# Specificity of the Proton Adenosinetriphosphatase of *Escherichia coli* for Adenine, Guanine, and Inosine Nucleotides in Catalysis and Binding†

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ABSTRACT: Specificity of the *Escherichia coli* proton ATPase for adenine, guanine, and inosine nucleotides in catalysis and binding was studied. MgADP, CaADP, MgGDP, and MgIDP were each good substrates for oxidative phosphorylation. The corresponding triphosphates were each substrates for hydrolysis and proton pumping. At 1 mM concentration, MgATP, MgGTP, and MgITP drove proton pumping with equal efficiency. At 0.1 mM concentration, MgATP was 4-fold more efficient than MgITP or MgGTP. Nucleotide-depleted soluble $F_1$ could rebind to $F_1$-depleted membranes and block proton conductivity through $F_0$; rebound nucleotide-depleted $F_1$ catalyzed pH gradient formation with MgATP, MgGTP, or MgITP. This showed that the nonexchangeable nucleotide sites on $F_1$ need not be occupied by adenine nucleotide for proton pumping to occur. It was further shown that no nucleotide was tightly bound in the nonexchangeable sites of $F_1$ during proton pumping driven by MgGTP in these reconstituted membranes, whereas adenine nucleotide was tightly bound when MgATP was the substrate. Nucleotide-depleted soluble $F_1$ bound maximally 5.9 ATP, 3.2 GTP, and 3.6 ITP of which half the ATP and almost all of the GTP and ITP exchanged over a period of 30–240 min with medium ADP or ATP. Also, half of the bound ATP exchanged with medium GTP or ITP. These data showed that inosine and guanine nucleotides do not bind to soluble $F_1$ in nonexchangeable fashion, in contrast to adenine nucleotides. Purified $\alpha$-subunit from $F_1$ bound ATP at a single site but showed no binding of GTP nor ITP, supporting previous suggestions that the nonexchangeable sites in intact $F_1$ are on $\alpha$-subunits.

The proton ATPase of *Escherichia coli* is a multisubunit enzyme that serves an essential role in oxidative phosphorylation and the ATP-driven formation of transmembrane proton electrochemical gradients. It consists of eight different subunits which form two structurally distinct sectors. The $F_1$ sector contains the catalytic site for ATP synthesis and hydrolysis, and the membrane sector ($F_0$) binds the $F_1$ sector to the membrane and forms a transmembrane pathway for proton conduction [reviewed in Senior & Wise (1983)].

Recently, much attention has been focused on the molecular mechanism of ATP synthesis and ATP hydrolysis in $F_1$-ATPases from various sources. A significant portion of this work has dealt with the binding of adenine nucleotides to soluble $F_1$-ATPases. There appear to be six total adenine nucleotide binding sites per mole of $F_1$-ATPase in both mitochondrial and *E. coli* enzymes (Cross & Nalin, 1982; Wise et al., 1983; this work). Of these, three sites correspond to catalytic or "exchangeable" sites (Cross & Nalin, 1982) and exchange with $t_{1/2}$ values on the order of minutes, whereas three sites exchange much more slowly (Cross & Nalin, 1982; Wise et al., 1983; this work), with apparent $t_{1/2}$ values of several hours, and appear to correspond to the "tightly bound" nucleotides previously described by Harris et al. (1973) in mitochondrial $F_1$-ATPase, by Abrams et al. (1973) in *Streptococcus faecalis* $F_1$-ATPase, and by Maeda et al. (1976) and Bragg & Hou (1977) in *E. coli* $F_1$-ATPase. The tightly bound nucleotides are also referred to as "endogenous", "nonexchangeable", or "noncatalytic" in the literature. Their role in mitochondrial or bacterial proton ATPase is not yet known.

It has been reported that the catalytic sites of mitochondrial $F_1$ exhibit a relatively broad nucleotide specificity, in terms of both binding and catalysis, whereas the tight-binding or nonexchangeable sites are relatively specific for adenine nucleotides (Rosing et al., 1975; Harris, 1978; Harris et al., 1978). Further, it was reported that some nucleoside triphosphates which did not appear to bind to nonexchangeable sites, e.g., GTP and ITP, did not participate in energy-linked reactions even though they were hydrolyzed by the proton ATPase (Harris et al., 1978), implying that hydrolysis of nucleoside triphosphate was not necessarily coupled to proton transport. However, in that work, proton transport was not assayed directly, only high (≥1.2 mM) concentrations of GTP or ITP were used to drive energy-linked reactions, and, at least in the case of GTP, some binding to nonexchangeable sites on membrane-bound $F_1$ in submitochondrial particles did occur.

