Recombinant Synthesis, Purification, and Nucleotide Binding Characteristics of the First Nucleotide Binding Domain of the Cystic Fibrosis Gene Product*

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The majority of mutations which lead to clinical cystic fibrosis are located within the two predicted nucleotide binding domains of the cystic fibrosis gene product. We have used a prokaryotic expression system to synthesize and purify the first nucleotide binding domain (NBD-1, amino acids 426-588) with and without the most common mutation associated with the disease (the deletion of phenylalanine at position 508, ΔF508). Both wild type and ΔF508 NBD-1 bind ATP-agarose in a quantitatively comparable manner; this binding was inhibited by excess Na₂ATP, trinitrophenol-ATP, or 8-azido-ATP. Irreversible NBD-1 labeling by an ATP analog was demonstrated using [³²P]8-azido-ATP. This covalent labeling was inhibited by preincubation with Na₂ATP, with half-maximal inhibition for Na₂ATP occurring at approximately 5 mM for both the wild type and ΔF508 nucleotide binding domain. These experiments are among the first to confirm the expectation that the cystic fibrosis transmembrane conductance regulator NBD-1 binds nucleotide. Since, under the conditions used in our study, NBD-1 without phenylalanine 508 displays very similar nucleotide binding characteristics to the wild type protein, our results support previous structural models which predict that the ΔF508 mutation should not alter an alternation in ATP binding.

The gene responsible for cystic fibrosis (CF)* has been identified (1-3), and increasing evidence suggests that the gene product (termed the cystic fibrosis transmembrane conductance regulator (CFTR) functions at least in part as a Cl⁻ channel (4-6). CFTR has a predicted structure which includes two putative nucleotide binding domains (NBDs) (2, 7, 8). A central role for nucleotide binding in normal CFTR function is anticipated, since the majority of mutations which lead to clinical disease occur within these domains (2, 3, 7-9). Furthermore, given the prevalence of clinically relevant mutations in the nucleotide binding domains, it is reasonable to imagine that defects in nucleotide binding or ATP hydrolysis may represent underlying biochemical mechanisms in some forms of the disease.

Nucleotide binding by a full-length CFTR NBD-1 has not been previously demonstrated. Recently, a 67-amino acid polypeptide corresponding to a portion of NBD-1 of the wild type CFTR was synthesized and shown to bind the ATP analog trinitrophenol ATP (8). In the current study, we used a prokaryotic expression system to synthesize and isolate the wild type CFTR NBD-1 and to study its nucleotide binding properties. The most common mutation leading to clinical CF is the deletion of phenylalanine at CFTR position 508 (ΔF508). In order to test the influence of this ΔF508 mutation on nucleotide binding by NBD-1, we purified and characterized the corresponding NBD-1 without the phenylalanine at CFTR position 508.

MATERIALS AND METHODS

Synthesis and Isolation of CFTR NBD-1—Synthesis and isolation of CFTR NBD-1 was performed as follows. The nucleotide binding domain was defined by two primers, 5' GCGCGAATTCATGACACAGCCTCTTCTTCAG3' and 5' GCCATCATTTTCAACACAGAATTTTCAAAA3' and amplified using polymerase chain reaction (PCR) in a 100-µl reaction mixture which included 2.5 units of Taq polymerase, 100 µl of each primer, 1 ng of template cDNA, and 200 mM of each dNTP in a buffer containing 50 mM KCl and 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, and 0.01% gelatin (weight/volume) (94 °C × 1 min denaturation, 50 °C × 2 min annealing, 72 °C × 3 min elongation). Template for the wild type NBD-1 amplification was American Tissue Culture Collection (ATCC) plasmid T16-1; the phenylalanine deleted (ΔF508) NBD-1 template was ATCC plasmid C1-1/5 (2). Both PCR products had the predicted size of approximately 520-523 base pairs. Products were digested with EcoRI (to generate ends compatible with in-frame insertion into the EcoRI site of pGEX-2T) (10, 11) and gel-purified (GeneClean, Bio101). pGEX 2T (Pharmacia LKB Biotechnology Inc.) was linearized with EcoRI and dephosphorylated (calf intestinal alkaline phosphatase, GIBCO/ BRL) and then ligated with NBD-1 cDNA to generate a plasmid coding for an in-frame glutathione S-transferase nucleotide binding domain-1 fusion protein. The design of pGEX-2T includes a thrombin cleavage site; in this case the cleavage site was engineered between nucleotides 6455.
ATP Binding by the CFTR Nucleotide Binding Domain

harvested using a slow speed centrifugation (2000 x g x 5 min). Inclusion bodies were then solubilized in 8 M urea and dialyzed against 50 mM Tris-HCl, pH 7.4 (13). After 5 changes of 50 x 50 ml volume, NaCl and CaCl2 were added to 150 and 2.5 mM, respectively. Human thrombin (Sigma) was added to a final concentration of 20 units/ml.O

Products of the cleavage reaction were then loaded on a preparative acrylamide gel, and the 21-kDa NBD-1 polypeptide was electrophoretized into a buffer containing 200 mM glycine, 25 mM Tris-HCl, pH 7.4. The isolated polypeptide was then dialyzed against 6 M urea, with 1% Dowex resin to absorb residual sodium dodecyl sulfate (6 x 200-ml dialysis changes). Finally, the sample was dialyzed (5 x 1 liter) into 10 mM Tris-HCl, pH 7.4, and studied using the nucleotide binding protocols described below. Utilizing this approach, 200 ml of E. coli starting material yielded approximately 100-200 mg of isolated, purified NBD-1. Amino acid sequence and compositional analyses were performed on a Beckman 7300 AAA instrument at the Yale University Protein and Nucleic Acid Sequencing Facility, New Haven, CT.

