A recombinant polypeptide model of the second predicted nucleotide binding fold of the cystic fibrosis transmembrane conductance regulator is a GTP-binding protein

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Abstract Association reactions of a recombinant CFTR-NBF-2 polypeptide fused to glutathione *S*-transferase with guanine nucleotides were monitored quantitatively by recording the fluorescence enhancement of excited trinitrophenol (TNP)-labelled GTP after binding to NBF-2. Binding of TNP-GTP to the recombinant NBF-2 polypeptide was characterized by a K_d value of 3.9 µM. The corrected K_d values for unlabelled guanine nucleotides were determined to be 33 µM for GTP, 92 µM for GDP and 217 µM for GMP. TNP-ATP bound to NBF-2 was competitively displaced by GTP indicating a common binding site for both nucleotides. The recombinant NBF-2 did not show an intrinsic GTPase activity above a detection limit of 0.007 min^{-1}. Our findings provide the first experimental evidence that NBF-2 can act as a GTP-binding subunit that would favor the release of GDP after GTP hydrolysis.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Nucleotide binding; Guanosine triphosphate; G-protein; GTPase activity

1. Introduction

Cystic fibrosis, the most common fatal autosomal recessive genetic disease affecting Caucasian populations [1], results from mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR). There is strong evidence that CFTR is a membrane chloride channel [2,3] 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 regulatory (R) domain [4]. Both CFTR nucleotide binding folds, NBF-1 and NBF-2, were identified by the occurrence of Walker A and B consensus sequences [5] characterizing NBFs in the ATP-binding cassette family [6]. It could be shown that synthetic peptides as well as recombinant polypeptides, delineated

from the amino acid sequence of the two NBFs, are capable of binding ATP [7–11]. Furthermore we could demonstrate that a NBF-2 polypeptide was able to bind all three adenine nucleotides (ATP, ADP and AMP) with high affinity [11]. There is now strong evidence that binding of adenine nucleotides is a crucial step in activation and inhibition of CFTR channel gating. Both events appear to be mediated via NBF-2, whereas NBF-1 seems to be involved only in ATP-mediated channel opening [12,13]. Recently, a similarity in the amino acid sequence of CFTR-NBFs and the sequence of heterotrimeric G proteins was noted [14]. This observation is of particular interest in view of convincing data that show that heterotrimeric G proteins are able to inhibit cAMP activation of chloride channels in CF airway epithelial cells [15]. In order to investigate whether CFTR itself could play a role in regulatory events as a GTP-binding protein we studied GTP-binding activity of a recombinant model of CFTR-NBF-2 showing the described adenine nucleotide binding activity. NBF-2 rather than NBF-1 was chosen due to its obvious functional parallel to a G protein in its behavior as a switch between an opened and a closed state of the CFTR channel. In contrast, NBF-1 appears to be involved only in channel opening by ATP hydrolysis, as discussed by Carson and Welsh [16].

In the present study we demonstrate binding of GTP to a recombinant NBF-2 polypeptide at the same site and with similar affinity as ATP. Furthermore we show significantly lower GDP- and GMP-binding activity. This is the first direct experimental evidence that NBF-2 is not only capable of binding ATP, but also can act as a GTP-binding subunit.

2. Materials and methods

2.1. Overexpression and purification of GST-NBF-2 fusion proteins
The CFTR sequence from Gly^{1208} to Leu^{1399} (numbering according to [4]) containing the entire predicted NBF-2 was expressed in fusion with GST in *E. coli* HB101 harboring the expression vector pGEX-NBF-2 [11] using low expression temperatures (25–28°C) and low inductor concentrations (0.1 mM IPTG) and purified as described previously [11]. For all studies described in this paper only the primary soluble fraction of the NBF-2 fusion protein was employed, which – in contrast to the insoluble fraction – should have achieved a properly folded state. In a final step the purified fusion protein was extensively dialyzed over 20 h against a 1000-fold excess of 50 mM Tris-HCl pH 7.5, which was exchanged 3 times in order to remove possible bound guanine nucleotides. Protein chemical characterization of the purified recombinant NBF-2 by SDS-PAGE and protein sequencing as well as quantifying the amount of fusion protein was done as described [11].

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2.2. Determination of nucleotide binding characteristics

The fluorescence enhancement of increasing concentrations of TNP-labelled GTP or ATP (Molecular Probes, Eugene, OR) in the presence of 1.96 µM GST-NBF-2 or an equivalent amount of GST as control in 50 mM Tris-HCl pH 7.5 was recorded at 25°C using a SPEX FluoroMax fluorometer (integration time 8 s). To keep the influence of distorting scattered light as low as possible, all titrations were carried out with vertically polarized excitation λ = 408 nm (slit width 2.35 mm) and horizontally polarized emission λ = 545 nm (slit width 3.5 mm) [17].

