The First Nucleotide Binding Fold of the Cystic Fibrosis Transmembrane Conductance Regulator Can Function as an Active ATPase*

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Cystic fibrosis is caused by mutations in the cell membrane protein called CFTR (cystic fibrosis transmembrane conductance regulator) which functions as a regulated Cl− channel. Although it is known that CFTR contains two nucleotide domains, both of which exhibit the capacity to bind ATP, it has not been demonstrated directly whether one or both domains can function as an active ATPase. To address this question, we have studied the first CFTR nucleotide binding fold (NBF1) in fusion with the maltose-binding protein (MBP), which both stabilizes NBF1 and enhances its solubility. Three different ATPase assays conducted on MBP-NBF1 clearly demonstrate its capacity to catalyze the hydrolysis of ATP. Significantly, the mutations K464H and K464L in the Walker A consensus motif of NBF1 markedly impair its catalytic activity. MBP alone exhibits no ATPase activity and MBP-NBF1 fails to catalyze the release of phosphate from AMP or ADP. The Vmax of ATP hydrolysis (−30 nmol/min/mg of protein) is significant and is markedly inhibited by azide and by the ATP analogs 2'-(3'-O-(2,4,6-trinitrophenyl)-adenosine-5'-triphosphate and adenosine 5'-O-(β,γ-imido)triphosphate. As inherited mutations within NBF1 account for most cases of cystic fibrosis, results reported here are fundamental to our understanding of the molecular basis of the disease.

Cystic fibrosis is the most common autosomal recessive disease in Caucasians affecting approximately 1 in 2000 people in the United States and Canada (1–3). The disease is caused by mutations in the CFTR* protein which impair its normal function as a regulated phosphorylation-dependent Cl− channel in epithelial cells (2, 3). CFTR, which is comprised within a single polypeptide chain of 1480 amino acids, is predicted to fold into five distinct domains, two nucleotide binding folds (NBF1 and NBF2), a regulatory domain (R), and two transmembrane spanning regions (4). Although inherited mutations throughout the CFTR protein are known to cause cystic fibrosis, there is a high frequency of such mutations within or surrounding NBF1 and NBF2, with those within NBF1, particularly if 508, being responsible for most cases of the disease (1–4). Therefore, in order to understand the molecular basis of the disease, it is both necessary and fundamental to elucidate the functions of NBF1 and NBF2.

There is considerable evidence that both phosphorylation of the CFTR protein, and its interaction with ATP, independent of phosphorylation events, are required for optimal functions (5–8). In the latter case, the finding that nonhydrolyzable ATP analogs like AMP-PNP fail to support CFTR function has led to the suggestion that ATP hydrolysis may be required (7, 8). Thus, unlike ATP, AMP-PNP fails to open prephosphorylated Cl− channels in patch clamp experiments using HeLa cells or 3T3 fibroblasts expressing CFTR (7, 8). Moreover, in single channel analysis of artificial lipid planar bilayers reconstituted with human CFTR, AMP-PNP, unlike ATP, failed to support channel activity (9).

Despite the above findings, it remains unresolved whether one or both of the two nucleotide binding folds of CFTR exhibit the capacity to hydrolyze ATP. In earlier reports from this laboratory (10–12), we have demonstrated that peptide segments of both NBF1 and NBF2 containing the Walker A consensus motif (13) can bind TNP-ATP and that this analog can be displaced by ATP. However, no ATP hydrolytic capacity for either the NBF1 or NBF2 segment could be demonstrated (10–12). Purified preparations of the complete NBF1, and of NBF1 in fusion with the maltose-binding protein (MBP-NBF1), have been obtained also (14, 15). Similar to the peptide segments, NBF1 and MBP-NBF1 bind ATP and/or TNP-ATP (14, 15). However, these preparations have not been examined in detail for their capacity to hydrolyze ATP.

