

Expression and functional properties of the second predicted nucleotide binding fold of the cystic fibrosis transmembrane conductance regulator fused to glutathione-S-transferase

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Received 1 March 1995; Revised version received 14 March 1995

Abstract CFTR-NBF-2 was expressed in *Escherichia coli* in fusion with glutathione-S-transferase, the soluble portion was purified and identified as a structured protein by its CD spectrum. Association reactions of the recombinant NBF-2 with adenine nucleotides were monitored qualitatively by demonstrating its ability to bind specifically to ATP-, ADP- and AMP-affinity agarose and quantitatively by recording the fluorescence enhancement of excited trinitrophenol (TNP)-labelled adenine nucleotides occurring as a result of binding to NBF-2. Best-fit monophasic binding curves to the fluorescence data indicated K_d values of 22 μ M for TNP-ATP, 39 μ M for TNP-ADP and 2.1 μ M for TNP-AMP. The corrected K_d values for unlabelled adenine nucleotides competing with the fluorophores were determined to be 37 μ M for ATP, 92 μ M for ADP and 12 μ M for AMP. The recombinant NBF-2 did not show any hydrolytic activity on ATP (detection limit 0.001 s⁻¹). Our findings support the concept of a central role of NBF-2 in CFTR activity regulation acting as an allosteric switch between channel opening and closing and give the first experimental evidence that the channel inhibitor AMP could act via NBF-2.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator (CFTR); Nucleotide binding; Fluorescence enhancement; Inner filter effect; ATPase activity

1. Introduction

Cystic fibrosis is the most common fatal autosomal recessive genetic disease affecting Caucasian populations [1]. The disease results from mutations in the gene that codes for the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, which has been predicted to be composed of two motifs of six transmembrane spanning segments followed by a nucleotide binding fold that are separated by a unique regula-

tory (R) domain [2]. Both CFTR nucleotide binding folds, NBF-1 and NBF-2, were identified by the occurrence of Walker A and B consensus sequences [3] characterizing NBFs in the ATP-binding cassette family [4]. CFTR channel activation requires both phosphorylation by cAMP-dependent protein kinases [5] and the presence of cytosolic hydrolyzable MgATP in millimolar concentrations [6,7]. Cytosolic ADP [8] as well as AMP [7] have been shown to act as inhibitors of channel activity. Electrophysiological data suggest that for channel activation by ATP both NBF-1 and NBF-2 are required [9], while channel inhibition by ADP seems to be a function of NBF-2 [8]. The latter domain is coded by exons 19–23 of the CFTR gene and defined as the CFTR sequence from Y1219 to R1386 [2]. It has recently been reported that a 51-amino acid synthetic peptide segment from NBF-2 (E1228 to T1278) is able to bind TNP-ATP and that comparatively high concentrations of ATP can displace TNP-ATP with a K_d of 0.46 mM. The capacity to bind TNP-ADP and TNP-AMP has been noted to be much less [10]. Furthermore there is evidence that the NBF-2 mutation G1349D corresponding to the CF-associated replacement of glycine 551 with aspartic acid in NBF-1 disrupts TNP-ATP and [γ -³²P]2-azido-ATP binding of a recombinant NBF-2 protein recovered from inclusion bodies [11]. Up to now no experimental data have been published describing the interactions of all three unlabelled adenine nucleotides with NBF-2.

In the present study we describe the expression, purification and characterization of a soluble recombinant protein comprising the entire predicted second nucleotide binding fold of CFTR fused to glutathione-S-transferase which shows non-hydrolytic interactions of high affinity with all three adenine nucleotides. First experimental evidence that the channel inhibitor AMP could act via NBF-2 is presented.

