The C-Terminal Nucleotide Binding Domain of the Human Retinal ABCR Protein Is an Adenosine Triphosphatase†

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ABSTRACT: The rod outer segment ATP binding cassette (ABC) transporter protein (ABCR) plays an important role in retinal rod cells presumably transporting retinal. Genetic studies in humans have linked mutations in the ABCR gene to a number of inherited retinal diseases particularly Stargardt macular degeneration and age-related macular degeneration (ARMD). The ABCR protein is characterized by two nucleotide binding domains and two transmembrane domains, each consisting of six membrane-spanning helices. We have cloned and expressed the 376 amino acid (aa) C-terminal end of this protein (amino acid residues 1898−2273) containing the second nucleotide binding domain (NBD2) with a purification tag at its amino terminus. The expressed protein was found to be soluble and was purified using a rapid and high-yield single-step procedure. The purified protein was monomeric and migrated as a 43 kDa protein in SDS−PAGE. The purified NBD2 protein had strong ATPase activity with a K_m of 631 μM and V_{max} of 144 nmol min^{-1} mg^{-1}. This ATPase activity on normalization was kinetically comparable to that observed for purified and reconstituted native ABCR. Nucleotide inhibition studies suggest that the binding of NBD2 is specific for ATP/dATP, and that none of the other ribonucleotides appeared to compete for binding at this site. These studies demonstrate that cloned and expressed NBD2 protein is a fully functional ATPase in the absence of the remainder of the molecule. The level of ATPase activity was comparable to that of *trans*-retinal-stimulated ABCR ATPase. The NBD2 expression plasmid was used to generate a Leu2027Phe mutation associated with Stargardt disease. Analysis of the ATPase activity of the mutant protein demonstrated that it had a 14-fold increase in binding affinity (K_m = 46 μM) with a corresponding 9-fold decrease in the rate of hydrolysis (V_{max} = 16.6 nmol min^{-1} mg^{-1}), indicating a significant alteration of the ATPase function. It also provided a molecular basis of Stargardt disease involving this mutation.

Members of the ATP binding cassette (ABC) superfamily are transmembrane proteins which transport various substances across cell membranes in an energy-dependent manner (*1*). Typically, the eukaryotic ABC proteins are comprised of tandem transmembrane domains with two Walker (*2*) type A and type B nucleotide binding motifs. The two repeated domains are joined by a "linker" region (*1*). ABC transporters are involved in active transport of a variety of hydrophobic substances across membranes including drugs (*3*), lipids (*4*), metabolites (*5*), peptides (*6*, *7*) and steroids (*1*). A retina-specific member of the ABC family (ABCR) has been identified in humans (*8*) and localized to chromosomal position 1p22.1-p21 by fluorescence in situ hybridization (*9*). This protein is homologous to the bovine (*10*) and *Xenopus* (*11*) rim proteins previously identified in rod out segments. Several studies have localized ABCR to the disk membrane of rod outer segments (*10*, *12*−*14*). In vitro reconstitution studies carried out using purified bovine ABCR suggest that a retinoid, specifically *trans*-retinal, is the substrate of ABCR (*15*). These findings were extended through ocular characterization of ABCR knock-out mice (*16*) which displayed delayed dark adaptation and increased levels of *all-trans*-retinaldehyde and phosphatidylethanolamine in the outer segments following light exposure.

The identification of the specific mutations associated with various visual diseases (*17*−*19*) has helped to formulate the analysis of their basis at a molecular level. Human genetic studies have correlated mutated forms of retina-specific ABCR gene with several inherited visual diseases, including Stargardt macular dystrophy (*9*, *20*−*24*), fundus flavimaculatus (*20*, *23*, *25*, *26*), age-related macular degeneration (*21*, *24*, *26*, *27*), retinitis pigmentosa (*22*, *28*−*30*), and cone−rod dystrophy (*22*, *28*). Nearly all ABCR mutations identified in patients with recessive Stargardt disease, age-related macular degeneration, and fundus flavimaculatus map within nucleotide binding domains 1 and 2 (NBD1[1] and NBD2),

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[1] Abbreviations: aa, amino acid(s); ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thio-β-D-galactoside; kDa, kilodalton(s); NBD, nucleotide binding domain; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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pointing to a defect in the ATP-driven energy transduction process. However, very little is known regarding the energy transduction process mediated by these two domains, nor is it known how the ABCR mutations observed in inherited visual diseases affect the mechanism of action of this protein.

