The Escherichia coli F₁F₀ ATP Synthase Displays Biphasic Synthesis Kinetics*

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The F₁F₀ proton-translocating ATPase/synthase is the primary generator of ATP in most organisms growing aerobically. Kinetic assays of ATP synthesis have been conducted using enzymes from mitochondria and chloroplasts. However, limited data on ATP synthesis by the model Escherichia coli enzyme are available, mostly because of the lack of an efficient and reproducible assay. We have developed an optimized assay and have collected synthase kinetic data over a substrate concentration range of 2 orders of magnitude for both ADP and Pi, from the synthase enzyme of E. coli. Negative and positive cooperativity of substrate binding and positive catalytic cooperativity were all observed. ATP synthesis displayed biphasic kinetics for ADP indicating that (1) the enzyme is capable of catalyzing efficient ATP synthesis when only two of the three catalytic sites are occupied by ADP; and (2) occupation of the third site further activates the rate of catalysis.

The F-type proton-translocating ATPase/synthases are a family of enzymes primarily responsible for the oxidative phosphorylation of ADP to form ATP (1–3). These membrane-bound enzymes are found in the inner membranes of mitochondria, the thylakoid membranes of chloroplasts, and the inner membranes of bacteria. The synthase uses energy from an electrochemical gradient of protons generated by a membrane-bound electron transport chain to drive the binding and catalysis of ADP and Pi, and the release of ATP. The actual synthesis (or hydrolysis in the reverse direction) reaction of the triphosphate nucleotide occurs in one of three catalytic sites under tightly bound conditions (the “tight” site or site 1) with a $K_{eq} \sim 1$; thus, the energy provided by the proton gradient is used primarily to alter the binding of the substrates and products, and hence the “binding change” mechanism.

Certain aspects of the enzymes are generally accepted. In the direction of hydrolysis, given an enzyme with three empty catalytic sites, MgATP binds to the first site with very high affinity (nanomolar range). Once bound, this nucleotide undergoes oxygen exchange with medium water (a process called unisite catalysis) as the nucleotide undergoes rapid cycles of hydrolysis and resynthesis. Release of ADP is very slow (4, 5).

As the concentration of ATP is increased and approaches the $K_d$ values for the remaining two sites, the rate of product release increases, a phenomenon called catalytic cooperativity (6). The affinity of the second site for MgATP (micromolar range) is lower than the first, and the affinity of the third site is lower still (range, 10–100 μM). Thus, the binding of nucleotide exhibits negative cooperativity.

There has been controversy recently regarding the necessity of substrate binding to subsequent sites for the activation of rapid catalysis. On one hand, it has been argued and demonstrated that some enzymes, particularly those from mitochondria and chloroplasts, display bisite activation, i.e. the binding of substrate(s) at the second site is necessary and sufficient to accelerate the enzyme from slow unisite catalysis to rapid rotational catalysis (7). This does not exclude the occupation of the third site by a nucleotide but argues instead that there is (at any given moment) a single site with a single $K_d$ that must be occupied for rapid catalysis to proceed. In contrast to this, studies of purified mutant F₁ sector from Escherichia coli suggest that for ATPase activity all three catalytic sites must be bound before rapid catalysis may proceed (8).

Mitochondrial and plastidic enzymes function primarily and perhaps exclusively as ATP synthases within their cellular contexts. Similarly, the enzyme from Paracoccus denitrificans, a photosynthetic bacterium, also functions almost exclusively as a synthase (9). Other bacterial enzymes, in particular the well-characterized enzyme from E. coli, have important roles both as a synthase and as an ATP-driven proton pump. This latter role is especially critical during growth on fermentable carbon sources when the cytoplasmic pH drops and rapid proton extrusion is necessary (10). A consequence of these varying roles has been a greater focus on the synthase activity of mitochondrial and plastidic enzymes and on the ATPase activity of the E. coli enzyme. Because of these differences in experimental systems the debate has often been conducted across the line between ATPase and ATP synthase mechanisms, although important distinctions exist between the two activities.

The ATP synthase assay has been used as a regular tool for many years in this laboratory, and over that time the protocol has been optimized so that we now obtain consistently high synthase activity from our E. coli membrane preparations. Here we present data on E. coli ATP synthase kinetics and determine the dependence on substrate concentration over a range of 2 orders of magnitude for both substrates, allowing comparison to previous data on the synthase activity of enzymes from mitochondrial and plastidic sources.

