Effects of the Inhibitors Azide, Dicyclohexylcarbodiimide, and Aurovertin on Nucleotide Binding to the Three F₁-ATPase Catalytic Sites Measured Using Specific Tryptophan Probes*

(Received for publication, August 18, 1998, and in revised form, September 14, 1998)

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Equilibrium nucleotide binding to the three catalytic sites of Escherichia coli F₁-ATPase was measured in the presence of the inhibitors azide, dicyclohexylcarbodiimide, and aurovertin to elucidate mechanisms of inhibition. Fluorescence signals of β-Trp-331 and β-Trp-148 substituted in catalytic sites were used to determine nucleotide binding parameters. Azide brought about small decreases in $K_d$(MgATP) and $K_d$(MgADP). Notably, under MgATP hydrolysis conditions, it caused all enzyme molecules to assume a state with three catalytic site-bound MgATP and zero bound MgADP. These results rule out the idea that azide inhibits by “trapping” MgADP. Rather, azide blocks the step at which signal transmission between catalytic sites promotes multisite hydrolysis. Aurovertin bound with stoichiometry of 1:8 (mol/mol of F₁) and allowed significant residual turnover. Cycling of the aurovertin-free β-subunit catalytic site through three normal conformations was indicated by MgATP binding data. Aurovertin did not change the normal ratio of 1 bound MgATP/2 bound MgADP in catalytic sites. The results indicate that it acts to slow the switch of catalytic site affinities (“binding change step”) subsequent to MgATP hydrolysis. Dicyclohexylcarbodiimide shifted the ratio of catalytic site-bound MgATP/MgADP from 1:2 to 1:6:1:4, without affecting $K_d$(MgATP) values. Like azide, it also appears to affect activity at the step after MgATP binding, in which signal transmission between catalytic sites promotes MgATP hydrolysis.

F₁-ATPase is the catalytic moiety of F₁,F₀-ATP synthase, the membrane enzyme that carries out ATP synthesis in mitochondria, bacteria, and chloroplasts and also ATP-driven proton pumping in bacteria. It contains three catalytic sites, located primarily on β-subunits, which show strong binding cooperativity with substrates MgATP and MgADP and show strong positive catalytic cooperativity. The recent discovery of ATP-driven rotation of the central γ-subunit relative to the α,β3 subunit hexagon proved that the catalytic sites act in sequential manner during catalysis and gave strong impetus to studies of the catalytic mechanism (for reviews see Refs. 1–3). Azide, dicyclohexylcarbodiimide (DCCD),¹ and aurovertin are three potent, commonly used, inhibitors of ATP hydrolysis by purified, soluble F₁ and membrane-bound F₁,F₀-ATP synthase. Their exact modes of action are not yet clear, so it is of interest to study them further for the purpose of understanding the inhibitory mechanisms.

We have developed substituted Trp probes that specifically report binding of nucleotides to catalytic sites in Escherichia coli F₁-ATPase under true equilibrium conditions and during catalysis. One is β-Trp-331. It has a very large fluorescence signal, which is totally quenched upon binding of MgATP or MgADP in catalytic sites (4–6). It can be used to measure parameters such as nucleotide binding stoichiometry and affinity under a wide variety of conditions. A second probe is β-Trp-148, whose fluorescence signal changes depending upon whether nucleoside di- or triphosphate is bound in the catalytic sites, thereby allowing discrimination of catalytic site-bound MgATP from MgADP (7). Combined use of these two Trp probes has led to a proposal for the catalytic mechanism of F₁-ATPase (2, 7) in which enzyme molecules cycle through four states, each containing two or three bound nucleotides, with the bound nucleotide species (MgATP or MgADP) in each of the three catalytic sites defined for each state.

In this paper, we have studied the effects of the inhibitors azide, DCCD, and aurovertin on nucleotide binding properties of F₁-ATPase using these two Trp probes. The results provide improved understanding of the mechanism of action of the inhibitors, and in the case of azide, the results are in clear contradiction of a current school of thought.