In this paper, we describe the first cloning, expression, and biochemical characterization of the C-terminal nucleotide binding domain of human ABCR protein (hABCR). The approach of molecular analysis of smaller fragments of a larger polypeptide has proved quite effective in the study of rhodopsin by Crouch and co-workers (*31*). Expression of NBD2 as an individual polypeptide will allow (i) determination of whether the putative nucleotide binding domain is also a ATPase enzymatic site, (ii) analysis of the enzymatic activity/structure of this domain, and (iii) development of a means of analyzing the specific biochemical consequences of observed genetic mutations on the ATPase activity of the domain. The knowledge gained in our studies will be helpful in understanding the relative contribution of this domain in the translocation process. Most importantly, it will establish a system for analyzing mutations identified in inherited visual diseases such as Stargardt disease, age-related macular degeneration, and fundus flavimaculatus.

MATERIALS AND METHODS

Nucleic Acids, Enzymes, and Other Reagents. The plasmid pH85972 containing wild-type cDNA corresponding to the C-terminal domain of the human ABCR gene was obtained from Genome Systems Inc., St. Louis, MO, and the full-length clone was obtained from Dr. Jeremy Nathans of Johns Hopkins University, Baltimore, MD. Ultrapure ribo- and deoxynucleotides were obtained from Pharmacia and were used without further purification. [α-^{32}P]ATP and [α-^{32}P]dATP were obtained from DuPont NEN (Boston, MA). Poly(ethylenimine)−cellulose TLC strips were from J. T. Baker Chemical Co. (Pittsburgh, PA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and were of high purity (≥95%), as determined by autoradiography of the phosphorylated products. Oligonucleotides used in the polymerase chain reaction (PCR) were used without additional purification. The T7 expression system vector pET29a and the S-protein agarose affinity resin were from Novagen (Madison, WI). *Pfu* DNA polymerase for PCR amplification was from Stratagene (La Jolla, CA).

Buffers. Buffer A contained 25 mM Tris-HCl (pH 7.9), 10% sucrose, 0.005% NP40, and 0.25 M NaCl. Buffer B contained 20 mM Tris-HCl, (pH 7.5), 150 mM NaCl, and 0.01% NP40. Buffer C contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/mL BSA, and 5 mM DTT.

Assay for ATPase Activity. The ATPase activity assays were carried out as previously described (*32*). The amount of NBD2 protein used in the assays was selected such that the rate of hydrolysis would be linear in the time range examined. A standard 10 μL reaction mixture contained 10 mM $MgCl_2$, 200 pmol of M13mp18 ssDNA (unless otherwise indicated), 100−500 μM (as indicated) [α-^{32}P]ATP, and purified NBD2 as indicated, in buffer C. Reactions were incubated at 37 °C for 30 min (unless stated otherwise) and terminated by addition of 2 μL of 200 mM EDTA followed by chilling on ice. Two microliter aliquots were applied to poly(ethylenimine)−cellulose strips which were prespotted with an ADP and ATP marker. The strips were developed with 1 M formic acid, 0.5 M LiCl and dried. The ADP and ATP spots were located by UV fluorescence. The portions containing ATP and ADP were excised and counted in a Beckman LS500 liquid scintillation counter using a toluene-based scintillator. In the kinetic analyses, reactions were carried out in a single tube and were initiated by the addition of NBD2 protein. At the indicated time points, aliquots (10 μL) were removed and transferred to tubes containing 2 μL of 200 mM EDTA, and held on ice until completion of the last time point. The remainder of the assay was carried out as described above.

