Nucleotide-binding Sites on *Escherichia coli* F$_1$-ATPase

SPECIFICITY OF NONCATALYTIC SITES AND INHIBITION AT CATALYTIC SITES BY MgADP$^*$

(Received for publication, August 3, 1994)

David J. Hyndman, Yakov M. Milgrom, Elizabeth A. Bramhall, and Richard L. Cross

From the Department of Biochemistry and Molecular Biology, State University of New York, Health Science Center, Syracuse, New York 13210

---

F$_1$-ATPase, the catalytic component of proton-translocating ATP-synthase complexes from eubacteria, mitochondria, and chloroplasts, has a subunit structure of $\alpha_6\beta_6\gamma_2\epsilon_6$ and a total of six nucleotide-binding sites. Three of these sites exchange nucleotide rapidly during catalytic turnover and are considered to be catalytic sites. The remaining three sites do not exchange rapidly and are referred to as noncatalytic sites (for reviews see Futai et al., 1989; Senior, 1990; Fillingame, 1990; Penefsky and Cross, 1991; Allison et al., 1992; Issartel et al., 1992; Boyer, 1993).

For mitochondrial and chloroplast F$_1$, it has been shown that MgADP can bind at a catalytic site to form an inhibitory complex. This is evident as a lag in ATP hydrolysis when these enzymes are preincubated with MgADP in the presence of Mg$^{++}$. This inhibition with Mg$^{++}$ alone has no effect on endogenous nucleotides that are previously removed. Azide prevents the subsequent reactivation of the enzyme, whereas anions such as sulfite activate the enzyme by facilitating release of the inhibitory MgADP (Vasilyeva et al., 1982b). In the dark, 2-azido-ADP gives inhibition with identical properties to that of MgADP. Photolysis of the inhibitory complex results in the covalent labeling of a catalytic-site peptide on CF$_1$, (Zhou et al., 1988) and MF$_1$ (Milgrom and Boyer, 1990; Chernyak and Cross, 1992).

The F$_1$-ATPase from *Escherichia coli* has been shown to bind Mg$^{++}$ tightly (Senior et al., 1980) and to be inhibited when magnesium is added in excess of ATP (Kanazawa et al., 1980). It has been argued that this inhibition is not due to inhibitory MgADP bound at a catalytic site since, unlike the mitochondrial and chloroplast enzymes, no dependence on ADP could be demonstrated (Senior et al., 1992). Although potential support for the presence of catalytic site inhibitory MgADP comes from the observation that azide inhibits EcF$_1$, this has been attributed to a direct effect of azide on subunit cooperativity (Noumi et al., 1987). Additional suggestive evidence is that sulfite activates the E. coli enzyme (Dunn et al., 1987). Factors that could mask an ADP-dependent lag include the presence of an inhibitory subunit, which can dissociate slowly upon dilution of EcF$_1$ into an ATP hydrolysis assay (Laget and Smith, 1979) and the presence of endogenous catalytic site ADP on the isolated enzyme.

Over the last 15 years, many studies of the nucleotide-binding sites on F$_1$-ATPases have relied on ligand specificity to distinguish between catalytic and noncatalytic sites and that MgADP inhibition should be taken into account in the kinetic analysis of EcF$_1$ mutants.

---

*This work was supported by Research Grant GM 25152 from the National Institutes of Health, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and [3H]adenine nucleotide contaminants as determined by anion-exchange HPLC in the presence of carrier ADP, ATP, GDP, and GTP. (γ-32P)ATP (4500 Ci/mmol) and (α-32P)ATP (3000 Ci/mmol) were obtained from ICN. (γ-32P)Azido-ATP was synthesized as described by Melese and Boguski (1985).

