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A recombinant polypeptide model of the second nucleotide-binding fold of the cystic fibrosis transmembrane conductance regulator functions as an active ATPase, GTPase and adenylate kinase

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Abstract CFTR–NBF-2 expressed and purified in fusion with the maltose-binding protein was shown to catalyse the reaction ATP → ADP+P_i by three different assays, monitoring ATP turnover, formation of ADP and release of P_i (K_m 86 μM, rate constant 0.37 min^{-1}). The reaction product ADP inhibits this ATPase activity. In a similar manner the hydrolysis of GTP to GDP and P_i was demonstrated (K_m 40 μM, rate constant 0.29 min^{-1}). In the presence of AMP the ATPase reaction was superseded by the formation of two ADP from ATP and AMP. As typical for adenylate kinases a distinct AMP-binding site could be verified for CFTR–NBF-2 by the inability of TNP–ATP and AMP to compete for binding. All three enzymatic activities were inhibited by the symmetric double-substrate-mimicking inhibitor Ap_5A. As NBF-2 plays a central role in CFTR channel opening and closing the results reported here are fundamental in understanding mechanisms of CFTR channel activity regulation.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; ATPase activity; GTPase activity; Adenylate kinase

1. Introduction

The most common fatal autosomal recessive genetic disease affecting Caucasian populations, cystic fibrosis [1], is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR). This protein has been demonstrated to be a membrane chloride channel [2,3]. It has been predicted to be composed of two transmembrane domains of six transmembrane spanning segments each, that are both followed by a nucleotide-binding fold, and a fifth domain — a unique regulatory (R) domain — linking both halves of the protein [4]. Both CFTR–NBFs were identified by sequence homologies to adenylate kinase and to other NBFs of the ATP-binding cassette family [5] including the occurrence of Walker A and B consensus sequences [6] present in the nucleotide-binding pockets of many proteins. In addition, a similarity in the amino acid sequences of CFTR–NBFs and the sequences of heterotrimeric G-proteins is evident [7]. CFTR channel opening requires both phosphorylation by cAMP-dependent protein kinases [8] and the presence of cytosolic hydrolyzable MgATP [9,10]. Since single mutations in both CFTR–NBFs can impair the rate of phosphorylated channels to open, the activating effect of MgATP seems to be mediated by both NBFs [11–14]. Cytosolic ADP [11] as well as AMP [10] have been shown to act as inhibitors of channel activity and evidence has been presented from electrophysiological data that inhibition by ADP is a function of NBF-2 [11]. Furthermore, it could be demonstrated that the closure of CFTR channels previously opened by ATP is coupled to ATP hydrolysis [15] providing the energy for the transition from a longer-lived, lower conductance open state to a shorter-lived, higher conductance open state, an event that is subsequently followed by transition to the closed state [16]. Indirect evidence that ATP hydrolysis closing the channel takes place at NBF-2 has been gained from the observation that single amino acid substitutions of highly conserved residues within the Walker motifs of NBF-2 produce channels with prolonged bursts of activity [13,14] by inhibiting the transition to the shorter-lived second open state [16]. In contrast to NBF-1, that appears to be only involved in channel opening, NBF-2 seems to regulate both channel opening and closing [11,13,14,16] and a CFTR construct with deleted NBF-2 has been shown to be not functional despite being properly localized in the plasma membrane [17]. In order to achieve a better understanding of the individual regulatory events that are required for CFTR channel opening and closing and of the effects of CF causing mutations the enzymatic properties of isolated CFTR domains have to be studied and related to known electrophysiological data. We recently demonstrated that a recombinant CFTR–NBF-2 polypeptide is able to bind all three adenine nucleotides with high affinity [18] and that there exists a common binding site for ATP and GTP [19]. Up to now — in contrast to a recombinant model of CFTR–NBF-1 exhibiting not only ATP-binding [20] but

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Abbreviations: ADK, adenylate kinase; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; Ap_5A, adenosine(5′)pentaphospho(5′)adenosine; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; ΔF, fluorescence enhancement; ΔF_m, maximal fluorescence enhancement (asymptotic maximum of a fitted curve); IPTG, isopropyl-β-D-thiogalactoside; LDH, lactic dehydrogenase; MBP, maltose-binding protein; MBP–NBF-2, maltose-binding protein–CFTR–NBF-2 fusion protein; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; NBF, nucleotide-binding fold; PK, pyruvate kinase; TNP–ATP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidine) adenosine 5′-triphosphate

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also ATPase activity [21] — no experimental data have been published directly demonstrating enzymatic activity of NBF-2.