Cloning of the Construct Containing NBD2. NBD2 was amplified from the human retinal cDNA clone pH87952 (Genome Systems, Inc., St. Louis, MO). The DNA containing this domain was isolated by polymerase chain reaction (PCR) under high-fidelity reaction conditions (use of Pfu DNA polymerase and high-fidelity reaction conditions: low dNTP concentration and use of a minimal number of cycles) using oligonucleotide primers containing a *Bam*HI site with an in-frame ATG (met) codon at the 5′-primer and a *Hin*dIII site after the stop codon in the 3′-primer. The region amplified spanned nucleotides 5692−7196, considering the ATG start codon as the first nucleotide of the ABCR gene. This region is slightly larger than the actual NBD2 ORF which corresponds to nucleotides 5692−6819. The PCR-amplified DNA was cloned into the expression vector pET29a (Novagen Corp., Milwaukee, WI) in the *Bam*HI/*Hin*dIII sites. Putative recombinants were analyzed by restriction mapping. The plasmid pET29aNBD2 was digested with *Afl*II and *Bgl*II, and a wild-type *Afl*II/*Bgl*II fragment was incorporated in order to remove any mutation arising from PCR. The *Afl*II/*Bgl*II fragment encompasses a major portion of the gene excluding a 90 bp region near the 3′-end which was verified by DNA sequencing. The resulting recombinant plasmid, pET29aNBD2, was used for expression of the polypeptide in *E. coli* [*BL21DE3*] following the procedure described below. The NBD2 construct contained 376 aa spanning the entire C-terminal soluble hydrophilic domain of ABCR and has a deduced molecular mass of 41.6 kDa. The cloning was designed such that NBD2 was expressed as an S-tag fusion protein in order to facilitate purification, which added an additional 34 aa to the polypeptide. The calculated molecular mass of the NBD2 protein with an S-tag is 45.3 kDa.

In Vitro Site-Directed Mutagenesis of the NBD2 Gene. Site-directed mutagenesis was carried out using a mutagenesis kit (Stratagene) as previously described (*32*). The NBD2 expression vector pET29aNBD2 was used as template, 12 cycles of PCR, and each cycle was 30 s at 95 °C, 30 s at 50 °C, and 15 min at 68 °C using the complementary oligonucleotides 5′-AGT TTG ATG CAA TCG ATG AGC TGT TCA CAG GAC GAG AAC ATC TTT AAC ATC TTT ACC TT-3′ and 5′-AAG GTA AAG ATG TTC TCG TCC TGT GAA CAG CTC ATC GAT TGC ATC AAA CT-3′) as mutagenic primers to generate NBD2 *L2027F*. Expression and purification of the mutant protein were as described above for the wild-type NBD2 protein. The authenticity of the mutation and the absence of other fortuitous mutations were confirmed by DNA sequencing carried out by the Nucleic Acid Core Facilities at the Kimmel Cancer Center of Thomas Jefferson University and the University of