Enzyme for Noncatalytic Site Specificity Studies—EcF, was isolated from E. coli strain AN1460 (Downie et al., 1980) by the method of Senior et al. (1979a, 1979b) as modified by Wise et al. (1981). Purified EcF, was stored at −70°C at a concentration of about 20 mg/ml in buffer containing 50 mM Tris-Cl, pH 7.4, 4 mM EDTA, 1 mM ATP, 1 mM dithiothreitol, 40 mM imidazole, 0.1% NP-40, 10% glycerol, and 0.5% acrylamide. Before use, EcF, was diluted to a concentration of about 2 mg/ml in buffer containing 20 mM MOPS/Tris, pH 8.0, 150 mM sucrose, and 0.2 mM EDTA and passed through two sequential 1-m1 Sephadex centrifuge columns (Penesky, 1977) equilibrated with the same buffer. This form of the enzyme (referred to as native or EcF,[2,1]) contained 2.8 mol of endogenous ADP/mol of which 2.0 mol/mol was not chaseable by GTP in agreement with Beharry and Bragg (1992). The nonchaseable nucleotides consisted of 0.9 mol of ADP and 1.1 mol of ATP.

Nucleotide-depleted enzyme (EcF,[0,0]) was prepared by published procedures (Carrett and Penesky, 1975; Senior et al., 1992). Two ammonium-sulfate precipitation steps preceded gel filtration. The enzyme was eluted from a 1.0 × 100 cm column of Sephadex G-75 (medium) at a flow rate of 0.6-1.2 ml/h with 100 mM Na2SO4, pH 8.0, 4 mM EDTA, 100 mM Na3SO4, and 50% glycerol. Selected fractions containing EcF, were combined and concentrated using a Centricon-10 microcentrifugal concentrator (Amicon). The enzyme was diluted 20-fold and recombined twice in order to transfer it to storage buffer containing 100 mM Tris-HCl, pH 8.0, 4 mM EDTA, and 50% glycerol. EcF,[0,0] was stored at a concentration of about 20 mg/ml at −20°C. The nucleotide content of EcF,[0,0] was less than 0.2 mol/mol of enzyme, as determined by subjecting a neutralized perchloric acid extract to ion-exchange HPLC (Milgrom and Cross, 1984).

Enzyme for MgADP Inhibition Studies—EcF, was isolated from strain JP17 containing the wild-type plasmid pJW1 and prepared to the solubilization stage as described by Senior et al. (1979a, 1979b). Chromatographic purification employed a Productive DC cartridge, and the enzyme was judged to be greater than 95% pure by SDS-polyacrylamide gel electrophoresis analysis. Enzyme was stored at 20-30 mg/ml at −70°C. Episomal-depleted enzyme, d-EcF,[2,1], was prepared as described (Dunn, 1986). Unlike the 5-subunit enzyme, we found that d-EcF,[2,1] could be depleted of endogenous nucleotides simply by ammonium sulfate precipitation. This involved adding 2 volumes of saturated ammonium sulfate to d-EcF,[2,1], in TE buffer (50 mM Tris-Cl, pH 8.0, 0.5 mM EDTA), incubating at 0°C for 2.5 h, pelleting 10 min in a Beckman microfuge, and redissolving in TE buffer followed by passage through a 1-m1 centrifuge column equilibrated in the same buffer. A luciferase assay of neutralized, perchloric acid extracts showed EcF,[2,1] to contain less than 0.1 mol AXAP. This produced enzyme, whereas EcF,[0,0] contained less than 0.1 mol APN/mol.

Methods
Protein concentration was determined by a modified Lowry method according to Peterson (1977). The concentrations of free and complexed metals and nucleotides were calculated using the program Bound and Determined (Brooks and Storey, 1992).

Noncatalytic Site Specificity Studies—[3H]Nucleotide binding was measured by a centrifuge column assay. Columns containing 1 or 2 ml of swollen Sephadex were equilibrated with MTSEM buffer (20 mM MgCl2, pH 8.0, 150 mM sucrose, 0.2% to 2% TFA, and 20% glycerol). When an ATP-regenerating system was present during incubation with [3H]GTP or [3H]ATP, it consisted of 50 mM KCl, 10 mM phosphoenolpyruvate, and 1 mg/ml pyruvate kinase. When the EcF, concentration was less than 3 pmol, 1 mg/ml bovine serum albumin was included in all buffers to enhance recovery of the enzyme through centrifuge columns (Cross and Nalin, 1982). Under these conditions, the recovery from 100-μl samples was 90% when applied to 1-ml columns and 85% when applied to 2-ml columns. 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 EcF, (usually less than 5%). The standard deviation for replicate measurements is ±10% using these procedures.