**EXPERIMENTAL PROCEDURES**

**Preparation of Enzymes—**Wild-type F₁, was from strain SWM1 (8), β331W mutant F₁, from strain SWM4 (4), and βF148W mutant F₁, from strain CB1 (7). Purification of F₁, was as described by Weber et al. (9). Before use, the enzymes were passed twice through 1-ml centrifuge columns of Sephadex G-50 in 50 mM Tris/SO₄, pH 8.0 as described (4, 5), which has been shown to effectively remove catalytic site-bound nucleotide. Nucleotide-depleted F₁, (depleted of both catalytic site-bound and noncatalytic site-bound nucleotide) was prepared as described by Senior et al. (10).

**Assay of Inhibition of ATPase Activity in the Presence of Azide or Aurovertin—**All manipulations were done at room temperature. Enzyme (20 µg/ml, 52 nm) was preincubated in 50 mM Tris/SO₄, pH 8.0, in the presence or absence of 10 mM sodium azide or 10 µM aurovertin for 15 min. (Nucleotide additions during preincubation are described below.) NaATP (10 mM) and MgSO₄ (4 mM) were added to start the reaction, which was terminated after 4 min by the addition of an equal volume of 10% (w/v) SDS. P, released was assayed colorimetrically (11).

**Covalent Modification of Enzymes with DCCD and Assay of ATPase Activity—**Covalent modification with DCCD was as described by Weber et al. (5). The assay of activity was as above except that DCCD-modified F₁, was added to buffer containing Tris/SO₄, NaATP, and MgSO₄ to start the reaction.

**Fluorescence Measurements—**We used the same conditions for fluorescence measurements as for ATPase assay, namely room temperature, 50 mM Tris/SO₄, pH 8.0, and an enzyme concentration of 50–100 nM. A SPEX Fluorolog 2 or a Amico-Bowman 2 spectrofluorometer was used. Excitation wavelength was 295 nm. For MgATP titration, two

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* Supported by National Institutes of Health Grant GM25349 (to A. E. S.). 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.

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¹ The abbreviations used are: DCCD, dicyclohexylcarbodiimide; MgAMPFPNP, Mg-5'-adenyl imidodiphosphate; MgATP, Mg-adenosine 5'-O-(thiotriphosphate).
**Table I**

**Inhibition of β-Trp-331 and β-Trp-148 enzymes by azide**

<table>
<thead>
<tr>
<th>F1</th>
<th>Additions during preincubation</th>
<th>Inhibition of ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Trp-331</td>
<td>Azide</td>
<td>96</td>
</tr>
<tr>
<td>β-Trp-331</td>
<td>Azide plus 0.7 μM MgATP</td>
<td>97.5</td>
</tr>
<tr>
<td>β-Trp-331</td>
<td>Azide plus 0.4 μM MgADP</td>
<td>99.5</td>
</tr>
<tr>
<td>β-Trp-148</td>
<td>Azide</td>
<td>92</td>
</tr>
<tr>
<td>β-Trp-148</td>
<td>Azide plus 0.7 μM MgATP</td>
<td>96</td>
</tr>
<tr>
<td>β-Trp-148</td>
<td>Azide plus 0.4 μM MgADP</td>
<td>97</td>
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</tbody>
</table>

Fig. 1. Titration of β-Trp-331 F1, with MgATP in the presence or absence of azide. A, NaATP and MgSO4 were added in a constant 2.5:1 concentration ratio to enzyme in 50 mM TrisSO4 pH 8.0, with or without 10 mM sodium azide, and the fluorescence signal was measured (see “Experimental Procedures” for details). B, MgATP was added to enzyme in 2.5 mM MgSO4, 50 mM TrisSO4, pH 8.0. , in the absence of sodium azide; ●, in presence of 10 mM azide. The lines are fits to the data using a model with three sites of different affinities.

Effect of Azide on MgATP and MgADP Binding to β-Trp-331 F1—Fluorescence titrations were carried out to determine binding characteristics for MgATP and MgADP in presence of 10 mM sodium azide. In all cases, enzyme and azide were preincubated together at least 15 min before nucleotide was added. Thus, the enzyme would in all cases be potently inhibited. In the presence of azide, upon the addition of nucleotide the fluorescence signal decreased within 2 min or less to a value that remained stable for at least 30 min.