In the present study we give the first direct experimental evidence that a recombinant NBF-2 polypeptide possesses a unique combination of enzymatic activities. We demonstrate that this domain is not only an active ATPase and GTPase but also an adenylate kinase with an additional AMP-binding site different to the ATP/GTP-binding site.

2. Materials and methods

2.1. Overexpression and purification of MBP–NBF-2 fusion proteins

The CFTR cDNA sequence from base 3754 to base 4331 [4] coding for the CFTR protein sequence from Gly-1208 to Leu-1399 (numbering according to Ref. [4]), that encloses the whole predicted NBF-2, was amplified by PCR from the expression vector pGEX-NBF-2 [18] using the oligonucleotides 5′-CTCGGGAATTCGGCCAAATGACT-GTCAAAGATC-3′ and 5′-GAGCCTGCAGTTATCAGAGAATT-ACTGTGCAATCAGC-3′ as primers. PCR amplified products were digested with *Eco*RI and *Pst*I restriction enzymes and finally ligated into the *Eco*RI/*Pst*I-cut pMAL™-c2 expression vector [22,23] (New England Biolabs®) in frame with the coding sequence for MBP according to standard procedures [24]. Plasmids of transformants showing positive inducible expression of a fusion protein of the appropriate size were isolated and both strands of the inserted DNA were completely sequenced using an Applied Biosystems automated sequencer to exclude amino acid substitutions in the CFTR–NBF-2 part of the expressed fusion protein due to fidelity constraints of the PCR reaction.

Overexpression of MBP–NBF-2 and preparing soluble *E. coli* proteins were performed as described before [18]. The latter were loaded on a column containing amylose resin previously equilibrated with the column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). After washing with 20 column volumes of column buffer the fusion protein was eluted with 10 mM maltose dissolved in the column buffer. In a final step the isolated fusion protein was extensively dialyzed over 20 h against a 1000-fold excess of 50 mM Tris-HCl, pH 7.5, that was exchanged 3 times. The amount of fusion protein was quantified spectrophotometrically (A_{280}) using a molar absorption coefficient of 64 720 M^{-1} cm^{-1}.

2.2. HPLC, amino acid sequence analysis and mass spectroscopy

HPLC, Lys-C digestion and amino acid sequencing were done as described [18] with an amount of 400 pmol of purified protein. Masses were determined with a tandem quadrupole instrument API III equipped with an atmospheric pressure ionization source (Sciex, Thornhill, Ontario, Canada). The instrument *m/z* scale was calibrated with the ammonium adduct ions of polypropylene glycol. The average molecular mass values of the Lys-C digestion products were calculated from the *m/z* peaks in the charge distribution profiles of the multiply charged ions [25].

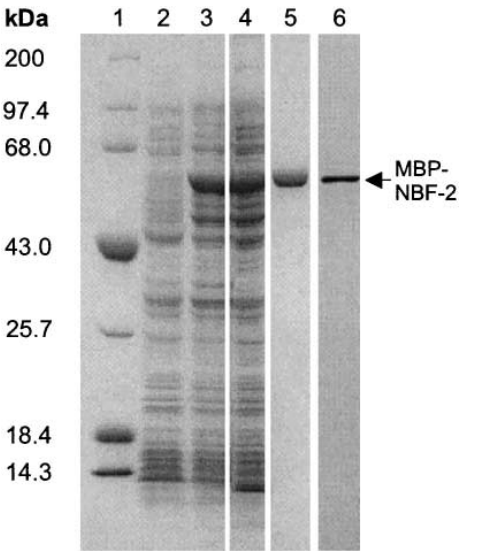

Fig. 1. Analysis by SDS-PAGE of overexpressed and purified MBP–NBF-2. Lane 1, molecular size markers are myosin H-chain (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68.0 kDa), ovalbumin (43.0 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa). Lane 2, whole-cell lysate of uninduced *E. coli* (3×10^8 cells). Lane 3, whole-cell lysate of *E. coli* (3×10^8 cells) after IPTG induction. Lane 4, soluble proteins of the induced *E. coli* of lane 3, which were subsequently further purified by affinity chromatography. Lane 5, MBP–NBF-2 (11.5 μg) purified by amylose affinity chromatography (Coomassie-stained 12.5% SDS-PAA-gel). Lane 6, 1.5 μg of the purified MBP–NBF-2 run on a silver-stained 12.5% PAA-gel.