In this report, we describe the specificity of the proton ATPase of *E. coli* for guanine, adenine, and inosine nucleotides in terms of binding to catalytic and nonexchangeable sites and as substrates for oxidative phosphorylation and formation of proton electrochemical gradients. We show directly that GTP and ITP can drive proton pumping, and we describe the GTP and ITP concentration dependence of proton pumping. We also show by direct binding measurements that GTP and ITP do not bind in a nonexchangeable fashion to soluble $F_1$, or to purified $\alpha$-subunit from $F_1$, and that membrane-bound $F_1$ catalyzes proton pumping under conditions where the nonexchangeable sites are not occupied by tightly bound nucleotide.

## Materials and Methods

*E. coli Strains.* The *E. coli* strains used were AN862 (*unc*$^+$) (Cox et al., 1978) (for preparation of nucleotide-depleted $F_1$) or AN1460 (*unc*$^+$) (Downie et al., 1980) (for all other preparations).

*Growth of E. coli Cells, Preparation of Membranes, and Purification of $F_1$.* Cells were grown to mid-log phase in 14-L batches as described by Cox et al. (1970). Cells were harvested, ruptured in a French pressure cell, and fractionated to give everted membrane preparations containing bound $F_1$ as described (Senior et al., 1979a,b). $F_1$-depleted membranes were prepared as described by Perlin et al. (1983) except that prior to the final resuspension, the membranes were suspended and centrifuged in 20 mM tris(hydroxymethyl)aminomethane

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hydrochloride (Tris-HCl),[1] pH 8.0, 1 mM EDTA, and 150 mM KSCN to remove residual $F_1$. $F_1$ was purified as described (Senior et al., 1979a,b; Wise et al., 1981).

*Purification of α-Subunit from $F_1$.* The procedure was as described by Dunn & Futai (1980).

*Preparation of Nucleotide-Depleted $F_1$.* The procedure was as described by Wise et al. (1983) and followed essentially that originally described by Garrett & Penefsky (1975). The preparations had $OD_{280}/OD_{260}$ ratios of 1.84–1.87.

*Binding of Nucleotides to Nucleotide-Depleted Soluble $F_1$ and to Purified α-Subunit.* The binding of radioactive nucleotides was measured as described by Wise et al. (1983). Essentially the method involved incubation of $F_1$ or α-subunit with nucleotide, followed by separation of bound and free nucleotides by the centrifuge column procedure described by Penefsky (1977). Additionally, for binding of nucleotide to α-subunit, equilibrium dialysis was also used as described by Dunn & Futai (1980).

*Analysis of Tightly Bound Nucleotide in Membrane-Bound $F_1$.* Nucleotide-depleted soluble $F_1$ (50 μg) was incubated with 2 mg of $F_1$-depleted everted membranes in 0.95 mL of medium containing 20 mM Tris-HCl, pH 7.5, 200 mM KCl, and 5 mM $MgSO_4$ at 30 °C for 10 min. Control samples contained no nucleotide-depleted $F_1$ in the incubation. Then 50 μL of 20 mM radioactive ATP or GTP (20 mM) was added, and after a 2.5-min incubation at room temperature, the membranes were centrifuged at top speed for 1 min in a Beckman microfuge. (Parallel experiments using the acridine orange assay showed that the membranes reconstituted with nucleotide-depleted $F_1$ generated a large transmembrane pH gradient during the 2.5-min incubation after addition of ATP or GTP.) The pellets were then washed 5 times by resuspension in 1 mL of 20 mM Tris-HCl, pH 8.0, 200 mM KCl, and 5 mM $MgSO_4$, and centrifugation in the microfuge. The final pellets were resuspended in the same buffer for analysis of bound nucleotide.

*Nucleoside Diphosphate Induced Enhancement of Bound Aurovertin Fluorescence.* The procedure was as described by Wise et al. (1981).

*Assay of Oxidative Phosphorylation.* NADH-driven ATP synthesis was measured following the procedure of Cox et al. (1973). The reaction medium (2 mL final volume) contained 25 mM TES–KOH, pH 7.5, 200 mM KCl, 5 mM $MgSO_4$ or 5 mM $CaCl_2$, 1 mM ADP, IDP, or GDP, 3 mM glucose, 50 units of hexokinase, 1 mM potassium [$^{32}$P]phosphate, and 1.5 mg of $F_1$-saturated AN1460 membranes. NADH (2 mM) was added to start the reaction, which was terminated by addition of 2 mL of 0.67 N HCl, 3% ammonium molybdate, 1% triethylamine, and 1% bromine water (Sugino & Miyoshi, 1964; Grubmeyer & Penefsky, 1981). After brief centrifugation, glucose 6-[$^{32}$P]phosphate was estimated in the supernatant by radioactivity counting.