The values were corrected for a portion reflecting unspecific binding of TNP-labelled nucleotide defined as the amount of fluorescence enhancement that was not further de-enhanceable by an excess of unlabelled nucleotide. It was calculated as the asymptotic minimum of the unlabelled nucleotide titration curves and was assumed to grow proportional to the concentration of TNP-labelled nucleotide present in the assay. Correction for inner filter effect and fitting of monophasic binding curves to the corrected fluorescence data were done according to the method described in [11].

2.3. Coupled assay for GTPase activity

A coupled GTP-regenerating enzyme system was employed following the method described by Gonzalo et al. [18]. 0.764 or 1.57 µM GST-NBF-2 was incubated together with 1 mM GTP, 90 µM NADH, 3 mM phosphoenolpyruvate, 8.3 µg/ml lactate dehydrogenase, 25 µg/ml pyruvate kinase (Boehringer Mannheim), 1.2 mM $MgCl_2$, 1.5 µM EDTA, and 6 mM KCl in 50 mM Tris-HCl pH 7.5 at 30°C. $\Delta A_{340}/\Delta t$ was monitored using an Ultrospec II (LKB Biochrom) spectrophotometer with a stability of ± 0.002 A/h. Addition of 130 µM GDP at the end of the reaction was used to verify that the assay would have indicated nascent GDP.

3. Results and discussion

3.1. Quantifying guanine nucleotide binding of NBF-2

Guanine nucleotide binding characteristics were obtained by measuring the fluorescence enhancement of GTP labelled with the extrinsic fluorophore trinitrophenol (TNP) in dependence on increasing concentrations, which occurs if the fluorophore is transferred into a less polar environment like a protein's nucleotide binding pocket as a consequence of an increase in quantum yield [19]. As illustrated in Fig. 1,

TNP-GTP was bound with apparently high affinity. It was competitively displaced by unlabelled GTP indicating that the observed fluorescence enhancement must be a consequence of GTP and not of TNP binding. No specific enhancement was recorded using GST instead of the GST-NBF-2 fusion protein (Fig. 1) or in the presence of 6 M urea (data not shown). We calculated a K_d value of 3.9 µM for TNP-GTP and a ratio of $\Delta F/\Delta F_m = 0.82$ at the end of titration after fitting our data to a binding curve using equations of a binding model published by Mullen et al. [20] with the assumption that the active binding portion of the recombinant NBF-2 was equal to the protein concentration (1.96 µM). In order to validate the latter assumption we had to estimate the magnitude of the active binding fraction of the NBF-2 fusion protein because we were not able to determine the amount of active binding protein directly by titration since we could not further increase the protein concentration in the assay for reasons of protein stability in solution of the recombinant NBF-2. Therefore we also established the best possible fit to the data without any assumption on the active binding protein concentration, the K_d value and ΔF_m. The corresponding curve fitting to the depicted data was characterized by the following parameters: $K_d = 3.3 \pm 0.53$ µM, active binding protein concentration 2.66 ± 0.63 µM and $\Delta F/\Delta F_m = 0.84$ at the end of titration. In addition, we tried to fit a monophasic binding curve following the equation $\Delta F = \Delta F_m/(1+K_d/c_{TNP-GTP})$. Here we assumed the concentration of the protein-bound TNP–GTP to be negligible in comparison with its total concentration. This would be the case if the concentration of the active binding fraction of the recombinant NBF-2 was at least one magnitude lower than the total protein concentration. The results, however, differed significantly from the best-fit curve and from the curve assuming an active binding NBF-2 concentration of 1.96 µM (calculated values for the curve corresponding to the depicted data: $K_d = 5.6$ µM; $\Delta F/\Delta F_m = 0.77$). Since the two curves derived from the nonclassical binding model of Mullen et al. [20] are nearly iden-
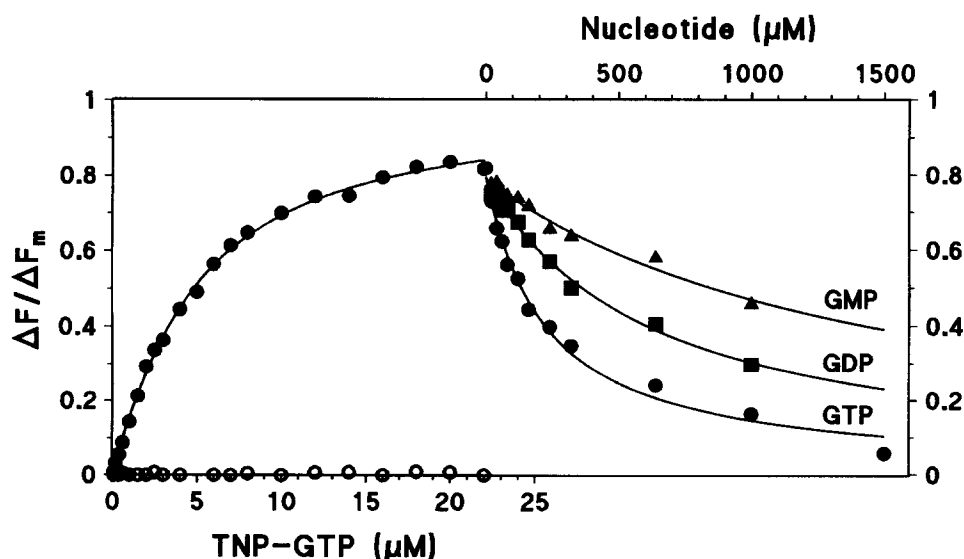

