Catalytic Properties of the *Escherichia coli* Proton Adenosinetriphosphatase: Evidence That Nucleotide Bound at Noncatalytic Sites Is Not Involved in Regulation of Oxidative Phosphorylation†

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ABSTRACT: Nucleotide-depleted F_1-ATPase from *Escherichia coli* was reconstituted with F_1-depleted membranes and shown to catalyze high rates of oxidative phosphorylation of ADP and GDP. Adenine nucleotide became bound to the nonexchangeable nucleotide sites on membrane-bound F_1 during ATP synthesis, but binding of guanine nucleotides to nonexchangeable sites during GTP synthesis was not detectable. It was possible to reload the nonexchangeable sites on nucleotide-depleted F_1 with radioactive adenine nucleotide prior to membrane reconstitution. The radioactive adenine nucleotide did not exchange significantly during oxidative phosphorylation of ADP or GDP. The amount of nonexchangeable adenine nucleotide found in membrane-bound F_1 was the same when the nonexchangeable sites were reloaded either prior to membrane reconstitution of the F_1 or after membrane reconstitution with nucleotide-free F_1 followed by a burst of oxidative phosphorylation of ADP. The results showed that occupation of the nonexchangeable sites on F_1 by tightly bound nucleotide is not required for oxidative phosphorylation of GDP (a physiological activity of F_1 in the bacterial cell). Also, the results confirm directly that the adenine-specific nonexchangeable sites on F_1 are noncatalytic sites. Using this experimental approach, it was possible to look for a regulatory effect of the nonexchangeable nucleotide on oxidative phosphorylation. Nucleotide-depleted F_1 was first reloaded with (i) ATP, (ii) ADP, (iii) 5′-adenylyl imidodiphosphate, or (iv) zero nucleotide, and was then reconstituted with F_1-depleted membranes. The reconstituted membranes were compared in respect to rates of oxidative phosphorylation of GDP and K_m values of GDP and P_i. No regulatory role for the nonexchangeable nucleotide was evident. In a further study, the rate of oxidative phosphorylation of GDP was varied down to low values by inhibiting oxidation with KCN. No differences in rate were noted when either (i) ATP-reloaded F_1 or (ii) nucleotide-free F_1 was used. The data strongly suggest that the nonexchangeable, adenine-specific nucleotide sites on *E. coli* F_1 play no regulatory role in oxidative phosphorylation.

The proton ATPase of *Escherichia coli* catalyzes ATP synthesis during oxidative phosphorylation and ATP-dependent formation of a transmembrane electrochemical proton gradient. It consists of two sectors, F_1 and F_0. F_1 is composed of five different protein subunits arranged in a complex structure ($\alpha_3\beta_3\gamma\delta\epsilon$) that carries the sites of ATP synthesis and hydrolysis. The F_0 sector is composed of three distinct subunits, it forms a pathway for conduction of protons through the membrane, and it binds F_1 to the membrane such that ATP hydrolysis and synthesis are coupled to proton translocation. For recent reviews on proton ATPase structure and function, see Senior & Wise (1983) and Walker et al. (1984).

ATP hydrolysis and synthesis occur at three catalytic sites on F_1 in both mitochondrial and *E. coli* enzymes (Cross & Nalin, 1982; Cross et al., 1982; Gresser et al., 1982; Wise et al., 1983, 1984). The catalytic sites exhibit relatively broad nucleotide specificity (Rosing et al., 1975; Harris, 1978; Harris et al., 1978; Sorgato et al., 1982; Perlin et al., 1984), and exchange of nucleotide bound at catalytic sites with nucleotide free in solution occurs relatively rapidly (Cross & Nalin, 1982; Wise et al., 1983; Perlin et al., 1984). The catalytic sites are probably located on F_1 β-subunits (Senior & Wise, 1983). Steady-state catalysis by F_1 involves very strong, positive catalytic cooperativity between catalytic sites (Grubmeyer et al., 1982; Cross et al., 1982) and requires $\alpha \leftrightarrow \beta$ intersubunit conformational interaction (Wise et al., 1981, 1984).

In addition to three catalytic sites, F_1 preparations from mitochondria and *E. coli* also contain three nucleotide binding sites that do not participate directly in catalysis, the so-called nonexchangeable or noncatalytic nucleotide binding sites (Cross & Nalin, 1982; Wise et al., 1983). In *E. coli* F_1, these sites are specific for adenine nucleotide (Perlin et al., 1984), and nucleotide bound at the nonexchangeable sites of soluble F_1 exchanges with nucleotide in solution extremely slowly. The nonexchangeable sites are probably located on F_1 α-subunits (Dunn & Heppel, 1981; Perlin et al., 1984). F_1 preparations from mitochondria and bacteria have been reported to contain "tightly bound" nucleotide (Abrams et al., 1973; Harris et al., 1973; Maeda et al., 1976; Bragg & Hou, 1977) which appears to correspond to nucleotide bound at nonexchangeable nucleotide binding sites. The role of the nonexchangeable nucleotide sites is unknown.