Titration with MgATP was first carried out under exactly the same conditions as the ATPase assay, with an ATP/MgATP concentration ratio of 2.5:1, which as noted above is optimal for activity. Fig. 1A shows the results. The data were analyzed as described under “Experimental Procedures” and the legend to Fig. 1. A binding model with three types of site with different affinities gave a good fit to the data, as seen previously (4, 5, 15). In the absence of azide (Fig. 1A, open symbols), three catalytic sites were filled by MgATP, with Kd1 = 50 nM, Kd2 = 2.5 μM and Kd3 = 33 μM, very similar to previous results (4–6).

In presence of azide (Fig. 1A, filled symbols), three sites were also filled, with Kd1 = 50 nM, Kd2 = 100 nM, and Kd3 = 16 μM. The major effect of azide was clearly to increase MgATP binding stoichiometry at low MgATP concentrations, by decreasing Kd2 and possibly also Kd3. However, under these experimental conditions it is difficult to calculate low MgATP concentrations accurately, which introduces uncertainty for Kd values below micromolar level.

We therefore carried out titration with MgATP in the presence of excess (2.5 mM) MgSO4 and azide. This allows accurate calculation of low MgATP concentrations, since one can assume that all ATP is complexed with Mg2+. In addition, nucleotide-depleted F1 (10) was used in these experiments to prevent any MgATP released from noncatalytic sites from interfering with calculation of MgATP concentration. The results are shown in Fig. 1B. The data again fit well to a model assuming three sites of different affinities. In absence of azide (open symbols), the following values were calculated: Kd1 = 53 nM, Kd2 = 1.8 μM, and Kd3 = 42 μM. In the presence of azide (filled symbols), the values were as follows: Kd1 = 21 nM, Kd2 = 0.39 μM, and Kd3 = 49 μM. Therefore, azide had the effect of increasing MgATP binding affinity at site one (by 2.5-fold) and at site two (by 5-fold).

It is apparent that azide had greater effect on nucleotide binding at low MgATP concentrations in Fig. 1A than it did in Fig. 1B. This could be due to effects of azide on free ATP binding, since free ATP would be present under the conditions...
of Fig. 1A but not those of Fig. 1B. In Fig. 2, free ATP binding in presence and absence of azide was measured, and it is clear that azide did enhance binding of free ATP at low concentrations of the nucleotide. For this reason we feel that the data from Fig. 1B give the more reliable estimates of the effects of azide on MgATP binding.

Titration with NaADP in presence of 2.5 m mM MgSO₄ is shown in Fig. 3. In absence of azide, two types of site were evident, with one site of higher and two sites of lower affinity, as has been seen previously (6, 15). This pattern was retained in presence of azide. Calculated $K_d$ values were as follows. Without azide, $K_{d1} = 0.11\, \mu M$ and $K_{d2} = 37\, \mu M$; with azide, $K_{d1} = 0.04\, \mu M$ and $K_{d2} = 20\, \mu M$. These results indicated that azide increased the affinity for MgADP binding at all three catalytic sites, although by relatively modest factors, namely by 3-fold at site one and by 2-fold at sites two and three. When the experiment was repeated with nucleotide-depleted F₁ (10), the same results were obtained.

Effect of Azide on MgATP and MgADP Binding to β-Trp-148 F₁—Previous work (7) has established that the β-Trp-148 enzyme shows a reduction in fluorescence at 350 nm upon binding of MgADP and an enhancement of fluorescence at 325 nm upon binding of MgAMPNNP or MgADP-beryllium fluoride complex (which is also an MgATP analog). We established first that similar fluorescence responses were seen when 10 mM sodium azide was added along with saturating concentration of the relevant nucleotide.

In Fig. 4, we carried out titration with NaATP/MgSO₄ at constant 2.5:1 concentration ratio in presence or absence of azide, under the same conditions as used for ATPase assay. Fig. 4A shows total bound nucleotide (MgATP plus MgADP) in presence of azide (filled symbols) as compared with its absence (open symbols). Consistent with the data in Fig. 1, stoichiometry of total bound nucleotide at saturation was 3 mol of nucleotide/mol of enzyme, and it can also be seen that at the lower MgATP concentrations, azide yielded higher binding stoichiometries due to higher affinity of the enzyme for MgATP. Fig. 4B shows bound MgATP in the presence (filled symbols) or absence (open symbols) of azide, as a function of increasing MgATP concentration. It is immediately apparent that azide induced a major shift toward an increased level of bound MgATP. This is amply confirmed by Fig. 4C, in which bound MgADP is presented as a function of added MgATP concentration, and it is seen that in the presence of azide, there was essentially no bound MgADP. The conclusion from Fig. 4 is that, at saturation with added MgATP, the distribution of bound nucleotide...
achieve total inhibition of hydrolysis, and the residual activity could be due to enzyme molecules with bound aurovertin showing slowed turnover or to enzyme molecules with no aurovertin bound.

