Nucleotide-depleted Beef Heart F$_1$-ATPase Exhibits Strong Positive Catalytic Cooperativity*

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**Catalytic cooperativity is a central feature of the binding change mechanism for F$_0$F$_1$-ATP synthases. However, in a recent publication (Reynafarje, B. D., and Pedersen, P. L. (1996) J. Biol. Chem. 271, 32546–32550), Reynafarje and Pedersen claim that cooperative effects are an artifact caused by endogenous nucleotides and that when such nucleotides are removed, the multiple catalytic sites on MF$_1$ behave independently during ATP hydrolysis. In contrast to this conclusion, we show here that when ATP is loaded at a single catalytic site on nucleotide-depleted MF$_1$, the rate of product release is accelerated by up to 5 × 10$^4$-fold by the binding of ATP at adjacent catalytic sites. Hence, nucleotide-depleted MF$_1$ is not an exception but does in fact show strong cooperative interactions. In addition, evidence is presented supporting a random order for product release during ATP hydrolysis.

F$_0$F$_1$-ATP synthases are found embedded in the membranes of mitochondria, chloroplasts, and bacteria. During oxidative phosphorylation and photo-phosphorylation, the synthases couple the movement of protons down an electrochemical gradient to the synthesis of ATP. The F$_0$ sector is composed of membrane-spanning subunits that conduct protons across the membrane (1, 2), whereas the F$_1$ sector is an extrinsic complex that contains catalytic sites for ATP synthesis (3). F$_1$ can be removed from the membrane in a soluble form and, once unplugged from the proton current, functions as an ATPase.

A model for energy coupling by F$_0$F$_1$-ATP synthases that has gained considerable support is the binding change mechanism. According to this proposal, the major energy requiring step is not the synthesis of ATP at the three catalytic sites on F$_1$ but rather the simultaneous and highly cooperative binding of substrates to and release of product from these sites (4, 5). Furthermore, the binding changes are believed to be coupled to proton transport by the rotation of a complex of subunits that extends through F$_0$F$_1$ (6), and a number of recent studies provide strong support for this concept (7–11).

Evidence for cooperative interactions between the multiple catalytic subunits on F$_1$ has come from diverse sources (12). Perhaps most compelling has been the demonstration that during ATP hydrolysis at a single site on soluble mitochondrial F$_1$ (MF$_1$),$^1$ the rate of product release can be accelerated by up to 5 orders of magnitude upon binding substrate to adjacent catalytic sites (13–15). In contrast to this view, Reynafarje and Pedersen (16) recently presented kinetic studies of nucleotide-depleted MF$_1$ (ndMF$_1$), which they interpret as evidence against the kind of strong positive catalytic cooperativity that has been observed in other laboratories using the native enzyme. Although ndMF$_1$ is known to have some altered properties, the lack of catalytic cooperativity would be difficult to reconcile with the binding change mechanism (12). In studies presented here, we have applied techniques that have successfully revealed details of subunit cooperativity in previous studies. The results demonstrate that like native MF$_1$, ndMF$_1$ exhibits strong positive cooperativity between catalytic sites.

**EXPERIMENTAL PROCEDURES**

Materials—ATP, phosphoenolpyruvate monopotassium salt, salt-free lyophilized pyruvate kinase, bovine serum albumin, and Sephadex G-50–80 were obtained from Sigma. [$\gamma$-$^32$P]ATP and [$\alpha$-$^32$P]ATP were obtained from Amersham.

MF$_1$ was prepared from beef heart mitochondria according to Knowles and Peneffsky (17) and depleted of tightly bound endogenous nucleotides according to Garrett and Peneffsky (18). Protein fractions with an $A_{280}/A_{400}$ ratio higher than 1.95 were used. The nucleotide content of ndMF$_1$ preparations was less than 0.1 mol/mol of enzyme as determined by anion exchange high pressure liquid chromatography analysis of the extracted nucleotides (19).

Methods—The hydrolysis of [$\gamma$-$^32$P]ATP was measured at 22 °C using charcoal to separate nucleotide from $^32$P, in acid-quenched samples. Details are described in the figure legends.

The dissociation of radiolabeled ligands from ndMF$_1$ was assayed at 22 °C using Sephadex centrifuge columns (20). Columns consisting of 1 ml of Sephadex G-50–80 equilibrated with MTEM buffer (20 mM Mops/Tris (pH 8.0), 0.2 mM EDTA, and 2.2 mM Mg(CH$_3$COO)$_2$) containing 1.1 mg/ml BSA were used for 100-μl samples. Before sample application, the columns were pre-spun for 40 s using an IEC Clinical Centrifuge (International Equipment Co.) at top speed. The separation of unbound ligands was carried out under the same conditions. The results obtained were corrected for a small amount of radiolabeled ligand that passed through the column in the absence of ndMF$_1$ (<1%).

Protein was determined by a modified Lowry method according to Peterson (21). A value of 371 kDa was used as the molecular mass of MF$_1$ (22).