The composition of [3H]nucleotides bound to EcF, was determined by anion-exchange HPLC using a Partisil PXS 10/25 SAX column (4.6 × 250 mm). Bound nucleotides were extracted by perchloric acid precipitation of protein in the presence of carrier ADP, ATP, GDP, and GTP. Extracts were neutralized with KHCO3, centrifuged, and diluted prior to loading on the HPLC column. Nucleotides were eluted using a phosphate gradient as described (Milgrom and Cross, 1983). Aliquots of the fractions were counted by liquid scintillation using 5-10 volumes of BioSafe II (Research Products International).

Results
Specificity of Noncatalytic Nucleotide-binding Sites—Incubation of nucleotide-depleted enzyme (EcF,[0,0]) with 2 mM Mg[3H]GTP for 2 min results in the binding of about 3 mol of [3H]GNP/mol of enzyme (Table 1, Experiment 1). If the enzyme is then incubated for 1 min without further addition, all of the [3H]GNP remaining bound during passage through a second centrifuge column (Experiment 2). However, if millimolar concentrations of unlabelled GTP (Experiment 3) or ATP (Experiment 4) are present during the second incubation, 1 mol of GNP/mol of enzyme is released. [3H]GNP displaced from catalytic sites during this cold-chase step consists of nearly equal amounts of GDP and GTP. In contrast, the 2 mol/mol remaining bound at noncatalytic sites is mostly GTP (−90%).

When the experiments described above were repeated using native enzyme (EcF,[2,1]), about 2 mol of [3H]GNP bound/mol of enzyme during incubation with 2 mM [3H]GTP (Table 1, Experiments 5 and 6). Again, close to 1 mol of labeled nucleotide is displaced from catalytic sites during subsequent incubation with unlabelled GTP (Experiment 7) or ATP (Experiment 8). However, native enzyme retained only 1 mol of labeled nucleotide at noncatalytic sites. Again this was mostly GTP. In summary, the results presented in Table 1 show that nucleotide-depleted enzyme can bind 2 GTP at noncatalytic sites, whereas the native enzyme binds GTP at a noncatalytic site.

A time course for binding GNP to EcF,[0,0] during incubation with 100 μM [3H]GTP in the presence of a GTP-regenerating system is presented in Fig. 1. A total of 2.7 mol of [3H]GNP bound/mol of enzyme (circles), with 1.7 mol/mol bound at noncatalytic sites (triangles), and 1.0 mol/mol bound at catalytic sites (squares). Binding reached maximal levels within 3–4 min. Bound nucleotides were extracted from aliquots removed at 10 min, and their composition analyzed by anion-exchange
The stoichiometry, composition, and location of [$^{3}$H]GTP bound to EcF,$[0,0]$ and EcF,$[2,1]$ during incubation with [$^{3}$H]GTP

EcF,$[0,0]$ at 5.5 μM and EcF,$[2,1]$ at 4 μM were incubated in MTSEM buffer with 2 mM Mg[$^{3}$H]GTP for 2 min. Unbound nucleotide was removed on a centrifuge column where indicated by a double slash (-). Some samples were subjected to a second incubation of 1 min without (Experiments 2 and 6) or with 3.8 mM MgGTP (Experiments 3 and 7) or 3.7 mM MgATP (Experiments 4 and 5). Bound [$^{3}$H]GTP was extracted and analyzed by anion-exchange HPLC as described under “Experimental Procedures.” Values represent mol/mol of enzyme.