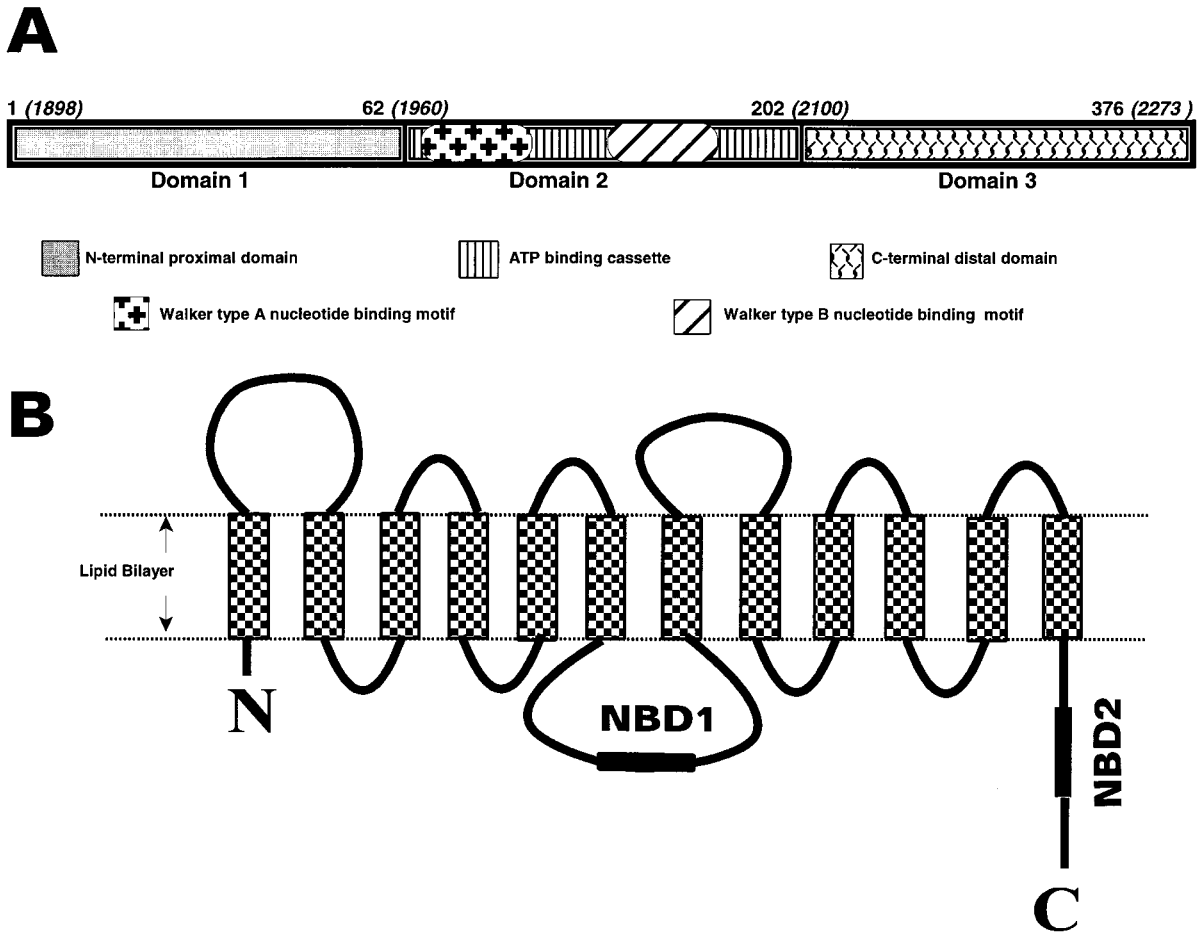

FIGURE 1: Schematic representation of the various domains of NBD2 and human ABCR. (A) Linear representation of the NBD2 polypeptide, dividing it into three subdomains. The locations of the Walker type A nucleotide binding motif (GXXGXGKT) at aa 72−82 (1970−1980) and the Walker type B nucleotide binding motif (LLLDEPXXXLD) at aa 196−205 (2094−2103) are indicated. The amino acid numbers as they pertain to full-length ABCR are italicized in parentheses. (B) The ABCR polypeptide with its 12 membrane-spanning helices (rectangles). The cytoplasmic domains referred to are defined as follows: NBD1 (aa residues 854−1357) and NBD2 (aa residues 5733−6900).

Medicine and Dentistry of New Jersey. The sequence of NBD2 wild type matched that of the published hABCR sequence (GenBank Accession Numbers GI:2959642, AF001945.1) (*9*).

Other Methods. Protein concentrations were determined by the method of Bradford (*33*) using bovine serum albumin as a standard. SDS−PAGE was carried out as described by Laemmli (*34*).

RESULTS

Design of the NBD2 Construct. Two cytoplasmic domains (*9*) which are thought to play a role in ATP hydrolysis based on the presence of Walker (*2*) type A (GXXGXGKT) and B (LLLDEPXXXLD) nucleotide binding motifs were identified by sequence analysis of the protein structure of human ABCR protein. The first cytoplasmic nucleotide binding domain comprises aa residues 854−1357, which we have defined as NBD1 (Figure 1). The second cytoplasmic nucleotide binding domain, aa residues 1898−2273, is defined as NBD2 (Figure 1) and is the focus of our current studies. Analysis of the amino acid sequence of this region revealed the presence of a Walker type B nucleotide binding motif as well as a second Walker type A nucleotide binding motif, not previously described in the literature, located at amino acids 1970−1980.