*Measurement of pH Gradients.* The formation of pH gradients was monitored as described (Perlin et al., 1983) using acridine orange. Where CaATP was substrate, in order to ensure that $Ca^{2+}$ did not liberate bound $Mg^{2+}$ from membranes during the assay, the membrane vesicles were first washed 2 times by resuspension and centrifugation in 2.0 mM EDTA, 5 mM *p*-aminobenzamidine, 100 mM KCl, and 10 mM Tris-HCl, pH 7.5, and then washed in the same way with 5 mM $CaCl_2$, 100 mM KCl, and 10 mM Tris-HCl, pH 7.5. Further, a specific interaction of free ATP with bound $Mg^{2+}$ was circumvented by using free $Ca^{2+}$ to initiate pH gradient formation.

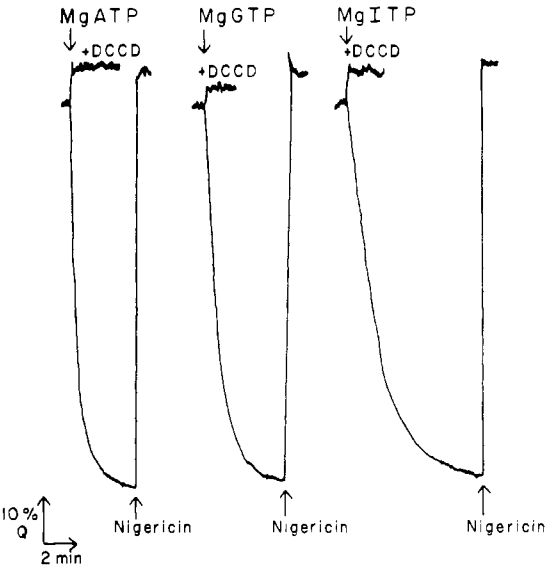


FIGURE 1: ATP-, GTP-, and ITP-driven pH gradient formation. The quenching ($Q$) of acridine orange fluorescence (see Materials and Methods) was used to monitor interior acid pH gradient formation in everted membranes. Fluorescence quenching was initiated by the addition of magnesium nucleotide (1.5 mM) to both control membranes and membranes pretreated with 100 μM DCCD (Perlin et al., 1983). As indicated, the DCCD-treated membranes exhibited a slight nonspecific fluorescence increase upon substrate addition. Nigericin (1 μg) and/or FCCP (2 μg) were added to collapse pH gradients.

*Analysis of Nucleotide Purity.* The procedure followed that of Bergkvist & Deutsch (1954, 1955) using Eastman Kodak cellulose plates with fluorescent indicator and developing for 4 h in saturated $(NH_4)_2SO_4$ solution. Spots were visualized under UV light. Typical $R_f$ values, for ATP, GTP and ITP were 0.25, 0.44 and 0.54. Typical $R_f$ values for ADP, GDP, and IDP were 0.25, 0.43, and 0.54, respectively. Contamination of the GTP, ITP, GDP, or IDP samples by adenine nucleotide was not detected. Contamination levels of 0.4% could be readily detected in "spiked" samples.

*Routine Procedures.* Protein was assayed by the method of Miller (1959) using sodium dodecyl sulfate [3% (w/v) final concentration] to solubilize membrane protein. ATPase assays were performed as described by Senior (1979) with incubation periods modified appropriately.

*Chemicals.* α-$^{32}$P-Labeled ATP and [8-$^{3}$H]GTP were obtained from Amersham; [8-$^{14}$C]ITP was from Schwarz/Mann; 5′-[8-$^{3}$H]guanylyl imidodiphosphate (GMPPNP) was from ICN. Nonradioactive ATP and GTP were from Sigma; nonradioactive GMPPNP and ITP were from P-L Biochemicals.

## Results

*Proton Transport Driven by GTP, ITP, and ATP.* Figure 1 illustrates directly that the hydrolysis of MgGTP and MgITP by everted $F_1$-containing membrane vesicles was accompanied by pH gradient formation. No pH gradient formation was seen if the membranes were pretreated with 100 μM DCCD, and in each case, the pH gradients formed were dissipated on addition of nigericin + $K^+$ or FCCP + valinomycin. Nucleoside triphosphate induced formation of pH gradients was seen to vary as a function of nucleotide concentration. A

[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; GMPPNP, 5′-guanylyl imidodiphosphate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DCCD, dicyclohexylcarbodiimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid.

