage through a centrifuge column (Penefsky,
1977) equilibrated with the same buffer. An
ion-exchange HPLC analysis of extracted nucleotides showed that
this form of the enzyme retained 0.07 mol of
ADP/mole ATP. MgADP-bound F1[3,1] was obtained by preincubating
F1[2,1] with 6 mM MgATP. Unbound nucleotides were removed by passage through three sequential centrifuge columns
(upper panel, open symbols) with 50 mM KCl, 10 mM phosphoenolpyruvate, 1 mg/ml pyruvate kinase, and 1.1 mg/ml bovine serum albumin. At the times indicated, aliquots
were applied to Sephadex centrifuge columns to remove unbound
ligands. Because of the low concentrations of F1 used in these assays,
centrifuge columns were equilibrated with buffer containing bovine
serum albumin to enhance recovery of enzyme through the column
(Cross and Nalin, 1982). When column effluents were to be counted,
they were collected directly in liquid scintillation vials. Data were
corrected for radioactivity eluting in the absence of F1; (usually less than 5%). The standard deviation for replicate measurements is ±
10% using these procedures.

Rate constants for reactivation and inhibition of ATP hydrolysis
were obtained from plots of ln (Vt - V0) or ln (Vt - V0) versus time,
where Vt is the rate at time t, and V0 is the steady-state rate reached
after full reactivation or inhibition. Rate constants for loading [3H]
ANP at catalytic sites were obtained from similar plots of binding data for various MgADP-inhibited forms of F1. Half-times were calculated from the measured rate constants.

The composition of [3H]GNP bound to F1 was determined by
anion-exchange HPLC using a Partisil px3 10/25 SAX column (4.6
× 250 mm). Aliquots of 90 μl containing 0.4–5.0 μM F1 were channeled with an equal volume of cold 0.6 M HClO4, and a mixture of 5–40
nmol of carrier ADP, ATP, GDP, and GTP was added in 12 Al. After
5 min on ice, samples were centrifuged to remove protein. One-third
volume of 1 M KHCO3 was immediately added to neutralize the
supernatants, and HClO4 was removed by centrifugation.
Samples were then diluted to 1 ml with water prior to loading onto the HPLC column. Nucleotides were eluted at a flow rate of 1 ml/min using the following program: solvent A for 5 min, a linear gradient of solvent B from 0 to 80% over 25 min, a linear gradient of solvent B from 50 to 100% over 10 min, and finally 100% solvent B for 10 min. Solvents
A and B were 0.05 M Na2HPO4 and 1.0 M NaH2PO4, respectively, adjusted to
pH 3.5 with H3PO4. This procedure gives good separation of ADP, GDP, ATP, and GTP, which elute at 29, 33, 43, and 47 min, respectively. Aliquots of the collected fractions were counted by liquid
scintillation in 5–10 volumes of Bio-Safe II (Research Products International). Data were corrected for quenching induced by eluting
solvents.

Protein was determined by a modified Lowry method according to

Reactivation of Different Forms of F1, after Inhibition by MgADP Bound at a Catalytic Site—The inhibition induced by preincubating mitochondrial F1[2,1] with Mg2+ (Moyle and Mitchell, 1975) can be prevented by removing endogenous
nucleotides (Drobinskaya et al., 1985). The addition of an equimolar amount of ADP to nucleotide-depleted enzyme (F1[0,0]) restores Mg2+-induced inhibition (Drobinskaya et al., 1985) due to the binding of ADP at a catalytic site (Milgrom and Boyer, 1980; Chernyak and Cross, 1992). Under conditions for ATP hydrolysis, the activity of MgADP-inhibited F1 recovers in a time-dependent manner (Hackney, 1979), accompanied by the dissociation of inhibitory ADP (Drobinskaya et al., 1985).

It remains uncertain, however, whether MgADP-inhibited F1 has any residual capacity for catalysis at the two remaining unoccupied catalytic sites. The presence of small amounts of uninhibited F1, combined with the very low rates possible for uni- and bi-site catalysis, precludes answering this question by direct measurement of enzyme activity. Instead, the approach employed here is to compare the rate of enzyme reactivation during hydrolysis of [3H]ATP with the rate of increase in the stoichiometry of catalytic sites having bound [3H]-labeled nucleotide. Transiently bound nucleotides are intermediates in the catalytic cycle, and one catalytic site/F1 is able to retain such nucleotide during passage through a
centrifuge column. Thus, the fraction of F1 in the column effluent having [3H]ANP bound at a catalytic site is a measure of the fraction of F1 capable of catalysis (Cunningham and Cross, 1988).