2. Experimental

2.1. Construction of the expression vector pGEX-NBF-2

RNA of the CFTR expressing [12] human colonic adenocarcinoma cell line CaCo-2 (American Type Cell Culture Company; Rockville, MD) was isolated by single step acid guanidinium thiocyanate/phenol/chloroform extraction [13]. NBF-2 coding sequence was gained from reverse transcribed CFTR-mRNA by PCR (performed in 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1 mg/ml gelatine, 200 μ M of each dNTP, 1.4 μ M of each primer and 0.02 U/ μ l Taq-polymerase from Amersham; cycle conditions: 1 min 94°C, 1 min 60°C, 1 min 40 s 72°C; 2 \times 30 cycles). For pre-amplification the oligonucleotides 5'-CACGTGAAGAAAGATGACATC-3' and 5'-CGAGCTCCAAT-TCCATGAGC-3' were used. The booster PCR was carried out with 5'-GGCCAAATGACTGTCAAAGATC-3' and 5'-CAGAGA-

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; G_{Cl} , Cl⁻ conductance; GST, glutathione-S-transferase; GST-NBF-2, glutathione-S-transferase-CFTR-NBF-2 fusion protein; IPTG, isopropyl- β -D-thiogalactoside; NBF, nucleotide binding fold; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; TNP-ATP, 2'-(3')-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate; TNP-ADP, 2'-(3')-O-(2,4,6-trinitrophenyl) adenosine 5'-diphosphate; TNP-AMP, 2'-(3')-O-(2,4,6-trinitrophenyl) adenosine 5'-monophosphate; AK, adenylate kinase.

ATTACTGTGCAATCAG-3' as primers defining and amplifying the CFTR coding sequence from base 3,754 to base 4,331 [2]. The final amplification product was introduced into the *Sma*I site of the expression vector pGEX-3X [14] (obtained from Pharmacia) according to standard procedures [15] using competent *recA*⁻ *E. coli* HB101. By this strategy the C-terminal coding region of the glutathione-S-transferase gene of the vector was fused in frame to the inserted NBF-2 coding DNA. Plasmids of transformants showing positive inducible expression of a fusion protein of the appropriate size were isolated and both strands of the inserted DNA including vector-insert junctions were totally sequenced using an Applied Biosystems automated sequencer to verify accordance with the published sequence [2] and to exclude amino acid substitutions in the CFTR-NBF-2 part of the expressed fusion protein due to fidelity constraints of the PCR reaction.

2.2. Overexpression and purification of GST-NBF-2 fusion proteins

E. coli HB101 harboring the expression vector pGEX-NBF-2 was grown at 32°C in dyt-medium (5 g NaCl, 10 g bacto yeast extract, 16 g bacto tryptone per liter, pH 7.0; 200 µg ampicillin/ml). They were induced with 0.1 mM IPTG at A_{550} of approx. 1.3 for 3.5 h at 25–28°C. Cells were harvested by centrifugation at 4000 × *g* for 20 min and washed once with ice-cold PBS [15]. They were resuspended in 10 ml ice-cold PBS/0.1 mM PMSF per gram wet weight and lysed by passing the suspension twice through a French Press at 3500 psi. Triton X-100 was added to a final concentration of 1% and after gentle mixing for 35 min at room temperature the lysate was centrifuged at 10,000 × *g* for 15 min at 4°C. For reasons discussed below the supernatant of soluble proteins was incubated with 5 mM MgATP for 30 min at 37°C. Immediately afterwards, 1 ml 50% glutathione-Sepharose 4B (Pharmacia, binding capacity 5 mg of fusion protein/ml of bed volume) per 40 ml supernatant was added, followed by an one-hour incubation on ice with occasional gentle mixing. After decanting the supernatant, the beads were washed once with 100 volumes PBS/1% Triton X-100 and five times with 100 volumes PBS without Triton X-100 before they were filled into a suitable column. The GST-NBF-2 fusion protein was eluted with 10 mM reduced glutathione dissolved in 50 mM Tris-HCl, pH 8.0 (final pH 7.5), at a flow rate of 0.5 ml/min. Fractions containing fusion proteins detected by their absorbance at 280 nm were pooled and applied in 500 µl portions to a Superose 12 preparative FPLC column (Pharmacia) pre-equilibrated with 50 mM Tris-PO₄, pH 7.5. Fractions eluted from the Superose 12 column were monitored by their absorbance at 280 nm and subsequently by SDS-PAGE. Fractions of different runs corresponding to the same reproducible, well-resolved peak of a 48 kDa protein (determined by comparison with runs of marker proteins) were pooled. The amount of fusion protein was quantified by determination of the absorbance at 280 nm using a molar absorption coefficient of 73,390 M⁻¹·cm⁻¹.

