

# A Heteromeric Complex of the Two Nucleotide Binding Domains of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Mediates ATPase Activity\*

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**The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a member of the ABC superfamily of transporter proteins. Recently, crystal structures of intact, prokaryotic members of this family have been described. These structures suggested that ATP binding and hydrolysis occurs at two sites formed at the interface between their nucleotide binding domains (NBDs). In contrast to the prokaryotic family members, the NBDs of CFTR are asymmetric (both structurally and functionally), and previous to the present studies, it was not clear whether both NBDs are required for ATP hydrolysis. In order to assess the relative roles of the two NBDs of human CFTR, we purified and reconstituted NBD1 and NBD2, separately and together. We found that NBD1 and NBD2 by themselves exhibited relatively low ATPase activity. Co-assembly of NBD1 and NBD2 exhibited a 2–3-fold enhancement in catalytic activity relative to the isolated domains and this increase reflected enhanced ATP turnover ( $V_{\max}$ ). These data provide the first direct evidence that heterodimerization of the NBD1 and NBD2 domains of CFTR is required to generate optimal catalytic activity.**

The cystic fibrosis transmembrane conductance regulator (CFTR)<sup>1</sup> belongs to the superfamily of ABC transport proteins (1, 2). Although it is a member of this transporter superfamily, it actually functions as a regulated chloride channel, and naturally occurring mutations cause the disease cystic fibrosis (3, 4). The CFTR protein, like other ABC proteins, consists of two transmembrane domains and two cytoplasmic nucleotide binding domains (NBDs). In addition, it contains a unique regulatory or “R” domain, which is phosphorylated at multiple sites by protein kinase A and protein kinase C (5, 6).

Up until very recently, there was no crystallographic information available concerning CFTR, and investigators drew parallels to CFTR from the structures that were available for prokaryotic NBDs. The more recent of these latter structures suggested that two identical catalytic sites were formed at the

interface between two identical NBDs arranged in a head-to-tail orientation (7–9). In this heterodimeric orientation, the signature motif of one NBD is juxtaposed against the “P” loop or Walker A of the other. However, unlike those of the prokaryotic ABC transporters, the two NBDs of CFTR have quite different amino acid sequences. There is clearly a lack of sequence conservation even within the Walker motifs implicated in direct interactions with nucleotides. Whereas NBD1 possesses the canonical Walker C (ABC signature) motif, the Walker A motif is modified (*i.e.* S/T substituted for T/S (10)); it lacks conservation of the glutamate residue implicated in interaction with the hydrolytic water of Walker B (11) and lacks a conserved histidine also implicated in this reaction (9). This degree of sequence degeneracy and biochemical studies showing stable interaction of azido ATP with NBD1 (12, 13) led to the suggestion that NBD1 by itself is a weak ATPase. In fact, the recent crystal structures of mouse CFTR-NBD1 did show ATP bound to the Walker A, or “P” loop but did not reveal coordination of the ATP with a hydrolytic base in the protein (14). On the other hand, NBD2 possesses sequence conservation in the canonical Walker A, B, and H motifs. However, it lacks conservation within the signature motif (Walker C). Molecular modeling of the NBDs of CFTR, using prokaryotic NBD dimers as a template, predicts the formation of one “conventional” site (comprising the Walker A and B motifs of NBD2 and the Walker C of NBD1) and one “unconventional” catalytic site (comprising the Walker A and B of NBD1 with the Walker C of NBD2) in the head-to-tail heterodimeric complex (10).

To date, there is no empirical evidence available to directly support the hypothesis that heterodimerization of the NBDs is necessary to confer the ATPase activity of CFTR. There have been several studies of the ATPase activity of isolated NBD1 peptides alone (15–18), peptides containing sequences corresponding to NBD1 and the R domain (19, 20), and NBD2 peptides alone (21). Unfortunately, variations in the experimental reagents preclude comparisons between studies and assessment of the relative roles of the two NBDs in ATPase activity. For example, even the intrinsic ATPase activity of NBD1 peptides appears to vary from study to study, depending on the domain boundaries employed and the incorporation of fusion proteins.

In this study, we compared the function of isolated NBD1 and NBD2 of human CFTR with co-reconstituted NBD1 and NBD2 using comparable expression and purification conditions in order to directly evaluate the hypothesis that both domains are necessary to confer optimal ATPase activity. We expressed both domains as recombinant proteins in Sf9 cells, purified them by virtue of hexahistidine tags, and compared their ATPase activities separately and following their co-assembly. We used the same purification system as we employ in our

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<sup>1</sup> The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide binding domains; PFO, penta-decafluorooctanoic acid; BSA, bovine serum albumin; TNP-ATP, 2’-(3’-O-(2,4,6-trinitrophenyl)adenosine 5’-triphosphate).

studies of the full-length membrane protein, since we found significant membrane association of the NBDs. This strategy permitted purification of sufficient quantities of NBD protein for biochemical studies as well as subsequent comparison of the activity of the NBDs.