Induction of Expression of pET29aNBD2 in E. coli Strain BL21(DE3). E. coli cells (strain *BL21*(*DE3*)) harboring the pET29aNBD2 plasmid were grown with shaking at 37 °C to OD_{600} 0.4. IPTG was then added to a final concentration of 0.5 mM, and incubation at 37 °C with shaking was continued for 1 h. The expressed polypeptide appeared to be of the anticipated size (~45 kDa) as determined by SDS−PAGE (Figure 2). The cells were harvested by centrifugation for 10 min at 5000*g* and then resuspended in 2.5% of the original culture volume of buffer A at 4 °C and stored at −80 °C until further use.

Preparation of E. coli Whole Cell Extract. Frozen cells were thawed in an ice/water bath, and the following components were added: 0.1 mg/mL lysozyme; 1.0 μg/mL each of the following protease inhibitors: leupeptin, pepstatin A, antipain, chymostatin; 0.1 mM each of benzamidine hydrochloride and $NaHSO_3$; and 2.5 μg/mL each of TPCK and TLCK. The cells were kept on ice for 30 min, placed in a 37 °C water bath for 5 min, and then returned to ice. Freeze−thaw treatment resulted in a greater degree of cell lysis than that observed with addition of lysozyme alone. The cell extract was then sonicated (Tekmar sonicator) using 3 × 60 s bursts at 80% total power. This greatly decreased the viscosity caused by the release of cellular DNA. Following sonication, the extract was clarified by centrifuga-

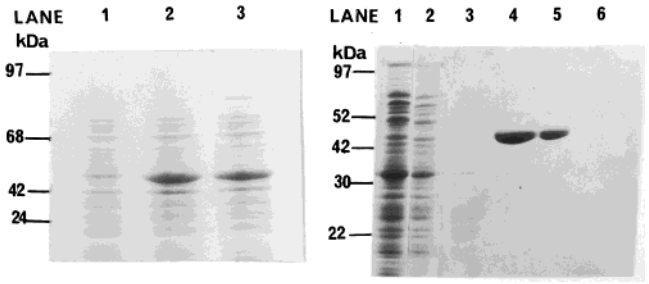

FIGURE 2: (Left) SDS−PAGE analysis of the expression of NBD2 polypeptide in *E. coli*. Expression of the recombinant protein was carried out as described under Results, in the presence of 0.5 mM IPTG. Equal amounts of cells before and after induction were analyzed by 5−18% SDS−PAGE followed by Coomassie Blue staining. Lane 1, BL21(DE3)/pET29aNDB2 (clone 2-1) cells before induction; lane 2, BL21(DE3)/pET29aNDB2 cells (clone 2-1) after induction; lane 3, lane 2 BL21(DE3)pET29aNDB2 cells (clone 2-2) after induction. Protein standards are as indicated. (Right) SDS−PAGE of S-protein agarose purification of NBD2. Aliquots (25 μL) of the indicated fractions were analyzed on a 10−15% SDS−PAGE gel which was stained with Coomassie Blue R250. Lane 1, fraction I; lane 2, flow through; lane 3, wash; lane 4, fraction 1; lane 5, fraction 2; lane 6, fraction 3. Protein molecular mass markers are as indicated.

tion at 30000*g* for 20 min at 4 °C. A second extraction of the cell pellet was carried out to remove any residual protein, and the two supernatants were combined. The NBD2 polypeptide appeared to be moderately soluble. The extract (fraction I) was further purified as described below.

Purification of NBD2 Protein by Immobilized S-Protein Agarose Chromatography. NBD2 protein carrying the S-tag was purified using immobilized S-protein agarose affinity resin (Novagen). Proteins which carry the S-tag bind to the ribonuclease S-protein which is coupled to resin. Recovery of the target protein is effected by elution with buffer containing 3 M $MgCl_2$. This affinity purification method yields highly purified, homogeneous preparations of protein in a single step.