Aurovertin quenched the fluorescence signal of $\beta$-Trp-331 $F_1$ by 30% (mean of 12 experiments) at 360 nm and had no effect (except for a strong inner filter effect) on the fluorescence signal of wild-type $F_1$. The quenching is expected, due to Förster resonance energy transfer, since the $\beta$-Trp-331 fluorescence emission spectrum extensively overlaps the aurovertin absorption spectrum ($\lambda_{\text{max}} = 368$ nm). The x-ray structure of mitochondrial $F_1$ complexed with aurovertin (19, 20) shows two molecules of aurovertin bound per enzyme molecule, one to the $\beta$-Trp and one to the $\beta$-subunit, in the cleft between the nucleotide domain and the C-terminal domain of the respective $\beta$-subunit. The conjugated double bond system of each aurovertin molecule comes within 10 Å of the $\beta$-331 residue of the $\beta$-subunit to which aurovertin is bound and is around 55 Å from the two other $\beta$-331 residues. Assuming a fluorescence quantum yield of 0.13 for each $\beta$-Trp-331 residue and a value of $k^2 = 0.667$, then the $R_0$ value for the donor-acceptor pair $\beta$-Trp-331 and aurovertin is calculated to be 30.5 Å. We can therefore conclude that the fluorescence of $\beta$-Trp-331 residues on the two aurovertin-containing $\beta$-subunits is completely quenched and that the remaining $\beta$-Trp-331 on the aurovertin-free $\beta$-subunit would not be quenched to a significant extent.

In $\beta$-Trp-331 mutant $F_1$, the introduced $\beta$-Trp-331 residues account for 50% of the total fluorescence. The 30% quenching of the total fluorescence seen with 10 μM aurovertin (above) therefore corresponds to 60% quench of the $\beta$-Trp-331 fluorescence and indicates that the stoichiometry of bound aurovertin was 1.8 mol/mol of $F_1$, consistent with the x-ray structure.

We used the residual fluorescence signal to analyze nucleotide-binding parameters in the presence of 10 μM aurovertin. Fig. 5A shows the MgATP titration curve, carried out at a constant ATP/Mg$^{2+}$ concentration ratio of 2.5:1. Two fits to the data are shown; the solid line is a fit to a model assuming three types of site, and the dotted line is a fit to a model assuming two types of site. The former model yields the better fit, and the calculated $K_d$ values are $K_{d1} = 50$ nM, $K_{d2} = 0.47$ μM, and $K_{d3} = 20$ μM, with total apparent stoichiometry $n = 1.2$. Since 1.8 of the $\beta$-Trp-331 residues are expected to be spectroscopically silent due to quenching by the bound aurovertin (1.8 mol/mol), the maximum apparent nucleotide binding stoichiometry expected from the remaining signal is 1.2 mol/mol. Thus, it is interesting that with MgATP, three different types of binding site are indicated, with $K_d$ values similar to the uninhibited enzyme. Our conclusion is that, in the aurovertin-inhibited enzyme, there is residual ATPase turnover, and the $\beta$-Trp-331 residue on the aurovertin-free $\beta$-subunit is continuously cycling between three different affinities or conformations, consistent with the rotational enzyme mechanism.

The results with the $\beta$-Trp-331 probe had established that the DCCD-inhibited enzyme shows normal MgATP binding, with no change in binding stoichiometry or affinity at each of the three catalytic sites (5). This indicated that DCCD acts subsequent to MgATP binding, at a step or steps involved in positive catalytic cooperativity.