| Experiment | Treatment and form of enzyme used | [$^{3}$H]GTP bound at all sites | [$^{3}$H]GTP displaced from catalytic sites | [$^{3}$H]GTP retained at noncatalytic sites |
|------------|----------------------------------|---------------------------------|------------------------------------------|------------------------------------------|
| 1          | EcF,$[0,0] + Mg[^{3}H]GTP$        | 3.4 0.8 2.6                     |                                          |                                          |
| 2          | EcF,$[0,0] + Mg[^{3}H]GTP + MgGTP$ | 3.3 0.8 2.5                     | 0.9 0.5 0.4                              | 2.4 0.3 2.1                             |
| 3          | EcF,$[0,0] + Mg[^{3}H]GTP + MgGTP$ | 1.8 0.5 1.3                     | 1.1 0.6 0.5                              | 2.2 0.2 2.0                             |
| 4          | EcF,$[2,1] + Mg[^{3}H]GTP$        | 1.7 0.5 1.2                     | 0.7 0.3 0.4                              | 1.0 0.2 0.8                             |
| 5          | EcF,$[2,1] + Mg[^{3}H]GTP + MgGTP$| 0.8 0.4 0.4                     | 0.0 0.1 0.0                              |                                          |

Fig. 1. Binding of [$^{3}$H]GTP to EcF,$[0,0]$ during hydrolysis of 100 μM [$^{3}$H]GTP and the effect of preincubating the enzyme with unlabeled ATP. EcF,$[0,0]$ was incubated at 100 μM with 100 μM [$^{3}$H]GTP in MTSEM buffer containing a GTP-regenerating system (“Experimental Procedures”). At the times indicated, aliquots were removed and applied to centrifuge columns equilibrated with MTSEM containing 1.1 mg/ml bovine serum albumin without (circles) or following (triangles) a 1-min incubation with 3.2 mM MgATP. Catalytic site binding (squares) was calculated as the difference between total and nonchaseable (noncatalytic) binding. An additional incubation (inverted triangles) contained enzyme that was preincubated for 5 min with 100 μM unlabeled ATP in the presence of an ATP-regenerating system and passed through a centrifuge column prior to incubation with [$^{3}$H]GTP.

Fig. 2. Binding of [$^{3}$H]GTP to EcF,$[0,0]$ during hydrolysis of 100 μM [$^{3}$H]ATP in the absence or presence of PP$\gamma$. EcF,$[0,0]$ was incubated at 60 nM with 100 μM [$^{3}$H]ATP in MTSEM buffer containing an ATP-regenerating system (“Experimental Procedures”), with (inverted triangles) or without (all other data) 1 mM MgPP$\gamma$. Nucleotide binding was measured as described for Fig. 1, except that 4 mM MgATP was used in the cold chase step.

Fig. 3. Binding of [$^{3}$H]ADP to EcF,$[0,0]$ and the effect of preincubating the enzyme with unlabeled ATP. EcF,$[0,0]$ was incubated at 80 nM with 100 μM [$^{3}$H]ADP in MTSEM buffer containing 1.1 mg/ml albumin. Nucleotide binding was measured as described for Fig. 1, except that 3.7 mM MgATP was used in the cold chase step. An additional incubation (inverted triangles) contained enzyme that was preincubated for 3 min with 100 μM ATP in the presence of an ATP-regenerating system and passed through a centrifuge column prior to incubation with [$^{3}$H]ADP.

HPLC. Again, nearly all of the [$^{3}$H]GTP bound at noncatalytic sites was GTP (95%), whereas catalytic sites contained a mixture of GDP and GTP.

In contrast to the results obtained with GTP, we were unable to detect significant GDP binding at noncatalytic sites. Incubation of EcF,$[0,0]$ with 100 μM [$^{3}$H]GDP for up to 90 min gave a total of 1.6 mol/mol bound with only 0.2 mol/mol bound at nonchaseable sites. This experiment demonstrates the efficacy of the conditions used to chase catalytic site-bound nucleotide.

In order to compare these results to the binding of adenine nucleotides, EcF,$[0,0]$ was incubated with 100 μM [$^{3}$H]ATP in the presence of an ATP-regenerating system (Fig. 2) or with 100 μM [$^{3}$H]ADP (Fig. 3). Binding stoichiometries were very similar in all cases, although the rate of binding adenine nucleotides to noncatalytic sites (Figs. 2 and 3, triangles) appears to be faster than the rate of binding GTP (Fig. 1, triangles). Bound nucleotides were extracted from aliquots, taken after 3 min of incubation of EcF,$[0,0]$ with [$^{3}$H]ATP and a regenerating system (Fig. 2), and their composition was analyzed by HPLC. ATP accounted for 96% of the bound nucleotide at noncatalytic sites.