#### MATERIALS AND METHODS

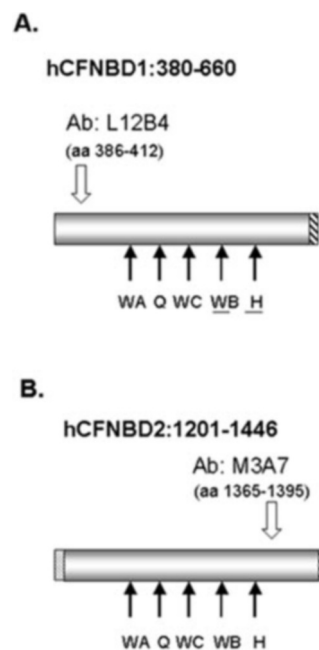
**Construction of an Expression Vector Containing NBD1 and NBD2 of CFTR**—We created recombinant baculovirus containing human NBD1 and NBD2 for expression in Sf9 cells. To do this, we used the Bac-To-Bac baculovirus expression system from Invitrogen. Briefly, the plasmid expressing the entire human CFTR coding sequence (22) was used as a template to amplify the regions: NBD1 380–660 and NBD2 1201–1446. Primers had a restriction site for EcoRI at the N terminus, one for Kpn at the C terminus, and a hexahistidine C-terminal sequence for purification purposes. For NBD1 the forward primer was 5'-CGG GAA TTC ATG TAT AAG ACA TTG GAA TAT AAC TTA AC-3'. The reverse primer was 5'-CCG GGT ACC CTA ATG ATG ATG ATG ATG ATG TCC TCC TGA ATT TCT TCT TTC TGC ACT AAA-3'. For NBD2, the forward primer (containing a hemagglutinin tag) was 5'-CGG GAA TTC ATG TAC CCA TAT GAC GTC CCA GAC TAC GCT GAT GAC ATC TGG CCC TCA GGG G-3'. The reverse primer was 5'-CCG GGT ACC CTA ATG ATG ATG ATG ATG ATG TCC TCC TCC CCT GTC GGA GGG GCT GAT GGC-3'. DNA coding for human NBD1 or NBD2 was ligated into pFastBac1 for subsequent generation of recombinant bacmid DNA and virus generation in Sf9 cell cultures. Successful expression was confirmed by Western blotting of cell extract with NBD1- and NBD2-specific antibodies. PCR analysis was performed on the baculovirus and sequenced to confirm that the NBD1 and NBD2 sequences were accurate.

**Purification and Renaturation of NBD1 and NBD2**—Recombinant NBD1 and NBD2 were produced by infection of Sf9 cell cultures grown in 75-ml flasks. Cells were incubated with 2.5 ml of harvested virus for 40 h at 26 °C. After this period, cells were harvested in phosphate-buffered saline, pH 8.0, and disrupted using a French press (1000 p.s.i.) and the fragments were centrifuged for 20 min at 500 × g at 4 °C. The subsequent purification is similar to that described for the full-length CFTR protein (25, 26). Briefly, the pellet produced by the above centrifugation was solubilized overnight in 20 ml of 8% pentadecafluorooctanoic acid (PFO) (Oakwood Products Inc., West Columbia, SC), 25 mM phosphate, pH 8.0, at 25 °C. Detergent-solubilized His-tagged NBD protein was then filtered through a 0.2-μm syringe filter and applied to a freshly generated nickel column at a rate of 2 ml/min. A pH gradient (pH 8.0–6.0) in 4% PFO and 25 mM phosphate was then applied using fast protein liquid chromatography, and 2-ml fractions were collected.

The fractions eluted from the column were assessed for the presence of NBD protein by dot blot analysis. Immunopositive fractions were selected and concentrated using a Microcon YM-10 concentrator (molecular mass cut-off of 10,000 Da; Millipore Corp., Bedford, MA) to a final volume of ~50 μl. Fractions containing pure protein (single silver-stained protein band on SDS-PAGE) were then combined and dialyzed in a Spectra/Por dialysis membrane (cut-off 10,000 Da) overnight against 4 liters of buffer containing 10% glycerol, 50 mM Tris-HCl, 50 mM NaCl, 0.1 μM dodecyl maltoside at pH 7.5. Renatured protein samples were concentrated 4-fold in a Centricon YM-10 concentrator (molecular mass cut-off 10,000 Da; Millipore). In studies where NBD1 and NBD2 were refolded together, equal amounts of purified NBD1 and NBD2 were mixed together in a Spectra/Por dialysis membrane, and the PFO was dialyzed off overnight into 4 liters of buffer containing 10% glycerol, 50 mM Tris-HCl, 50 mM NaCl, 0.1 μM dodecyl maltoside at pH 7.5.