The results with the $\beta$-Trp-148 enzyme reported here show that DCCD-inhibited enzyme follows the normal catalytic path-

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**Fig. 4. Titration of $\beta$-Trp-148 $F_1$ with MgATP in the presence or absence of azide.** NaATP and MgSO$_4$ were added in a constant 2.5:1 concentration ratio to enzyme in 50 mM Tris/SO$_4$, pH 8.0, and the fluorescence signal was measured at 325 and 350 nm, allowing calculation of bound MgATP and MgADP (see “Experimental Procedures” for details). Each data point represents a single experiment. A, total bound MgATP plus MgADP; B, bound MgATP; C, bound MgADP. Filled symbols represent data in the presence of 10 μM azide; open symbols are in the absence of azide (these latter data include new experiments together with previous data points from Ref. 7).
Nucleotide Binding to Inhibited F$_1$-ATPase

way for MgATP hydrolysis as described in the catalytic mechanism in Fig. 6 (2, 7). In normal enzyme, under steady-state hydrolysis conditions, the most populous state of the enzyme molecules is one with one MgATP and two MgADP bound, corresponding to “State A,” resulting from the fact that release of MgADP is the overall rate-limiting step. In DCCD-inhibited enzyme there was a shift toward a higher ratio of bound MgATP/bound MgADP, demonstrating a higher population of molecules in “State A.” In Fig. 6, immediately after binding of MgATP to the loosest (L) of the three catalytic sites (D → A) there is intersubunit signal transmission (A → B), which promotes hydrolysis of MgATP at the highest affinity site (H), which in turn triggers the “binding change,” whereby the three sites switch their affinities (arrows in State B). In previous work using MgATP as substrate, we also saw accumulation of State A (7). This can be readily explained as due to slowing of the actual hydrolysis step by the nucleotide analog, which has been demonstrated to occur under both multisite and unisite hydrolysis conditions (22). On the other hand, because DCCD inhibits unisite hydrolysis to a significantly lesser extent than multisite hydrolysis, and because its binding site is remote from the catalytic sites as shown by the x-ray structure (20), we conclude that the step in the mechanism that is slowed by DCCD is the conformational change that occurs immediately after binding of MgATP to the loosest site on the enzyme, which is relayed to the high affinity site and which signals the high affinity site to hydrolyze its bound MgATP rapidly.

Aurovertin was found to be less inhibitory than DCCD in both β-Trp-331 and β-Trp-148 mutant enzymes, consistent with previous literature. Stoichiometry of aurovertin binding could be measured using the β-Trp-331 F$_1$ fluorescence and was close to 2 mol/mol F$_1$, as was seen in the x-ray structure of mitochondrial F$_1$ (19). There was significant quenching of Trp fluorescence by bound aurovertin in both mutant enzymes, due to Förster resonance energy transfer, also consistent with and expected from the x-ray structure. Nevertheless, we were able to use the residual Trp fluorescence signals to obtain information about nucleotide binding. The interesting result with aurovertin-inhibited β-Trp-331 enzyme was that the MgATP binding curve indicated cycling of one β-subunit through each of the three different states, corresponding in MgATP binding affinity to the three different β-subunit conformations seen in normal enzyme, whereas with MgADP only one state was seen. This is consistent with the conclusion that the aurovertin-inhibited enzyme carries out normal rotational catalysis but at a reduced rate. With aurovertin-inhibited β-Trp-148 enzyme, we showed that upon the addition of MgATP, the ratio of bound MgATP/MgADP in the enzyme population remained at 1:2, as in normal enzyme. Thus, the step that is slowed in the presence of aurovertin could be any of those steps subsequent to the hydrolytic step, namely the “binding change” of the catalytic sites, the release of product P$_i$ or the release of product MgADP (see Fig. 6). From the x-ray structure analysis, van Raaij et al. (19) suggested that it is the “binding change” that is impeded by aurovertin. Our data ($K_p$ values and MgATP/MgADP distribution) support this conclusion.