Studies described in this report have focused on the MBP-NBF1 fusion protein which was originally overexpressed in Escherichia coli and purified in this laboratory (15). Below, compelling evidence is provided that NBF1 within the MBP-NBF1 complex is an active ATPase.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of the MBP-NBF1 Fusion Protein—Methodology is essentially as described previously (15), with the modifications that 1) the lysis buffer included also per 25 ml, 1000 units protein kinase A, and 5 mM ATP, and 2) the final protein product was stored as an ammonium sulfate pellet. The expression cassette polymerase chain reaction (16, 17) was used to synthesize the NBF1 of CFTR, which included base 1429 (Phe-433) to base 1899 (Ser-589). A pMal-cr1 expression plasmid containing the cDNA for NBF1 in frame with MBP, and under control of the tac promoter, was used to transform TB1 E. coli cells. After induction with isopropyl-1-thio-b-D-galactopyranoside, incubation, lysis, subfractionation, and precipitation with ammonium sulfate, the final pellet was suspended in column buffer (10 mM sodium phosphate, 0.5 mM NaCl, and 1 mM EDTA, pH 7.2) and loaded onto an amylose column. Elution was carried out with 20 mM maltose in column buffer. The purified MBP-NBF1 fusion protein was stored at −80 °C as a 55% ammonium sulfate pellet.

Preparation of Mutant Proteins—Mutations in the lysine residue at position 464 in the primary sequence of NBF1 within the MBP-NBF1 fusion protein were introduced by use of the Mutagenol oligonucleotide

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§ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; NBF1, the first nucleotide binding fold of CFTR; NBF2, the second nucleotide binding fold of CFTR; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 3'-O-(β,γ-imido)triphosphate; TNP-ATP, 2'-(3'-O-(2,4,6-trinitrophenyl)-adenosine-5'-triphosphate.
directed in vitro mutagenesis kit (Bio-Rad). This kit is based on the highly efficient and simple method of Kunkel (18, 19). The E. coli vector, pMal-c2 containing NBF1 DNA was used as template. The mutagenic oligonucleotides used and the respective changes introduced (underlined) are as follows.

K464H: 5’-ACT GGA GCA GGC CAC ACT TCA CTA-3’
K464L: 5’-ACT GGA GCA GGC CTC ACT TCA CTA-3’

The identity of the two base changes was confirmed by DNA sequencing (20). Mutant proteins were overexpressed and purified as described above for wild type MBP-NBF1.

SDS-PAGE—This was carried out by the method of Laemmli (21). Gels were stained with Coomassie dye to determine the degree of overexpression of recombinant wild type and mutant proteins and to estimate their purity.

Assay for ATP Hydrolytic Activity—Three different methods were used to monitor the ATP hydrolytic capacity of wild type and mutant MBP-NBF1 fusion proteins. In the first method, the disappearance of ATP was followed by monitoring the loss of the chemiluminescence signal in an assay system containing luciferin and luciferase. The reaction was performed at 25 °C at pH 6.7 in a 1.2-mI system containing 10 mM KPi, 50 mM KCl, 1 mM MgSO4, 10 μM of luciferin/luciferase (Bio-orient kit, Turku, Finland), and the indicated amounts of ATP, MBP-NBF1, MBP, or mitochondrial F0F1-ATPase in membrane bound form. The specific activity of 3.0 μmol/min/mg of protein and prepared as described previously (22). In the second assay method the formation of ADP coupled to the enzyme pair pyruvate kinase and lactic dehydrogenase was monitored spectrophotometrically at 25 °C by following the decrease in absorbance of NADH at 340 nm. The system contained in a volume of 1 ml, 50 mM Tris-Cl, 5 mM MgCl2, 0.6 mM phosphoenolpyruvate, 0.3 mM NADH, 3.9 units of pyruvate kinase, 1.2 units of lactic dehydrogenase, and the indicated concentrations of ATP and MBP-NBF1. In the third assay, the release of inorganic phosphate was monitored at 25 °C in a 0.5-mI system containing 50 mM Tris-Cl, 5 mM ATP or 5 mM AMP, 5 mM MgCl2, pH 7.4, and the indicated amount of MBP-NBF1. After 75 min, 3.5 ml of water was added to 0.4 ml of the reaction mixture followed by addition of acid molybdate and Elon reagent as described previously (23). After 15 min the optical density at 660 nm was measured.