Perlin et al. (1984) demonstrated that membrane-bound *E. coli* F_1 catalyzes GTP-driven proton translocation under conditions where nonexchangeable nucleotide sites are not occupied by tightly bound nucleotide. Therefore, occupancy of the nonexchangeable sites seemed not to be obligatory for catalysis. Myers & Boyer (1983) used pyruvate kinase or hexokinase to manipulate medium ATP/ADP concentration ratios under conditions that promoted exchange of tightly bound nucleotide in submitochondrial particles and observed differential occupancy of sites for tightly bound ATP and ADP under the different incubation conditions. No effects on ATP hydrolysis activity or ^{18}O-exchange reactions were observed, however, suggesting that nucleotide bound at nonexchangeable

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sites was not regulating catalysis.

We conceived that by extending the use of techniques described previously by Perlin et al. (1984) in which nucleotide-depleted *E. coli* F_1 was shown to rebind to F_1-depleted membranes and to catalyze oxidative phosphorylation, it should be possible to clearly demonstrate a regulatory effect of nucleotide binding at nonexchangeable sites on oxidative phosphorylation, if such an effect actually existed. This study presents the results of experiments wherein kinetic features of oxidative phosphorylation were measured by using membrane-reconstituted F_1 in which the nonexchangeable, nucleotide binding sites were reloaded with ATP, AMPPNP, ADP, or zero nucleotide, looking for changes in activity which might reflect a regulatory effect of the bound nonexchangeable nucleotide.

Materials and Methods

Materials. [α-^{32}P]ATP, ^{32}P-labeled inorganic phosphate, [^{3}H]ADP, and [^{3}H]GDP were purchased from Amersham. Nonradioactive ADP, GDP, and ATP were from Sigma. Nonradioactive 5′-adenylyl imidodiphosphate (AMPPNP)[1] was from P-L Biochemicals. All other materials were purchased from commercial sources.

E. coli Strains. E. coli strain AN862 (*unc*$^+$) (Cox et al., 1978) was used to prepare native and nucleotide-depleted F_1. *E coli* strain AN1137 (*uncA453*) (Senior et al., 1979a) was used to prepare F_1-depleted membranes.

Preparation of Cell Membranes, Purification of F_1, and Preparation of Nucleotide-Depleted F_1. Everted cell membranes and purified F_1 were prepared as described previously (Senior et al., 1979a,b; Wise et al., 1981). F_1-depleted everted membranes were prepared as in Senior et al. (1979a,b) except that membranes were washed an additional time with 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, containing 5 mg/mL BSA (fatty acid free). F_1-depleted membranes were resuspended in 25 mM TES–KOH, pH 7.5, and stored at −70 °C in small aliquots. Nucleotide-depleted F_1 was prepared as described by Wise et al. (1983) as modified from Garrett & Penefsky (1975) and had OD_{280}/OD_{260} ratios of 1.90–1.95.

Reloading of Nucleotide-Depleted F_1 with Nucleotide. This was performed as described previously (Wise et al., 1983; Perlin et al., 1984). Essentially, radioactive nucleotides were incubated with nucleotide-depleted F_1, followed by centrifuge column separation of F_1-bound and free ligand (Penefsky, 1977). Exchange of bound radioactive nucleotide with unlabeled medium nucleotide was then performed by incubating F_1 samples in medium containing nonradioactive nucleotide for 2 h. Free nucleotide was then separated from F_1-bound nucleotide by passage through a second centrifuge column.

Reconstitution of F_1-Depleted Membranes with Nucleotide-Depleted F_1. The procedure followed that of Perlin et al. (1984) except that nucleotide-depleted F_1 was incubated with F_1-depleted membranes in the ratio of 0.25 mg of F_1/mg of membrane protein to saturate the F_1 binding sites in the AN1137 (*uncA453*) membranes.