Circular dichroism (CD) spectra were recorded with an AVIV 62DS spectropolarimeter interfaced to a personal computer (80386) and measured every 0.5 nm with 2.0 nm bandwidth. A 0.01-cm pathlength cell was used for far-UV spectra. Spectra were signal-averaged by adding at least 40 scans (-5 pmol) which is induced (lane C) is glutathione S-transferase. After transfection with recombinant plasmid coding for the glutathione S-transferase NBD-1 fusion protein (lane D), induction leads to the appearance of a recombinant product which migrates with an apparent molecular mass of 44 kDa. The anticipated disappearance of the 28-kDa glutathione S-transferase is also observed. Lane E shows a partial purification of the fusion protein by inclusion body isolation (see "Materials and Methods"). In lane F, the fusion protein has been dialyzed into cutting buffer. Lane G shows cleavage with thrombin, yielding the predicted polypeptides of 28 kDa (glutathione S-transferase) and 21 kDa (CFTR NBD-1). Final purification of the NBD-1 after electrophoresis and dialysis is shown in lane H. Lane A shows molecular mass standard; 12% acrylamide gel.

Fig. 1. Recombinant synthesis and purification of CFTR NBD-1. E. coli transfected with pGEX-2T with and without the CFTR NBD-1 insert are shown. Cell lysates representing total protein from 50 x 106 of E. coli culture prior to induction (lane B) or 4 h after induction (lane C) are demonstrated for bacteria containing pGEX 2T without CFTR NBD-1 insert. The prominent 28-kDa polypeptide which is induced (lane C) is glutathione S-transferase. After transfection with recombinant plasmid coding for the glutathione S-transferase NBD-1 fusion protein (lane D), induction leads to the appearance of a recombinant product which migrates with an apparent molecular mass of 44 kDa. The anticipated disappearance of the 28-kDa glutathione S-transferase is also observed. Lane E shows a partial purification of the fusion protein by inclusion body isolation (see "Materials and Methods"). In lane F, the fusion protein has been dialyzed into cutting buffer. Lane G shows cleavage with thrombin, yielding the predicted polypeptides of 28 kDa (glutathione S-transferase) and 21 kDa (CFTR NBD-1). Final purification of the NBD-1 after electrophoresis and dialysis is shown in lane H. Lane A shows molecular mass standard; 12% acrylamide gel.

RESULTS

Nucleotide Binding Domain Synthesis and Purification—The pGEX vector system was utilized to purify the CFTR NBD-1 (amino acids 426-588) (Fig. 1). After synthesis and partial purification of a glutathione S-transferase NBD-1 fusion protein (lanes B–D), the sample was dialyzed into cutting buffer (lanes E and F). Cleavage with human thrombin resulted in the appearance of two predicted polypeptide fragments, a 28-kDa glutathione S-transferase segment and a 21-kDa CFTR NBD-1 (lane G). The NBD-1 was electrophoretically and renatured as described under "Materials and Methods" (lane H). Amino acid compositional analysis and amino-terminal sequencing of the first 13 residues of the 21-kDa polypeptide confirmed identity with that predicted for CFTR NBD-1 (426-D-S-L-F-F-S-N-F-S-L-L-G-T-P-439). Far-UV CD spectra of wild type and ΔF508 NBD-1 revealed highly structured polypeptides which were very similar in solution (67-70% β sheet, 11-12% α helix, and 0-3% β turn for both polypeptides). The finding of high β sheet content in NBD-1 is consistent with the report of a predominantly β sheet secondary structure for a synthetic 67-amino acid peptide corresponding to the first portion of the wild type CFTR NBD-1 (8).
equilibrated ATP-agarose (Pharmacia, type III) was incubated with An experiment using wild type NBD-1 is shown; identical results were also obtained using the ΔF508 NBD-1. In lane B, 20 μl of equilibrated ATP-agarose (Pharmacia, type III) was incubated with 120 μl of NBD-1 (20 μg/ml) in 10 mM Tris-HCl, pH 7.4; matrix was washed and then loaded on a 12% acrylamide gel. Lanes C–E demonstrate identical experiments in which 10 mM Na₂ATP, 10 mM 8-azido-ATP, or 10 mM trinitrophenol-ATP were added to the ATP-agarose prior to addition of NBD-1. In lanes F and G, conditions were as those described for lane B, except that ATP-agarose type II (lane F) or type IV (lane G) was used. Lane A, molecular mass standard, 12% acrylamide gel stained with Coomassie Blue. Arrow, 21-kDa marker.