**TNP-ATP Binding**—Binding of TNP-ATP to NBD1 or NBD2 was analyzed by steady state fluorescence measurements in a fluorescence spectrophotometer (Hitachi). 100 ng of protein was prepared in 1 ml of buffer containing 10% glycerol, 50 mM Tris-HCl, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.1 μM dodecyl maltoside, pH 7.5, in 1 ml (1 × 1-cm optical path) fluorescence cuvettes. TNP-ATP was added at varying final concentrations. Experiments were performed at 25 °C with continuous stirring. The samples were excited at 410 nm, and the emission intensity at 550 nm was recorded. A proteinless sample was used for subsequent correction of the inner filter effect on the fluorescence of TNP nucleotides as described by Garboczi *et al.* (23). Where indicated, ATP was added at the concentrations shown to displace TNP-ATP. *K<sub>i</sub>* was calculated by fitting a competitive binding curve as described (24).

**ATPase Measurements**—ATPase activity was measured as the production of [ $\alpha$ -<sup>32</sup>P]ADP from [ $\alpha$ -<sup>32</sup>P]ATP by the NBDs as described previously (26, 29). The assay was carried out in a reaction mixture (15 μl) containing 1 μg of NBD1 or -2, 10% glycerol, 50 mM Tris-HCl, 50 mM



**FIG. 1. Diagrams depicting the boundaries and motifs of the human NBD1 (hCFNBD1) and NBD2 (hCFNBD2) domains.** A, the NBD1 protein consists of amino acids (aa) 380–660 of the human CFTR sequence. In addition, it has a C-terminal His<sub>6</sub> tag (hatched box). The Walker A (WA), the Q motif (Q), Walker B (WB), Walker C (WC), and the or Switch (H) motifs have been indicated with the solid arrows. Those sequences in NBD1 that are not canonical motifs have been underlined (i.e. WB and H). The epitope for the monoclonal antibody L12B4 is also marked (open arrow). B, the NBD2 protein encompasses amino acids 1201–1446 of the human CFTR sequence. It also has a C-terminal His<sub>6</sub> tag (hatched box) and an N-terminal HA tag (speckled box). Of the Walker A, B, C, and H motif in NBD2, the Walker C (WC) is not canonical. The epitope for the monoclonal antibody M3A7 is shown (open arrow).

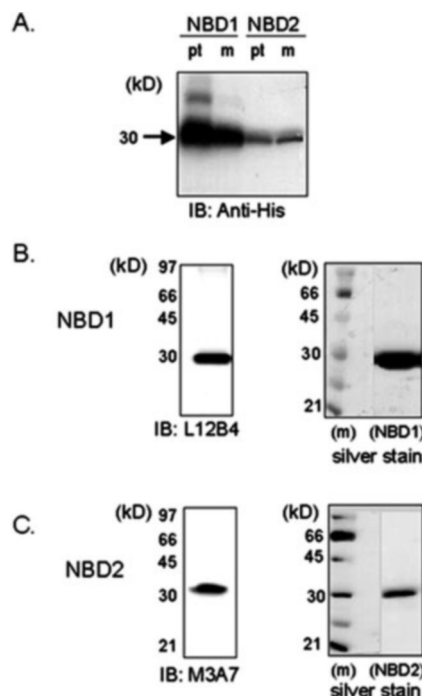
NaCl, 2 mM MgCl<sub>2</sub>, 0.1 μM dodecyl maltoside, and 8 μCi of [ $\alpha$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and 1.0 mM unlabeled ATP, pH 7.5.

**Analyses**—The ATP dose-response curves for the ATPase activities by NBD2 and NBD1 plus NBD2 were fit with the Michaelis-Menten equation using the curve-fitting program Prism (GraphPad). The dose response of TNP-ATP binding to NBD1 was analyzed similarly.