Assay of Oxidative Phosphorylation. NADH-driven ATP or GTP synthesis was measured by using the procedure described by Cox et al. (1973). The reaction contained 25 mM TES–KOH, pH 7.5, 200 mM KCl, 5 mM $MgSO_4$, 3 mM glucose, 50 units of hexokinase, ADP or GDP and ^{32}P-labeled inorganic phosphate as indicated, and F_1-saturated AN1137 membranes (0.125–0.25 mg/mL) in magnetically stirred plastic tubes. Reaction was initiated by addition of NADH (2 mM) and terminated by addition of 1.5 N perchloric acid to 0.3 N final concentration. Glucose 6-[^{32}P]phosphate was determined by addition of 0.85 volume of 0.067 N HCl, 3% ammonium molybdate, 1% triethylamine, and 1% bromine water (Sugino & Miyoshi, 1964; Grubmeyer & Penefsky, 1981) to the terminated reaction. After brief centrifugation to remove ^{32}P-labeled inorganic phosphate, glucose 6-[^{32}P]-phosphate was estimated in the supernatant by scintillation counting. Steady-state ATP and GTP synthesis rates were determined by least-squares fitting of time course data obtained between 0 and 2 min of reaction.

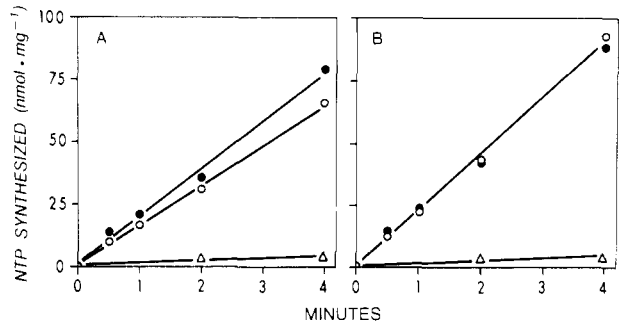

FIGURE 1: NADH-driven ATP and GTP synthesis by native F_1 and nucleotide-depleted F_1 reconstituted membranes. Assays were performed as described under Materials and Methods with ADP, GDP, and P_i concentrations equal to 1 mM and with 0.25 mg/mL reconstituted membranes. Where indicated, gramicidin was added prior to initiation of the reaction at 4 μg/mL. (A) Nucleotide-depleted F_1 reconstituted membranes. (B) Native F_1 reconstituted membranes. (●) ADP as substrate (ATP synthesis); (○) GDP as substrate (GTP synthesis); (△) plus gramicidin.

Estimation of Nonexchangeable Adenine or Guanine Nucleotide Which Became Bound to Membrane-Bound Nucleotide-Depleted F_1 during the Oxidative Phosphorylation Assay. Oxidative phosphorylation was performed as described above with the following modifications. [2,8-^{3}H]ADP or [2,8-^{3}H]GDP was substituted for nonradioactive ADP or GDP, and nonradioactive P_i was substituted for [^{32}P]P_i in the reaction medium. At the end of the incubation, the reaction medium was diluted 6-fold with ice-cold 20 mM Tris-HCl, pH 8.0, 200 mM KCl, and 5 mM $MgSO_4$ and immediately centrifuged (75000*g*, 10 min, 2 °C). Pelleted membranes (0.125–1.0 mg) were resuspended in cold 20 mM Tris-HCl, pH 8.0, 200 mM KCl, and 5 mM $MgSO_4$ (2 mL), and the wash procedure was repeated 5 times. Control experiments were run in parallel using F_1-depleted membranes which had not been reconstituted with F_1, and nucleotide bound to membrane-bound F_1 was calculated as the difference between "plus F_1" and "no F_1" membranes.

Routine Procedures. Protein was assayed by the method of Miller (1959) using sodium dodecyl sulfate [0.3% (w/v) final concentration] to solubilize membrane protein. Nucleotide purity was assessed as in Perlin et al. (1984) by the procedure of Bergkvist & Deutsch (1954, 1955). All other methods were as described in Wise et al. (1984).

Results

NADH-Driven ATP and GTP Synthesis by F_1-Reconstituted Membranes. Figure 1 demonstrates that high rates of both ATP and GTP synthesis were observed when nucleotide-depleted F_1 (panel A) or "native" F_1 (panel B) was reconstituted with F_1-depleted AN1137 (*uncA453*) membranes. In each case, observed phosphorylation of GDP and ADP was

[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; BSA, bovine serum albumen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; GMPPNP, 5′-guanylyl imidodiphosphate.