2.3. Amino acid sequence analysis

For N-terminal sequencing 100–200 pmol of the purified fusion protein were used directly. For sequencing internal portions of the fusion protein 100 pmol of the purified protein were incubated with 1 µg endoproteinase Lys-C (Boehringer Mannheim, sequencing grade) for 14 h at 37°C. Digestion products were isolated by HPLC as follows: samples were loaded onto a Supersphere 60 RP-SELECT B column (125 mm × 2 mm; Merck) and eluted using a linear gradient of 0–60% (by vol.) acetonitrile in 0.1% (by vol.) trifluoroacetic acid, within 60 min, at a flow rate of 0.3 ml/min. Automated amino acid sequencing was performed using a gas-phase sequencer 473A (Applied Biosystems).

2.4. Circular dichroism spectroscopy (CD)

CD spectra (190–250 nm) of 0.8 µM GST-NBF-2 in 50 mM Tris-PO₄, pH 7.5, were obtained at 20°C using an Auto-Dichrograph Mark IV (Jobin Yvon Division d'Instruments S.A.) spectropolarimeter and a 0.1 cm pathlength demountable cell. Analysis of the smoothed average of ten separate spectra for secondary structure was done with the CONTIN FIT program [16].

2.5. Qualitative adenine nucleotide binding studies

1 nmol of glutathione-S-transferase or GST-NBF-2 was incubated with 50 µl ATP-, ADP- or AMP-agarose (Sigma) in 500 µl 50 mM Tris-HCl, pH 7.5/0.1% Triton X-100 for 50 min on ice. Beads were washed twice with 1 ml of the incubation buffer before they were

resuspended in 50 µl SDS sample buffer [17] and boiled for 10 min. The supernatant was subjected to SDS-PAGE followed by staining with Coomassie blue. Specific nucleotide binding was demonstrated by carrying out the described binding assay in the presence of 20 mM ATP, ADP or AMP (from Boehringer Mannheim, stock solutions dissolved in 50 mM Tris, pH adjusted to pH 7.5).

2.6. Determination of nucleotide binding characteristics

The fluorescence enhancement of varying concentrations of TNP-labelled adenine nucleotides (Molecular Probes Inc., Eugene, OR) in the presence of 1.8 µM GST-NBF-2 or an equivalent amount of glutathione-S-transferase as control in 50 mM Tris-HCl, pH 7.5, was recorded at 25°C using a SPEX FluoroMax fluorometer (integration time 8 s). All titrations were carried out with vertically polarized excitation $\lambda = 408$ nm (slit width 2.35 mm) and horizontally polarized emission $\lambda = 545$ nm (slit width 3.5 mm). Fluorescence data were corrected for inner filter effect using the equation

$$E(c) = E_0(c) \cdot e^{-\mu c} \quad (1)$$

where c is the concentration of the fluorophore, μ is an attenuation coefficient dependent on terms of application, $E(c)$ is the emitted light and $E_0(c)$ is the emitted light without filter effect that should be proportional to c in a constant solvent [18]:

$$E_0(c) = k' \cdot c \quad (2)$$

(k' is a constant). Concentration dependent correction factors $e^{\mu c}$ were calculated after determination of μ for an individual experiment using the algebraic transformation of Eq. 1 with $E_0(c)$ substituted by Eq. 2

$$\ln [E(c)/c] = -\mu c + k \quad (3)$$

(k is a constant) that presents μ as slope of a regression line fitted to the transformed data.