Fig. 1 shows that preincubation of F1[0,0] with stoichiometric amounts of ADP in the presence of Mg2+ to give an
inhibited form of the enzyme causes a significant decrease in the initial rate of hydrolysis of 1 μM ATP compared with enzyme lacking this pretreatment (trace 2 versus trace 1). The rate of reactivation was determined to be 0.068 min−1 (t1/2 = 10.3 min, Table I). Fig. 1 also shows that the addition of 5
mM K2SO4 greatly accelerates the rate of reactivation (trace 3), whereas 0.2 mM NaN3 completely prevents reactivation (trace 4). These results are in accord with an earlier report (Vasilyeva et al., 1982).

Fig. 2 presents the kinetics of [3H]ANP binding to F1[0,0] (open symbols) during hydrolysis of 1 μM [3H]ATP in the presence of an ATP-regenerating system. The binding of [3H] ANP at noncatalytic sites (upper panel, open circles) is seen to increase steadily, reaching about 1 mol/mol F1 after 30 min. In contrast, catalytic site occupancy (lower panel, open
The different forms of F₁ shown in the first column were prepared as described under "Methods." The MgADP-inhibited form of F₁[0,0] was obtained by incubating the enzyme with stoichiometric amounts of ADP as described for Fig. 1. Since the other forms of F₁ already had an ADP bound at a catalytic site, the inhibited form was obtained simply by storing the enzyme in buffer containing excess magnesium. Fully active enzyme for use in measuring the rate of azide inhibition was obtained by including Pi in the storage buffer or in the case of F₁[0,0], by omitting preincubation with ADP.

<table>
<thead>
<tr>
<th>Initial form of F₁</th>
<th>Half-time*</th>
<th>Loading [³H]ANP at catalytic sites</th>
<th>Azide inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₁[0,0]</td>
<td>10.3</td>
<td>9.0</td>
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<tr>
<td>F₁[1,1]</td>
<td>8.5</td>
<td>8.9</td>
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<td>4.4</td>
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<tr>
<td>F₁[2A+1G,1A]</td>
<td>1.5</td>
<td>1.6</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*The initial nucleotide content of enzyme used to produce the MgADP-inhibited state as described.

Half-time values were calculated from measured rate constants.

Results shown in Fig. 3 lend further support to this conclusion. Azide, which prevents reactivation (Fig. 1) and dissociation of the inhibitory ADP (Vasilyeva et al., 1982; Milgrom and Murataliev, 1986; Murataliev et al., 1991), also substantially reduces the binding of [³H]ANP at catalytic sites on MgADP-inhibited F₁ during incubation with [³H]ATP (Fig. 3, circles). Sulfite, which accelerates reactivation of MgADP-inhibited F₁ (Fig. 1), also accelerates the binding of [³H]ANP to a catalytic site on the MgADP-inhibited enzyme during hydrolysis of [³H]ATP (triangles). Preincubation with Pi, which is known to reverse MgADP-induced inhibition (Yalamova et al., 1982; Drobinskaya et al., 1985), also reverses the effect of inhibitory MgADP on the binding of [³H]ANP to catalytic sites during turnover (squares).

To verify that these properties were not unique to MgADP-inhibited F₁, prepared from F₁[0,0], some experiments were repeated using native enzyme (F₁[2,1]). Since this form of the enzyme already has one catalytic site occupied (Kironde and Cross, 1986), preincubation with Mg⁺⁺ alone is sufficient to obtain the inhibited state. Subsequent incubation with 1 μM ATP results in reactivation with a t₁/₂ of 8.5 min (Table I). Again, this is similar to the half-time for binding [³H]ANP at catalytic sites during hydrolysis of 1 μM [³H]ATP (8.9 min, Table I).