Table I: Kinetic Constants for GTP and ATP Synthesis[a]

F_1	K_m(GDP)[b] (mM)	V_{max}(GDP)[b] (nmol·min^{-1}·mg^{-1})	K_m(P_i)[c] (mM)	V_{max}(P_i)[c] (nmol·min^{-1}·mg^{-1})
ATP reloaded	0.42	60.9	1.7	46.8
AMPPNP reloaded	0.45	58.9	1.5	45.4
ADP reloaded	0.45	59.2	2.7	49.0
nucleotide depleted[d]	0.49	48.0	1.8	40.8
native[e]	0.09	69.8	2.5	61.3
native	0.02[f]	75.1[f]	ND[g]	ND[g]

[a] Nonlinear regression analysis (Cleland, 1967) of the data of Figure 2 was performed for the two equations $v = V_mS/(K_m + S)$ and $v = V_{m_1}S/(K_{m_1} + S) + V_{m_2}S/(K_{m_2} + S)$ where v = initial velocity and S = substrate concentration. In each case, the single-component curve provided the best fit. [b] Assayed in Figure 2A-D (GTP synthesis). [c] Assayed in Figure 2E (GTP synthesis). [d] Nucleotide-depleted F_1 was rebound directly to membranes without prior incubation with nucleotide. [e] Data not shown in Figure 2 (GTP synthesis). GDP concentration ranged from 0.01 to 2 mM at 8.8 mM P_i [K_m(GDP) and V_{max}(GDP) assays]. P_i concentrations ranged from 0.125 to 10 mM at 1 mM GDP [K_m(P_i) and V_{max}(P_i) assays]. [f] Please note these are values of K_m(ADP) and V_m(ADP) from Figure 2F where ATP synthesis was assayed. [g] ND = not determined.

totally dependent on added F_1 and was abolished by the addition of uncoupler.

Binding of Guanine and Adenine Nucleotides to Nonexchangeable Sites on Membrane-Bound Nucleotide-Depleted F_1 during NADH-Driven GTP and ATP Synthesis Assays. Nucleotide-depleted F_1 was reconstituted directly with F_1-depleted membranes, and oxidative phosphorylation was assayed by using 1 mM [^{3}H]ADP or [^{3}H]GDP as described under Materials and Methods. The membranes were then washed 5 times by repeated centrifugation and resuspension in buffer (also described under Materials and Methods) before determination of bound nucleotide. The difference between the plus F_1 and no F_1 washed membranes was taken as the amount of nucleotide bound at nonexchangeable sites on F_1. When [^{3}H]ADP was used in the oxidative phosphorylation assay, the amount of adenine nucleotide bound was 0.30 nmol/mg of membrane protein (±0.13 SD, 21 experiments). When [^{3}H]GDP was used, the amount of guanine nucleotide bound was zero (eight experiments).

Additional experiments were performed to test whether the bound radioactive adenine nucleotide retained by the washed membranes was truly nonexchangeable. First, one batch of washed membranes containing radioactive adenine nucleotide was incubated with 1 mM nonradioactive ATP for 2 min at 37 °C; another batch was incubated under similar conditions but without ATP. Both sets of membranes were then centrifuged 1 additional time and resuspended in buffer as described under Materials and Methods. The no ATP membranes retained 0.32 nmol of radioactive adenine nucleotide per milligram of membrane protein (±0.05 SD, five experiments). The plus ATP membranes retained 0.27 nmol/mg (±0.15 SD, five experiments). Therefore, under these ATP hydrolysis–proton pumping conditions (1 mM ATP and 5 mM $MgSO_4$, 2 min, 30 °C) where an estimated 3000 turnovers per F_1 of nonradioactive ATP hydrolysis would have occurred, 84% of the bound radioactive nucleotide did not exchange with medium (substrate) nucleotide.

Second, soluble nucleotide-depleted F_1 was reloaded with [α-^{32}P]ATP before being rebound to the F_1-depleted membranes. Initially, the F_1 reloaded 5.8 mol of [^{32}P]ATP/mol of F_1. After incubation for 2 h with nonradioactive ATP to allow exchange of the [^{32}P]ATP bound at catalytic sites, the F_1 contained 3 mol of [^{32}P]ATP/mol of F_1, which was therefore bound at nonexchangeable sites. This enzyme was then bound to F_1-depleted membranes, the reconstituted membranes were washed once by centrifugation and resuspension in 20 mM Tris-HCl, pH 8.0, 200 mM KCl, and 5 mM $MgSO_4$, and the amount of bound radioactive adenine nucleotide was determined to be 0.30 nmol/mg of membrane protein (±0.06 SD; five experiments). The fact that this value was the same as that determined in experiments in which binding of adenine nucleotide occurred during the oxidative phosphorylation assay, after binding of nucleotide-depleted F_1 to membranes, further substantiated that the centrifuge wash procedure properly measured nonexchangeable nucleotide.