To determine whether prior binding of ATP at noncatalytic sites on EcF,$[0,0]$ blocks subsequent binding of GTP, we preincubated the enzyme with 100 μM ATP and an ATP-regenerating system. After unbound nucleotides were removed, the enzyme was incubated with 100 μM [$^{3}$H]GTP in the presence of a GTP-regenerating system. The results show that prior exposure to ATP prevents [$^{3}$H]GTP binding at noncatalytic sites (Fig. 1, inverted triangles versus triangles), while having no effect on
binding at catalytic sites (data not shown). This suggests that GTP binds at the same two noncatalytic sites that bind ATP or that cooperative interactions prevent the binding of more than two nucleoside triphosphates at the three noncatalytic sites.

Preincubation of EcF, [O, O] with unlabeled ATP was also very effective in slowing [3H]ADP binding at noncatalytic sites (Fig. 3, inverted triangles versus triangles), again having no effect on catalytic site binding (data not shown). It was of interest, however, that in contrast to [3H]GTP (Fig. 1, inverted triangles), [3H]ADP appears either to slowly exchange for noncatalytic site-bound ATP or to bind at the third noncatalytic site (Fig. 3, inverted triangles).

In order to distinguish between these possibilities, EcF,[O, O] was incubated briefly with 100 µM ATP and an ATP-regenerating system to bind 1.4 mol ATP at noncatalytic sites. Unbound ligand was removed, and the enzyme was further incubated with 100 µM ADP for 1 h. This resulted in the binding of 1.5 mol of ADP at nonexchangeable sites, with loss of only 0.1 mol of the ATP to give a total of 2.5 mol of ANP bound at noncatalytic sites/mol EcF,. It was also shown in Table I (Experiments 7 and 8) that native enzyme, which has one noncatalytic site filled with ADP and one with ATP ("Experimental Procedures"), can still bind one GTP at the third noncatalytic site. Hence, the results show that although only 2 mol/mol of GTP (Fig. 1), ATP (Fig. 2), or ADP (Fig. 3) can bind at noncatalytic sites during incubation with 100 µM nucleotide for up to 1 h, all three sites can be filled by a combination of nucleoside di- and triphosphates.

GTP bound at noncatalytic sites on EcF, is quite stable. In MTSEM buffer, the half-time for dissociation is 175 min (Table II). The addition of EDTA in excess of Mg2+ causes a 2-fold increase in the rate of release. A 5-8-fold increase is obtained with the addition of MgPPi, MgADP, or EDTA. Approximately 3.3 mol of Mg2+ were removed, and the enzyme was further incubated with 100 µM Mg2+ and unbound nucleotides were removed on a centrifuge column as described ("Experimental Procedures"). The effluent was incubated without further addition (Experiment 1) or with the additions shown.

Half-times were calculated from the measured rate constants.

| Experiment Additions | t1/2 (min) | Fold increase |
|----------------------|-----------|---------------|
| 1                    | 4 mM EDTA | 80            | 2             |
| 2                    | 1 mM MgPPi| 34            | 5             |
| 3                    | 2 mM MgADP| 29            | 6             |
| 4                    | 4 mM EDTA + 2 mM ATP | 23 | 8 |
| 5                    | 100 µM MgATP + ATP-regenerating system | 8 | 23 |

**Table II**

Half-times for dissociation of [3H]GTP from noncatalytic sites on EcF,.

EcF,[O, O] was incubated at 60 nM for 10 min in MTSEM buffer containing 100 µM [3H]GTP and a GTP-regenerating system (Table II). [3H]GTP was displaced from catalytic sites by incubating with 3.3 mM Mg2+ and unbound nucleotides were removed on a centrifuge column as described ("Experimental Procedures"). The effluent was incubated without further addition (Experiment 1) or with the additions shown. Half-times were calculated from the measured rate constants.