Thus, in the case of MgADP-induced inhibition, the rates of increase in occupancy of catalytic sites during hydrolysis closely parallel the rates of reactivation for both F₁[0,0] and F₁[2,1]. However, reactivation rates measured in the present work were considerably slower than those reported by Chernyak and Cross (1992) for MgADP-inhibited enzyme formed from F₁[2,0] and F₁[3,0]. These differences might be caused by differences in the level of saturation of noncatalytic nucleotide binding sites. Both F₁[0,0] and F₁[2,1] have at least one vacant noncatalytic site. In contrast, all noncatalytic sites on F₁[3,0] are filled and, as shown in a later section, F₁[2,0] actually has a GNP bound at the third noncatalytic site. To test for an effect of noncatalytic site occupancy, we incubated F₁[2,1] with MgATP to load ANP at the vacant noncatalytic site (F₁[2,1]) or with MgGTP followed by MgATP to load GNP (F₁[2A+1G,1A]). As shown in Table I, this treatment caused the half-time for reactivation of the MgADP-inhibited enzyme to drop to 1.5 min for F₁[3,1] and to 1.5 min for two unoccupied catalytic sites from functioning in either uni- or bi-site catalysis.

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F_{1}[2A+1G,1A]. As anticipated, F_{1}[3,1] and F_{1}[2A+1G,1A] also bind [3H]ANP rapidly at catalytic sites during turnover, exhibiting half-times of 2.0 and 1.6 min (Table I).

Two conclusions may be drawn from the results presented in this section. First, the appearance of nucleotide transiently bound at catalytic sites does not occur at a rate faster than the rate of recovery of activity as measured in a coupled-enzyme assay. This indicates that MgADP-inhibited F_{1} has no residual capacity for uni- or bi-site catalysis. Second, noncatalytic site occupancy affects the rate of reactivation of MgADP-inhibited F_{1}.

Effects of Noncatalytic Site Occupancy on Rate of Formation of the MgADP-inhibited State during ATP Hydrolysis—Incubating F_{1} with Mg^{2+} under conditions where at least one catalytic site is occupied by ADP is not the only way to obtain MgADP-inhibited enzyme. When fully active enzyme is added to a coupled-enzyme assay lacking an activating anion such as sulfite, the kinetics of hydrolysis are biphasic, with the rate slowing with time (Fig. 1, trace 1 versus trace 3; Vasileva et al., 1982; Zhou et al., 1988; Murataliev et al., 1991). This is because of a slow increase in the fraction of F_{1} having inhibitory MgADP bound at a catalytic site. At steady state, the rates of formation and ATP-dependent dissociation of the inhibited complex become equal. However, the binding of azide appears to stabilize the inhibited complex to such an extent that its rate of formation can be measured without interference from the reverse reaction (Vasileva et al., 1982; Murataliev et al., 1991).

Since filling the vacant noncatalytic site on F_{1}[2,1] was found to accelerate the rate of dissociation of the inhibited complex, it was of interest to determine whether this treatment might also affect the rate of formation of the complex during ATP hydrolysis. The last column of Table I gives half-times for inhibition of fully active F_{1} during hydrolysis of 1 mM ATP in the presence of azide. When F_{1} has one or more vacant noncatalytic sites (F_{1}[0,0] and F_{1}[2,1]), the half-time for inhibition was found to be only 2.6 min, whereas it was 4.4 min when all noncatalytic sites were filled with ANP (F_{1}[3,1]) and 5.7 min when two were filled with ANP and one with GNP (F_{1}[2A+1G,1A]). Hence, full occupancy of the noncatalytic sites destabilizes the MgADP-inhibited F_{1} complex by both increasing its rate of dissociation and decreasing its rate of formation.

Full occupancy of noncatalytic sites also had an effect on the enzyme’s specific activity. When assayed at 2 mM ATP, preloading the vacant noncatalytic site on F_{1}[2,1] with either ANP or GNP increased the rate of hydrolysis by about 80%. However, at higher ATP concentrations (0.1–1 mM), only bound GNP caused an increase in the rate (40%). Filling all noncatalytic sites with ANP also increased the rate of hydrolysis of 0.2 mM GTP by 50% and elevated its sensitivity toward inhibition by azide (data not shown).