**RESULTS**

Under conditions where substoichiometric ATP can bind rapidly at one of the three catalytic sites on ndMF$_1$ (Scheme 1, Step 1), ATP hydrolysis exhibits an initial burst followed by a slow phase (Fig. 1, circles). The rapid phase is due to the establishment of an equilibrium between bound substrate and bound products (Scheme 1, Step 2), whereas the slow phase is due to product release (Scheme 1, Step 3). The overall reaction (Scheme 1, Steps 1–3), referred to as uni-site catalysis, is a very slow process limited by the rate of product release in Step 3. A brief incubation with a large excess of unlabeled ATP just prior to acid quenching (Fig. 1, squares) has two effects. First, upon binding ATP to additional catalytic sites, the release of radiolabeled product from the first site is accelerated (Scheme 1,
Step 3’). This is demonstrated by the fact that all of the bound \( [\gamma-^{32}\text{P}]\text{ATP} \) is hydrolyzed during the chase. Second, it prevents any remaining unbound \( [\gamma-^{32}\text{P}]\text{ATP} \) from binding. Because only a few percent of the “cold chase” ATP is hydrolyzed during the 5-s incubation, any remaining unbound \( [\gamma-^{32}\text{P}]\text{ATP} \) would be trapped. The fact that all of the radiolabel appears as \( ^{32}\text{P} \) in the cold chase indicates that the \( [\gamma-^{32}\text{P}]\text{ATP} \) initially added had all bound to catalytic sites prior to the first time point shown and that none of it dissociated as radiolabeled ATP during the cold chase.

The promotive effect of excess ATP on the hydrolysis of radiolabeled ATP preloaded at a single catalytic site (Fig. 1) indicates that the three catalytic sites on ndMF, behave in a cooperative manner. As a more direct means of assessing the ability of substrate binding at one site to promote product release from a second site, we used a Sephadex centrifuge column binding assay for the experiments reported in Figs. 2 and 3. In Fig. 2, we follow the dissociation of \( ^{32}\text{P} \) by measuring the amount of \( [\gamma-^{32}\text{P}]\text{ATP} \) plus \( ^{32}\text{P} \) remaining bound to a site initially loaded under uni-site conditions. The addition of low nanomolar concentrations of unlabeled ATP results in a measurable acceleration in the rate of release of \( ^{32}\text{P} \). The same effect is observed when we follow the dissociation of \( [\alpha-^{32}\text{P}]\text{ADP} \) by measuring the amount of \( [\alpha-^{32}\text{P}]\text{ATP} \) and \( [\alpha-^{32}\text{P}]\text{ADP} \) remaining bound to a site also loaded under uni-site conditions (Fig. 3). Acceleration of product release by ATP (Figs. 2 and 3) is due to nucleotide binding at remaining unoccupied catalytic sites because ATP binding to noncatalytic sites is too slow (23) to occur to a significant extent during these experiments. Hence, the promotive effect of excess ATP on the rates of release of bound \( ^{32}\text{P} \) and \( [\alpha-^{32}\text{P}]\text{ADP} \) are a direct demonstration of positive catalytic cooperativity.

In Fig. 4, rates obtained from Figs. 2 and 3 in the absence (closed symbols) or the presence (open symbols) of excess unlabeled ATP for the dissociation of \( ^{32}\text{P} \) (circles) and \( [\alpha-^{32}\text{P}]\text{ADP} \) (squares) are plotted versus the concentration of cold chase ATP. A linear regression analysis gives a value of \( 10.6 \pm 1.6 \times 10^{6} \text{M}^{-1} \text{s}^{-1} \) for the ATP concentration dependence of the rate of product release. This is in agreement with the rate constant for ATP binding at a second site (Scheme 1, \( k' \)) determined from the rates of hydrolysis of nanomolar concentrations of ATP (data not shown). This correlation provides further evidence that ATP binding at one catalytic site promotes product release from an adjacent site.

The degree of cooperativity between catalytic sites can be quantitated by measuring the acceleration of product release in going from uni- to multi-site turnover conditions. Values obtained for the ndMF, preparation used in this study are presented in the first line of Table I. The results show that substrate binding can increase the rate of product release from a separate but interacting site by up to 50,000-fold under maximal velocity conditions. This is similar to the degree of rate acceleration previously observed for native MF (Table I, sec-
Additional information not related to subunit cooperativity. This is in accordance with the binding change mechanism where the exergonic tight binding of cold chase ATP to an unoccupied catalytic site would be expected to drive the release of tightly bound product from an adjacent occupied site.