Fig. 1. Binding characteristics of guanine nucleotides to the NBF-2 fusion protein. Data of fluorescence enhancement (ΔF) were normalized referring to the asymptotic maximum of the fitted curve (ΔF_m). Comparable binding curves were obtained in three independent sets of experiments. The following K_d values (± S.E.M.) were calculated assuming a concentration of functional active protein of 1.96 µM: 3.9 ± 0.16 µM for TNP-GTP (●), 33 ± 2.5 µM for GTP (●), 92 ± 5.0 µM for GDP (■), and 217 ± 15.4 µM for GMP (▲). As control GST was used, which could not cause a specific fluorescence enhancement of TNP-GTP (○).
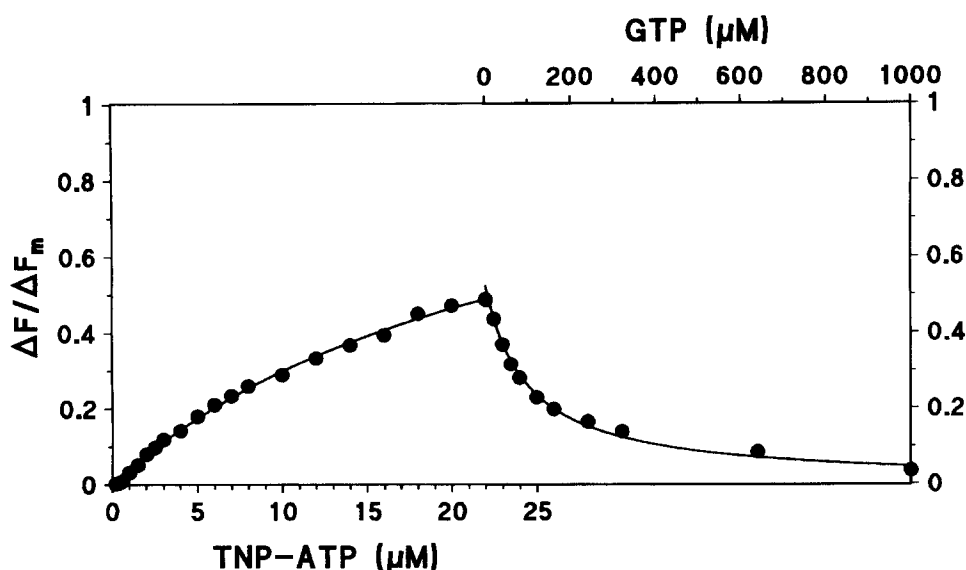
Fig. 2. Competitive displacement of TNP-ATP by GTP. For TNP-ATP a dissociation constant of 22 μM was calculated (±1.8 μM S.E.M., three experiments). The fluorescence enhancement due to binding of TNP-ATP was readily de-enhanceable by an excess of GTP displaying a corrected K_d value of 48 μM (±2.2 μM S.E.M.).

tical, we can conclude that the concentration of the active binding portion of the recombinant NBF-2 is of the same magnitude as the total protein concentration in the assay. With a K_d value of 3.9 μM for TNP-GTP the corrected K_d value for unlabelled GTP was determined to be 33 μM.

The capability of GDP to displace TNP-GTP was about three times less compared to GTP and that of GMP was one magnitude lower. From these competition experiments the following corrected K_d values were calculated: GDP 92 μM and GMP 217 μM.