#### RESULTS

**Expression of hNBD1 and hNBD2 in Sf9 Cells**—Constructs coding for human CFTR NBD1 and NBD2 were designed on the basis of structural information generated for prokaryotic ABC transporter by x-ray crystallography (7, 8). The boundaries were based on the alignment of CFTR NBDs with others shown in the recent crystal structure of BtuCD. Specifically, they were chosen to include all of the secondary structural elements revealed for the NBDs in BtuCD (8). In addition, the amino terminus of NBD1 was extended to include residues that comprise the epitope (amino acids 386–412) recognized by CFTR-specific monoclonal antibody, L12B4 (27). The construct representing human NBD1 included residues 380–660, and these boundaries correspond well with those used for the recently obtained mouse NBD1 crystal structure (14), namely residues 389–673. The present human NBD2 construct spans residues 1201–1446 and includes the epitope (amino acids 1365–1395) recognized by a distinct CFTR-specific antibody, M3A7, permitting convenient identification of the two generated proteins. As shown in Fig. 1, both constructs were designed to include a C-terminal hexahistidine tag for subsequent purification.

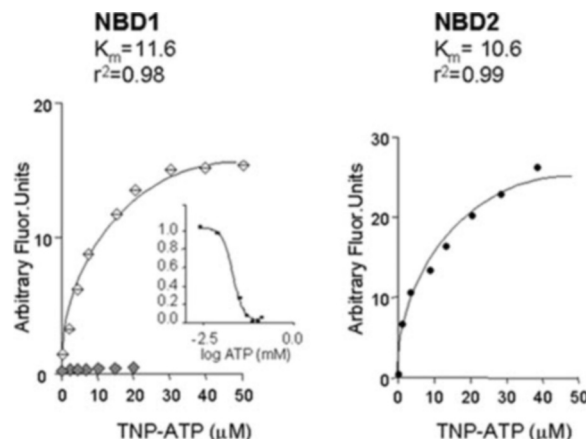
Sf9 cells were infected for 40 h with baculoviruses containing either NBD1 or NBD2, and the expression of either protein was determined following cell fragmentation using a French press and centrifugation. As shown in Fig. 2A, most of the NBD



**FIG. 2. Expression of NBD1 and NBD2 in Sf9 cells.** *A*, left two lanes, His-tagged NBD1 protein expressed in both the low speed pellet (*pt*) from broken Sf9 cells (containing inclusion bodies) and membranes (*m*) enriched from supernatant by sucrose density centrifugation. Western blot probed using anti-His antibody. *Right two lanes*, His-tagged NBD2 protein also expressed in both low speed pellet and membranes. Immunoblot (*IB*) was probed using anti-His antibody. *B*, left panel, NBD1 expression in detergent lysates of Sf9 cells confirmed using NBD1-specific antibody L12B4. *Right panel*, purification of NBD1 as an ~30-kDa protein from Sf9 cells confirmed by SDS-PAGE and silver stain analysis. *C*, left panel, NBD2 expression in detergent lysates of Sf9 cells confirmed using NBD2-specific antibody M3A7. *Right panel*, purification of NBD2 as an ~30-kDa protein from Sf9 cells confirmed by SDS-PAGE and silver stain analysis.

proteins (assessed by immunoblotting using a monoclonal anti-His antibody) were present in the low speed pellet (containing larger organelles and inclusion bodies). Membranes (including microsomal and plasma membranes) were enriched from the supernatant of the previous low speed spin by sucrose density centrifugation. As shown in Fig. 2*A*, both NBD1 and NBD2 proteins, expressed as proteins with molecular mass of ~30 kDa, were associated with the low speed pellet and with the microsomal and plasma membrane-enriched fraction.

**Purification of NBD1 and NBD2 from Sf9 Cells**—In order to purify NBD1 and NBD2, 100-ml cultures of Sf9 cells expressing either protein were harvested and lysed at 1000 p.s.i. Blots were incubated with L12B4 to assess expression of NBD1 and M3A7 for NBD2 (Fig. 2, *B* and *C*, respectively). The finding that the NBDs were associated with cellular membranes prompted us to extract the NBDs from lysed Sf9 cells with detergent as in our previous purifications of intact CFTR. A low speed spin was employed to first eliminate debris and nuclei, and the remaining cellular material was collected by high speed centrifugation and then solubilized in 8% PFO. The detergent lysate was applied to a nickel affinity column, and NBD1 and NBD2 proteins were specifically eluted using a pH gradient at pH 6.04 and 6.12 respectively. Fig. 2 shows assessment of fractions using SDS-PAGE. The silver stain of the purified fraction of NBD1 shows a single band migrating at the molecular mass expected for NBD1, 31.6 kDa (Fig. 2*B*, right panel). The silver stain of the purified NBD2 also shows one band migrating at the molecular mass expected for monomeric NBD2, 27.7 kDa (Fig. 2*C*).