Third, nonexchangeable sites on nucleotide-depleted F_1 were reloaded by incubation with [^{32}P]ATP prior to membrane binding as described above. The F_1 was then rebound to membranes, and nonradioactive ATP and GTP synthesis assays were initiated and allowed to continue for 2 min such that at least 200 catalytic turnovers per F_1 occurred. The membranes were then washed (once) by centrifugation and resuspension. It was found that 96% (GTP synthesis assay) and 97% (ATP synthesis assay) of the bound ^{32}P-labeled nucleotide were retained on the membranes at the end of the experiment. This experiment demonstrated directly that adenine nucleotide bound at nonexchangeable sites on membrane-bound F_1 does not exchange with medium (substrate) nucleotide during oxidative phosphorylation. It should be noted that the total concentration of GDP plus GTP present during the GTP synthesis assay was approximately 10^4-fold that of the F_1-bound ^{32}P-labeled adenine nucleotide.

ADP Binding to Nucleotide-Depleted Soluble F_1. We have previously shown that nucleotide-depleted F_1 may be reloaded with either ATP or AMPPNP such that the three nonexchangeable sites become saturated (Wise et al., 1983; Perlin et al., 1984). Therefore, this presented the opportunity to study the possible regulation of oxidative phosphorylation as a function of occupation of the nonexchangeable sites by the adenine nucleotides ATP, AMPPNP, and ADP. We had not previously, however, shown that nucleotide-depleted F_1 could be fully reloaded with ADP. It was therefore necessary to demonstrate this before proceeding.

Nucleotide-depleted F_1 was found to bind 5.8 mol of ADP/mol of F_1 after 1-h incubation under the conditions described under Materials and Methods. This was equal to the binding of AMPPNP and ATP found previously and assures that the nonexchangeable sites were saturated with ADP.

Search for Regulation of Oxidative Phosphorylation by Nucleotide Bound at Nonexchangeable Sites on F_1. According to the nucleotide binding procedure described under Materials and Methods, soluble nucleotide-depleted F_1 preparations were reloaded by incubation with ATP, ADP, AMPPNP, or zero nucleotide. (The last preparation will be referred to simply as "nucleotide-depleted F_1" in the text.) These preparations, or native F_1, were then bound to F_1-depleted membranes, and NADH-driven oxidative phosphorylation of GDP or ADP by the reconstituted membranes was studied. Figure 2 shows the results of these experiments, and Table I summarizes the calculated kinetic parameters.

Effects of varying GDP concentration at constant (10 mM) P_i concentration in the GTP synthesis assay are shown in Figure 2A–D. As the figure and Table show, the rates of GTP synthesis by ATP-, ADP-, and AMPPNP-reloaded F_1 recon-