In contrast to L3H1GTP (Fig. 1, inverted triangles), [3H]GTP hydrolysis was enhanced by Mg2+ during GTP and ITP hydrolysis (Fig. 5, squares and triangles). However, the trapped azido-adenine dinucleotide was stripped of endogenous nucleotides by ammonium sulfate precipitation ("Experimental Procedures"). A burst in activity of EcF, [O, O] was seen after preincubation with either EDTA (Fig. 7, open squares) or Mg2+ (open circles), whereas MgADP resulted in a lag (closed circles). Preincubation with MgATP under conditions that allowed complete conversion to ADP also produced a lag (data not shown).

Azide has been shown to trap the MgADP complex formed of catalytic sites on MF, and CF, (Vinay Kumar et al., 1982). Preliminary experiments with native EcF, indicated that retention of labeled ANP during a cold chase could be enhanced by azide. With increase in azide concentration during cold chase, an increase in azide-trappable ANP showed a linear correlation with the loss of hydrolytic activity (Fig. 8). Extrapolation to complete inactivation corresponded to 0.45 mol of azide-trappable ANP/mol of enzyme.

In order to identify the nucleotide site occupied by the azide-trappable ADP, 2-azido-adenine nucleotide was used. In the dark, this photoaffinity analog is trapped by azide during a cold chase in a similar manner to ADP. The trapped azido-adenine nucleotide was covalently incorporated into the protein by photolysis, and reversed-phase HPLC elution profiles of digested samples were determined (Fig. 9). Photoincorporation following a cold chase in the presence of azide resulted in significant labeling of a catalytic site peptide (Cat) eluting at 23% acetonitrile (top panel). In contrast, a cold chase in the presence of
The first was based on affinity. Noncatalytic sites were thought to be very tight and nearly constant. The positions of catalytic and noncatalytic site peptides in the elution profile were assigned based on controls where EcF1 was selectively labeled at either catalytic (pY331) or noncatalytic (pY354) sites as described by Wise et al. (1987). Also, using anion-exchange HPLC which separates peptides labeled by the di- and triphosphates forms of the probe, it was found that the azide-trappable catalytic site peptide is labeled exclusively by 2-azido-ADP (data not shown).

**DISCUSSION**

Past studies of the nucleotide-binding sites of F$_1$-ATPases have relied on three different criteria for distinguishing between catalytic and noncatalytic sites. The first was based on affinity. Noncatalytic sites were thought to be very tight and catalytic sites to be loose (Harris, 1978). The three tightly bound endogenous nucleotides retained during purification of MF$_1$ were assumed to be bound at the three noncatalytic sites.

However, this was later shown not to be the case. One of the endogenous nucleotides exchanges rapidly during catalytic turnover (Kironde and Cross, 1986), and the first catalytic site to bind ADP (K$_d$(ADP) = 1 nM, Cunningham and Cross, 1988) is tighter than the loosest noncatalytic site (K$_d$(ADP) = 50 nM, Kironde and Cross, 1987). Another reason for not considering catalytic sites to be loose comes from the finding that the first catalytic site on MF$_1$ to bind ATP does so with a remarkably high affinity (K$_f$(ATP) = 10$^{-12}$ M, Grubmeyer et al., 1982).

In recent years few laboratories have relied on affinity as a means for distinguishing between catalytic and noncatalytic nucleotide sites.

A second widely used criterion is based on the nucleotide specificities of the sites. Catalytic sites clearly tolerate variations in the structure of the base moiety as GTP and ITP are
ATP may also explain why binding to the isolated α-subunit, been thought to be highly specific for adenine nucleotide that GTP bound at noncatalytic sites would also have dissociated. It was recently shown that CF₁, binds GTP at noncatalytic sites (Harris, 1978; Harris et al., 1978; Perlin et al., 1984). However, it was recently shown that CF₁ binds GTP at noncatalytic sites (Guerrero et al., 1990; Milgrom et al., 1991), and we find the same is true for MF₁ (Milgrom and Cross, 1993) and EcF₁ (Table I and Fig. 1; see also Weber et al., 1994). In addition, it appears that pyrophosphate can bind at noncatalytic sites on EcF₁ (Figs. 2 and 4) and MF₁ (Milgrom and Cross, 1993).