2.3. Assays for detecting and measuring enzymatic activity

The consumption of ATP in the presence of MBP–NBF-2 was detected with the ATP Bioluminescence Assay Kit CLS II (Boehringer-Mannheim) at 25°C. Luciferase reagent (100 μl) from the kit (containing D-luciferin and luciferase from *Photinus pyralis*) were mixed with 100 μl of a 2×10^{-6} M MgATP solution. After 5 min the indicated amount of MBP–NBF-2 or the control reagents, respectively, was added and stirred for 30 s. The flow of photons directly proportional to the ATP concentration in the assay was monitored continuously at 562 nm via the emission pathway of a SPEX FluoroMax fluorometer (slit width 7.0 mm).

The assays to detect nascent ADP and GDP, respectively, were

Table 1
Characterization of the Lys-C digestion products of the recombinant CFTR–NBF-2 by mass spectroscopy and amino acid sequencing

Fragment	Amino acid position	Theoretical mass (MH^+)	Determined mass (MH^+)	N-terminal sequencing
1 (linker)	−28– 6	3697.85 (av)	3697.8 (av)	−28 to −22
2	7– 11	547.31	n.d.	n.d.
3	12– 43	3280.67 (av)	3279.6 (av)	n.d.
4	44– 77	3920.46 (av)	n.d.	44– 46
5	78– 85	859.5	859.6	78– 80
6	86– 95	1201.67	1201.6	86– 92
7	96–110	1950.89	1951.4	n.d.
8	111–127	1843.99	n.d.	111–114
9	128–144	1795.92	1796.2	n.d.
10	145–156	1348.74	1348.0	145–150
11	157–158	218.15	n.d.	n.d.
12	159–182	2806.33 (av)	2805.4 (av)	n.d.
13	183–192	1080.54	1080.6	183–187

Amino acid positions of the 192 CFTR amino acids containing NBF-2 were numbered with positive numbers (positions 1–192 correspond to amino acids 1208–1399 of CFTR [4]). Negative numbers refer to the MBP fusion partner with −1 being the C-terminus of the linker motif. Masses above 2000 are given as average (av) and not as monoisotopic masses. N-terminal sequencing was performed where indicated (n.d. = not determined).

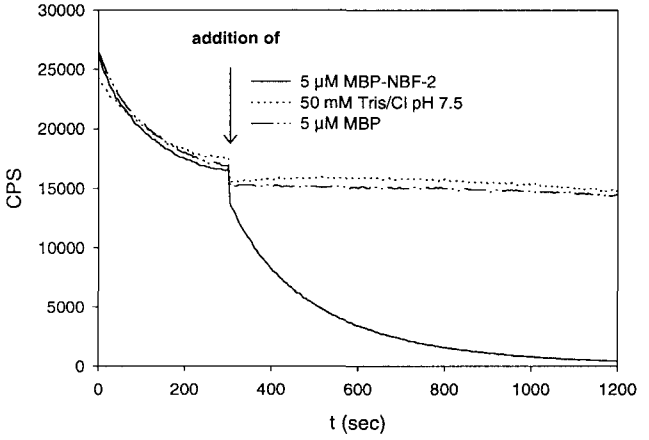

Fig. 2. ATPase activity of MBP–NBF-2 monitored by following the disappearance of ATP in the luciferin–luciferase chemiluminescence assay. No decrease in the chemiluminescence signal measured as counts per second (cps) at 562 nm was recognized after adding MBP or Tris-buffer. All experiments were repeated at least twice and similar results were obtained.