**FIG. 3. TNP-ATP binding to NBD1 and NBD2.** *A*, the fluorescence increase of TNP-ATP upon binding to NBD1 was measured at an excitation wavelength of 410 nm and emission wavelength of 550 nm at varying TNP-ATP concentrations (mean of duplicate values shown, open squares). Data were fit using the Michaelis-Menten equation ( $r^2 = 0.98$ ) to yield a  $K_m$  of 11.6. The mean  $K_m$  obtained from assessment of two different NBD1 preparations, each with duplicate or triplicate studies, respectively, was 7.7  $\mu\text{M}$ . Denatured NBD1 protein (in the presence of 8% PFO detergent) failed to show TNP-ATP-dependent binding and fluorescence (mean values of duplicate studies shown, diamond). Approximately 100 ng/ml NBD1 protein was used in both cases (renatured and denatured protein). *Inset*, displacement of TNP-ATP by ATP. The quenching of the fluorescence by ATP confirms that TNP-ATP binding was specific. Competition experiments resulted in  $K_i$  for ATP of  $6.7 \pm 0.3$  mM (mean  $\pm$  S.E. of triplicate studies, solid squares overlay error bars). *B*, fluorescence increase of TNP-ATP upon binding to NBD2. The mean  $\pm$  S.E. values of triplicate studies of a NBD2 protein preparation (solid circles overlay S.E. values) were fit using the Michaelis-Menten equation ( $r^2 = 0.99$ ) to yield a  $K_m$  of 10.6  $\mu\text{M}$ .

**TNP-ATP Binds to Both NBD1 and NBD2**—Following purification, PFO detergent was removed from NBD1, NBD2, and NBD1 + NBD2 preparations (containing equimolar concentrations of NBD1 and NBD2, by dialysis overnight in the presence of 10% glycerol, 50 mM Tris-HCl, 50 mM NaCl, 0.1  $\mu\text{M}$  dodecyl maltoside, pH 7.5. The protein was then concentrated and employed in studies of ATP binding and ATPase activity.

In order to assess whether the NBD1 and NBD2 protein were folded correctly and functional, TNP-ATP binding studies were performed (Fig. 3). NBD1 bound TNP-ATP with a  $K_m$  of 7.7  $\mu\text{M}$  (mean,  $n = 2$ ; range 3.3–11.6, as shown in Fig. 3). This is in the same range as other studies of TNP-ATP binding to NBD1 (20, 28). In addition, binding was specific, since protein denatured in 8% PFO failed to show any evidence of binding. Furthermore, the natural ligand ATP was demonstrated to compete with the TNP-ATP binding with a  $K_i$  of  $6.7 \pm 0.3$  mM (mean  $\pm$  S.E.,  $n = 3$ , Fig. 3, *inset*). Purified NBD2 bound TNP-ATP with a  $K_m$  of 10.6  $\mu\text{M}$ , comparable with that of NBD1. Therefore, our findings suggest that both NBDs are functionally reconstituted and can bind this poorly hydrolyzable ATP analogue with similar affinity.

**ATPase Activity of NBD1, NBD2, and NBD1/2**—ATPase was measured as the production of [ $\alpha$ - $^{32}\text{P}$ ]ADP from [ $\alpha$ - $^{32}\text{P}$ ]ATP by the NBDs as described previously (29). One microgram of NBD protein was used, and initially, all reactions were carried out in the presence of 1.0 mM unlabeled ATP. One microgram of bovine serum albumin (BSA) was used as a negative control protein in parallel studies. As shown in the phosphor image of Fig. 4*A*, *i* (upper panel), the production of radiolabeled ADP ([ $\alpha$ - $^{32}\text{P}$ ]ADP) from ([ $\alpha$ - $^{32}\text{P}$ ]ATP, ATPase activity) by NBD1 was slightly increased ( $9.4 \pm 0.8$  nmol/mg/min (S.E.) relative to the paired negative controls, BSA ( $8.0 \pm 0.8$  nmol/mg/min), and this difference was repeatable (four different protein preparations, duplicate samples) and statistically