**GTP Binding at Noncatalytic Sites**—The observation that preincubation of F_{1}[2,1] with MgGTP altered the interaction of catalytic sites with inhibitory MgADP, combined with the recent report that noncatalytic sites on CF_{1} can bind guanine nucleotides (Guerrero et al., 1990), prompted us to reinvestigate the specificity of noncatalytic nucleotide binding sites on the mitochondrial enzyme. Table II shows that a 30-s incubation of F_{1}[2,1] with Mg[^3H]GTP results in the binding of about 2 mol of guanine nucleotides/mol of F_{1} (Experiment 1). These remain bound during passage through a second centrifuge column (Experiment 2). Bound label was nearly equally distributed between GTP and GDP. When the Mg[^3H]GTP-treated enzyme was incubated for 1 min with unlabeled MgGTP (Experiment 3) or MgATP (Experiment 4) before passage through the second centrifuge column, nearly all of the [^3H]GNP displaced from catalytic sites was GDP. In contrast, the 1 mol of [^3H]GNP/mol of F_{1} remaining bound at noncatalytic sites was nearly all GTP.

Table II also shows the effects of using GTP hydrolysis to deplete catalytic sites of ANP in the preparation of F_{1}[2,0] by the procedure of Cunningham and Cross (1988). Since the enzyme retains 1.2 mol of bound guanine nucleotides, represented mostly by GTP (Experiment 5), the enzyme is not in the form it was thought to be. In the future, this form of the enzyme should be referred to as F_{1}[2A+1G,0] rather than F_{1}[2,0] since the latter designation stipulates only the adenine nucleotide content.

Data presented in Table II show that F_{1}[2,1] can bind GTP at its vacant noncatalytic site. The kinetics of [^3H]GTP binding to this site during hydrolysis of 100 mM [^3H]GTP in the presence of a GTP-regenerating system are presented in Fig. 4 (upper panel, open circles). It is seen that binding is relatively fast, being completed within 2–3 min. When repeated using F_{1}[0,0], we observed the binding of 1.5–1.7 mol of [^3H]GNP versus about 2.5 mol of [^3H]ANP at noncatalytic sites after a 20-min incubation with 100 mM H-labeled nucleotide and an NTP-regenerating system (data not shown). It appears, therefore, that at least two noncatalytic sites on F_{1} are capable of binding GNP provided they are empty.

GTP bound at a noncatalytic site does not dissociate during a 30-min incubation in MTSEM buffer at an F_{1} concentration of 100 nM. The addition of 10 mM F_{1}, 100 mM ADP, 1 mM MgPP_{i}, or 100 mM ATP plus EDTA also failed to induce dissociation of the GTP. However, in the presence of 100 mM ATP and an ATP-regenerating system, the noncatalytic site GTP dissociated with t_{1/2} of 10 min. GTP hydrolysis also

### Table II

<table>
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<tr>
<th>Treatment of F_{1}[2,1]</th>
<th>[^3H]GNP bound at all sites</th>
<th>[^3H]GNP displaced from catalytic sites</th>
<th>[^3H]GNP bound at noncatalytic sites</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>GDP</td>
<td>GTP</td>
</tr>
<tr>
<td>EXP. 1 Mg[^3H]GTP //</td>
<td>2.0</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>EXP. 2 Mg[^3H]GTP //</td>
<td>2.1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>EXP. 3 Mg[^3H]GTP // MgGTP //</td>
<td>0.8</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>EXP. 4 Mg[^3H]GTP // MgATP //</td>
<td>1.0</td>
<td>0.9</td>
<td>0.1</td>
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<td>EXP. 5 Native F_{1}, treated as for preparation of F_{1}[2,0]</td>
<td>1.2</td>
<td>0.3</td>
<td>0.9</td>
</tr>
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</table>

**The stoichiometry, composition, and location of [^3H]GNP bound to mitochondrial F_{1}, during incubation with [^3H]GTP**

In Experiments 1–4, native F_{1} was incubated in MTSEM buffer with 3.7 mM [^3H]GTP for 30 s. Unbound nucleotides were removed, where indicated by a double slash (//), on a centrifuge column equilibrated with the same buffer. Some samples were subjected to a second incubation of 1 min without (Experiment 2) or with 3.8 mM MgGTP (Experiment 3) or 3.7 mM MgATP (Experiment 4). In Experiment 5 F_{1}[2,0] was prepared as described (Cunningham and Cross, 1988). Bound [^3H]GNP was extracted and analyzed by anion-exchange HPLC as described under "Methods."
stimulated release of noncatalytic site GTP, but at a rate one-sixth that obtained with an equal concentration of ATP (data not shown).

