Catalytic Properties of Beef Heart Mitochondrial ATPase Modified with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole

EVIDENCE FOR CATALYTIC SITE COOPERATIVITY DURING ATP SYNTHESIS*

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We have studied the incorporation of water oxygens into the γ-phosphoryl group of ATP during ATP synthesis (intermediate ATP = HOH exchange) by a preparation of mitochondrial inner membranes reconstituted with F₁-ATPase modified by NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole). These particles allow ATP synthesis although ATP hydrolysis is largely blocked. The incorporation of water oxygens into each ATP molecule synthesized increased significantly as the medium ADP concentration was lowered, as has been observed with native mitochondrial and chloroplast membranes. However, at each ADP concentration tested, the extent of exchange was less than that catalyzed by control preparations that were not modified by NBD-Cl. Further, the concentration of ADP required for a half-maximal rate of ATP synthesis (sₐₐ) is approximately 10-fold greater than that observed with native submitochondrial particles. These data suggest that the effects of NBD-Cl are not limited to ATP hydrolysis. More importantly, although the NBD group appears to alter the catalytic properties, alternating in separate catalytic sites or loci in ATP synthesis and hydrolysis. A residual ATPase activity observed in the presence of NBD-Cl has been shown to result largely from a slow, spontaneous reactivation process that can be observed after removal of medium NBD-Cl.

The accumulated experimental findings on ATP synthesis by the membrane-bound ATP synthase complexes and on ATP hydrolysis by the corresponding isolated ATPases appear to be consistent with the view that the same catalytic sites or loci participate in both the hydrolysis and synthesis reactions. Some observations, however, have suggested that additional consideration should be given to the possibility of separate sites for synthesis and hydrolysis, as noted in some reviews (1, 2), and in recent publications on the effect of NBD-Cl. This work was supported by Grant GM 11094 from the Institute of General Medical Sciences, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (also known as 4-chloro-7-nitrobenzofurazan); PSBA, 5'-P-fluorosulfonylbenzoyladenosine; NBD-F₁, F₅ which has been modified by treatment with NBD-Cl; NBD-F₁-ASU and F₁-ASU, ASU particles which have been reconstituted with NBD-F₁ and unmodified F₁, respectively; OSCP, oligomycin sensitivity conferring protein; S₁₀, 5-chloro-3'-butyl-2'-chloro-4'-nitrosalicylanilide.

The F₁-ATPase is regarded as having an α₃β₃γ or an α₃β₃γδ structure with the catalytic sites likely on the β-subunit (see Ref. 8 for review). Until the stoichiometry of the ATPase subunits is definitely settled, it will remain uncertain whether the catalytic cooperativity shown by F₁-ATPase and the ATP synthase is best described as catalysis by a cooperative alternating two-site or three-site model.
Standard Procedures—[14C]Pi, 97%, was prepared by the hydrolysis of PCl3 in [14CH3]OH, 95% (9, 10). Beef heart mitochondria were isolated essentially by the method of Smithies (11) and used as starting material for the preparation of F1 (12), OSCP (13), and F1-depleted submitochondrial particles (3). Protein was assayed by the Bradford procedure (14) or by a modified Lowry procedure (15) that included a quantitative precipitation of the protein in the presence of Na dodecyl sulfate serum albumin was used as a standard in both cases. Phosphate was assayed by the Sumner method (17) or the method described by Ohnishi and Gall (18).

Modification of F1 by NBD-CL—Reactions were carried out exactly as described by Steinmeier and Wang (3). F1, stored at 4 °C as the ammonium sulfate precipitate, was rehydrated by centrifugation and resuspended in a buffer containing 0.2 mM sucrose, 50 mM triethanolamine HCl, pH 7.5, 4 mM ATP, and 2 mM EDTA (STEA buffer). The enzyme was desalted by centrifuging the mixture through a Sephadex G-50 fine column (19) previously equilibrated with the same buffer. Typically, inactivation was done in 1 ml of STEA buffer which contained 1-3 mg of F1. Reactions were initiated by the addition of NBD-CL from a freshly prepared ethanolic solution to a final concentration of 100 μM. The inactivation mixture was incubated in the dark for 2 h at 25 °C, unless otherwise noted. The inactivation reaction was stopped by centrifuging the enzyme through a 6-mL column with STEA buffer. The buffer, NBD-F1, was either rapidly frozen and stored in liquid nitrogen, or left at room temperature for immediate use.