performed as described [18,19] with the following alterations of assay buffer composition; the indicated amount of ATP or GTP, respectively, and the protein (2–4 µM) were incubated together with 3 mM phospho*enol*pyruvate, 0.27 mM NADH, 6.5 mM magnesium acetate, 34 mM KCl, 4.17 µg (1.9 U) lactate dehydrogenase and 12.5 µg (1.9 U) pyruvate kinase (Boehringer-Mannheim) in 50 mM Tris-HCl, pH 7.5, in a total volume of 360 µl at 25°C. The ADP detection assay containing 1 mM ATP and no ATPase was also used to determine AMP concentrations. For this purpose 5 µg (1.8 U) adenylate kinase from hog muscle (Boehringer-Mannheim) was added. The addition of AMP resulted in formation of two ADP per molecule AMP leading to a decrease in A_{340} proportional to the concentration of AMP. The same amount of adenylate kinase was added if traces of AMP present in the enzyme and ATP preparation had to be consumed in order to assume c_{AMP} to be zero.

The release of inorganic phosphate was measured according a method described by Webb [26] using the EnzChek™ Phosphate Assay Kit (Molecular Probes). The indicated amount of the protein of interest was incubated together with the specified nucleotides at 25°C in a 360-µl system of 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM sodium azide, 0.2 mM MESG substrate and 1 U of purine nucleoside phosphorylase. $\Delta A_{360}/\Delta t$ was monitored, which is a linear function of the rate of phosphate release, since in the presence of P_i purine nucleoside phosphorylase catalyses phosphorolysis of MESG to ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine resulting in an increase of absorbance at 360 nm (change in extinction coefficient at pH 7.6: 11 000 $M^{-1} \times cm^{-1}$) [26]. Without protein and nucleotides this assay was used to determine P_i concentrations of sample aliquots as described by Webb [26].

2.4. Nucleotide-binding studies

Fluorescence measurements recording the binding of TNP–ATP to MBP–NBF-2 and competition of this binding by unlabelled nucleotides, corrections for unspecific binding of TNP–ATP and for inner filter effect, and fitting of monophasic binding curves to the corrected fluorescence data were done according to the methods described in [18,19] with a protein concentration of 6.75 µM.

3. Results and discussion

The second nucleotide-binding fold (NBF-2) of CFTR was expressed in fusion with the maltose-binding protein (MBP) in *E. coli* as described in Section 2. In contrast to our earlier studies [18,19] the initial washing step of the purification protocol with PBS containing 1% Triton X-100 was omitted since we could show that this extensive exposure to Triton X-100 was the main reason for our previous failure to demonstrate ATPase and GTPase activity [18,19]. As shown in Fig. 1, the expression of a 64.2 kDa protein (42.71 kDa for MBP +21.49 kDa for NBF-2) under the control of the *tac* promotor after induction with IPTG is strikingly evident. Most of the overexpressed protein was found in the soluble protein fraction. The effectiveness of the employed purification procedure resulting in 5–6 mg of purified NBF-2 fusion protein per liter of *E. coli* culture is illustrated in Fig. 1. Silver staining revealed no other proteins larger than the cutoff molecular mass of the gel. HPLC and N-terminal sequence analysis detected only a single N-terminal sequence in agreement with the seven N-terminal residues of the MBP fusion partner. In addition, the masses of nine internal protein fragments expected to cover 70% of the NBF-2 sequence were determined and found to be in excellent agreement with their theoretical masses. For