MATERIALS AND METHODS

Membrane Preparation—Evoked E. coli membranes were prepared from strain LE392. Cells were grown in LB medium to an $A_{600}$ of 1.5, harvested by centrifugation at 5000 $\times g$ for 5 min, and resuspended in

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Preincubation with NADH significantly improves reaction rates. We tested a range of NADH concentrations and preincubation times and measured reduced ATP synthesis at either higher (5 mM, 7.5 mM) or lower (1.25 mM) NADH concentrations and for longer preincubation times (data not shown). A 1-min preincubation in 2.5 mM NADH produced the highest rates of synthesis. We routinely measure synthesis activity of 0.40–0.50 μmol/min/mg at room temperature (22 °C). We have also confirmed that there is negligible ATP present or synthesized 1) when the membranes are not energized, 2) without the addition of substrates, or 3) when the membranes are from strain LE392 Δ(uncL-uncC) (data not shown).

The proton gradient behaves as a third substrate in the synthetic reaction. Therefore, before beginning a comprehensive kinetic analysis, we determined whether the proton gradient established by the electron transport chain was limiting. Research on plasticic enzymes, reproducibly energized with light, has shown that the Vmax of the synthesis reaction, but not the substrate Ks values, is affected by variation of the ΔpH (13). Furthermore, extensive studies of mitochondria using the electron transport chain energized with either NADH or succinate have shown this method of energization is sufficient for valid kinetic analyses (14).

To examine the relationship between the proton gradient and the synthase activity, we performed our standard ATP synthase assay in the fluorimetric cuvette with ACMA to measure the proton gradient (Fig. 2). The experiment shown was performed in one-tenth the volume and with 10 times the amount of membrane and NADH to increase the signal to noise ratio. However, we also performed the experiment under conditions essentially identical to those for measuring synthesis rates (data not shown), and except for the higher noise, the results were the same. After pre-energizing with NADH, the addition of the synthase substrates (ADP + P) results in normal, maximal synthesis of ATP with no detectable deflection of the steady state proton gradient, as indicated by an unchanging fluorescence. This experiment demonstrates that once a proton gradient is established, the respiratory chain can respond to additional proton translocation during maximum ATP synthesis so that as the enzyme synthesizes ATP the resultant proton movement does not diminish the transmembrane proton-motive force under the conditions of these fluorescence quenching assays.

Velocity Dependence on Concentration of ADP and P—To examine the effect of substrate concentrations on the rate of synthesis we conducted 49 ATP synthase assays, a matrix of seven concentrations for each substrate, covering a concentration range of 2 orders of magnitude for each. The primary velocity data are shown by the symbols in Fig. 3, A and B. Shown beneath the primary velocity plots are the analytical
plots: double-reciprocal (C and D) and Eadie-Scatchard (E and F). We provide these for easy comparison with previously published literature. It is immediately obvious that for ADP the analytical plots are not linear and are apparently biphasic. The concave-down pattern in the double-reciprocal plots and the concave-up pattern in the Eadie-Scatchard plots typically indicate negative cooperativity of binding. For Pi, the double-reciprocal plots appear linear, but the Eadie-Scatchard plots reveal that at high concentrations of ADP, Pi appears to show negative cooperativity with either no cooperativity or positive cooperativity at lower concentrations of ADP.

The affinity of the enzyme for both ADP and Pi depends on the concentration of the other substrate. From linear regression analysis of the double-reciprocal plots, we calculated an apparent $K_m$ of 150–450 $\mu$M, depending on the [ADP] (Fig. 3D). These values are lower than previously reported values (15–18). For ADP we calculated separate apparent $K_m$ values for [ADP] of $\approx 166$ $\mu$M and for [ADP] of $\approx 166$ $\mu$M because of the apparent biphasic nature of the secondary plots. The apparent $K_m$ values are $K_m^{ADP=166} \approx 10$ $\mu$M and $K_m^{ADP=450} \sim 60$ $\mu$M, although this latter value rises sharply for [Pi] of $\approx 100$ $\mu$M (Fig. 3G). These $K_m^{ADP}$ values are similar to those reported under conditions of saturating P$_i$ (6, 18–20). The apparent $V_{\text{max}}$ as a function of [Pi] is fairly constant (400 nmol of ATP/min/mg of protein) when [ADP] is $> 83$ $\mu$M; below that level, it drops sharply. The apparent $V_{\text{max}}$ values for ADP at low and high [ADP] both show a smooth increase from low to high [Pi]. At the highest [Pi] for [ADP] of $\approx 166$ $\mu$M, however, the apparent $V_{\text{max}}^{[P_i]=3200}$ = 341 nmol of ATP/min/mg of protein, whereas for [ADP] $\approx 166$ $\mu$M the apparent $V_{\text{max}}^{[P_i]=3200}$ = 471 nmol of ATP/min/mg of protein. As seen in the data shown in Fig. 1 we occasionally have preparations with slightly higher activities.