Reconstitution—Mixtures typically contained, at pH 7.5, 5.5 mM of ASU protein, 0.3 mg of F1, or NBD-F1/mg of ASU, 0.05 mg of final volume 8.0 ml. The reaction mixture contained 0.5 M sucrose, 20 mM succinate, 2 mg of bovine serum albumin, 75 units of hexokinase, and 10 mM [14C]Pi (46.6 c.p.m.) at pH 8.0. Dithiothreitol was present where indicated at 2 mg/ml. The amount of particle protein added varied from 100 to 700 μg. Reactions were initiated by the addition of ADP at the final concentrations shown. Final volumes were 0.5 ml. Rapid stirring was maintained throughout the entire reaction period. Reactions were quenched after 20 min with 0.5 ml of 1 M perchloric acid.

Glucose 6-phosphate (4 μmol) was added as carrier along with 7.5 mg of activated charcoal to adsorb nucleotides. Following centrifugation to remove charcoal, 2 ml of 1.75 M HCl and 3 ml of isobutanol alcohol/benzene (1:1) were added. Then, while vortexing the sample, 1 ml of 50 mM ammonium molybdate was added. This order of addition minimized the precipitate that tended to form at the solvent interface. The samples were extracted for a total of 6 times with the addition of 1 μmol of F1, chase after the second extraction. After bubbling nitrogen through the lower layer to remove traces of isobutanol alcohol/benzene, the glucose 6-phosphate was hydrolyzed as described with alkaline phosphatase (21). The F1 produced was removed as the phosphomolybdate complex into isobutanol alcohol/benzene (3 extractions). The organic layers were combined and vortexed with 10 ml of 200 mM Tris base which brought the F1 into the aqueous phase. The aqueous fraction was diluted to 100 ml and applied to a Bio-Rad AG 1-X4 100-200 mesh (0.7 × 5 cm) column. The columns were developed by adding 10 ml of water, 4 ml of 33 mM MgCl2 to move the F1 down the column away from molybdate, sufficient 10 mM HCl to bring the sample effluent to pH 2, and then 10 ml of 30 mM HCl to elute the F1. The specific activity of the F1 was measured and the sample lyophilized to dryness. The H2PO4 was diluted and converted to KH2PO4 by the addition of KOH. The sample was rereplicated and the 18O content was measured by mass spectroscopy after converting the phosphate oxygen to CO2 by pyrolysis in the presence of guanidine HCl (22). The dilution of 18O due to the addition of carrier was corrected by comparing the activities of the 32P in the reaction mixture and after workup. Exchange was measured as the loss of enrichment compared to the starting F1.

RESULTS

Properties of Particles Reconstituted with NBD-F1—Mitochondrial ASU particles, prepared as described by Steinmeier and Wang (3), are capable of high rates of ATP synthesis when reconstituted with native F1 and OSCP. Table I compares the activities of several different preparations. When native F1, is added back to these depleted membranes, ATP synthesis rates comparable to those observed in many sub-mitochondrial particle preparations can be obtained; we routinely observed activities well over 150 nmol min⁻¹ mg⁻¹. The rate of ATP hydrolysis seen with these preparations is higher than usually observed with intact particles and probably reflects the absence of the mitochondrial inhibitor protein (23). A rapid P1 = ATP exchange is also evident upon rehydrolysis modified F1, back to the membrane.