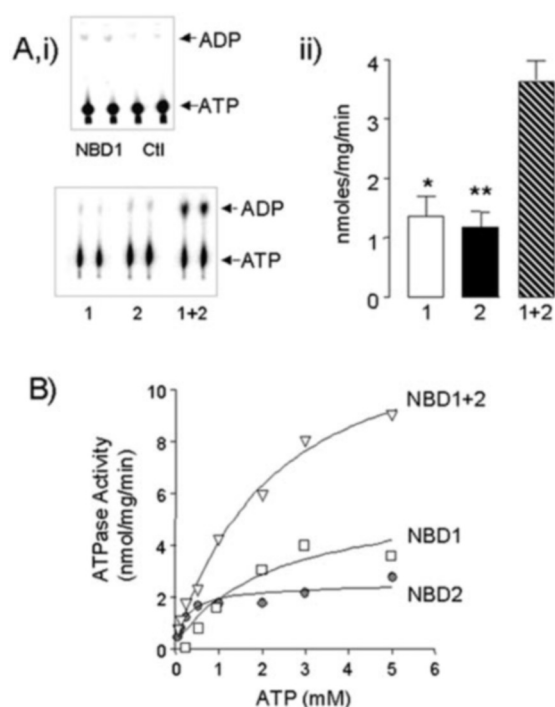


FIG. 4. **ATPase activity of NBD1, NBD2, and combination (NBD1 + NBD2).** A, *i*, top panel, phosphor images showing separation of radiolabeled ADP ( $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ ) from  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  or ATPase activity by thin layer chromatography. ATPase activity is greater by purified NBD1 (duplicate samples) than by BSA (control) (*Ctl*), duplicate samples. A, *i*, bottom panel, phosphor images show that ATPase activity is considerably less by isolated NBD1 and isolated NBD2 than by co-reconstituted (NBD1 + NBD2) (duplicate samples for each). A, *ii*, a bar graph shows the mean and S.E. of the ATPase activity of NBD1 (open bars), NBD2 (solid bars), and the mixture of NBD1 and NBD2 (NBD1 + NBD2) (hatched bars). NBD1 was purified from four different reconstitutions, NBD2 from three reconstitutions, and NBD1 + NBD2 from three co-reconstitutions. ATPase activities (duplicates for each preparation), conferred by 1  $\mu\text{g}$  of total NBD protein, were determined in the presence of 1 mM ATP, 1 h after initiation of the reaction. These values were corrected for background, by subtraction of activity determined in reaction mixtures containing 1  $\mu\text{g}$  of BSA instead of the CFTR NBDs. The activity conferred by the co-reconstituted NBD1 + NBD2 was significantly greater than that measured for either NBD1 ( $p = 0.022$ , \*) or NBD2 ( $p = 0.0004$ , \*\*) alone. B, the graph shows a comparison of ATP dependence of NBD1 + NBD2 (open triangle), NBD1 (open square), and NBD2 (filled circle) ATPase activity (performed in duplicate; mean shown). One microgram of purified protein was used in each reaction (1-h duration). Nucleotide dependences of NBD ATPase activity were fit using the Michaelis-Menten equation ( $r^2 = 0.92$ ): NBD1 ( $r^2 = 0.84$ ) and (NBD2 ( $r^2 = 0.77$ )).

significant ( $p = 0.005$ ). Similarly, there was a slight but statistically significant difference between the ATPase activity determined for NBD2 relative to the paired negative BSA control:  $9.8 \pm 0.2$  versus  $8.7 \pm 0.3$  (3 different protein preparations, duplicate samples,  $p = 0.007$ ).

ATPase activity of each NBD alone and following coreconstitution (NBD1 + NBD2) is shown as the production of radiolabeled ADP ( $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ ) from  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  in the lower panel of Fig. 4A, *i*. This phosphor image shows very clearly that co-reconstitution of NBD1 with NBD2 leads to enhanced ATPase activity. The stimulation of ATPase activity upon co-reconstitution of NBD1 and NBD2 was observed consistently in multiple protein preparations. In Fig. 4A, *ii*, we report the specific ATPase activity of the NBD peptides after subtraction of background values (BSA controls) in the presence of 1 mM ATP. Specific ATPase activity (nmol/mg/min) of either NBD (NBD1,  $1.33 \pm 0.33$  (S.E.),  $n = 8$ , duplicates of four different protein preparations; NBD2,  $1.12 \pm 0.25$ ,  $n = 6$ , duplicates of three different protein preparations) alone was significantly less

TABLE I  
Relative kinetic parameters

Protein	$K_m(\text{ATP})$	$V_{\max}$ nmol/mg/min
NBD1	2.2	5.9
NBD2	0.3	2.5
NBD1 + NBD2	2.1	12.9
CFTR <sup>a</sup>	1.0	50.0

<sup>a</sup> Previously reported in Ref. 29.

than the co-reconstituted mixture of NBD1 + NBD2:  $3.60 \pm 0.38$  (nmol/mg/min,  $n = 6$ , duplicates of three different protein preparations), with  $p$  values of 0.022 and 0.0004, respectively. The ATP dependence of the ATPase activity of NBD1, NBD2, and NBD1 + NBD2 could be fit using the Michaelis-Menten equation and the relative kinetic parameters, apparent affinity ( $K_m$ ) and  $V_{\max}$ , shown in Table I. It is clear from these analyses that the increase in the ATPase activity observed for the co-reconstituted NBD1 + NBD2 over NBD1 or NBD2 alone is associated with a marked increase in rate of ATP turnover or  $V_{\max}$  (by approximately 2–5-fold, respectively) (Fig. 4B, Table I). Further, the apparent affinity of co-reconstituted NBD1 and NBD2 for nucleotide is low, with a  $K_m$  of  $\sim 2$  mM, consistent with its increased activity as an ATPase and enhanced nucleotide off rate.