**DISCUSSION**

Studies of mitochondria and plastids have dominated research on the kinetics of ATP synthesis by the F-type proton-translocating ATPase/synthases. Here we examine synthesis kinetics by the model enzyme found in *E. coli*. These data compare favorably with what has been observed previously, and they provide a new perspective for understanding the mechanism of the ATP synthase.

The interactions of the enzyme with ADP and phosphate are obviously complex and inter-related. We have attempted to use our data to develop a detailed kinetic model for ATP synthesis with limited success, so we do not present it at this time. In addition to showing negative cooperativity and the biphasic nature of the enzyme-ADP interaction, our data clearly demonstrate the dependence of the $K_m$ for each substrate on the concentration of the other substrate. Increasing the concentration of either ADP or Pi reduces the $K_m$ for the other substrate. We replotted the data from Fig. 3, G and H, to measure how the concentrations of ADP and Pi affected the specificity constant, $V_{\text{max}}/K_m$ for the other substrate. Fig. 4 shows that at high ADP concentrations the $V_{\text{max}}/K_m$ for ADP does not change with increasing Pi, but at lower ADP concentrations the $V_{\text{max}}/K_m$ is strongly affected by the concentration of Pi. Because Pi increases the affinity of the enzyme for ADP and competes for binding to the enzyme with ATP but not ADP, these data support the idea that under certain physiological conditions phosphate can stimulate ATP synthesis even under conditions of relatively high intracellular ATP concentrations (3). We also plotted the $V_{\text{max}}/K_m$ for ADP versus [ADP] at two saturating concentrations of Pi. Fig. 5 shows that the $V_{\text{max}}/K_m$ for ADP increases as the concentration of ADP decreases. Therefore, the enzyme is a better enzyme for ADP at lower ADP concentrations.

We believe that this assay can help distinguish differences between the first order hydrolytic reaction and the second order synthetic reaction. During hydrolysis, it has been shown that all three sites of the *E. coli* F$_1$-ATPase are occupied by nucleotide under conditions of rapid catalysis (8, 21). Whether this occupation is necessary for catalysis has been a topic of much debate (22–24). For the synthetic direction, having two substrates complicates the matter of site occupation. The biphasic nature of our data shows that whereas binding of ADP at the third site increases the $V_{\text{max}}$ of the enzyme this is not necessary for the enzyme to proceed with catalysis. Full occupation of two sites appears sufficient to promote complete catalysis and release of ATP especially because the $V_{\text{max}}/K_m$ for ADP is higher at lower ADP concentrations whereas binding to the third site would be minimal. Occupation of the third site by ADP would further stimulate the rate. We therefore conclude that the enzyme can operate as a synthase in essentially two states: 1) under conditions of low intracellular ADP the enzyme has a lower $V_{\text{max}}$ but a significantly higher $V_{\text{max}}/K_m$ for ADP, i.e. it is a better enzyme for ADP and is activated by increased Pi; and 2) under conditions of higher intracellular ADP the enzyme has a lower $V_{\text{max}}/K_m$ for ADP and does not respond to changes in Pi, but has a higher $V_{\text{max}}$.

Work on both the mitochondrial and plastidic enzymes have demonstrated a similar phenomenon. Research using submitochondrial particles from bovine heart mitochondria have demonstrated a two-$V_{\text{max}}$ two-$K_m$ phenomenon similar to that which we observed for *E. coli* (14, 20). Studies on plastids have also shown that binding of ADP to the third site increases the $V_{\text{max}}$ of the enzyme (7, 23).

Experimentally, conditions necessary to achieve a high, “three-site” $V_{\text{max}}$ would probably depend on the $\Delta \mu_{\text{H}^+}$, which is a function of the rate of proton gradient formation by the respiratory chain and the rate of consumption by the ATP synthase as well as the volume of the membrane vesicles, vesicle leakiness, and the density of gradient-generating and -consuming proteins per unit area of membrane. The steady state $\Delta \mu_{\text{H}^+}$ may, however, be only slightly affected as seen in the ACMA fluorescence-quenching data in Fig. 2 and in studies by other laboratories (14).