As previously reported (3), reconstitution of ASU particles with NBD-F1 (NBD-F1-ASU) yields a preparation capable of high rates of ATP synthesis, however, ATP hydrolysis is inhibited. In this experiment, F1, whose ATPase activity was

| Preparation | ΔATP synthesis* | ΔATP hydrolysis | P1 = ATP exchange |
|-------------|-----------------|-----------------|------------------|
| NBD-F1-ASU | 90              | 180             | 1                |
| NBD-F1-ASU + dithiothreitol | 165           | 2400            | 30               |
| F1- ASU    | 180             | 6600            | 78               |

*Sucinate as substrate.
95% inhibited due to modification, retained 50% of its capacity to synthesize ATP. Comparison of the P\textsubscript{i} = ATP exchange activities of the native and modified system shows that, as seen previously (9), this ATP-dependent reaction is eliminated within the sensitivity of the method. The addition of dithiothreitol, which removes the covalently bound NBD, restores both ATP hydrolysis and the P\textsubscript{i} = ATP exchange. However, full restoration of activity has not been observed by us with either the soluble or membrane-bound NBD-F1.

We explored the possibility that energization of the membranes might be stimulating the removal or rearrangement of the NBD group, rendering it ineffective at inhibiting ATP synthesis. However, a significant stimulation of ATPase activity was still observed upon addition of dithiothreitol after exposure of particles reconstituted with NBD-F1; to state 3 conditions, followed by centrifugation and resuspension (data not shown).

**ADP Requirement for Synthesis with Reconstituted NBD Particles**—We determined if kinetic and exchange characteristics of the ATP-synthesis reaction catalyzed by the modified system were the same as in the native system. Double reciprocal plots of substrate versus velocity data did not yield reliable kinetic constants since they were not linear. However, an estimate of that substrate required for half-maximal velocity (s\textsubscript{0.5}) can be obtained from the data shown in Fig. 1. The results show that modification by NBD-C1 has a pronounced effect on the s\textsubscript{0.5} of ADP observed for ATP synthesis. Half-maximal velocities required ADP concentration in the range of 250-300 \mu M, about 10 times the reported values for ADP in the native system (24, 25).

The low ATPase activity detectable with the NBD-F1-ASU preparation was inhibited approximately 90% by oligomycin. In spite of this, the modified system was able to generate only a very low phosphate potential. This could reflect an increase in the affinity of the modified enzyme for ATP, resulting in product inhibition. Consistent with this view, the addition of dithiothreitol brought about a 2-fold increase in the steady state level of ATP attained when assayed in the absence of hexokinase/glucose. Further assessment was complicated because the dithiothreitol led to the appearance of an oligomycin-insensitive ATPase rate that was far in excess of the initial rate of ATP synthesis (results not shown).

**Oxygen Exchanges by Reconstituted NBD Particles.** We also examined the capacity of the modified system to catalyze the exchange of phosphate oxygens during the synthesis of ATP (intermediate ATP = HOH exchange). This measures the reversibility of the bond-forming step during ATP synthesis. Highly labeled \(^{18}O\) phosphate was added to the medium phosphate pool and exchange was measured by the loss of \(^{18}O\) from the phosphate after incorporation into ATP. The \(\gamma\)-phosphoryl group of ATP was converted to glucose \(\delta\)-phosphate by the addition of hexokinase/glucose to the reaction mixture. This not only trapped the \(\gamma\)-phosphoryl in a form more suitable for analysis, but it prevented the medium P\textsubscript{i} = HOH exchange from occurring (21). Medium exchange would cause significant loss of \(^{18}O\) from the phosphate pool without net ATP synthesis. The inhibition of medium exchange by product removal can be explained by the binding change mechanism for ATP synthesis and is discussed elsewhere (21).

The data in Table II show that P\textsubscript{i} undergoes a substantial loss of \(^{18}O\)-labeled oxygens upon incorporation into ATP by the NBD-modified system. Upon formation of ATP, a maximum of 3 of the original 4 phosphate oxygens could be incorporated, as 1 oxygen is lost in the form of H\textsubscript{2}O. However, as indicated in the table, only about 1 of the original P\textsubscript{i} oxygens remains in the \(\gamma\)-phosphoryl group of ATP when synthesis occurs at 750 \mu M ADP. This loss is taken to mean that the catalytic step of ATP formation undergoes many reversals during each turnover of the modified enzyme.