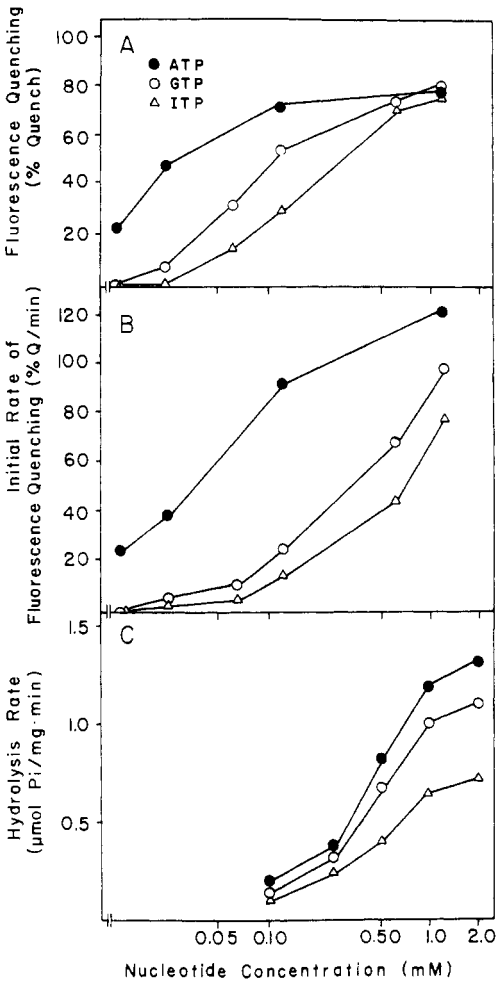


FIGURE 2: Effect of nucleoside triphosphate concentration on pH gradient formation and nucleoside triphosphate hydrolysis. Nucleoside triphosphates at the indicated concentrations were added rapidly to everted membranes (1.5 mg of protein) suspended in pH gradient assay medium (see Materials and Methods). The formation of pH gradients was evaluated in terms of final steady-state acridine orange fluorescence quenching values (A) and as initial rates of fluorescence quenching (B). Nucleoside triphosphate hydrolysis (C) was measured under the same conditions described above (A and B), except that a 1-mL volume was used and only 150 μg of protein was present. Values presented in panel C were calculated from linear initial rates. Maximal standard errors calculated for each of the data points were ±10% of the values reported (three to six experiments for each point).

concentration of 19 μM ATP (in the presence of equimolar Mg) was required for half-maximal saturation of steady-state pH gradient formation (Figure 2A). The corresponding concentrations for GTP and ITP were 84 and 180 μM, respectively. Initial rate measurements of pH gradient formation at varying nucleotide concentrations are shown in Figure 2B. Figure 2C shows the initial rates of hydrolysis of ATP, ITP, and GTP under the same conditions. Hydrolysis of ATP, GTP, and ITP by soluble "native" $F_1$ was also studied (data not shown), and the same order of preference (ATP > GTP > ITP) and concentration dependence was seen as in Figure 2C.

Dividing the initial rates of pH gradient formation (Figure 2B) by the initial rates of $P_i$ release (Figure 2C) gives a measure of the "efficiency" of proton pumping. At 0.1 mM concentration of nucleotide, proton pumping appeared about 4 times as efficient with ATP as with GTP or ITP. However at 1 mM nucleotide and above, the three nucleotides appeared equally efficient.

It was of interest to test whether $F_1$-depleted membranes which had been reconstituted with nucleotide-depleted soluble

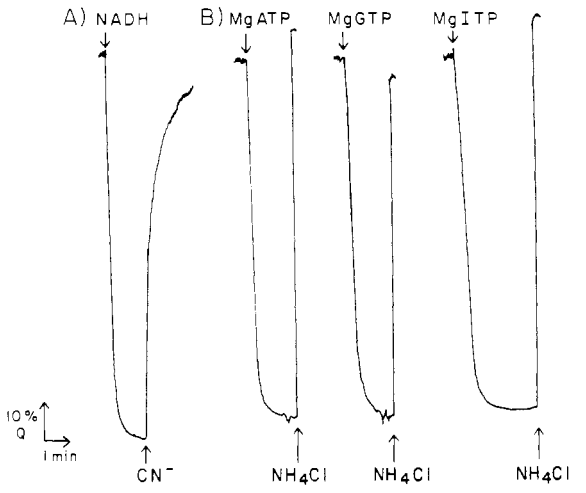


FIGURE 3: Reconstitution of $F_1$-depleted membranes with nucleotide-depleted $F_1$. (A) Nucleotide-depleted $F_1$ (25 μg) was incubated with $F_1$-depleted everted membranes (1 mg) for 10 min at 30 °C in the pH gradient assay medium. Acridine orange fluorescence quenchging ($Q$) was initiated by the addition of 2 mM NADH. (B) Magnesium nucleoside triphosphate (2.5 mM) was added to initiate acridine orange fluorescence quenching in $F_1$-depleted membranes reconstituted with nucleotide-depleted $F_1$.