All procedures were carried out at 4 °C unless otherwise indicated. Starting from 2 L of induced cell culture, the volume of fraction I was adjusted to decrease the conductivity to that of buffer B. The extract was loaded slowly at a rate of 0.5 mL/min onto a 1.0 mL affinity column equilibrated with buffer B. The column was then washed with 10 column volumes of buffer B. The NBD2 protein was eluted isocratically with buffer B containing 3 M $MgCl_2$. One milliliter fractions were collected. The fractions were assayed for protein and analyzed by SDS−PAGE (Figure 2). The protein is homogeneous following elution from the S-protein column. The peak fractions were pooled, and $MgCl_2$ was removed by dialysis against buffer B containing 25% glycerol. The yield of NBD2 from this single-step purification was 0.5 mg.

ATPase and dATPase Activities of NBD2. The availability of highly purified homogeneous preparations of the NBD2 allowed us to examine the role of this domain in the ATPase activity of the ABCR protein. As described above, NBD2 contains both Walker type A and type B nucleotide binding motifs. At present it is not known (i) whether NBD2 has ATPase activity and (ii) the exact contribution of the various nucleotide binding motifs to the overall nucleotide hydrolysis, to determine whether this domain contains an entire ATPase catalytic site or whether other functional domains of the ABCR protein are also required for ATPase activity. It was

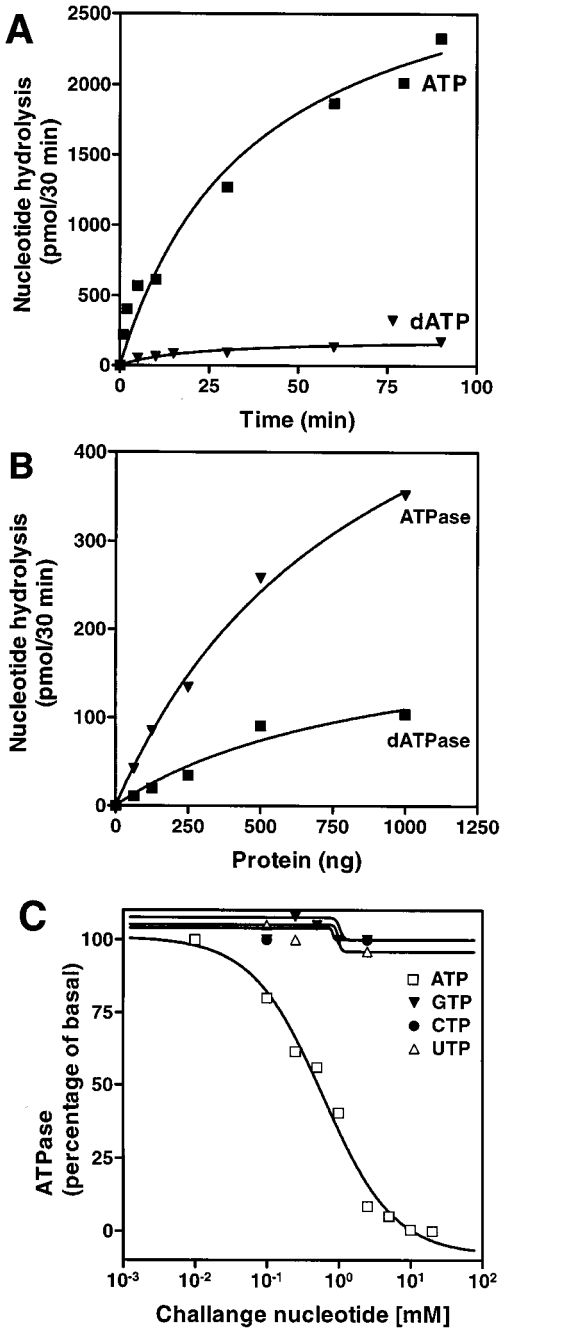

FIGURE 3: Analysis of the ATPase activity of NBD2. (A) ATP/dATP hydrolysis by NBD2. Protein titration of purified NBD2 in a standard ATPase assay was carried out as described under Materials and Methods at 37 °C for 30 min using the indicated nucleotide triphosphate. (B) Time course analysis of ATP/dATP hydrolysis. Standard ATPase assays were carried out as described under Materials and Methods at 37 °C for the times indicated using the respective nucleotide triphosphate and 250 ng of purified NBD2 protein. (C) Nucleotide inhibition analysis of ATP hydrolysis. Inhibition of ATP hydrolysis by varying concentrations of unlabeled ATP (□), GTP (▼), CTP (●), and UTP (△). The ATPase assay was carried out as described under Materials and Methods, using 250 ng purified NBD2.