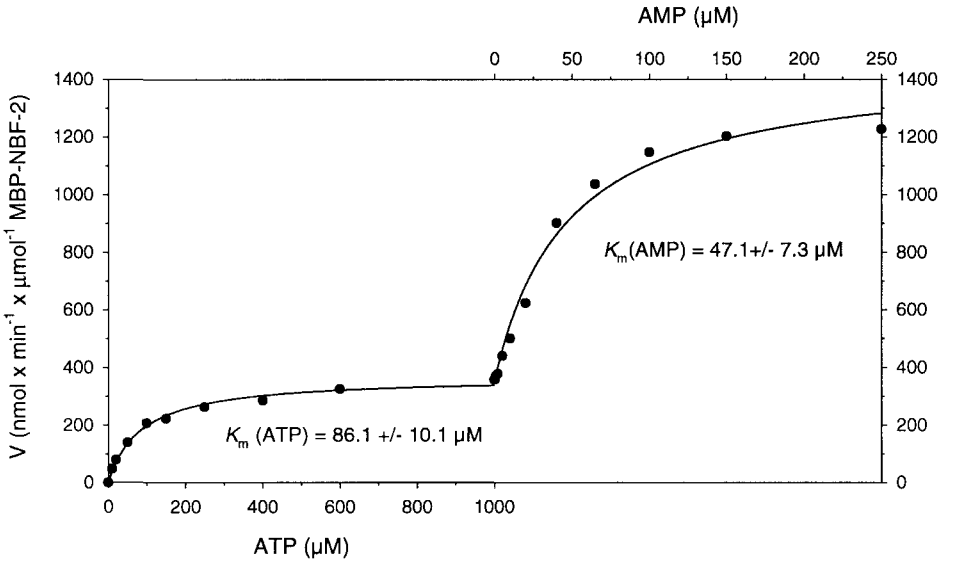

Fig. 3. Enzymatic activity of MBP–NBF-2 monitored by following the formation of ADP in a 'coupled' (PK/LDH) ATP-regenerating enzymatic assay. Left panel: no AMP was added. Right panel: the effect of increasing amounts of AMP at a constant concentration of 1 mM ATP on ADP formation is illustrated. This experiment was repeated 3 times with similar results.

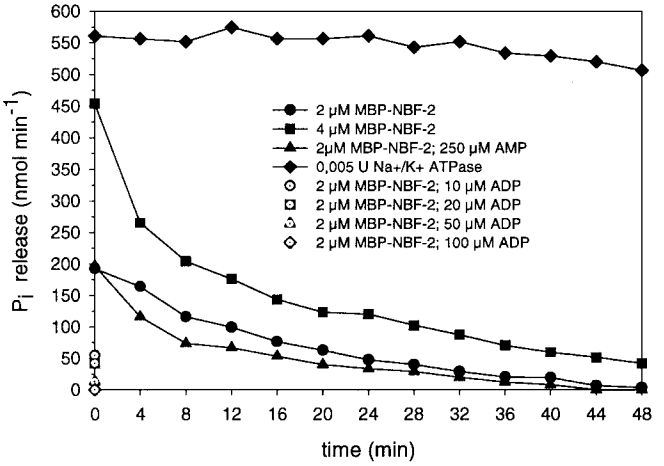

Fig. 4. ATPase activity of MBP–NBF-2 and of (Na^+,K^+)ATPase at 1 mM ATP monitored by following the release of P_i, and the influence of AMP and ADP on ATP hydrolysis by MBP–NBF-2. All experiments were repeated, and similar results were obtained.

seven of these Lys-C digestion products N-terminal sequencing was performed and no aberration to the expected NBF-2 sequence was detected (Table 1). Based on these experiments we estimate the protein employed in the studies reported here to be more than 95% pure.

In order to determine directly whether MBP–NBF-2 has the capacity to catalyse the hydrolysis of ATP, three completely different assays were used: one to detect the disappearance of the substrate ATP, one to detect nascent ADP and the third to detect nascent P_i. Fig. 2 illustrates the first assay based on the disappearance of ATP, which includes the luciferin/luciferase reaction to monitor ATP levels. Upon addition of ATP to the luciferin/luciferase system, a chemiluminescence response is detected that levels off to a near zero decay rate and remains stable over more than 30 min. In contrast to a control experiment with the isolated fusion partner MBP, addition of the NBF-2 fusion protein causes a rapid decrease of the chemiluminescence signal indicating the disappearance of ATP. In order to prove the turnover of ATP to ADP and P_i and to determine V_{max} of this reaction, which has not been directly possible in the bioluminescence assay since this assay is limited in the amount of ATP that can be added, we used a second, ATP-regenerating assay that couples the formation of ADP via the pyruvate kinase and lactic dehydrogenase reactions to an equimolar decrease in NADH that can be monitored by photometric analysis. Fig. 3 summarizes our experiments with different concentrations of MBP–NBF-2 and increasing concentrations of ATP. In data not presented here, MBP alone as well as a fusion protein of MBP and the α-subunit of β-galactosidase expressed and purified under identical conditions as MBP–NBF-2 failed to cause any decrease in A_{340} that would indicate nascent ADP. In contrast, the ATP hydrolysis rate of MBP–NBF-2 was found to be linear with the employed protein concentration, and a typical Michaelis-Menten plot is obtained when ATPase activity is plotted versus ATP concentration, resulting in a K_m of 86 μM and a rate constant of 0.37 min^{-1}. From the experimental conditions the specific activity of the preparation was determined to be 5.7×10^{-3} U/mg. Using the same assay, K_m and first-order rate constant of (Na^+,K^+)ATPase (from dog kidney, specific activity 1.2 U/mg, Sigma) were determined to be 191 μM and 12.71 min^{-1}, respectively.