To the data corrected for volume dilution and filter effect monophasic binding curves were fitted using equations of a non-classical binding model allowing for depletion of free ligand by complex formation, as published by Mullen et al. [19]. In the unlabelled nucleotide titrations the data were fitted by assuming binding competition between the nucleotide and its TNP-analogue at a single site. Thereby the concentration of the protein-bound unlabelled nucleotides was considered to be negligible in comparison with its total concentration.

2.7. Coupled assay for ATPase activity

A coupled ATP-regenerating enzyme system was employed following a method described by Nørby [20]. In a total volume of 1 ml, 1.8 µM (87.46 µg/ml) GST-NBF-2 was incubated together with 3 mM ATP, 0.12 mM NADH, 3 mM phosphoenolpyruvate, 10 µg lactate dehydrogenase and 30 µg pyruvate kinase (Boehringer Mannheim) in 50 mM Tris-HCl, pH 7.0/4 mM MgCl₂ at 30°C and $\Delta A_{340}/\Delta t$ was monitored. Addition of 3 mM ADP at the end of the reaction should verify that the assay would have indicated nascent ADP. In order to detect nascent AMP, 10 µg (3.6 U) adenylate kinase, catalyzing the reaction $\text{ATP} + \text{AMP} \leftrightarrow 2 \text{ADP}$, was added to the assay. Addition of 0.04 mM AMP at the end of the incubation period demonstrated that the assay would have indicated nascent AMP.

3. Results and discussion

3.1. Overexpression, purification and characterization of soluble GST-NBF-2

The expression vector pGEX-NBF-2 was constructed as described in section 2 to express the CFTR sequence from G1208 to L1399 (numbering according to [2]). We used a cytoplasmic expression system, since the C-terminal nucleotide binding fold seems to be intracellular [21]. As shown in Fig. 1, the expression of a 48 kDa (26 kDa for GST + 22 kDa for NBF-2) fusion protein under the control of the *tac* promoter after induction with IPTG is strikingly evident. Most of the overexpressed