There are several reasons why this lack of specificity was not previously detected. The first is that GTP binding is not as tight as ATP binding. Under conditions of catalytic turnover, GTP bound at noncatalytic sites on EcF₁ dissociates with a half-time of 8 min (Table II), whereas little dissociation of ATP could be detected (Page and Senior, 1980). In testing for GTP binding at noncatalytic sites, Perlin et al. (1984) used a 30-min incubation with unlabeled ATP to chase [³²P]GNP from catalytic sites. In light of the data presented in Table II, it can be predicted that GTP bound at noncatalytic sites would also have dissociated under these conditions. A lower affinity for GTP than for ATP may also explain why binding to the isolated subunit, which appears to retain part of the noncatalytic binding domain (Dunn and Futai, 1980), was not detected (Senda et al., 1983; Perlin et al., 1984).

A second reason is that the affinity of noncatalytic sites for GDP is much lower than for GTP, just as ADP binds less tightly than ATP. This may preclude detection of GDP binding by a centrifuge column assay ("Results," and Kironde and Cross, 1986) or when the enzyme is incubated with GTP over a period of hours under conditions where the GTP is rapidly converted to GDP (Perlin et al., 1984).

A final explanation for the failure to readily detect guanine nucleotide binding at noncatalytic sites relates to the very slow rate of dissociation of adenine nucleotides from these sites. With EcF₁, having ANP prebound at noncatalytic sites, Senior and co-workers (Perlin et al., 1984; Senior et al., 1992) observed no exchange for medium GTP. Even when GTP is maintained by a regenerating system, no exchange is seen over a 20-min period if enzyme is pretreated with ATP (Fig. 1, inverted triangles).

A third criterion for distinguishing between catalytic and noncatalytic sites is based on the fact that catalytic sites on fully active MF, exchange bound ligand rapidly during turnover, whereas noncatalytic sites do not (Cross and Nalin, 1982). The validity of this criterion is supported by the finding that 2-azido-ATP labels a single, specific β-tyrosyl residue when loaded at exchangeable sites and a single, but different, β-tyrosyl residue when loaded at nonexchangeable sites on MF₁ (Cross et al., 1987), CF₁ (Xue et al., 1987), and EcF₁ (Wise et al., 1987).

However, this criterion also has limitations. The first is due to the fact that noncatalytic sites are not nonexchangeable. The noncatalytic site on MF₁, having the lowest affinity for ADP releases bound ligand with a half-time of 3 min during catalytic turnover (Kironde and Cross, 1987). However, as originally defined (Cross and Nalin, 1982), exchangeable (catalytic) sites exchange with medium ATP very rapidly during a brief episode of catalytic turnover. Typically ATP is added in a 1000:1 molar ratio to F₁ and cleavage is complete within a few seconds. Under these conditions, noncatalytic sites do not exchange. However, it should be noted that pH values below 7 (Harris et al., 1978) and nonaqueous solvents such as glycerol (Garrett and Penefsky, 1975) can accelerate release of nucleotide from noncatalytic sites.

A second limitation results from the fact that inhibitory MgADP bound at a single catalytic site on CF₁ (Feldman and Boyer, 1985; Zhou et al., 1988) or MF₁ (Drobinskaya et al., 1985; Milgrom and Boyer, 1990; Chernyak and Cross, 1992) cannot dissociate slowly, under certain conditions, upon addition of MgATP. Hence, nucleotide bound at catalytic sites does not always exchange rapidly. However, with MF₁, this can be avoided simply by use of buffers containing 1 mM Mg²⁺ (Grubmeyer et al., 1982).

In contrast, the formation of an inhibitory catalytic site MgADP complex has not been thought to play a significant role in the kinetics of EcF₁, making this enzyme unique. Instead it has been argued that Mg²⁺ inhibits directly and that azide inhibition of the enzyme is related to changes in subunit cooperation.

The ability to demonstrate MgADP inhibition of EcF₁ has been complicated by the presence of the inhibitory ε-subunit. When EcF₁ is incubated with MgADP, it is inhibited in an ATP hydrolysis assay to a concentration below the K₅₅ (2-10 nM) for the ε-subunit (Sternweis and Smith, 1980; Wood et al., 1987), its slow dissociation results in a steady increase in activity (Lagert and Smith, 1979). This lag would mask a delay caused by the slow release of inhibitory MgADP such as that observed with MF₁ (Minkov et al., 1979; Drobinskaya et al., 1985) and CF₁ (Feldman and Boyer, 1985).