Our previous studies indicated a surprisingly high affinity of NBF-2 for AMP [18] which has been shown to exercise a strong inhibitory effect on CFTR channels [10]. Since there is evidence that ATP consuming enzymatic activity of NBF-2 mediates channel closing [13,14], we were interested in the influence of AMP on the catalytic activity of NBF-2. As illustrated in Fig. 3, the addition of AMP causes a dramatic increase in the formation of ADP. There is also a Michaelis-Menten plot obtained when the rate of ADP formation is plotted versus AMP concentration at a constant saturating concentration of ATP. This second plot was characterized by a K_m of 47.1 μM and a rate constant of 1.47 min^{-1}. In both cases enzymatic activity could readily be inhibited by AMP-PNP (K_i = 158 μM without and 135 μM in the presence of 30 μM AMP, respectively). AMP had no effect on the catalytic activity of (Na^+,K^+)ATPase (data not shown).

Two possible hypotheses could be delineated from the observed results. First, AMP could be an allosteric effector that enhances hydrolysis of ATP to ADP and P_i or, second, AMP could be a second substrate of NBF-2 as it would be the case

Table 2
Turnover of AMP, ADP and P_i in dependence of increasing AMP start concentrations

MBP–NBF 2 (μM)	AMP start conc. (μM)	AMP end conc. (μM)	AMP turnover (μM)	ADP released (μM)	P_i released (μM)
2	–	–	–	14.67	12.87
2	30	11.06	18.94	57.88	8.73
2	60	28.89	31.11	72.72	6.63
2	100	48.42	51.58	99.43	1.53

The ATP concentration of 1 mM was high enough to remain practically constant. All reactions were carried out for 48 min and monitored in the 'coupled' PK/LDH-containing enzymatic assay in order to avoid inhibition of the reactions by nascent ADP and to determine the amount of released ADP directly. Aliquots taken at begin and after stopping the reactions by quick freezing were used to determine the amounts of consumed AMP and of released P_i as described in Section 2. All experiments were repeated at least once with similar results.

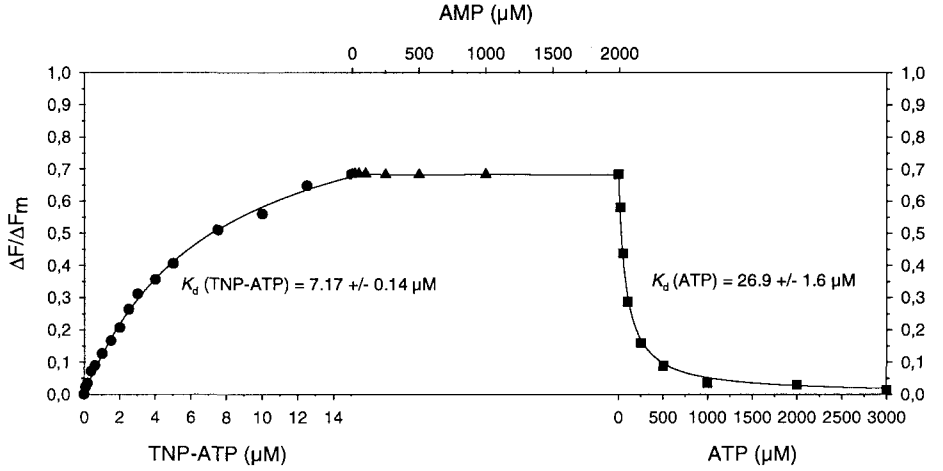

Fig. 5. Evidence for two distinct binding sites for ATP and AMP. The fluorescence enhancement (ΔF) at 545 nm of excited (408 nm) TNP–ATP due to binding to NBF-2, normalized referring to the asymptotic maximum of the fitted curve (ΔF_m), is illustrated. AMP was not able to displace TNP–ATP since it could not diminish its fluorescence enhancement even in a 150-fold excess. This fluorescence signal was indeed due to nucleotide binding since it was readily de-enhancable by unlabelled ATP, an experiment performed as control. From this competition experiment a similar corrected K_d value (26.9 ± 1.6 μM SEM, three experiments) for ATP could be determined as previously described for NBF-2 fused to glutathione-*S*-transferase (37 μM [18]).