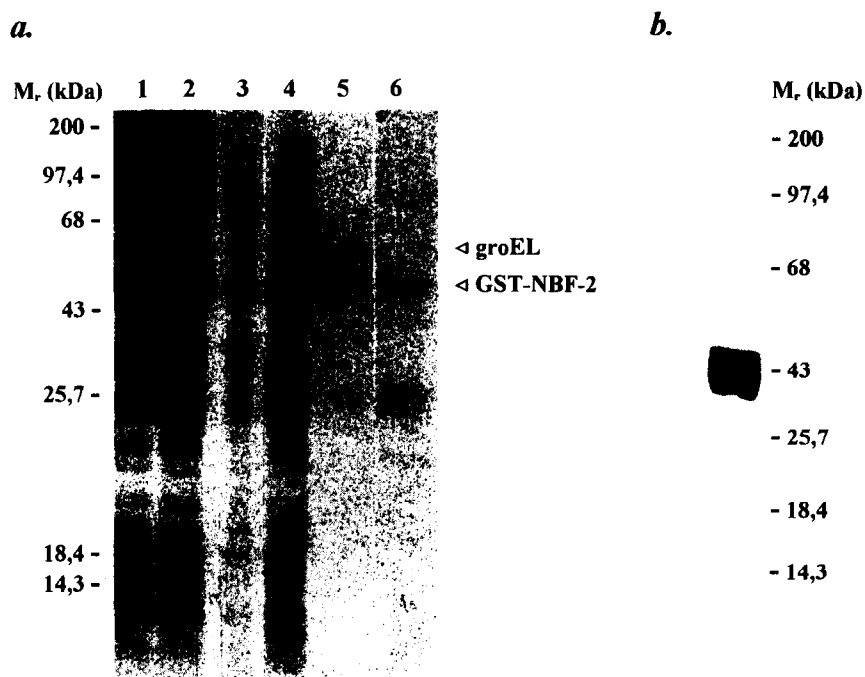


Fig. 1. Expression and purification of recombinant GST-NBF-2. (a) Lane 1, lysate of uninduced *E. coli* (3×10^8 cells) bearing the expression vector pGEX-NBF-2; lane 2, lysate of *E. coli* carrying pGEX-NBF-2 induced for 3.5 h with 0.1 mM IPTG (3×10^8 cells); lane 3, insoluble proteins of the induced *E. coli* employed in lane 2 (not used in further purification); lane 4, soluble proteins of the induced *E. coli* of lane 2, which were subsequently further purified by affinity chromatography; lane 5, 6, eluates from glutathione-Sepharose: in difference to lane 5, *E. coli* protein extract pretreated with 5 mM MgATP (30 min, 37°C) was employed in lane 6 (Coomassie stained 12.5% SDS-PAA-gel). (b) 6 μ g of the final gel filtration purified fraction was run on a silver stained 12.5% SDS-PAA-gel.

protein was found to be insoluble. In order to increase the soluble fraction of the NBF-2 fusion protein we lowered induction temperatures to 25–28°C and inducer concentrations to 0.1 mM thus allowing a higher proportion of protein to achieve a folded state [22]. As GST exhibits high affinity for glutathione, binding of GST-NBF-2 to glutathione-Sepharose provided an effective purification step. Interestingly, a second protein of 60 kDa was eluted from the glutathione-Sepharose affinity column by an excess of free reduced glutathione in addition to the 48 kDa fusion protein (Fig. 1) and was not found in a control experiment expressing GST without fusion partner. A tryptic digestion fragment of this protein revealed the amino acid sequence L-Q-E-R-V-A-K, which was found to be specific for the *E. coli* heat shock protein groEL [23] where it is preceded by lysine thus identifying the peptide as a tryptic digestion fragment. GroEL is a bacterial folding complex of distinct quaternary structure with 14 subunits of 60 kDa arranged in two stacked heptameric rings [24] which is able to bind one or two protein molecules per 14-mer in the conformation of a 'molten globule' [25]. Folding and release of the bound protein requires ATP hydrolysis [25]. Because of the mentioned stoichiometry, less than 15% of the soluble recombinant NBF-2 seemed to be bound by groEL, estimated by the amount of each protein visible on a SDS gel after Coomassie blue or silver stain. As expected, groEL was missing in elution fractions when the extracts of soluble *E. coli* proteins were pretreated with MgATP. Therefore this step was included in the purification protocol. A very small amount of contaminating proteins of M_r 25 to 30 kDa were present in the elution fraction of the gluta-

thione affinity column. GST-NBF-2 was separated from these contaminants by gel filtration on Superose 12. The effectiveness of the major purification steps is illustrated in Fig. 1a,b. To demonstrate the purity of the NBF-2 fusion protein, a 12.5% gel was heavily loaded and stained with silver (Fig. 1b). No other proteins larger than the cutoff molecular weight of the gel were detectable. N-terminal sequence analysis revealed only one N-terminal sequence in agreement with the 5 N-terminal residues of the GST fusion partner. In addition, we sequenced an internal protein fragment of 11 residues (L-I-E-G-R-G-I-P-G-Q-M) that was identified as the predicted connecting link between GST and NBF-2.

Deconvolution of circular dichroism spectra using the CONTIN FIT program [16] indicated a structured protein with secondary structural elements being distributed as approximately 21% helix, 28% β -sheet and 51% elements belonging to the 'remainder' class (including turns and random coil). The overall yield of natively folded GST-NBF-2 fusion protein was approximately 100 μ g (2 nmol) per liter of *E. coli* culture.

3.2. Specific binding of CFTR-NBF-2 to adenine nucleotide affinity agarose

Fig. 2 shows binding of GST-NBF-2 to ATP-, ADP- and AMP-agarose. GST failed to be retained by either agarose. In the presence of an excess of unbound nucleotides binding of the NBF-2 fusion protein was markedly reduced. This emphasizes that the observed binding to the affinity agarose is due to a specific interaction of NBF-2 with the agarose-attached adenine nucleotides. However, these results do not allow to make