PP, Binding at Noncatalytic Sites—PP, was shown previously to slow the rate of azide-induced inhibition of F, (Kalashnikova et al., 1988a). We have repeated these measurements and find the effect of PP, to be very similar to that obtained from filling vacant noncatalytic sites with GTP or ANP. The t1/2 for inhibition of F, [2,1] was increased 2-fold by 1 mM PP, (compare with data presented in Table I). This suggested the possibility that noncatalytic sites may also bind PP,. If this were the case, then prefilling vacant noncatalytic sites with nucleotide should prevent PP, from further slowing the rate of azide-induced inhibition. This prediction was confirmed as the kinetics of azide inhibition of F, [3,1] and F, [2A+1G,1A] were not affected by PP,.

As a further test, we measured the ability of PP, to compete with "H-labeled nucleotide for binding at noncatalytic sites. The results for GTP binding are shown in the upper panel of Fig. 4. The addition of 1 mM MgPP, to the binding assay nearly completely prevents "H[GTP binding to the vacant noncatalytic site on F, [2,1] (solid versus open circles), while having little effect on binding to catalytic sites (lower panel). Similarly, 1 mM MgPP, prevents "H[GTP binding to noncatalytic sites on F, [0,0] (data not shown).

We also tested the ability of PP, to compete with adenine nucleotide for binding at noncatalytic sites. The results show that upon incubation of F, [2,1] with 10 mM "H[ATP (Fig. 5) or 100 mM "H[ADP (Fig. 6), 1 mM MgPP, again has little effect on nucleotide binding at catalytic sites (upper panels) but significantly slows nucleotide binding at noncatalytic sites (lower panels). Under the same conditions (Fig. 5), 2 mM K,SO, affects neither catalytic nor noncatalytic site binding (data not shown).

The data of Figs. 4-6 indicate that PP, competes with GTP, ATP, and ADP for binding at noncatalytic sites. To assess the affinity of these sites for PP, F, [2,1] was preincubated with PP, but PP, was omitted from the nucleotide binding assay. If PP, binding were weak and the rate of dissociation rapid, then preincubation with PP, would not be expected to affect the rate of nucleotide binding. However, as shown in Fig. 7, prior exposure to PP, substantially reduces the initial rate of "H[ANP binding at noncatalytic sites during hydrolysis of 100 mM "H[ATP. Again, no effect on binding at catalytic sites was observed (data not shown).

FIG. 4. Binding of "H[GNP to F, [2,1] during hydrolysis of 100 mM "H[GTP in the absence and presence of PP,. F, [2,1] was incubated at 32 nM with 100 mM "H[GTP in MTSEM buffer containing a GTP-regenerating system ("Methods") without (open symbols) or with (closed symbols) 1 mM MgPP,. Nucleotide binding at noncatalytic (upper panel) and catalytic (lower panel) sites were measured as described for Fig. 2, except that 3.3-3.7 mM MgATP was used in the cold-chase step.

DISCUSSION

The main conclusions emerging from these studies are: 1) that blocking one catalytic site with inhibitory MgADP prevents catalysis at the other two sites on beef heart mitochondrial F,; 2) that noncatalytic sites are not specific for adenine nucleotides; and 3) that the occupancy of noncatalytic sites affects the properties of catalytic sites.

MgADP Bound at One Catalytic Site Prevents Catalysis at the Two Remaining Sites—Previous studies have shown that the binding of MgADP at a single catalytic site on F, in the absence of medium P, results in an inhibited form of the enzyme (Drobinskaya et al., 1985; Zhou et al., 1988; Milgrom and Boyer, 1990; Chernyak and Cross, 1992). ATP-promoted recovery from this inhibited state is a slow time-dependent
process that is easily measured in a coupled-enzyme assay for ATP hydrolysis. It has been shown that reactivation is accompanied by the dissociation of the inhibitory MgADP (Feldman and Boyer, 1985; Drobinskaya et al., 1985). However, it remained possible that prior to dissociation, the two unblocked catalytic sites might retain some capacity for uni- or bi-site catalysis. Such activity could well be too low to detect over the background of activity resulting from uninhibited F$_1$. For example, a residual bi-site activity at the two open sites equivalent to 5% of that of fully active enzyme might easily be overlooked. Since multi-site activity is up to 100,000-fold faster than uni-site catalysis, this residual activity would represent a 5,000-fold acceleration over the rate of catalysis at a single site. This would indicate a very considerable level of positive catalytic cooperativity between the two remaining catalytic sites and would argue against a mechanism where the firing sequence of the three sites is strictly sequential.