in the adenylate kinase reaction where two molecules of ADP result from the transfer of the γ-phosphate of ATP to AMP. In order to further distinguish between these two possibilities a third assay was performed, in which the release of inorganic phosphate is measured continuously. Results presented in Fig. 4 show that phosphate is released in the presence of ATP and MBP–NBF-2. The rate of phosphate release is a linear function of the amount of added NBF-2 fusion protein. No phosphate release was obtained in control experiments using MBP or the above mentioned fusion protein of MBP and the α-subunit of β-galactosidase instead of MBP–NBF-2 (data not shown). In contrast to (Na^+,K^+)ATPase the rate of phosphate release catalysed by the NBF-2 fusion protein decreased rapidly over time indicating inhibition by a reaction product. Since nascent P_i is immediately consumed by the purine ribonucleoside phosphorylase reaction it must be the nascent ADP that causes the inhibition — a phenomenon that could not be observed in the coupled PK/LDH assay which regenerates ATP from ADP. Further confirmation of the inhibitory effect of ADP was obtained by pre-incubating the assay with increasing concentrations of ADP resulting in a strong, concentration-dependent decrease of the initial rate of phosphate release (Fig. 4).

In the presence of AMP, phosphate release from ATP was significantly reduced (Fig. 4) which rules out the hypothesis that AMP could be an allosteric effector of the ATPase reaction but favors the view that both the ATPase and an adenylate kinase reaction are catalysed by NBF-2 in competition and that the portion of ADP resulting from simple ATP hydrolysis rapidly decreases with increasing amounts of AMP present in the assay. In order to prove this hypothesis we determined the amounts of both consumed AMP and of the nascent products ADP and P_i under conditions of increasing AMP concentrations at an ATP concentration high enough to saturate NBF-2 completely and to remain practically constant over the time of reaction. Results given in Table 2 prove that AMP is a substrate of NBF-2 and that per molecule of AMP two molecules of ADP are released. This adenylate kinase reaction (ATP+AMP ↔ ADP) is in competition with an ATPase reaction that predominates only at low AMP concentrations, a fact that rules out as well the possibility that a contaminating protein, either another ATPase or a non-specific phosphatase, is responsible for the observed ATP hydrolysis.

Taking our previous results, that both ATP and AMP bind to NBF-2 [18], together with the observation that both nucleotides are substrates in the adenylate kinase reaction of NBF-2 implies that there must exist two distinct binding sites for ATP and AMP as it has been established for adenylate kinases [27,28]. To verify this assumption we tried to compete bound TNP–ATP by unlabelled AMP which should be readily possible with regard to their affinities to NBF-2 [18], if both

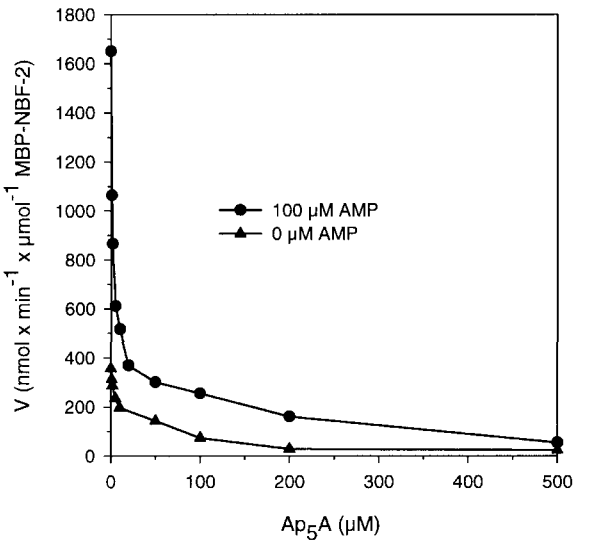

Fig. 6. Inhibition of ATPase (▲) and ADK (●) activity of the NBF-2 fusion protein by Ap_5A. Enzymatic activity at 1 mM ATP was monitored by following the formation of ADP in the 'coupled' (PK/LDH) ATP-regenerating enzymatic assay. Controls with ADP instead of MBP–NBF-2 and ATP assured that Ap_5A had no effect on the assay enzymes. All experiments were repeated at least once, and similar results were obtained.