A significant characteristic of interacting catalytic sites is the increased oxygen exchange per ATP formed as medium ADP concentration is decreased. As shown in Table II, lowering the ADP concentration causes this greater loss of labeled phosphate oxygens during ATP synthesis by the NBD-modified particles. At 5 \mu M ADP, only an average of about 0.3 of the original phosphate oxygens remains in the \(\gamma\)-phosphoryl group. This modulation of catalytic events by substrate concentration is also evident when the exchange is assayed at various ADP concentrations in the presence of dithiothreitol. However, removal of the NBD-group increases the extent of exchange at each of the ADP concentrations tested, indicating that the presence of the inhibitor significantly affects some rate constants involved in catalysis in the direction of ATP synthesis. As anticipated, exchange catalyzed by a preparation reconstituted with native F1 showed a similar loss of phosphate oxygens at the ADP concentrations tested. These levels of exchange observed in the absence of the NBD group closely correspond to those reported by Hackney and Boyer (21) for the intermediate ATP = HOH exchange catalyzed by intact submitochondrial particles. Similar results were obtained with two different particle preparations.

**Extent of ATPase Inactivation by NBD-C1.**—To determine if there might be limited capacity for ATP hydrolysis in the modified system, we assessed the extent of inactivation of the soluble F1-ATPase after prolonged periods of exposure to NBD-C1. Fig. 2 shows a time course for inactivation in the presence of 100 \mu M NBD-C1. The initial rate of loss of ATPase activity is rapid, with a half-time of 11 min. This is consistent with reported inactivation rates in the presence of similar

![Fig. 1. Effect of ADP concentration on the velocity of ATP synthesis catalyzed by particles reconstituted with NBD-F1.](http://www.jbc.org/)
levels of NBD-Cl (3). However, it was noted that at longer periods of inactivation the ATPase activity of the NBD-F₁ levels off. The activity of the enzyme after a 3-h exposure to NBD-Cl is stable at about 0.2 µmol min⁻¹ mg⁻¹. This residual rate of hydrolysis was 85-90% inhibited by the addition of 10 µg of efrapeptin, a potent inhibitor of F₁-ATPase (24), showing that this remaining activity was F₁-dependent. The lack of complete inhibition of the ATPase activity was not due to depletion of the NBD-Cl in the reaction mixture. As noted in Fig. 2, native enzyme readily lost activity when added to the inactivation mixture at 3.5 h. The small decrease in the rate of inactivation may be attributed to dilution of the NBD-Cl upon addition of more F₁.

As observed in the studies of ATP synthesis, the addition of dithiothreitol did not result in full recovery of ATPase activity. Even after relatively short periods of exposure to the NBD-Cl, only 60-70% of the original activity was recoverable. The Spontaneous Reversal of NBD-Cl Inactivation—During our attempts to characterize the residual ATPase activity as shown in Fig. 2, we noted that the covalent modification by NBD-Cl is not stable. Removal of the modified enzyme from buffer containing NBD-Cl resulted in a slow reactivation of the ATPase. Fig. 3 shows a typical time course for this reactivation phenomenon. In this experiment, the F₁ was exposed to 100 µM NBD-Cl for 3 h prior to the removal of the reagent through the use of a centrifuge column. After this treatment, the enzyme had an activity of 0.14 µmol min⁻¹ mg⁻¹. Incubation in the absence of NBD-Cl led to a gradual return of activity, which was greater than 6 µmol min⁻¹ mg⁻¹ after 26 h. All activities were efrapeptin-sensitive. The rate of reactivation was found to be variable and on some occasions more rapid recovery rates were observed. The factors leading to enhanced reactivation were not examined.

If the reactivation resulted from removal of the NBD residue and formation of the original unmodified enzyme, the partially reactivated enzyme should be inhibited by readdition of NBD-Cl. Fig. 3 shows that the addition of 100 µM NBD-Cl to the reactivated enzyme at approximately 4.5 and 25.5 h resulted in a rapid decrease in ATPase activity to levels similar to that observed before the removal of inhibitor. The addition of dithiothreitol to the reinhibited enzyme increased the activity to approximately 60% of the control value. A control sample incubated for 27 h at room temperature in STEA buffer lost less than 5% of its activity.