Determination of Protein—Protein was estimated using the Coomassie plus dye binding assay kit from Pierce.

RESULTS

All studies reported here were carried out with the first nucleotide binding fold (NBF1) of CFTR in fusion with the MBP. The MBP-NBF1 fusion protein was prepared by an earlier method developed in this laboratory (15) and modified as described under “Experimental Procedures.” Fig. 1 summarizes results of experiments in which this procedure was used to obtain highly purified MBP-NBF1 for those studies described in this report. The overexpression of the fusion protein in E. coli under control of the tac promoter after induction with isopropyl-1-thio- β-D-galactopyranoside, its elution from an amylose column, and its electrophoretic profile on SDS-PAGE are illustrated in Fig. 1, A–C, respectively. To assure maximal purity of the product only a single peak fraction was selected from the amylose column, which from SDS-PAGE is estimated to be >95% pure. Purified MBP-NBF1 has the molecular mass expected of ~60 kDa (42 kDa for MBP + 18 kDa for NBF1) and remains stable for several months when stored as a 55% ammonium sulfate pellet at −80 °C.

To determine directly whether MBP-NBF1 has the capacity to catalyze the hydrolysis of ATP, two completely different assays, one based on the disappearance of the substrate ATP and the other on the formation of the product ADP were used initially. The assay based on the disappearance of ATP, which includes the luciferin/luciferase reaction to monitor ATP levels, was employed first. This is one of the most sensitive ATPase assays known, detecting ATP concentrations in the nanomolar to micromolar range via chemiluminescence. The assay is illustrated in Fig. 2A in a control experiment with a known ATPase, the mitochondrial F0F1-ATPase. Here, it can be seen that upon addition of 3.3 μM ATP to a buffered system containing only luciferin and luciferase a light flash (chemiluminescence response) is detected which peaks and levels off to a near zero decay rate. Upon addition of F0F1-ATPase, the chemiluminescence signal rapidly decreases as ATP disappears upon its conversion to ADP and Pi. In Fig. 2B, it can be seen that when

![Fig. 1. Overexpression and purification of MBP-NBF1. A, overexpression in E. coli. Cells expressing MBP-NBF1 were grown as described under “Experimental Procedures.” Where indicated cells were induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C. After dissolving in SDS sample buffer, the whole cell lysate (300- or 75-μI aliquots of 2 × 109 cells/ml for uninduced and induced cells, respectively) was subjected to SDS-PAGE. Molecular size markers are from top to bottom, phosphorylase b (97.4 kDa), bovine albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The arrow indicates the position of overexpressed MBP-NBF1. B, purification of MBP-NBF1. The ammonium sulfate precipitate (see “Experimental Procedures”) from 4 liters of cells was dissolved in a buffer containing 20 mM Hepes, 50 mM NaCl, 1 mM EGTA, and 3 mM MgSO4, pH 7.4, and adsorbed onto a 2.5 × 5-cm amylose column. Elution was carried out at 25 °C in the same buffer containing 20 mM maltose at a flow rate of 1 ml/min. Fractions (3 ml) were collected and monitored at 595 nm using the Coomassie plus dye binding assay. Fraction 4 in this and identical experiments was collected. C, SDS-PAGE of purified MBP-NBF1. Fraction 4 (3.4 μg) was subjected to SDS-PAGE by the method of Laemmli (21). Molecular weight markers are identical to those in A.