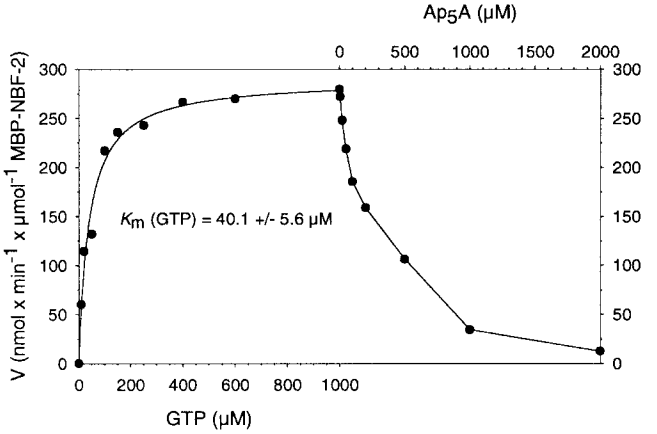

Fig. 7. GTP hydrolytic activity of the NBF-2 fusion protein monitored by following the formation of GDP in the 'coupled' PK/LDH containing assay. Ap_5A could readily inhibit GTPase activity of NBF-2. This experiment was repeated once with similar results.

molecules bind to the same site. As shown in Fig. 5, AMP failed to replace bound TNP–ATP, which clearly indicates two distinct binding sites.

Adenylate kinases are readily inhibited by adenosine(5')-pentaphospho(5')adenosine (Ap_5A) that mimics the two substrates ATP and AMP since it needs a three- and a monophosphate position for binding [29,30]. As shown in Fig. 6 ADK activity of the recombinant NBF-2 is strongly inhibited by Ap_5A. In addition, the mere ATPase activity seen in the absence of AMP is likewise inhibited by Ap_5A (Fig. 6). In data not presented, Ap_5A had no capacity to inhibit the enzymatic activity of (Na^+,K^+)ATPase.

We have previously shown for NBF-2 that ATP and GTP bind to the same site. In order to reveal any intrinsic GTPase activity of the purified recombinant NBF-2 we employed a coupled GTP regenerating enzyme system detecting nascent GDP. In contrast to MBP or the α-subunit of β-galactosidase fused to MBP and expressed in *E. coli*, which did not show any enzymatic activity, the MBP–NBF-2 fusion protein demonstrated a rate of hydrolysis linearly increasing with the amount of protein in the assay. Fig. 7 shows a Michaelis-Menten plot obtained by plotting GTPase activity versus GTP concentration with a K_m value of 40 μM and a first-order rate constant of 0.29 min^{-1}. Addition of AMP or GMP did not influence the observed enzymatic activity (data not shown). The GTPase activity of NBF-2 could be completely inhibited by Ap_5A (Fig. 7).

The fact that all three observed enzymatic activities are inhibited by Ap_5A while the control ATPase, the (Na^+,K^+)ATPase, is not, is an additional very strong argument that the ATPase and GTPase activities described here are properties of the same molecule exhibiting ADK activity and not contributions of different, not detected contaminants of the purified MBP–NBF-2 fraction.

This is the first report to provide direct evidence in vitro that the second nucleotide-binding fold of CFTR can function as an active ATPase, GTPase and adenylate kinase, which represents a unique combination of enzymatic properties. Beside the ATP/GTP-binding site a distinct AMP-binding site exists. With increasing concentrations of AMP in the presence of ATP the ADK activity quickly surpasses the ATPase activity. ADP inhibits the ATPase activity. It is tempting to speculate that the inhibitory effect of ADP on CFTR channel gating [11] is the consequence of interference with activating ATP hydrolysis. The strong inhibition of CFTR Cl^- currents in the presence of cytoplasmic AMP [10] could be the result of high ADP formation at NBF-2 that consecutively inhibits ATP hydrolysis.

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