That removal of the NBD-group was the basis for reactivation was further supported by studies of the intermediate F₁ = HOH exchange accompanying ATP hydrolysis by a preparation of NBD-F₁ which had been incubated for 2-4 h in the absence of NBD-Cl. The cleavage patterns of the 32P phosphate species and the dependence of exchange on medium ATP concentrations were quite similar to those observed with native F₁ or NBD-F₁ in the presence of dithiothreitol (data not shown). Some kinetic characterization of the residual ATPase activity was attempted, but was not deemed useful because of the continuous reactivation which was apparent even during the periods of assay. A preliminary report of modified kinetic characteristics was made prior to our recognition of the spontaneous reactivation phenomenon.⁴

The residual ATPase activity detectable after exposure to NBD-Cl thus appears to reflect an equilibrium level of unmodified enzyme resulting from a balance of the inactivation with a continuous loss of the NBD group from the modified enzyme. The observed residual activity of 0.1 to 0.2% of total corresponds to the level expected with a half-time of inactivation of about 0.2 h and a reactivation reaction that can be estimated roughly to have a half-time of 100-200 h.

**DISCUSSION**

We confirm the interesting report of Steinmeier and Wang (3) showing that NBD-modified F₁, when bound to F₁-depleted particles from the inner mitochondrial membrane, can catalyze rapid ATP synthesis. It does not appear that synthesis results from a reactivation of the inhibited ATPase activity during the time and conditions of the synthesis experiment. Additional experiments, thus, were directed toward finding if cooperative catalytic sites still participated in ATP synthesis by the NBD-modified enzyme, and testing some kinetic properties of ATP synthesis by the modified enzyme.

Pertinent to the interpretations of the NBD-Cl inhibition is the suggestion that the inhibitor binds at the catalytic site. This has been the view adopted by Steinmeier and Wang (3)...

⁴ W. E. Kohlbrener and F. D. Boyer, presented at the First European Bioenergetics Conference, June 29–July 5, 1980, Urbino, Italy.
and subsequently by Ting and Wang (4). However, this view appears to overlook the earlier data of Ferguson et al. (6, 26), which suggested that the tyrosine group modified by the NBD-C1 was not at the catalytic site. More recent observations from Allison’s laboratory (27) suggest that 2 tyrosine residues are distinguishable on each β-subunit, 1 at the catalytic site labeled by FSBA and another remote from the catalytic site, labeled by NBD-C1. Along similar lines, Cross and Nalin (28) did not find any detectable effect of NBD-C1 modification on the stoichiometry of binding of nucleotides or nucleotide analogues to F1.

At least two cooperative sites on one enzyme molecule are required to explain the ADP modulation of intermediate ATP ⇌ HOH exchange. Other results show that native F1 has at least two interacting and apparently identical sites that participate in hydrolysis (29-31). Separate sites or loci for synthesis and hydrolysis would then mean that each ATPase molecule would be required to have four catalytic sites, a possibility that, on the basis of the number of binding sites that interchange nucleotides rapidly with the medium (see Ref. 8 for review), seems quite unlikely.

A more likely interpretation to us is that the F1-ATPase molecule, like CF1-ATPase, is so constructed that, under appropriate conditions, the enzyme can be effectively a one-way catalyzer. Indeed, there are varieties of substrates that induce this property of apparent one-way catalysis. The CF1-ATPase, as present on isolated thylakoid membranes, will catalyze synthesis much more readily than hydrolysis. A more recent and rather striking example comes from Vinogradov’s laboratory (32). They have demonstrated that the membrane-bound F1-ATPase, after incubation with low concentrations of ADP, is capable of catalyzing rapid synthesis of ATP coupled to succinate oxidation without any lag, but regain of the capacity for ATP hydrolysis shows a pronounced lag. The slow and tight ADP binding apparently inhibits hydrolysis but not synthesis capacity. Such results suggest that ligand binding or group modification, other than at the catalytic site, is able to change the characteristics of the catalytic site so as to sharply curtail capacity for hydrolysis.