$F_1$ could sustain NADH-driven or nucleoside triphosphate driven pH gradient formation. Figure 3 shows that nucleotide-depleted $F_1$ did rebind to $F_1$-depleted membranes and did block the proton conduction through $F_0$, since significant NADH-driven pH gradient formation was seen. (Under the same conditions, $F_1$-depleted membranes alone gave no NADH-driven pH gradient formation). Further, addition of 2.5 mM MgATP, MgGTP, or MgITP to the reconstituted membranes induced pH gradient formation (Figure 3) which was indistinguishable in rate and magnitude from that seen in parallel experiments (not shown) where native $F_1$ was used to reconstitute the $F_1$-depleted membranes.

*Oxidative Phosphorylation of ADP, GDP, and IDP.* Membranes containing bound $F_1$ synthesized ATP from ADP and $[^{32}P]P_i$ when supplied with NADH to cause proton electrochemical gradient formation. ATP synthesis was prevented by FCCP and saturated at an ADP concentration of around 1 mM. GDP and IDP were also phosphorylated in an FCCP-sensitive manner. At 1 mM concentration of nucleotide, the rates of phosphorylation measured were 5.4 (ADP), 3.8 (IDP), and 2.9 (GDP) nmol $mg^{-1}$ $min^{-1}$. The comparatively lower rate of synthesis of GTP relative to ITP and ATP appeared to be a real property of the proton ATPase. The possibility that this could be an artifact due to the lower affinity of hexokinase for GTP was ruled out by the demonstration that the same rate of GTP synthesis occurred when a 10-fold higher hexokinase concentration was used.

*CaATP and CaADP as Substrates for Proton Transport and Oxidative Phosphorylation.* We found that in the concentration range of 0.5–2.5 mM, CaATP drives pH gradient formation with nearly the same effectiveness as MgATP. CaADP (1 mM) was effectve as a substrate in oxidative phosphorylation, giving rates of 80% of the corresponding rate with MgADP.

*Binding of Inosine and Guanine Nucleotides to Nonexchangeable Nucleotide Sites on $F_1$.* The data reported above (Figure 3), in which it was shown that MgGTP and MgITP drive proton transport in membranes which had been reconstituted with nucleotide-depleted $F_1$, established for the first time that the nonexchangeable nucleotides sites on $F_1$ need not be occupied by *adenine* nucleotide for proton pumping to