Previously, Etzold et al. (18) used the luciferase assay to measure the turnover number of the ATP synthase during
Fig. 3. Velocity data and secondary plots for both ADP and P_i with the best fit of the model. The velocity data (symbols) represent the average of two complete seven by seven experiments; vertical error bars represent one S.D. Additional data from smaller scale experiments are not included but displayed the same trends as the data presented. A single membrane preparation was used for each of the 49 assay experiments that examined a range of seven different ADP concentrations (20.75, 41.5, 83, 166, 322, 664, and 1328 μM) against seven different...
synthesis by membrane vesicles of E. coli. They measured ATP synthesis rates of −0.060−0.10 μmol/min/mg of membrane protein at room temperature and 0.20 μmol/min/mg of membrane protein at 37 °C. They found that F₁F₀ makes up 3.3% of the E. coli membrane proteins, giving a turnover number of 13−33 s⁻¹, considerably lower than those of other F-type ATPase complexes measured under optimal conditions. Specifically, they cited several studies of submitochondrial particles that gave turnover values of 400−600 s⁻¹ and also studies in chloroplasts that produced turnover numbers of 200 s⁻¹. Using an innovative approach they tested the hypothesis that the lower value produced by the E. coli ATPase was caused by an imbalance between the amount of enzyme and the respiratory capacity of the vesicles and that far more synthase existed than could be activated by the respiratory chain. In support of this hypothesis, they demonstrated that the turnover could be increased to 270 s⁻¹ by inactivating a percentage of the ATPase with DCCD in order to produce a greater respiratory driving force for the remaining active, unmodified enzymes. Additionally, in vivo studies on growth rate and yield of E. coli containing increased or decreased amounts of ATPase also concluded that cells contain an excess capacity of enzyme and that decreasing the amount of ATPase resulted in an increase in the turnover number during synthesis (25). In comparison, Al-Shawi et al. (15) measured turnover numbers in wild type membranes of 82 s⁻¹ for ATP synthesis in non-DCCD-treated membranes and 425 s⁻¹ for ATP hydrolysis.

In our in vitro system, the NADH-driven steady state protomotive force appears unaffected by maximum synthesis rates. After the protomotive force has been established during the preincubation with NADH, the respiratory chain is able to respond to proton translocation by the synthase by pumping additional protons essentially in a one-for-one exchange. Furthermore, using the values from Etzold et al. (18) of 3.3% for the fraction of membrane protein made up of F₁F₀, our value of ~0.5 μmol/min/mg of membrane protein translates into a turnover number at room temperature of 160 s⁻¹. At 37 °C the value becomes greater than 200 s⁻¹ (data not shown), which is comparable with the value cited for the chloroplast enzyme. It therefore appears as if, under these conditions, the ATPase is not present in excess of the respiratory capacity of the membrane vesicles. We cannot say what accounts for the significant (although less than 10-fold) difference in turnover number between these and previous studies (15, 18). The coupled assay depends on the activities of the ATPase and the enzymes of the respiratory chain in addition to the permeability of the membrane vesicles. Therefore, strain-specific differences in enzyme concentrations and activities and differences in the growth of cells or preparation of membrane vesicles, combined with the enhanced activity resulting from preincubation of vesicles with NADH, might all contribute to the differences in activities. Whatever the reason(s), this improved ATP synthase assay uses a NADH-driven protomotive force that is unchanged even during maximum rates of ATP synthesis, enabling us to measure and dissect the kinetics of ATP synthesis in more detail than has previously been possible for E. coli.

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Pᵢ concentrations (50, 100, 200, 400, 800, 1600, and 3200 μM). The velocity versus [substrate] curves are shown for ADP (A) and phosphate (B); concentrations of the second substrate are shown in the listed symbol keys (boxes). The consumption of substrate is indicated by the left error bars. Except for conditions of high [Pᵢ] and low [ADP], the consumption of substrate was less than 10% of either substrate during the 40-s time course. Double-reciprocal plots are shown for ADP (C) and Pᵢ (D); Eadie-Scatchard plots are shown for ADP (E) and Pᵢ (F). The apparent Vₘₐₓ ((bullet) and apparent Kᵢ ((square) for each substrate as a function of the other substrate concentration, calculated by linear regression from the plots in C and D, are shown for ADP (G) and Pᵢ (H). For ADP (G) two apparent Vₘₐₓ and two apparent Kᵢ were calculated; [ADP] is ≤166 μM (dotted lines, open symbols) and [ADP] is ≥166 μM (solid lines, filled symbols).
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