As a means of detecting such residual activity, we made use of one of the most striking features of catalysis by F$_1$: the enzyme's exceptionally tight binding of substrate ($K_{\text{M,ATP}} = 10^{-12}$ M, Grubmeyer et al., 1982) and product ($K_{\text{M,ADP}} = 10^{-9}$ M, Cunningham and Cross, 1988). The high affinity for product is responsible, at least in part, for the substantial rate enhancements observed when multiple sites turn over. Under uni-site conditions, the half-time for dissociation of tightly bound product is in the order of 5 min. However, when ATP binding occurs at an adjacent catalytic site, release of tightly bound product occurs within about 5 ms. Hence, during the 5 s it takes for enzyme to pass through a centrifuge column, the occupancy of catalytic sites is quickly reduced to one per F$_1$ regardless of whether one, two, or three sites were occupied prior to removal of unbound substrate (Cunningham and Cross, 1988). It can thus be predicted that if the two open catalytic sites on MgADP-inhibited enzyme are capable of uni- or bi-site catalysis, they should retain one molecule of bound substrate or product through the centrifuge column. Hence, the rate of filling a catalytic site with $^3$H label upon initiating an incubation of MgADP-inhibited enzyme with $[^3H]$ATP would be expected to be faster than the rate of full recovery of enzyme activity. In contrast, our results show that the rate of filling a catalytic site on various forms of MgADP-inhibited F$_1$ closely parallels the rate of recovery of activity.

There are several possible limitations in this type of measurement. The first is related to our measuring catalytic site binding as sites that exchange rapidly during a cold chase. The two open catalytic sites on MgADP-inhibited enzyme might be incapable of catalytic cooperativity but still able to bind ATP rapidly while releasing products at very slow uni-site rates. In this case, the bound labeled nucleotide would not have been displaced during the cold chase step. Such a process, however, would result in the apparent rapid binding of label at nonexchangeable sites, and this was not observed. A second potential limitation is the possibility that the open catalytic sites on MgADP-inhibited enzyme might retain the ability to bind and hydrolyze ATP but have a product dissociation rate too fast to allow ligand to remain bound during passage through a centrifuge column. If this were the case, however, the properties of such catalysis would be very different from those attributed to uni-site and bi-site catalysis. Also, it might be predicted that the extent of inhibition would decrease as the concentration of ATP decreased. This was not observed down to the lowest ATP concentration tested (10 nM).

Inhibition of F$_1$ by the naturally occurring inhibitor protein which traps bound nucleotide at a single catalytic site (Milgrom, 1991) was also found to prevent subsequent binding of nucleotide at the two remaining open sites (Kalashnikova et al., 1988). If all inhibitors gave the same result, it would indicate a strict sequential mechanism for the three sites. However, 2-azido-ANP labeling of a single catalytic site (Melese et al., 1988; Xue et al., 1988) or the binding of 1 mol of diadenosine oligophosphates (Vogel and Cross, 1991) appears to allow residual activity at the unblocked catalytic sites. Hence, some inhibitors may freeze one catalytic subunit in a state that prevents the other two from undergoing conformational transitions required for catalysis, whereas others may allow such transitions, albeit at reduced rates (Cross, 1992). This question would appear to merit further investigation.

**Noncatalytic Sites on Mitochondrial F$_1$, Do Not Bind Adenine Nucleotides Exclusively**—Noncatalytic nucleotide sites were long believed to be highly specific for adenine nucleotides (Harris, 1978). However, this appears not to be the case. One reason why previous studies may have failed to discover this lack of specificity is that nucleotide diphosphates were commonly used in binding studies. In contrast, in the current studies, Mg$[^3H]$GTP was incubated with low concentrations of F$_1$, in the presence of a GTP-regenerating system. Under these conditions, noncatalytic sites bind GTP almost exclusively. Noncatalytic sites are known to bind ATP much more tightly than ADP (Pagan and Senior, 1990; Milgrom et al., 1991), and the same may be true for other nucleotides.