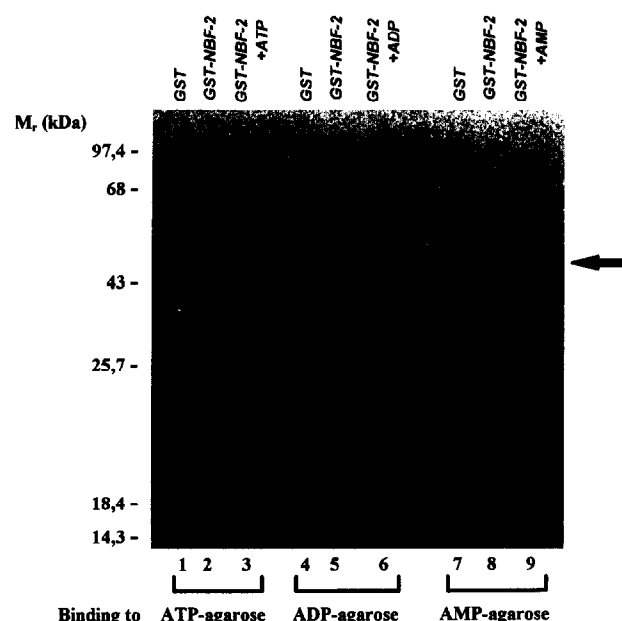


Fig. 2. Binding of the recombinant NBF-2 fusion protein to ATP-, ADP- and AMP-affinity agarose. Lanes 1, 4, 7: binding of glutathione-S-transferase (GST) to ATP- (lane 1), ADP- (lane 4) and AMP- (lane 7) agarose. Lanes 2, 5, 8: binding of the NBF-2 fusion protein to ATP- (lane 2), ADP- (lane 5) and AMP- (lane 8) agarose. Lanes 3, 6 and 9: binding of the NBF-2 fusion protein to ATP- (lane 3), ADP- (lane 6) and AMP- (lane 9) agarose in the presence of an excess (20 mM) of the corresponding nucleotide (Coomassie stained 12.5% SDS-PAA-gel).

quantitative statements concerning nucleotide binding of NBF-2, since the three applied affinity matrices exhibit different unspecific protein binding properties.

3.3. Quantifying nucleotide binding of NBF-2

Nucleotide binding characteristics were obtained by measuring the fluorescence enhancement of adenine nucleotides labelled with the extrinsic fluorophore trinitrophenol (TNP). Concentration-dependent enhancement as a consequence of increased quantum yield occurs if the fluorophore is transferred into a less polar environment like a protein's nucleotide binding pocket [18]. A specific signal due to nucleotide binding is expected to be readily de-enhancable by an excess of the corresponding unlabelled nucleotide. Since NBF-2 contains hydrophobic amino acid sequences, aggregate formation during fluorescence measurements may cause light scattering as a result of increasing turbidity. Therefore vertically polarized excitation (408 nm) and horizontally polarized emission (545 nm) were used to minimize scattered light, an effect that could be controlled by looking at the Raman scatter [26]. In addition, protein concentrations were kept low (1.8 μ M).

As illustrated in Fig. 3, all three TNP-labelled adenine nucleotides bound to the NBF-2 fusion protein and were effectively displaced by the corresponding unlabelled nucleotide. In the presence of 6 M urea the capacity to bind adenine nucleotides was completely lost. No specific enhancement was observed using GST instead of the GST-NBF-2 fusion construct (data not shown). All three adenine nucleotides were bound with high affinity displaying K_d values of 22 μ M for TNP-ATP, 39 μ M for TNP-ADP and 2.1 μ M for TNP-AMP. The corrected K_d

values for unlabelled adenine nucleotides were determined to be 37 μ M for ATP, 92 μ M for ADP and 12 μ M for AMP. The calculated K_d values for TNP labelled nucleotides are in good agreement with those obtained for the corresponding unlabelled nucleotides. This strongly suggests that indeed a nucleotide interaction has been observed with negligible influence of the TNP group on protein ligand association. Recently published work investigating nucleotide binding of a 51-amino acid synthetic peptide corresponding to the CFTR sequence from E1228 to T1278 (numbering according to [2]), containing the Walker A motif of NBF-2, indicated much lower capacity of ATP to replace TNP-ATP, low ability to bind TNP-ADP and very low ability to bind TNP-AMP [10]. In our opinion, this discrepancy emphasizes that the whole predicted NBF-2 sequence is necessary to provide the structural and functional integrity of that domain.