Azide is a much studied inhibitor of F$_1$-ATPase. It is well established that it inhibits steady-state hydrolysis (apparent $K_p$ in wild-type E. coli F$_1$ = 25 μM, Ref. 23) but does not inhibit single turnover (unisite) catalysis, which led previous workers...
to conclude that it does not affect the hydrolytic step of catalysis per se, but rather that it interferes with subunit interactions and cooperativity (24–26). On the other hand, a second school of thought holds that azide “traps” MgADP permanently at a catalytic site, thus preventing product release and leading to a population of inactive molecules (e.g., Refs. 27–30). Most of the evidence for “trapping” of MgADP comes from studies of inhibition kinetics of steady-state hydrolysis. Analysis of bound catalytic site nucleotides, e.g., by high pressure liquid chromatography, was also reported, but since this is not a true equilibrium technique it does not quantitatively report nucleotide binding parameters under the actual conditions of enzyme assay. Hyndman et al. (29) studied the stoichiometry of “trapping” of adenine nucleotide by azide in E. coli F1 and found that at full inhibition of ATPase the trapped catalytic site nucleotide amounted to just 0.38 mol/mol of F1, which is inconsistent with the idea that azide brings about inhibition by trapping MgADP in a catalytic site. Importantly, where corroboration was sought from the use of other techniques, the evidence has been ambiguous or contrary. For example, Mueller et al. (31) found that in yeast mitochondrial F1, azide had no significant effect on the ratio of bound MgATP/bound MgADP, averaged over the whole population, which is inconsistent with the idea that MgADP bound on the enzyme catalytic sites. Obviously, inhibition occurred in the absence of any MgADP bound on the enzyme catalytic sites. This result supports the idea that azide brings about inhibition by trapping MgADP in a catalytic site, but it seems too small to account for the degree of inhibition seen. When enzyme was titrated with MgATP, we found that the binding affinity at sites one and two was somewhat higher in the presence of azide than in its absence (Fig. 1, A and B), similar to what was seen with MgADP. Fig. 2 showed that azide increased the affinity for free ATP at site one.

The surprising finding, however, was that when MgATP was added to F1 in the presence of azide, the bound nucleotides on the enzyme at saturation consisted of three MgATP and zero MgADP (Fig. 4). Next, we carried out experiments where we added azide to F1 that was already hydrolyzing MgATP. Immediately after the addition of MgATP to initiate hydrolysis, the ratio of bound MgATP/bound MgADP was 1:2, as reported before (7), but after the addition of azide it changed to three bound MgATP/zero bound MgADP. These experiments completely rule out the idea that azide inhibits by trapping MgADP at a catalytic site; obviously, inhibition occurred in the absence of any MgADP bound on the enzyme catalytic sites.

As noted above, the residual ATPase activity seen in the presence of azide (Table I) could be due to a small fraction of F1 molecules with no bound azide or to residual turnover of azide-complexed molecules. We favor the first alternative. Calculation shows that 3–4% of fully active F1 molecules (with one MgATP/two MgADP bound in catalytic sites) would yield a total bound MgADP stoichiometry of 0.06–0.08 MgADP per F1, averaged over the whole population, which is clearly within the experimental variation seen in Fig. 4C. Also, it is germane to consider the potential effects of unisite catalysis, which is unaffected by azide as noted above. If one assumes a value of 1.0 for the equilibrium constant (Kaz) of the reaction F1-MgATP ↔ F1-MgADP-Pi in unisite catalysis (which is representative of the value measured in several laboratories), then catalytic site one (highest affinity site) would be expected to contain, on average, 0.5 MgADP per F1. This was clearly not seen (see “Results,” Fig. 4 and text). Under conditions where the three catalytic sites were filled (and in the presence of azide), the reaction equilibrium at site one was clearly shifted toward F1-MgATP.

As to the mechanism of inhibition by azide, it is clear that the enzyme species that accumulates in the presence of azide and MgATP (containing three bound MgATP plus zero bound MgADP) is not a normal catalytic intermediate state (see Fig. 6). The catalytic sites appear to be in free equilibrium with medium nucleotide, because the complement of three bound MgATP plus zero bound MgADP reflects the ambient concentrations of medium MgATP and MgADP, taking into account the respective Kd values for these nucleotides at the three catalytic sites. Our results strongly support the conclusion that azide blocks the conformational signal transmission between the catalytic sites that leads to normal positive catalytic cooperativity (and hydrolysis of bound MgATP in multisite conditions). DCCD acts upon the same step, but less effectively, as judged by the complement of bound catalytic site nucleotides. The higher inhibition of ATPase seen with DCCD as opposed to azide can be explained as due to the fact that DCCD is a covalent and azide a noncovalent inhibitor.

Acknowledgments—We thank Sean Hammond, Rachel Shaner, and Rachael Laundry for excellent technical work.

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doi: 10.1074/jbc.273.50.33210

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