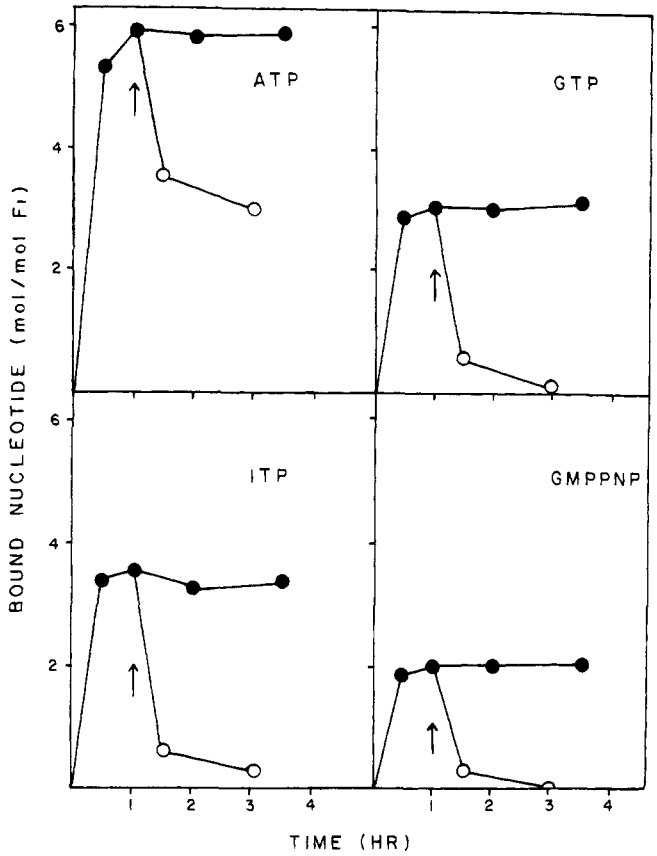


FIGURE 4: Binding of nucleotides to nucleotide-depleted soluble *E. coli* $F_1$ and exchange of bound nucleotide on addition of ATP or ADP. (●) Nucleotide-depleted purified $F_1$ was incubated with 1–2 mM radioactive nucleotide in buffer containing 50 mM Tris–sulfate, 0.5 mM EDTA, and 2.5 mM $MgSO_4$, pH 8.0 at room temperature. At the times indicated, free and bound nucleotides were separated by the centrifuge column technique, and $F_1$-bound nucleotide was estimated. (○) Samples of $F_1$ which were maximally reloaded with radioactive nucleotide were freed of nonbound ligand by the centrifuge column procedure, then nonradioactive ATP (1 mM) or ADP (10 mM) was added (arrows), and incubation was continued for 30 min or 2 h. The buffer for the second incubation was the same as in the first incubation described above. Remaining bound radioactive nucleotide was estimated as above at 30 min or 2 h. Under exactly the same conditions, Wise et al. (1983) noted that when $F_1$ was maximally reloaded with AMPPNP (6 mol/mol), addition of ATP (1 mM) or ADP (10 mM) induced exchange of 2.1, 2.9, and 3.0 mol of AMPPNP per mol of $F_1$ at 30 min, 2 h, and 4 h, respectively. In the experiments shown here, as in the previous studies, ATP (1 mM) or ADP (10 mM) had similar effects on the amount of nucleotide exchanged. All data shown here are means of at least quadruplicate experiments.

occur. It was clearly of interest to find out whether inosine or guanine nucleotide could bind to these sites. Our first experiments utilized soluble $F_1$.

When nucleotide-depleted $F_1$ was incubated with radioactive nucleotides for up to 3.5 h at 1–2 mM concentration (Figure 4, closed circles), it was found that ATP bound maximally to 5.9 sites, GTP bound maximally to 3.2 sites, ITP bound maximally to 3.6 sites, and GMPPNP bound maximally to 2.1 sites. Increasing the GTP (or ITP) concentration in the incubation to 5 mM led to only a slight increase in the amount of nucleotide bound. It was confirmed that nucleotide-depleted $F_1$ retained full ATPase activity after incubation with ATP, GTP, or ITP for 3.5 h under the conditions of Figure 4. Experiments in which nonradioactive ATP or ADP was added to the nucleotide-saturated enzyme to cause exchange of the bound radioactive nucleotide (Figure 4, open circles) showed that whereas only 54% (3.1 mol/mol of $F_1$) of the total bound adenine nucleotide exchanged in 2 h, almost all of the bound guanine and inosine nucleotides exchanged. We have previously shown that AMPPNP binds to six total sites on *E. coli* $F_1$, of which three correspond to exchangeable catalytic sites and three correspond to nonexchangeable sites (Wise et al., 1983). The results for ATP binding and exchange shown in Figure 4 are in complete agreement with those previous findings.

Additional experiments were also carried out as follows. Nucleotide-depleted soluble $F_1$ was reloaded with radioactive ATP as in Figure 4 to a level of 5.8 mol of ATP bound per mol of $F_1$. Then 1 mM nonradioactive GTP, ITP, or GMPPNP was added to promote exchange of bound ATP following the procedure described in the Figure 4 legend (open circles). The results were as follows. In the ITP experiment, the $F_1$ retained 2.83 (after 30 min) or 2.74 (2 h) mol of ATP/mol of $F_1$. With GTP, the ATP retained was 3.15 (30 min) or 2.7 (2 h) mol of ATP/mol of $F_1$; with GMPPNP, the ATP retained was 2.82 (30 min) or 2.7 (2 h) mol of ATP/mol of $F_1$. All experiments were done in duplicate.

The binding experiments suggest strongly that GTP, ITP, and GMPPNP bind to the catalytic, exchangeable sites and can displace ATP from these sites, whereas they do not bind to the nonexchangeable sites and cannot displace ATP from these sites. The fact that GTP and ITP are hydrolyzed by the *E. coli* proton ATPase, as has been previously shown by others and is also confirmed here (see, e.g., Figure 2), of course also establishes that binding at catalytic sites occurs. Some caution must be exercised, however, in considering whether the guanine and inosine nucleotides are unable to bind to the nonexchangeable sites. In the centrifuge column procedure of Penefsky (1977) used here, nucleotide binding sites with dissociation rate constants ($k_{off}$) greater than approximately 0.1 $s^{-1}$ would not be detected. Thus, it cannot be stated with absolute certainty from our data that guanine and inosine nucleotides do not bind to the three nonexchangeable sites on soluble $F_1$. However, it can be stated with confidence that if they do so, then they exchange out of the sites with dissociation rates considerably higher ($\geq 10^4$-fold) than those for adenine nucleotide.