Differential inhibition of ATP synthesis has also been reported. 3′-O-naphthyl derivatives of ADP and ATP are much more potent inhibitors of oxidative phosphorylation and photophosphorylation than of ATP cleavage (33, 34). Pertinent to these results is the recent finding by Franek and Strotmann (35) that the affinities the chloroplast ATPase has for these analogues changes significantly upon energization, illustrating the important role that energized conformational changes may play in promoting the differential effects that some inhibitors have on synthesis and hydrolysis. An energy-linked decrease in binding affinity can largely explain why the imido-ATP analogue (AMP-P-N-P) is a strong inhibitor of ATP hydrolysis but has little effect on net ATP synthesis (36).

Differential changes in rate constants, which alter maximum velocities in the forward and reverse direction, must be accompanied by shifts in apparent $K_m$ values for substrates and products in accordance with the appropriate Haldane equations. The mitochondrial transhydrogenase illustrates this requirement. In the absence of energization, the reduction of NAD by NADPH is favored. Upon energization, the maximum rate of NADP reduction becomes 5- to 10-fold greater, a change that is accompanied by considerable shifts in $K_m$ values (37).

The results given in Fig. 1 show that the apparent $K_m$ for ADP during net oxidative phosphorylation by particles reconstituted with NBD-F1, is about 10-fold greater than for catalysis with untreated F1. Changes in the $K_m$ for ATP during hydrolysis would also be anticipated, but the continual reactivation of F1, as noted in Fig. 3, did not allow a reasonable estimate of this value.

Similar considerations apply to the changes in relative rates of ATP synthesis and ATP-dependent reactions when alternate nucleotides and analogues are used as substrates. Separate sites for synthesis and hydrolysis have been proposed to explain these data (for discussion, see Refs. 1, 2, 38). However, a differential alteration of rate constants, leading to a shift in maximum velocities and $K_m$ values, would be expected in the presence of different substrates. In experiments that will be reported elsewhere, we have noted that modulation of oxygen exchange still occurs when other nucleotide substrates are used, as anticipated for catalysis by cooperative catalytic sites.

We also note a recent report (39) that purified F1 ATPase will catalyze a very slow incorporation of $^{32}$P into ATP after cold inactivation or treatment with various reagents. These results were interpreted as questioning whether ATP synthesis occurs essentially by the reversal of hydrolysis. We do not feel these observations bear on the question of separate sites for synthesis and hydrolysis. The detection of very small quantities of labeled ATP over a long time period by a sensitive assay could result from some reaction reversal that must occur regardless of the number of sites or mechanism involved.

An interesting characteristic of the NBD-C1 modification of F1-ATPase that was encountered during these studies was the spontaneous reversibility of inhibition. Several groups have reported a loss or transfer of the NBD group from the β-subunit tyrosine under various conditions. Ferguson et al. (40) showed that alkaline pH promotes a transfer of the NBD to an amino group. Irradiation of NBD-F1 with light also promotes this shift (41). Some loss of the bound reagent appears to occur during transfer at high pH, as shown by the slight regain of ATPase activity noted by Lunardi et al. (42). Activity also increases slowly upon incubation of NBD-F1 at lower pH values (see Ref. 40, Fig. 4). As developed under “Results,” such a spontaneous release of the NBD group best accounts for the low level of residual ATPase activity we observed during prolonged incubation of the F1-ATPase with NBD-C1. It appears that an equilibrium is reached between continued inactivation and spontaneous release of the NBD group. The NBD inhibition is known to be rapidly released by thiols (6), probably because of the ready transfer of the NBD moiety to the RS− group. The spontaneous reactivation could result from a slow hydrolysis. The rapid inhibition of recovered activity by readdition of NBD-C1 indicates that the NBD group is actually removed.

In summary, Steinmeier and Wang have uncovered a very interesting differential inhibition of ATPase and ATP synthesis activities of F1-ATPase by reaction with NBD-C1. How this is achieved remains to be explained, but at this stage, the results cannot be taken as evidence of separate catalytic sites or loci for synthesis or hydrolysis.

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W. E. Kohlbrenner and P. D. Boyer, manuscript in preparation.
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W E Kohlbrener and P D Boyer

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