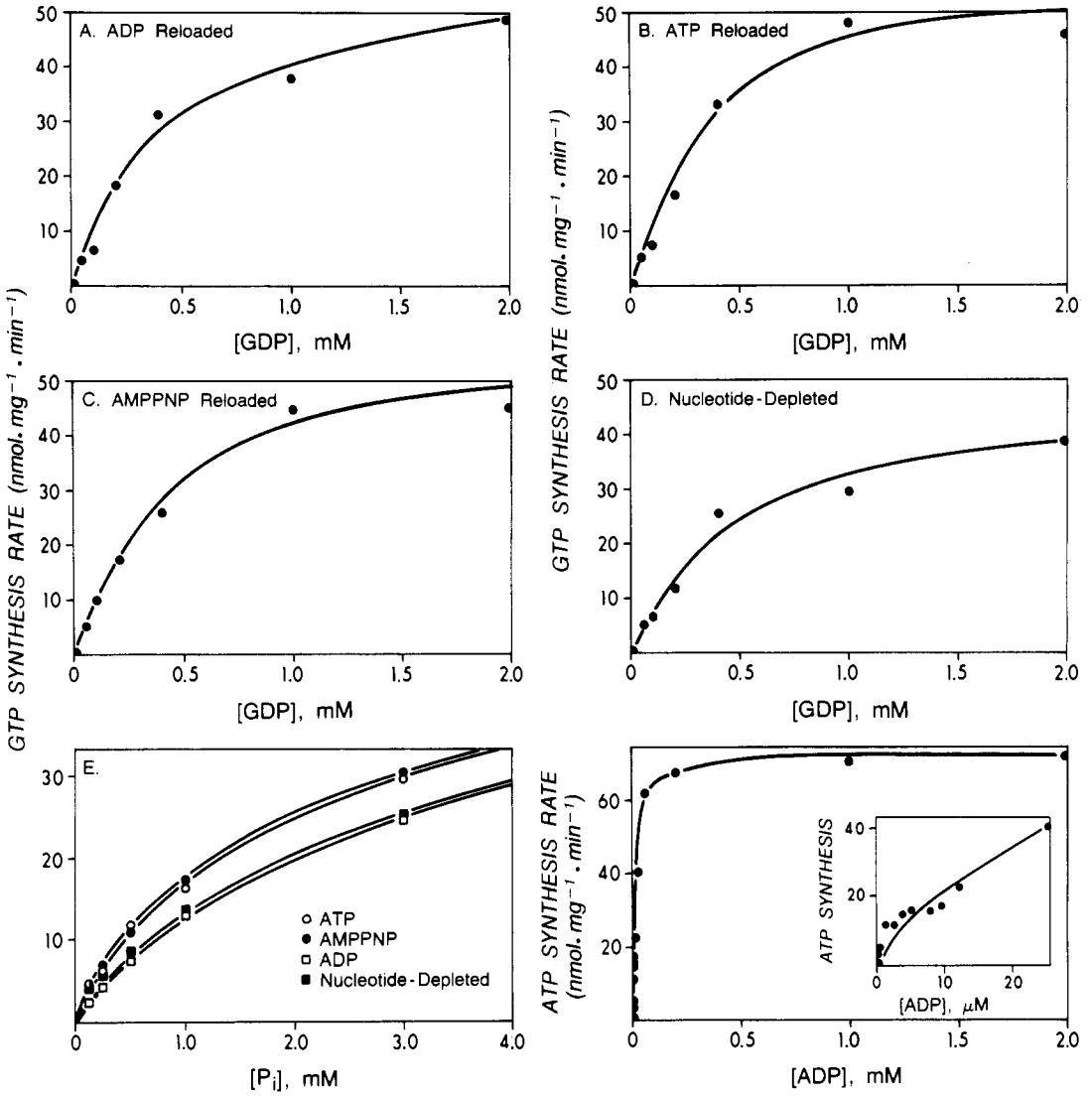

FIGURE 2: Rates of oxidative phosphorylation in reconstituted membranes. Assays were performed with reconstituted membranes at 0.125 mg/mL as described under Materials and Methods. Phosphorylation rates were linear with time at each substrate concentration. Curves shown were calculated as described in Table I. In all cases, the y axis represents the rate of nucleoside triphosphate synthesis. (A) ADP-reloaded F_1, 0.05–2.0 mM GDP, 10 mM [^{32}P]P_i; (B) ATP-reloaded F_1, 0.05–2.0 mM GDP, 10 mM [^{32}P]P_i; (C) AMPPNP-reloaded F_1, 0.05–2.0 mM GDP, 10 mM [^{32}P]P_i; (D) nucleotide-depleted F_1, not reloaded, 0.05–2.0 mM GDP, 10 mM [^{32}P]P_i; (E) as in panels A–D, except 1.0 mM GDP, 0.125–3.0 mM [^{32}P]P_i; (F) native F_1 reconstituted membranes, 0.02–2000 μM ADP, 10 mM [^{32}P]P_i. The inset shows ATP synthesis by native F_1 at low ADP concentrations.

stituted membranes were very similar, suggesting that there was no significant regulatory effect of nonexchangeably bound nucleotide at high or low GDP concentrations. The slightly lower V_{max} of GTP synthesis catalyzed by nucleotide-depleted F_1 reconstituted membranes (Figure 2D; see Table I) was reproducible and may reflect the lower stability of this enzyme [see Tiedge et al. (1983)]. Figure 2E shows the rate of GTP synthesis by nucleotide-depleted F_1 and ATP-, ADP-, and AMPPNP-reloaded F_1 reconstituted membranes as the concentration of P_i was varied at constant (1 mM) GDP concentration. Similar maximal GTP synthesis rates were observed with ATP-, ADP-, and AMPPNP-reloaded F_1 reconstituted membranes, with $K_m(P_i)$ slightly larger for ADP-reloaded F_1 relative to ATP-reloaded and AMPPNP-reloaded F_1 reconstituted membranes. As above, nucleotide-depleted F_1 reconstituted membranes showed slightly lower V_{max} values.

For comparison with the nucleotide-depleted and nucleotide-reloaded F_1, GTP synthesis assays were also performed with native F_1-reconstituted membranes. The data are not shown in Figure 2, but the calculated kinetic parameters are included in Table I, row 5. The calculated values of V_{max}-(GDP), $K_m(P_i)$, and $V_{max}(P_i)$ during GTP synthesis with native F_1 were similar to those seen with nucleotide-depleted and nucleotide-reloaded F_1. However, the value of K_m(GDP) (0.09 mM) was lower, for reasons that are unclear. Figure 2F shows the effect of varying ADP concentration at constant (10 mM) P_i concentration in ATP synthesis assays with native F_1-reconstituted membranes for comparison with GTP synthesis rates. Table I, row 6, shows that the K_m(ADP) is lower than the K_m(GDP). The maximal velocity of ATP synthesis was slightly higher than the maximal velocities of GTP synthesis (Table I).