We then tested whether membrane-bound $F_1$ would bind ATP or GTP at nonexchangeable sites. To do this, we first reconstituted nucleotide-depleted soluble $F_1$ with $F_1$-depleted membranes, and then added radioactive ATP or GTP under conditions which gave proton gradient generation, measuring the amount of tightly bound ATP or GTP as that which remained bound to the membranes after five washes by centrifugation and resuspension in buffer. Control experiments were run in parallel using $F_1$-depleted membranes only (not reconstitued with $F_1$), and the nucleotide remaining tightly bound to $F_1$ was calculated as the difference between the "plus $F_1$" and "no $F_1$" membranes. The results were as follows. There was no significant tight binding of GTP to $F_1$ (quintuplicate experiments). With ATP, the binding to the plus $F_1$ membranes was 0.71 nmol/mg of protein (±0.06 SD, $n$ = 6); binding to the no $F_1$ membranes was 0.25 nmol/mg of protein (±0.02 SD, $n$ = 4). The difference, 0.46 nmol/mg of protein, represents ATP tightly bound to $F_1$ and corresponds to 1.4 mol/mol of $F_1$ using a ratio of 1/7 for the ratio of $F_1$ to membrane protein (w/w) calculated previously by Perlin et al. (1983). Our conclusion is that proton pumping, driven by GTP, may occur in the absence of any tightly bound nucleotide in the nonexchangeable sites.

*Binding of GTP and ITP to Purified α-Subunit.* Using the centrifuge column binding assay, it was found that at 10 μM concentration of nucleotide, in 50 mM Tris–sulfate, pH 8.0, 0.5 mM EDTA, and 2.5 mM $MgSO_4$, purified α-subunit

(64–87 μg/mL) bound 0.87 mol of ATP/mol of α-subunit and negligible amounts of radioactive GTP or ITP after a 2-h incubation at 20 °C. Similar results were obtained at 250 μM nucleotide concentration. If $MgSO_4$ was omitted from the buffer, 0.64 mol of ATP/mol of α-subunit was bound, and no GTP or ITP was bound. Equilibrium dialysis experiments were performed also and showed that no binding of GTP or ITP to purified α-subunit occurred at concentrations of nucleotide up to 16 μM. Previously Dunn & Futai (1980) showed purified α-subunit binds ATP (1 mol/mol) with a high affinity (calculated $K_d$ was 0.1 μM). Our results show this binding site is specific for adenine nucleotide. The work of Senda et al. (1983), in which it was shown that ATP, in contrast to GTP and ITP, was able to protect α-subunit from trypsin proteolysis, had suggested that this was the case.

*Nucleoside Diphosphate Induced Enhancement of Bound Aurovertin Fluorescence.* Aurovertin binds to the β-subunit of *E. coli* $F_1$ and fluoresces upon binding [reviewed in Wise et al. (1981)]. We have previously shown that ADP enhances the fluorescence of aurovertin bound to normal soluble $F_1$. The $K_m$(ADP) for this effect was around 1 μM, and the fluorescence response was shown to be due to an $\alpha \leftrightarrow \beta$ intersubunit conformational interaction. We suggested this conformational interaction is required for normal catalysis (Wise et al., 1981), and subsequent experiments (Wise et al., 1984) have strengthened this proposal. Here we repeated the earlier experiments, substituting IDP and GDP for ADP. Both IDP and GDP gave the same amount of enhancement of bound aurovertin fluorescence as did ADP. The $K_m$(IDP) was 25.5 μM; the $K_m$(GDP) was 22.4 μM. Therefore, both IDP and GDP can induce intersubunit conformational interaction in $F_1$, and from data presented in Figure 4 and the text above, it appears that they do so by binding at catalytic sites.

## Discussion

The data presented here are relevant in several ways to the understanding of catalysis in the *E. coli* proton ATPase. We have described properties of the enzyme concerning catalytic specificity for nucleotides. We show here for the first time that the enzyme catalyzes oxidative phosphorylation of CaADP, MgIDP, and MgGDP and it catalyzes proton pumping coupled to hydrolysis of MgITP, MgGTP, and CaATP. We have also described properties of the enzyme concerning binding specificity for nucleotides. It has previously been shown by several workers that guanine and inosine nucleotides bind at the catalytic sites of $F_1$, and we confirm those findings. Additionally, we show by using a direct binding assay that guanine and inosine nucleotides do not bind in a nonexchangeable fashion to noncatalytic sites on soluble $F_1$ and do not displace bound ATP from the nonexchangeable sites of soluble $F_1$. In order to study binding of nucleotides to nonexchangeable sites of membrane-bound $F_1$, we used the procedure of reconstituting nucleotide-depleted solbule $F_1$ to $F_1$-depleted membranes. The fact that such reconstituted membranes catalyze proton pumping driven by ITP or GTP shows that proton pumping can occur in the absence of any bound *adenine* nucleotide at the nonexchangeable sites. We then measured the tight binding of GTP and ATP to the membrane-bound $F_1$ after a burst of proton pumping and found that ATP became tightly bound to $F_1$ but GTP did not. We conclude, therefore, that proton pumping driven by GTP may occur in the absence of *any* nucleotide tightly bound at the nonexchangeable sites.