## DISCUSSION

*Independently, the NBDs of CFTR Can Bind Nucleotide but Exhibit a Low Level of Intrinsic ATPase Activity*—There have been several different estimates of the ATPase activity mediated by isolated recombinant NBD1 (14, 15, 30). These estimates are highly variable, possibly reflecting distinct boundary choices, the presence of fusion proteins, and/or the functional assay employed. The boundaries that we used in our study of human NBD1 (residues 380–660) were chosen based on the alignment published by Locher *et al.* and should cover all of the secondary structural elements described for BtuD (8) and the recent mouse NBD1 crystal structure (14). However, the boundaries of NBD1 in this study exclude the entire carboxyl-terminal helix, H9b (residues 655–669), modeled in the recent crystal structure of mouse NBD1 (14). The authors of the crystal structure study speculate that this helix may represent either the carboxyl-terminus of NBD1 or the amino terminus of the phosphorylation-regulated R domain.

The NBD1 peptide described in the present study was capable of binding nucleotide, both the nonhydrolyzable nucleotide analogue, TNP-ATP, and ATP. These data argue that the protein has been functionally reconstituted and that the carboxyl-terminal helical segment revealed in the above crystal structure (encompassing residues 660–669) may not be required for NBD1 folding and function. Consistent with some but not all studies, we could measure a low but statistically significant level of ATPase activity associated with NBD1 peptide (15, 17, 31). A glutamate residue in the Walker B motif, shown to interact with the hydrolytic water in structures of prokaryotic NBDs, is replaced by a serine residue in NBD1 of CFTR (11). This may account for the low activity observed for NBD1. The partial activity measured may reflect some compensation of the glutamate by another residue. A similar suggestion was recently advanced by Gros and co-workers (32) in their studies of Walker B mutants of the highly related mammalian ABC protein PgP.

Our finding that isolated NBD2 (extending from residue 1201 to 1446) exhibits a low but detectable level of ATPase activity is consistent with studies reported by Randak *et al.* (33) of an NBD2-MBP fusion protein comprising CFTR residues 1208–1399. Like NBD1, the NBD2 protein analyzed in the

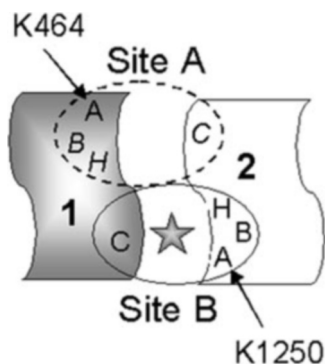


FIG. 5. **Schematic diagram showing putative NBD1 and NBD2 heterodimer.** The putative heterodimeric interface for NBD1 and NBD2 was based on modeling of MJ096 dimer (9, 10). Site A was composed of Walker A (A), Walker B (B), and H (H) motif donated by NBD1 and noncanonical Walker C (C) donated from NBD2. The Walker lysine 464 resides in Site A. Site B is composed of canonical motifs Walker A (A), Walker B (B), and motif H (H) from NBD2 juxtaposed against Walker C (C) from NBD1. The Walker lysine 1250 resides in Site B. The canonical catalytic site is indicated by star.

present study is capable of binding TNP-ATP with micromolar affinity, suggesting that it has been functionally reconstituted. The low level of catalytic activity measured for NBD2 can be described using the Michaelis-Menten function, leading to a  $K_m$  for ATP of  $\sim 300 \mu\text{M}$  and a  $V_{\text{max}}$  of  $\sim 2.5 \text{ nmol/min/mg protein}$ . This  $K_m$  is less than that determined for NBD1 reported in this study and suggests that, at least in isolation, NBD2 confers a higher affinity binding site for ATP than NBD1. However, the ATP dependence of the catalytic activity of NBD2 reaches a plateau at submillimolar ATP concentrations, yielding a  $V_{\text{max}}$  that is  $\sim 20\%$  that of the complex of NBD1 and NBD2. The structural basis for this residual activity is probably conferred by the conventional Walker A, B, H, and Q motifs of NBD2.

**NBD1 and NBD2 of CFTR Can Hydrolyze ATP Most Effectively as a Complex: Implications for Formation of ATPase Site(s) by NBD1 and NBD2**—We found that co-reconstitution of purified NBD1 with NBD2 led to the formation of an active enzymatic complex, possessing enhanced ATPase activity relative to each isolated domain. These data provide the first direct evidence that the optimal catalytic site(s) in CFTR are formed by interaction of the two NBDs.