It was possible that nucleotide bound at nonexchangeable sites might alter the "efficiency" of oxidative phosphorylation at low protonmotive force values. To test this, F_1-depleted membranes were preincubated with KCN in the presence of 0.2 mM NADH prior to reconstitution with F_1. As shown in Figure 3A,B, this procedure reduced the rate of GTP synthesis by reconstituted membranes. However, ATP-reloaded F_1 and nucleotide-depleted F_1 behaved comparably in the assays, showing that nonexchangeable nucleotide binding did not affect the catalytic efficiency of F_1 at low oxidation rates.

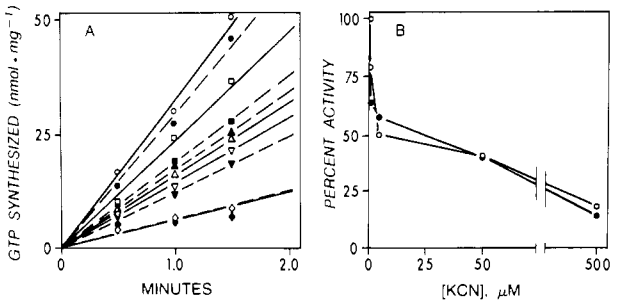

FIGURE 3: Effect of adenine nucleotide bound at nonexchangeable sites at low rates of oxidative phosphorylation. F_1-depleted membranes were preincubated with KCN (0–500 μM) and 0.2 mM NADH for 15 min at 30 °C to inhibit NADH oxidation, reconstituted with ATP-reloaded (open symbols) or nucleotide-depleted F_1 (closed symbols), and assayed for NADH-driven GTP synthesis (1.0 mM GDP, 10 mM P_i, and 0.125 mg/mL membrane protein) as described under Materials and Methods. (A) Time courses at varying KCN concentration in the preincubation: (●, ○) no KCN; (■, □) 0.5 μM KCN; (▲, △) 5 μM KCN; (▼, ▽) 50 μM KCN; (◆, ◇) 500 μM KCN. (B) Inhibition of GTP synthesis as a function of KCN concentration. One hundred percent represents the rate in the absence of KCN.

DISCUSSION

Physiological Catalytic Activities of E. coli Proton ATPase. The F_1 sector of the bacterial proton ATPase is in direct contact with the cytoplasmic nucleotides in vivo. This work and our previous work (Perlin et al., 1984) show that the enzyme catalyzes ITP- and GTP-driven proton pumping and oxidative phosphorylation of IDP and GDP at substantial rates. Mathews (1972) found intracellular concentrations of GTP and ATP to be 1.1 and 2.7 mM, respectively, in cells of *E. coli* B. Therefore, the measurements of GTP synthesis presented here apparently reflect a true physiological activity of the enzyme. It is likely that in vivo the enzyme uses a range of nucleotide substrates.

Stability of Adenine Nucleotide Bound at Nonexchangeable Sites of Membrane-Bound F_1. This work shows directly that adenine nucleotide bound at the nonexchangeable sites on membrane-bound F_1 does not significantly exchange with medium nucleotide during oxidative phosphorylation. This supports the previous work of Maeda et al. (1977) and Zlotnick & Abrams (1984), who studied bacterial systems, but is contrary to some other work on mitochondrial ATPase [e.g., see Harris et al. (1977, 1978)] in which exchange of tightly bound nucleotide was observed during catalysis. A possible explanation is that these last authors were actually measuring exchange at a high-affinity catalytic site such as the one described by Grubmeyer et al. (1982). This question has also been studied by Smith et al. (1983), by Myers & Boyer (1983), and by Cross et al. (1984). While it is clear that exchange of nucleotide bound at nonexchangeable sites may be induced to a variable extent under certain laboratory conditions, our data show that such exchange is not essential for oxidative phosphorylation. Our data also suggest that stable binding of fluorescent adenine nucleotide derivatives to the nonexchangeable sites of the proton ATPase might be possible, thereby providing a means of probing conformational changes during catalysis.