The observed affinity for nucleotides was even better than expected from electrophysiological data [6–8]. A hypothetical explanation would be that the NBF-2 nucleotide binding pocket of native, membrane inserted CFTR is not as well acces-

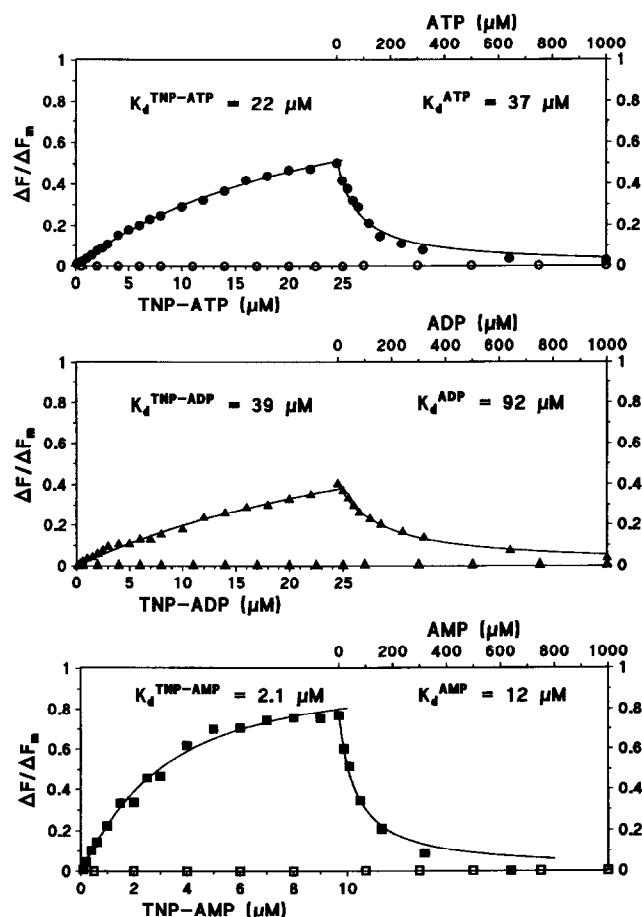


Fig. 3. Adenine nucleotide binding characteristics of the NBF-2 fusion protein. The fluorescence enhancement (ΔF) of TNP-labelled adenine nucleotides due to binding to NBF-2 is illustrated in the absence (solid symbols) or in the presence of 6 M urea (open symbols). Data were normalized referring to the asymptotic maximum of the best-fit curve (ΔF_m). Comparable binding curves were obtained in three separate sets of experiments. K_d values were calculated from the depicted sets of data giving the best fit to the theoretical binding equation [19], as indicated by the lowest standard deviations (1.48 μ M, 6.95 μ M, 0.32 μ M for K_d of TNP-ATP, TNP-ADP, TNP-AMP, and 4.39 μ M, 4.65 μ M, 1.93 μ M for K_d of ATP, ADP, AMP, respectively).

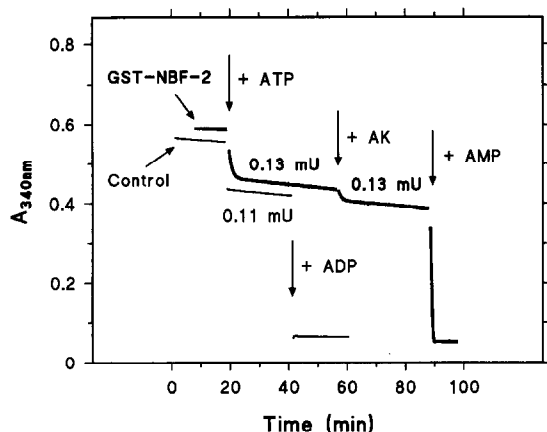


Fig. 4. Absence of ATP-hydrolysing activity of the NBF-2 fusion protein in a coupled enzymatic assay. 'AK' indicates addition of adenylate kinase to detect nascent AMP. Control: performance of the assay without GST-NBF-2. At the end of an experiment, the adenine nucleotide of interest (ADP or AMP) was added in order to verify that the assay was still functional.

sible as that of the isolated and solubilized domain. It is tempting to speculate that NBF-2 might be at least partially immersed into the membrane, as it is reasonable to assume in the case of NBF-1 [27].