In order to investigate whether the GTP-binding site is identical to the ATP-binding site we tried to compete the binding of TNP-ATP with GTP. As illustrated in Fig. 2 TNP-ATP was bound with a K_d of 22 μM which is identical to the K_d value determined in earlier experiments [11]. TNP-ATP could readily be displaced by GTP allowing to calculate a corrected K_d value for GTP of 48 μM. This value is in good agreement with the corrected K_d value obtained from the competition of TNP-GTP. In previous experiments we have been able to determine a similar corrected K_d value for ATP (37 μM [11]). These combined observations are consistent with the hypothesis of a common nucleotide binding pocket that binds GTP and ATP with affinities of the same magnitude.

The observed GTP-binding affinity of NBF-2 (10^{-5} M) is lower as compared to known GTPases having K_d values between 10^{-11} and 10^{-7} M [21]. Since we extensively dialyzed the purified NBF-2 protein against a nucleotide-free buffer the estimated magnitude of the affinity constant is the correct one and is not the consequence of titrating TNP-GTP against a protein that already has bound GTP or is even saturated with GTP at the beginning of the titration. With regard to the magnitude of cytoplasmic concentrations of GTP ($>10^{-4}$ M according to [21]) GTP saturation of NBF-2 could therefore theoretically depend on changes in the actual concentration of GTP in the cytoplasm of the cell. The cytoplasmic concentrations of GDP are about one magnitude less than those of GTP [21] which means that under physiologic conditions GDP-binding to NBF-2 would be negligible. Therefore

in a hypothetical GTPase cycle involving CFTR the release of nascent GDP and rebinding of GTP to NBF-2 would be favored.

3.2. Investigation of possible intrinsic GTPase activity of the recombinant NBF-2

For detection of intrinsic GTPase activity of the purified recombinant NBF-2 fusion protein a coupled GTP-regenerating enzyme system was employed. This assay takes advantage of the fact that pyruvate kinase can phosphorylate nascent GDP in the presence of phosphoenolpyruvate [18]. This reaction is followed by oxidation of NADH into NAD^+ which is monitored by photometric analysis. GTP was finally added in a concentration of 1 mM, expected to be sufficient to saturate NBF-2 according to its K_d. Over a time period of 30 min and with two different NBF-2 concentrations, 0.764 μM and 1.57 μM, no significant intrinsic GTPase activity was observed above the detection limit of 0.007 min^{-1} (6.16 pmol/min in a 575 μl solution). The assay should have been sensitive enough to detect slow rates of GTP hydrolysis of about 0.02 min^{-1} that are typical for purified GTPases [21] considering the evidence given above, that the concentration of the functional active fraction of the recombinant NBF-2 appears to be of the same magnitude as the employed protein concentration. Therefore we assume that, if there is any intrinsic hydrolytic activity, this activity must be at least one magnitude less than 0.02 min^{-1}.

Recent crystallographic studies of GTPases like p21^ras [22], $G_{i\alpha1}$ [23] and $G_{t\alpha}$ [24] have suggested that the conserved glutamine in the consensus sequence D-X-[G/A]-G-Q of the G-3 region [21], just N-terminal to the conserved Walker B sequence, plays a key role in hydrolysis but not in binding of GTP [25]. In the corresponding region of CFTR-NBF-2 the motif 1347-L-S-H-G-H-1350 fits best to the consensus sequence [16]. In p21^ras an intrinsic GTPase activity of 0.028 min^{-1} has been reported, while the oncogenic mutation of the conserved glutamine to histidine (variant Q61H) decreased the rate of GTP hydrolysis to 0.0019 min^{-1} [26]. Transferring these findings to the NBF-2 sequence, one would predict no or

an only very slow rate of GTP hydrolysis since amino acid 1350 in NBF-2 is histidine instead of glutamine. This may explain the failure to demonstrate GTPase activity of recombinant NBF-2 in our assay. Interestingly, it was recently shown that mutation of H1350 to glutamine in CFTR-NBF-2 produced channels with a decreased mean burst duration [16]. In view of our findings this observation would also be consistent with the hypothesis of a higher rate of switching into an inactive state as a consequence of GTP hydrolysis [21].

3.3. Conclusions

Our data show that a polypeptide corresponding to the second nucleotide binding fold of CFTR can bind GTP at the same site and with similar affinity as ATP, suggesting that CFTR-NBF-2 is also a GTP-binding subunit. The observed in vitro affinity for GTP and the reported range of cytoplasmic GTP concentrations may suggest that GTP saturation of NBF-2 depends on the actual cytoplasmic GTP concentration in vivo. Due to the observed affinity for GDP together with reported cytoplasmic GDP concentrations our model would favor the release of GDP after GTP hydrolysis. It is therefore tempting to speculate that hydrolysis of GTP at NBF-2 in the presence of some GTPase activating protein or allosteric GTP binding in dependence on the local GTP/GDP ratio or both might play an important regulating role in the timing of channel closing and opening.

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