![Fig. 2. ATP hydrolytic activity of MBP-NBF1 monitored by following the disappearance of ATP in the luciferin-luciferase chemiluminescence assay. After addition of 3.3 μM ATP, ATPase activity was monitored as a decrease in the chemiluminescence signal following subsequent addition of A, F0F1-ATPase (3 μg); B, MBP-NBF1 (435 μg); and C, MBP (580 μg). In the inset B the ATP hydrolytic rate is plotted versus ATP concentration. Details of the assay procedure are summarized under “Experimental Procedures.” All experiments were repeated at least twice and similar results were obtained.
this experiment is repeated with MBP-NBF1 a slower but significant rate of disappearance of ATP is observed. Moreover, this rate is ATP-dependent (Fig. 2B, inset) and remains linear at the very low concentrations of ATP employed. Finally, in Fig. 2C it can be seen that MBP alone at concentrations higher than MBP-NBF1 has no capacity to catalyze the hydrolysis of ATP, implicating NBF1 as the catalytic unit. (It should be noted that MBP has no nucleotide binding domains and neither binds nor hydrolyzes ATP (24).)

Although the chemiluminescence assay clearly demonstrates that MBP-NBF1 and not MBP alone exhibits the capacity to hydrolyze ATP, the assay is limited in the amount of ATP that can be added. Thus, V_max values must be extrapolated rather than determined directly. For this reason, the second assay noted above, which monitors the formation of ADP coupled to the pyruvate kinase and lactic dehydrogenase reactions, was employed. In this coupled system, the ADP formed in the ATPase reaction is monitored by the decrease in absorption of NADH when pyruvate is converted to lactate. Fig. 3A shows that when increasing amounts of MBP-NBF1 are added to the assay the rate of disappearance of NADH increases. Fig. 3B shows that this increased rate of hydrolysis is a linear function of the amount of MBP-NBF1 added assuring that the increased rate is not due to a non-protein contaminant. In data not presented, MBP alone had no capacity to catalyze ATP hydrolysis as indicated above for the luciferin-luciferase based assay. Results presented in Fig. 3C show that a typical Michaelis-Menten plot is obtained when ATPase activity is plotted versus ATP concentration resulting in K_m and V_max values, respectively, of 0.11 mM and 30 nmol/min/mg of protein (first order rate constant = 0.5 min^{-1}NBF1).

Results from the two different assay procedures described

![Figure 3](image-url) ATP hydrolytic activity of MBP-NBF1 monitored by following the formation of ADP in a "coupled" spectrophotometric assay. ATPase activity was assayed by coupling the release of ADP to the pyruvate kinase, lactic dehydrogenase reactions, and measuring the decrease in absorbance at 340 nm exactly as described under "Experimental Procedures." A, chart tracings showing the absorbance change at 340 nm under conditions where the following amounts of MBP-NBF1 were added to the assay: □, none; ○, 0.24 mg; □, 0.48 mg; ●, 0.75 mg; ■, 0.96 mg; ◆, 1.44 mg. B, initial rates in A plotted versus mg of MBP-NBF1. C, plot of ATPase specific activity of MBP-NBF1 versus ATP concentration.