These conclusions are in contrast to those reached by Reynafarje and Pedersen (16). They reported that under conditions for uni-site catalysis, the rate of substrate binding measured by a luciferase assay was equal to the rate of product release measured by a charcoal quench/centricon filtration assay during a 2-s incubation. Data presented in Figs. 1–3 clearly show that this is not the case. One possible explanation of their results is that the addition of charcoal at 2 s in their assay for product release was ineffective in stopping uni-site catalysis. Although charcoal would prevent further binding of ATP, it would not be expected to prevent cleavage of enzyme-bound ATP with subsequent dissociation of products. Indeed, we found that uni-site catalysis continued for up to 2 min in the presence of charcoal when we bound substoichiometric \([\gamma^{32}P]ATP\) to \(F_1\) under conditions described for Fig. 1, incubated for 4 s, added a charcoal suspension (pH 7.5) in the buffer because \(P_i\) is known to significantly reduce MgADP inhibition of \(MF_1\) (24, 25). It is also consistent with the fact that any endogenous inhibitory MgADP bound to the enzyme would have been removed during the preparation of ndMF1. Second, the results directly demonstrate positive catalytic cooperativity. This is in accordance with the binding change mechanism where the exergonic tight binding of cold chase ATP to an unoccupied catalytic site would be expected to drive the release of tightly bound product from an adjacent occupied site.

Additional details of our results warrant comment. First, ndMF1 shares a number of properties in common with native \(MF_1\) that contains endogenous nucleotides (13). Second, the observed rate constant for the slow phase of \([\gamma^{32}P]ATP\) hydrolysis (1.1 \(\times\) \(10^{-2}\) s\(^{-1}\), Fig. 1), is significantly higher than either of the observed rate constants for unpromoted product release (\([32P]_i = 4.6 \times 10^{-3} s^{-1}\), Fig. 2, and \([\alpha^{32}P]ADP = 6.8 \times 10^{-3} s^{-1}\), Fig. 3). Instead, the ATP hydrolysis rate under uni-site conditions is equal to the sum of the product dissociation rates (i.e. \((4.6 + 6.8) \times 10^{-3} = 1.1 \times 10^{-2} s^{-1}\)) as expected if the dissociation of products occurs in random order. A similar conclusion can be made from a reassessment of data reported for native \(MF_1\) that contains endogenous nucleotides (13, 15).

**DISCUSSION**

The finding that nanomolar concentrations of ATP accelerate the rate of product release from a catalytic site on ndMF1, initially loaded under uni-site conditions establishes two features of catalysis by this form of the enzyme. First, because the ATP-promoted rates of release of \(P_i\) and ADP equal the rate of ATP binding, it can be concluded that the enzyme is not subject to product inhibition. This is consistent with the presence of \(P_i\) in the buffer because \(P_i\) is known to significantly reduce MgADP inhibition of \(MF_1\) (24, 25). It is also consistent with the fact that any endogenous inhibitory MgADP bound to the enzyme would have been removed during the preparation of ndMF1. Second, the results directly demonstrate positive catalytic cooperativity. This is in accordance with the binding change mechanism where the exergonic tight binding of cold chase ATP to an unoccupied catalytic site would be expected to drive the release of tightly bound product from an adjacent occupied site.

The uni-site rate for ndMF1, was preincubated at 108 nm with 16 nm \([\alpha^{32}P]ATP\) (23.6 \(\times\) \(10^7\) cpm/pmol) in MTEMP buffer containing 10 nm potassium acetate, 0.5 nm phosphoenolpyruvate, 1 mg/ml pyruvate kinase, and 0.3 mg/ml BSA for 10 s. Samples were diluted 667-fold at zero time by the MTEMP buffer containing 1.1 mg/ml BSA in the absence (circles) or the presence of 1 (squares), 2 (diamonds), or 3 nm (triangles) ATP. At the times indicated, 100-\(\mu\)l aliquots were passed through 1-ml Sephadex G-50–80 centrifuge columns equilibrated with MTEMP buffer containing 1.1 mg/ml BSA. Each point is an average of duplicate samples. The lines were drawn as described for Fig. 2.

**FIG. 3.** Effect of ATP binding at a second site on the rate of dissociation of \([\alpha^{32}P]ADP\) from an occupied site. ndMF1 was preincubated at 108 nm with 16 nm \([\alpha^{32}P]ATP\) (23.6 \(\times\) \(10^7\) cpm/pmol) in MTEMP buffer containing 10 nm potassium acetate, 0.5 nm phosphoenolpyruvate, 1 mg/ml pyruvate kinase, and 0.3 mg/ml BSA for 10 s. Samples were diluted 667-fold at zero time by the MTEMP buffer containing 1.1 mg/ml BSA in the absence (circles) or the presence of 1 (squares), 2 (diamonds), or 3 nm (triangles) ATP. At the times indicated, 100-\(\mu\)l aliquots were passed through 1-ml Sephadex G-50–80 centrifuge columns equilibrated with MTEMP buffer containing 1.1 mg/ml BSA. Each point is an average of duplicate samples. The lines were drawn as described for Fig. 2.
ing are all very similar for the two forms of the enzyme. One rather minor difference is the 4-fold faster rate of uni-site catalysis observed with ndMF1. Second, the finding that the rate of ATP cleavage during uni-site hydrolysis is equal to the sum of the product release rates indicates a random order release of ADP and P$_i$. This is consistent with previous evidence that ADP and P$_i$ bind in a random order during ATP synthesis (5).

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F$_1$ Subunit Cooperativity