One potential limitation in these experiments is the fact that some commercial sources of guanine nucleotide contain adenine nucleotide contaminants. However, in our experiments, this was not a problem. Tritium-labeled nucleotide bound at noncatalytic sites during incubation with $[^3H]$GTP; was extracted and mixed with unlabeled ADP, GDP, ATP, and GTP; and analyzed by anion-exchange HPLC. The adenine nucleotide peaks contained no tritium label, eliminating the possibility that noncatalytic sites had selectively bound contaminating adenine nucleotides.

It was recently reported that GTP binds at noncatalytic sites on CF$_1$ (Guerrero et al., 1990; Milgrom et al., 1991). One difference noted in our current experiments is that this resulted in a stimulation of the ATP hydrolysis activity, whereas with CF$_1$, the rate was inhibited. We also find that E. coli F$_1$ can bind GTP at noncatalytic sites. Therefore, the lack of

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absolute specificity of noncatalytic sites for adenine nucleotides should be considered to be universal among F1-ATPases, and such a presumed specificity should no longer be used as a criterion for distinguishing between catalytic and noncatalytic sites.

PP, binding to F1, has been studied in several laboratories. Results recently obtained by Vignais and co-workers were interpreted as evidence that the PP, binding site may overlap both the noncatalytic and catalytic nucleotide binding sites (Peinénequin et al., 1992), whereas Jaltou and Allison (1993) presented evidence for PP, binding at both catalytic and noncatalytic sites. In this study, however, we found no effect of PP, on nucleotide binding at catalytic sites, but a strong inhibition of binding at noncatalytic sites. Similar results were recently reported by Murataliev (1992).

An interaction of PP, with noncatalytic sites was foreshadowed by work done by Slater and co-workers (Roveri et al., 1980). In preparing nucleotide-depleted F1, they found that removal of endogenous nucleotides was facilitated by the addition of 10 mM PP, during dialysis of the enzyme in the presence of glycerol. Most of the endogenous nucleotide removed by this procedure was bound at noncatalytic sites.

The Occupancy of Noncatalytic Sites Affects the Properties of Catalytic Sites—The finding that increased occupancy of noncatalytic sites decreases the stability of inhibitory MgADP bound at catalytic sites supports results obtained from studies of CF, (Xue and Boyer, 1989; Guerrero et al., 1990; Milgrom et al., 1990; Murataliev and Boyer, 1992) and mitochondrial F1 (Vogel and Cross, 1991; Jaltou and Allison, 1993), which suggest that these sites may have a regulatory function or that they may facilitate catalysis (Harris, 1993). The results may also help explain several previous observations. For example, the finding that preincubation of MgADP-inhibited, membrane-bound F1, with concentrations of ADP above 20 μM accelerated subsequent reactivation during ATP hydrolysis (Bulygin and Vinogradov, 1988) probably resulted from the filling of vacant noncatalytic nucleotide sites on the enzyme. Also, since we find that filling noncatalytic sites with adenine nucleotide increases the sensitivity of GTP hydrolysis to azide inhibition, it seems likely that differences in the azide sensitivity of hydrolysis reported for nucleoside triphosphates of CF, (Xue and Boyer, 1989; Guerrero et al., 1990) suggest that these sites may have a regulatory function or that they may facilitate catalysis (Harris, 1993). The results may also help explain several previous observations. For example, the finding that preincubation of MgADP-inhibited, membrane-bound F1, with concentrations of ADP above 20 μM accelerated subsequent reactivation during ATP hydrolysis (Bulygin and Vinogradov, 1988) probably resulted from the filling of vacant noncatalytic nucleotide sites on the enzyme. Also, since we find that filling noncatalytic sites with adenine nucleotide increases the sensitivity of GTP hydrolysis to azide inhibition, it seems likely that differences in the azide sensitivity of hydrolysis reported for nucleoside triphosphates of CF, (Xue and Boyer, 1989; Guerrero et al., 1990) suggest that these sites may have a regulatory function or that they may facilitate catalysis (Harris, 1993).

Interactions between noncatalytic and catalytic sites work in both directions. It was reported previously that the rate of release of ADP from one noncatalytic site on F1 increased 10-fold during ATP hydrolysis (Kirondre and Cross, 1987). Here we find a significant increase in the rate of GTP release from a noncatalytic site during ATP hydrolysis. This suggests that cooperative interactions between catalytic sites alter the properties of noncatalytic sites.

REFERENCES