**ATP Binding by the Wild Type CFTR NBD-1**—Covalent labeling of CFTR NBD-1 by [³²P]8-azido-ATP is shown in Fig. 2. Lane B shows labeling with 10 μM [³²P]8-azido-ATP; lane C shows inhibition of covalent labeling when the same experiment is performed in the presence of excess Na₂ATP. Lanes A and K show labeling with 1 and 5 μM [³²P]8-azido-ATP, respectively. 100 μM MgCl₂ (lane D) or 100 μM CaCl₂ (lane E) had little effect on labeling by the [³²P]8-azido-ATP. An attempt to label NBD-1 after electrophoresis but prior to renaturation (see “Materials and Methods”) was unsuccessful (lane F). As shown in lanes G–I, labeling required the presence of UV light and was only obtained when both NBD-1 and [³²P]8-azido-ATP ligand were present at the time of UV excitation. Lane J shows the result of an experiment performed at room temperature in which no labeling was observed. Temperature-dependent photoactivation of this type is typically observed with [³²P]8-azido-ATP (16).

**Fig. 3.** Binding of CFTR NBD-1 to ATP affinity-agarose. Utilizing a fluorescent ATP analog (trinitrophenol-ATP) have indicated quantitatively comparable nucleotide binding by the normal and ΔF508 mutant NBD-1 (17).

**Comparison of ATP Binding by Wild Type and ΔF508 CFTR NBD-1**—Fig. 4A shows the inhibition of [³²P]8-azido-ATP labeling by NBD-1 at concentrations between 0 and 10 mM. Half-maximal inhibition for both wild type and ΔF508 polypeptides occurs at about 5 mM Na₂ATP. The ΔF508 NBD-1 also exhibited an ATP affinity-agarose binding profile identical to that seen in Fig. 3 for the wild type NBD-1. Binding of wild type and ΔF508 polypeptides to ATP-agarose was quantitatively comparable (2 μg per 20 μl of type III ATP-agarose under conditions such as those shown in Fig. 3, lane B). In addition, orientation-specific binding of wild type and mutant polypeptides appears similar (Fig. 4B), based on differential binding to types II, III, and IV ATP-agarose. Furthermore, recent preliminary experiments in our laboratory

**DISCUSSION**

Using two independent protocols, we have demonstrated nucleotide binding by the recombinant CFTR NBD-1. This binding can be displaced by excess Na₂ATP. The mechanism by which this displacement occurs (competitive versus non-competitive) has not yet been determined. However, under the conditions described here, half-maximal inhibition of labeling occurs at about 5 mM Na₂ATP using a photoligand displacement assay. In addition, recombinant NBD-1 displays a steric preference for ATP immobilized on agarose via C8 of the adenine ring. In contrast, substantially less binding of the NBD-1 was observed when ATP was covalently attached to agarose by either ribose or by N6 of the nucleic acid base.
These data indicate that the full-length CFTR NBD-1 binds ATP and other nucleotides, an assumption which formerly has been based upon nucleic acid sequence homology of this domain of CFTR with members of an ATP binding cassette superfamily of gene products (7). Previously, a 67-amino acid synthetic polypeptide corresponding to a portion of the CFTR NBD-1 has been shown to bind trinitrophenol-ATP (8). Trinitrophenol-ATP binding to this polypeptide could be displaced by excess ATP with half-maximal displacement of binding occurring at about 300 \( \mu M \). The discrepancy in ATP binding that we report here could relate to added constraints in the binding of ATP by the complete NBD-1 (174 amino acids), compared with a smaller, 67-amino acid portion of it. Under the conditions used in our study, NBD-1 without phenylalanine 508 displays very similar nucleotide binding characteristics to wild type NBD-1. Our findings, therefore, are compatible with previous computer models of CFTR NBD-1 (7), particularly concerning the predicted effects of the phenylalanine 508 deletion on nucleotide binding (7, 8, 18). However, while our results support the notion that \( \Delta F508 \)-related CF does not result from an alteration in nucleotide binding, detailed studies with purified, full-length (170 kDa) CFTR will be required to formally exclude this possibility.

The precise mechanism by which deletion of the phenylalanine at CFTR position 508 leads to clinical disease is not known. It has been proposed that CFTR possessing the \( \Delta F508 \) mutation is recognized as abnormal during cellular processing and that disease results because the mutant protein is retained and prematurely degraded within the endoplasmic reticulum (19). Improper cellular processing of this type does not preclude normal nucleotide binding (or even normal anion transport function) by isolated, purified CFTR polypeptides in cell-free systems. On the other hand, since many of the NBD-1 mutations that cause CF do not appear to disrupt CFTR cellular processing, one can argue that the molecular pathogenesis of CF may be heterogeneous and that some NBD-1 mutations (particularly those which occur within the so-called “Walker” sequences (2, 7, 20) which are likely to be critical in the NBD-1-ATP interaction) might act by abolishing anticipated NBD-1 functions such as nucleotide binding or ATP hydrolysis. The experimental design described here provides a means of testing the influence of diverse CFTR NBD-1 mutations on nucleotide binding and perhaps also an approach for further delineating heterogeneity in CF pathogenesis.

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