Harris et al. (1978) previously suggested that inosine and guanine nucleotides did not bind to nonexchangeable sites on mitochondrial $F_1$, and our results concur with theirs. However, we believe our evidence is stronger because we have measured nucleotide binding by direct assays, and we show no tight binding of GTP to membrane-bound $F_1$, whereas Harris et al. (1978) actually reported a low, but significant, binding of GTP to submitochondrial particle membranes. Previously (Leimgruber & Senior, 1976a,b, 1978) it was suggested that occupancy of nonexchangeable nucleotide sites on $F_1$ by adenine nucleotide was essential for adenine nucleotide linked energized processes catalyzed by the mitochondrial proton ATPase. While, strictly speaking, the results presented here are not directly in disagreement with that conclusion (since we have not yet found conditions where adenine nucleotide linked catalysis can be measured without the nonexchangeable sites being occupied by adenine nucleotide), the fact that, as shown here, GTP-driven proton pumping occurs in the absence of any tightly bound nucleotide at the nonexchangeable sites certainly suggests occupancy of the nonexchangeable sites is not essential for proton pumping.

In relation to the location of nonexchangeable nucleotide sites on intact $F_1$, we found that purified α-subunit of *E. coli* $F_1$ bound ATP (in confirmation of previous work; Dunn & Futai, 1980; Dunn, 1980) but that it did not bind GTP or ITP. These data support previous suggestions of Dunn & Heppel (1981) that the nonexchangeable nucleotide sites of intact $F_1$ are located on α-subunits.

Harris et al. (1978) also suggested that the catalysis of energy-coupled reactions was less efficient with GTP and ITP as substrates than with ATP. In fact, they concluded that GTP and ITP did not support coupled reactions, except for transhydrogenase which was weakly supported by both nucleotides. This conclusion seems to have gained acceptance [e.g., see Cross (1981)]. We show here that if proton pumping is assayed directly, at the concentrations of nucleotide used by Harris et al. (1978) (>1 mM), no difference in "efficiency" between GTP, ITP, and ATP is seen. It should be borne in mind that Harris et al. studied mitochondrial proton ATPase, whereas we have studied *E. coli* proton ATPase. Nevertheless, we feel a likely explanation of the results of Harris et al. is that the rates of reactions such as nucleoside triphosphate driven reduction of NAD by succinate, ATP–$P_i$ exchange, and nucleoside triphosphate driven transhydrogenase may respond to different "threshold" levels of the proton electrochemical gradient and may therefore show different dependence on the nucleoside triphosphates according to the rate of proton translocation supported. However, we did see a lowered efficiency of proton pumping at low concentrations of GTP and ITP. Such lowered efficiency has been discussed previously by Harris et al. (1978) and by Cross (1981) in terms of differences between the ability of ATP on the one hand and GTP and ITP on the other hand to bind tightly at the catalytic site and to elicit conformational changes involved in catalysis. Evidence reported here showed GDP and IDP, like ADP, were able to induce an intersubunit conformational interaction which is strongly implicated in catalysis (Wise et al., 1981, 1984). Therefore, it is our conclusion that the lowered efficiency seen is more likely related to catalytic site binding properties of GTP and ITP.

Finally, the data presented here are relevant to the model of $F_1$ catalysis presented recently by Senior & Wise (1983), in which it was suggested that the positive catalytic cooperativity between catalytic sites on separate β-subunits, which is essential for normal rates of ATP hydrolysis (Grubmeyer et al., 1982; Cross et al., 1982), is mediated by an $\alpha \leftrightarrow \beta$ intersubunit conformational interaction (Wise et al., 1981, 1984). In the model, it is proposed that the nonexchangeable nu-

cleotide sites are located on the $\alpha$-subunit and that ADP interacts at catalytic sites on the $\beta$-subunit to enhance bound aurovertin fluorescence. Data presented here further support both of these proposals, thereby giving additional support for the model. However, although the data presented here describe more clearly the characteristics of the nonexchangeable nucleotide sites on the $F_1$ sector of *E. coli* proton ATPase, their function remains unknown.

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**Registry No.** ATPase, 9000-83-3; MgADP, 7384-99-8; CaADP, 75679-20-8; MgGDP, 7277-99-8; MgIDP, 7219-40-1; MgATP, 1476-84-2; MgGTP, 22139-68-0; MgITP, 22139-67-9; ATP, 56-65-5; GTP, 86-01-1; ITP, 132-06-9; hydrogen ion, 12408-02-5.

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