Evaluation of the dimeric models of prokaryotic NBDs in a head to tail orientation suggests that there may be two sites at which ATP interacts, with each site including structural elements of both NBD1 and NBD2 (Fig. 5). If NBD1 and NBD2 interact in a head to tail orientation, (10), one nucleotide binding site (Site A) may be composed of Walker A and B, the Q and H loops from NBD1, and the Walker C motif from NBD2. This Site A would possess a slightly modified Walker A (10). It would also lack a glutamate residue in the Walker B motif shown to interact with hydrolytic water in structures of prokaryotic NBDs, since it is replaced by a serine residue in NBD1 (9). Furthermore, the canonical LSGGQ, which is implicated in accelerating ATP hydrolysis by interaction with the transition state, is replaced by LSHGH from NBD2 (35). Our data do not allow assessment of the catalytic activity of the putative Site A; however, it has been proposed that this site may hydrolyze ATP suboptimally on the basis of the sequence alignments discussed above. Indeed, a number of biochemical experiments on full-length CFTR have shown that ATP interacts stably with NBD1 (36, 37).

Another nucleotide binding site (Site B, in the model shown in Fig. 5) would be composed of Walker C from NBD1 and Walker A and B, Q loop, and H loop from NBD2 in the heterodimer (10, 14). All of the critical residues for ATP binding

and hydrolysis in the Walker A, B, and C are conserved at Site B. Interestingly, the isolated NBD2 domain also possesses conserved Walker A and Walker B motifs, but the ATPase activity ( $V_{\text{max}}$ ) of the isolated NBD2 was considerably less (20%) that of the complex. Together, these findings suggest that the conserved Walker C (LSGGQ), donated by NBD1 to Site B, in the heteromeric complex may be absolutely required for optimal catalytic activity. In fact, Moncalian *et al.* (38) showed that dimerization of Rad50 nucleotide binding domains and ATPase activity required a conserved Walker C motif.

Studies of the ATPase activity of the full-length protein have also supported the idea that the two NBDs of CFTR functionally interact. Mutation of the canonical Walker A lysine in either NBD1 (K464A, targeting Site A) or NBD2 (K1250A, targeting Site B) decreased ATPase activity of the whole protein by greater than 50% (39). Interestingly, the most severe effects on ATPase activity and channel gating were observed in the K1250A mutant, wherein ATPase activity was virtually abolished, consistent with this conventional site playing a more important role in generating the overall catalytic function of CFTR. Unfortunately, there are no ATPase data available for the NBD2 Walker B mutations D1370N and E1371S, which would be expected to severely impair hydrolysis. However, an electrophysiological study found that these mutations caused a marked slowing of channel closing from bursts as did K1250A, which is known to reduce the ATPase activity of CFTR to around 1% of wild type (40). Furthermore, the corresponding mutations abolished hydrolytic activity in several other ABC ATPases (41, 42). Our data suggest that the nonconventional site to which Lys<sup>464</sup> contributes (Site A) also participates in the overall activity, since the K464A mutation causes a significant, albeit partial, decrease in function of the full-length protein. Nucleotide interaction with Site A may exert a modulatory role, a hypothesis to be tested in our future work.

Although the co-reconstituted NBD1 + NBD2 complex exhibited 2–5 times the activity of each isolated domain, it is still considerably less active than the full-length protein (Table I). This difference may reflect somewhat less than complete co-reconstitution of the domains in a complex or the addition of an HA tag on the amino terminus of NBD2 or, alternatively, implicate a role for other domains of CFTR in the regulation of the ATPase activity of the intact membrane protein. Functional studies of the ATPase activity of the full-length CFTR found that modulators of the pore in the membrane influenced the ATPase activity (43). Moreover, the crystal structure of the intact vitamin B<sub>12</sub> transporter, BtuCD, revealed an interaction between the NBDs and membrane spanning domains via helical segments (L1-L2) extending from the membrane domain (8). In the corresponding region of CFTR (ICL4), mutations result in altered gating, suggesting that this region couples ATP hydrolysis by the NBDs to opening and closing of the channel pore (34).

In conclusion, in this paper, we have described the successful expression, purification, and functional reconstitution of NBD1 and NBD2 of human CFTR. Functional measurements shed light on the ATPase mechanism of CFTR and support the hypothesis that optimal ATPase activity is mediated through association of NBD1 with NBD2. These domains will provide an opportunity to test further predictions of how they function in the intact CFTR protein.

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