![Figure 4](image-url) ATPase Function of the First CFTR Nucleotide Binding Fold

Evidence that the ATP hydrolytic capacity of MBP-NBF1 is not the result of a contaminating phosphatase and is a property of NBF1. A, relative capacity of MBP-NBF1 to hydrolyze the release of Pi from ATP and AMP. Phosphate release was monitored colorimetrically exactly as described under "Experimental Procedures" in an assay system containing 5 mM ATP or 5 mM AMP, 5 mM MgCl_2, and 220 μg of MBP-NBF1. (In data not presented, ADP when substituted for ATP, resulted in no phosphate release.) All assays were carried out in duplicate. B, comparison of the purity of wild type and mutant MBP-NBF1 proteins (K464H and K464L). SDS-PAGE by the method of Laemmli (21) was carried out on 4 μg of wild-type MBP-NBF1 and on 5 μg of the indicated mutant proteins. C and D, the effect of the mutations K464H and K464L within NBF1 on the ATP hydrolytic activity of MBP-NBF1. ATP hydrolytic activity was assayed using the coupled spectrophotometric method exactly as described under "Experimental Procedures." In C 550 μg of wild type and 670 μg of mutant protein were included in the assay, whereas in D 270 μg of wild type and 170 μg of mutant protein were included. (Note: specific activity is plotted so that differences in protein concentrations are normalized.) All experiments were repeated, and similar results were obtained.
above demonstrate that the highly purified MBP-NBF1 fraction exhibits the capacity to catalyze ATP hydrolysis. However, they do not rule out the possibility that a contaminating protein, either another cell ATPase or a nonspecific phosphatase, is responsible for the results obtained. For this reason two different experiments were performed. In the first, ATP hydrolysis was monitored by a third assay in which phosphate release is measured. Results presented in Fig. 4A show that in the presence of MBP-NBF1, phosphate is released from ATP as expected, but no phosphate is released from AMP, and in data not presented from ADP; phosphate release from AMP or ADP would be expected if a nonspecific phosphatase were contaminating the preparation. Even more convincing are results presented in Fig. 4, C and D, where it is seen that the mutations, K464H and K464L, in the Walker A nucleotide binding motif (26) as well as by sodium azide. However, in contrast to mitotinhibited by the ATP analogs TNP-ATP (25) and AMP-PNP

relative to other proteins that catalyze the hydrolysis of ATP, the V_{max} rate of MBP-NBF1 of ~30 nmol/min/mg is slightly higher than that observed for chaperones (32) but considerably lower than that observed for the CFTR related MDR-protein (multidrug resistance protein) (33, 34). The much lower rate characteristic of NBF1 of CFTR may reflect a role in using ATP hydrolysis to only open Cl^- channels, as opposed to transporting a drug, as in the case of MDR. However, it remains possible that the activity observed here for CFTR NBF1 may be a minimal value and that in vivo hitherto unknown substrates are transported by CFTR, in which case a higher ATP hydrolytic rate may be required. Significantly, it has been reported recently that in addition to Cl^-, CFTR can also translocate ATP (27, 35).

**DISCUSSION**

This is the first report to provide direct evidence in vitro that the first nucleotide binding fold (NBF1) of the CFTR protein can function as an active ATPase. Assays conducted on the MBP-NBF1 fusion protein monitored either disappearance of the substrate ATP (Fig. 2B) or appearance of the products ADP (Fig. 3) and P_i (Fig. 4A) leaving no doubt that the purified protein catalyzes the hydrolysis of ATP. The additional experimental results demonstrating that MBP alone has no catalytic capacity (Fig. 2C) and that mutations (K464H and K464L) within the Walker nucleotide binding motif GX_KK1 markedly inhibit ATPase activity (Fig. 4, C and D) localize the catalytic site to NBF1. These results do not exclude the possibility that within intact CFTR the NBF1 domain may require stabilizing interactions with another domain. Thus, it should be noted that in the F_1, moiety of the F_0,F_1-ATPase, the b subunit, although containing all residues essential for catalysis, is itself not catalytic (29, 30). Rather, interaction with the noncatalytic a subunit is required to elicit ATP hydrolysis (31).

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**Fig. 5. The effect of known ATPase modulators on the ATPase activity of MBP-NBF1.** ATPase activity was assayed by coupling the release of ADP to the pyruvate kinase, lactic dehydrogenase reactions, and measuring the decrease in absorbance at 340 nm exactly as described under “Experimental Procedures.” In all cases controls were run using ADP instead of MBP-NBF1 and ATP to assure that the modulators tested had no effect on the coupled enzymes. The indicated concentrations of modulators are shown. MBP-NBF1 (1.3 mg) was present in all assays, and all assays were carried out in duplicate. Experiments were repeated at least once with all modulators.