The successful demonstration of MgADP inhibition of EcF₁ obtained in the present study was aided by the use of ε-depleted enzyme. With four-subunit EcF₁, elimination of endogenous ADP by preincubation with an ATP regenerating system (Fig. 6) or by removal of all bound nucleotides (Fig. 7) resulted in a burst of ATP hydrolysis activity which could be prevented by MgADP but not by Mg²⁺ alone. In addition, only ε-deEcF₁,0,0] gave equal rates for hydrolysis and the loading of a catalytic site with labeled nucleotide (results not shown). All other enzyme forms loaded one catalytic site at a rate too slow to be consistent with a population of fully active enzyme. This reinforces the proposal that native EcF₁[2,1] is a heterogeneous mixture of active and inactive enzyme as suggested by kinetic studies from Allison's laboratory (Muneyuki et al., 1991), as shown by the lag in Fig. 6 (closed squares) and as quantitated by the extrapolation to 0.5 mol of azide-trappable ANP/mol of enzyme at zero activity (Fig. 8). Presumably the other half of the enzyme is already inhibited by unlabeled endogenous ADP.

A second criterion for demonstrating MgADP inhibition of MF₁ and CF₁ has been its nucleotide specificity. The ability of different nucleotides to differentially inhibit ε-deEcF₁ with in-
creases free Mg\textsuperscript{2+} (Fig. 5) is a strong indication that Mg\textsuperscript{2+} is not solely responsible for the observed inhibition. In a similar manner, sub mitochondrial particles are inhibited by preincubation with ADP but not by GDP or IDP (Vasilyeva et al., 1980).

A third criterion to show the presence of inhibitory MgADP at catalytic sites has been the photolabelling of a catalytic-site peptide by Mg\textsuperscript{2+}-azidoADP (Zhou et al., 1988; Milgrom and Boyer, 1990; Chernyak and Cross, 1992). Using azide to trap 2-azido-ADP on EcF\textsubscript{1}, photolysis of the inhibitory complex resulted in the modification of a catalytic-site peptide (Fig. 9). We exploited this property to trap inhibitory MgADP (Fig. 8) and Mg(2-azido)ADP (Fig. 9) under cold chase conditions.

Structural and mechanistic features of EcF\textsubscript{1} have been extensively studied by site-directed mutagenesis. Knowledge of the fact that EcF\textsubscript{1} is inhibited by catalytic site-bound MgADP is thus of major importance in addition to its sharing this property with MF\textsubscript{1} and CF\textsubscript{1}. The relative susceptibility of each mutant enzyme to inhibition by MgADP should be taken into account before binding or kinetic analyses are interpreted. One mutant EcF\textsubscript{1} analyzed in our laboratory, \( \beta_{2211D} \), shows significantly lower activity than wild-type enzyme under normal assay conditions, but this has been shown to be due to a heightened sensitivity to MgADP inhibition rather than to a catalytic defect.\textsuperscript{2} When assaying ATP hydrolysis, we currently minimize the fraction of enzyme in the \( \epsilon-F \) form by diluting below the \( K_d \) for \( \epsilon \) and by adding lauryldimethylamine oxide which helps reverse \( \epsilon \) inhibition (Lotscher et al., 1984). To minimize the level of \( F^{-} \cdot ADP \), we prefilt EcF\textsubscript{1} with ATP, add sulfite or selenite to the assay mixture, and maintain low concentrations of free Mg\textsuperscript{2+}.

In summary, with the proper precautions, measuring differences in the exchangeability of bound ligands can provide a reliable means of distinguishing catalytic from noncatalytic sites on F\textsubscript{1}-ATPases, whereas differences in substrate specificities or affinities may not. Also, in analyzing EcF\textsubscript{1} mutants, it is important to distinguish between alterations in sensitivity to inhibition by MgADP and changes in catalytic efficiency.

\textsuperscript{2} T. M. Duncan and R. L. Cross, unpublished data.