Binding of Nucleotide at Nonexchangeable Nucleotide Sites on F_1 May Not Be a Requirement for Oxidative Phosphorylation. The data showed that after a burst of GDP phosphorylation in membranes reconstituted with nucleotide-depleted F_1, no nonexchangeable nucleotide was detected in the membrane-bound F_1. Therefore, it can be stated with certainty that the nonexchangeable sites need not be occupied by *adenine* nucleotide for oxidative phosphorylation to occur and it would appear that the nonexchangeable sites need not be occupied by *any* nucleotide for oxidative phosphorylation to occur. An objection to the latter statement would be that during phosphorylation of GDP, guanine nucleotide interacts weakly with the nonexchangeable sites but that it is released during the washing procedure used to separate membrane-bound from free ligand. Of course, were this the case, the sites would not be acting as nonexchangeable sites toward guanine nucleotides. It is difficult to eliminate this possibility. However, it may be noted that the nonexchangeable sites are thought to reside on the α-subunits of F_1 [discussed by Perlin et al. (1984)] and that evidence from trypsin hydrolysis studies (Senda et al., 1983) and from equilibrium dialysis studies (Perlin et al., 1984) suggested that GTP or GDP did not bind to purified α-subunit. No binding of GTP or GMPPNP to nonexchangeable sites on soluble nucleotide-depleted F_1 was detected by Perlin et al. (1984), who calculated that if any binding of guanine nucleotide did occur at these sites, then the dissociation rate (k_{off}) must be at least 10^4-fold that of the corresponding rate of adenine nucleotide dissociation. Summarizing this section, one can state with confidence that oxidative phosphorylation of GDP occurs at normal rates in the absence of any nucleotide tightly bound at the nonexchangeable sites and the balance of the current evidence suggests that catalysis may proceed even when the sites are vacant.

Lack of a Regulatory Effect of Nucleotide Bound at the Nonexchangeable Sites on Oxidative Phosphorylation. We saw no significant effects of nonexchangeable site nucleotide binding on the rates of oxidative phosphorylation, K_m(GDP) or $K_m(P_i)$, or the "efficiency" at low NADH oxidation rates. The only differences we noted were small and occurred when nucleotide-free F_1 was reconstituted with F_1-depleted membranes. A physiological role of this effect is not obvious. It was observed that the procedure used to deplete F_1 of nucleotide caused the K_m(GDP) to rise from the value of 0.09 mM seen in native F_1 to around 0.42–0.49 mM (see Table I). Nevertheless, the design of the experiments should, we feel, have uncovered a physiologically relevant regulatory effect of bound nonexchangeable adenine nucleotide if such an effect exists.

Role of Nonexchangeable Nucleotide Sites on F_1. Previous authors have shown that the nonexchangeable nucleotide sites do not act directly in catalysis (Rosen et al., 1979; Gresser et al., 1979; Cross & Nalin, 1982), and our work confirms that notion. We have also pointed out previously (Senior & Wise, 1983) that there was no evidence in the literature to support a physiologically operative regulatory role for these sites. Subsequently, the paper of Myers & Boyer (1983) supported this view, and we believe the work presented here substantiates the idea that the nonexchangeable nucleotide sites are not regulatory. What then could be the role of these sites? It is to be expected that immediately after translation, newly synthesized α-subunit would bind ATP (K_d = 0.1 μM at pH 7.0; Dunn & Futai, 1980), undergo an ATP-induced conformational change (Dunn, 1980) to give a more compact molecule, and then become assembled into F_1. One hypothesis to explain the presence of the nonexchangeable sites would be that binding of ATP to the α-subunit is required prior to F_1 assembly to achieve the correct F_1 structure (yielding the properties of negative cooperativity for substrate binding and positive cooperativity for catalysis at the three catalytic sites of F_1). One could hypothesize that once the correct F_1 structure is assembled, it is stabilized by subunit interactions, and this would explain why nucleotide-depleted F_1 is active in oxidative phosphorylation of GDP and in GTP-driven proton

pumping. A test of this hypothesis would be to study the assembly of F_1 under conditions where nucleotide binding to the α-subunit was experimentally controlled. Studies of this topic are currently in progress.

ADDED IN PROOF

Under conditions of limited ATP production, when the ADP/ATP concentration ratio is high, *E. coli* cells might be expected to regulate nucleotide triphosphate hydrolysis by the proton ATPase. Under such conditions, ADP would be expected to be bound at the three noncatalytic sites. Additional experiments were therefore performed in which ADP-reloaded F_1 (all three noncatalytic sites contained ADP) or GDP-reloaded F_1 (control, no nucleotide bound at noncatalytic sites) was reconstituted with F_1-depleted membranes. GTPase activity was then assayed (GTP concentration = 0.1–5.0 mM). No difference in K_m(GTP) or V at any GTP concentration was seen between the two enzymes. Therefore, ADP bound at noncatalytic sites does not regulate GTP hydrolysis.

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Registry No. P_i, 14265-44-2; ATPase, 9000-83-3; ADP, 58-64-0; GDP, 146-91-8.

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