3.4. Investigation of possible enzymatic activity of recombinant NBF-2 regarding ATP hydrolysis

A coupled ATP-regenerating enzyme system was employed that is able to detect nascent ADP by a decrease in the concentration of NADH present in the assay, monitored by photometric analysis [20]. ATP was added last in a concentration of 3 mM, estimated to be sufficient to saturate NBF-2 with regard to its K_d . The small diminution of A_{340} observed immediately after adding ATP is due to the presence of a small amount of ADP in the ATP preparation (Fig. 4). Interestingly, the change is significantly slower when NBF-2 is present than in the control lacking NBF-2. This difference may be explained by binding of ADP to NBF-2 delaying its entry into the coupled enzymatic assay reactions. Over a time period of 20 min no significant ATPase activity was observed above the detection limit of 0.11 mU (turnover 0.001 s^{-1}) which is imposed by baseline activity of the control due to some small ATPase contamination of the employed assay enzymes. Using the same assay, ATPase activity of $(\text{Na}^+, \text{K}^+)\text{ATPase}$ (from dog kidney, specific activity 1.3 U/mg, Sigma) could readily be detected with an enzyme concentration of $0.1 \mu\text{g}/\text{ml}$ as lower detection limit (data not shown). Finally, adenylate kinase was added in order to detect AMP that would result if NBF-2 should catalyze hydrolytic liberation of pyrophosphate from ATP. Over a period of 30 min no significant decline in A_{340} was observed (see Fig. 4). The very small but slightly delayed diminution of A_{340} immediately after adding adenylate kinase indicates traces of AMP present in the enzyme preparation, the delay pointing out an initial competition in AMP binding between NBF-2 and adenylate kinase.

These findings do, however, not totally exclude the possibility that there is a weak hydrolytic activity of less than 0.1 mU or that enzymatic activity might be uncovered with different ionic buffer compositions. Nevertheless, regulation mecha-

nisms of CFTR activity based on non-hydrolytic nucleotide binding [7] to NBF-2 appear to be more likely.

3.5. Conclusions

Our data show that the second predicted nucleotide binding fold of CFTR can gain a structure providing ability to bind ATP and ADP with high and comparable affinities. They are consistent on a single domain level with electrophysiological data obtained with CFTR wild type and mutants [8,9] suggesting that NBF-2 plays a crucial role in CFTR channel regulation, regarding ATP-mediated activation of chloride currents as well as ADP-mediated channel inhibition. Moreover, our results provide the first direct experimental evidence that the observed strong inhibitory effect of AMP on chloride conductance [7] could be a function of NBF-2.

Acknowledgements: The authors are grateful to L. Moroder, Max-Planck-Institut für Biochemie, Martinsried, for using his CD spectropolarimeter as well as to H. Domdey and B. Obermeier-Skrobranek, Laboratorium für molekulare Biologie, Martinsried, for DNA sequencing. They wish to thank E. Weyher-Stingl, C. Huber and R.J. Soares Torquato for technical assistance and R. Mentele for N-terminal amino acid sequencing, performed as a guest in the laboratory of F. Lottspeich. C.R. is in particular grateful to H. Fritz, Abteilung für Klinische Chemie und Klinische Biochemie der Chirurgischen Klinik Innenstadt, München, for generously supporting this work and providing laboratory space. This work was supported by the Sonderforschungsbereich 207 of the Ludwig-Maximilians-University of Munich and from the Dr. von Haunersches Kinderspital.

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