hoped that purified NBD2 could be used to help resolve these questions, provided that the recombinant protein was enzymatically active. A protein titration of NBD2 versus the ATPase/dATPase activity is shown in Figure 3B. Recombinant NBD2 could hydrolyze both forms of this nucleoside triphosphate, but appeared to be less effective with dATP.

A time course analysis of ATP hydrolysis demonstrated that, under the conditions studied, the rate of hydrolysis is fairly linear over a 60 min time period, after which it begins to reach equilibrium (Figure 3A). The ATPase activity of NBD2 was salt-insensitive, as no inhibition of the activity was observed in the presence of NaCl up to 1.8 M (data not shown). The lack of influence of high salt on enzyme activity is consistent with the ionic strength in which the protein functions.

Analysis of the Specificity of Nucleotide Hydrolysis. To investigate the specificity of nucleotide binding, we have carried out nucleotide inhibition studies of the ATPase activity. These studies suggest that binding is specific for ATP, as no inhibition was observed with other nucleotides over the concentration range investigated (0.1−20 mM). Half-maximal inhibition was observed at approximately 200 μM ATP (Figure 3C). Previous nucleotide inhibition studies carried out using native bovine ABCR demonstrated that GTP as well as ATP could inhibit ATP photoaffinity labeling (*10*). This suggests that GTP binding may be mediated through the first nucleotide binding domain (NDB1).

Kinetic Analysis of ATP Hydrolysis. Kinetic analysis of ATP and dATP hydrolysis by NBD2 is shown in Figure 4A,B. Analysis of the ATPase activity of NBD2 in the presence of varying concentrations of ATP demonstrates simple Michaelis−Menten kinetics. The kinetic parameters of NBD2 for ATP hydrolysis are K_m= 631 μM, V_{max} = 144 nmol min^{-1} mg^{-1}. When this value was normalized to account for the difference in molecular mass between NBD2 (43 kDa) and ABCR (220 kDa), the rate of ATP hydrolysis [29 nmol min^{-1} (mg of NBD2)$^{-1}$] was comparable to that observed with reconstituted preparations of bovine ABCR (278 μM and 27 nmol min^{-1} mg^{-1}). Kinetic analysis of dATP hydrolysis indicated that NBD2 binds the deoxy form of the nucleotide triphosphate more tightly (K_m = 116 μM); however, it carries out the hydrolysis with approximately 10-fold less efficiency (14.6 nmol min^{-1} mg^{-1}; the normalized value was 2.92 nmol min^{-1} mg^{-1}).

Influence of a Leu2027Phe Mutation on ATP Hydrolysis by NBD2 Polypeptide. The mutation *L2027F* has been previously reported in some patients suffering from Stargardt macular degeneration (*9*, *21*). Consequently, it was of interest to examine the effect of this mutation on the ATPase activity of NBD2 (Figure 5). Using a site-directed mutagenesis protocol, the mutation (*L2027F*) was introduced into the pET29aNBD2 plasmid, following which the mutant protein was expressed and purified. The kinetic parameters (K_m and V_{max}) of the mutant and wild-type NBD2 proteins were then compared. Our results indicated a drastic change in the ATPase activity of the mutant protein. The *L2027F* point mutation in the NBD2 polypeptide increased the affinity of binding K_m (46 μM) and resulted in a decrease in the rate of ATP hydrolysis, 16.6 nmol of ATP min^{-1} mg^{-1}. This corresponds to a 9-fold decrease in V_{max} and a 14-fold decrease in K_m from those observed with the wild-type NBD2 polypeptide. Observation of a decrease in the rate of ATP hydrolysis as a result of the site-directed mutagenesis further substantiates that the observed ATPase activity was due to the NBD2 polypeptide and not a trace impurity undetectable by SDS−PAGE.

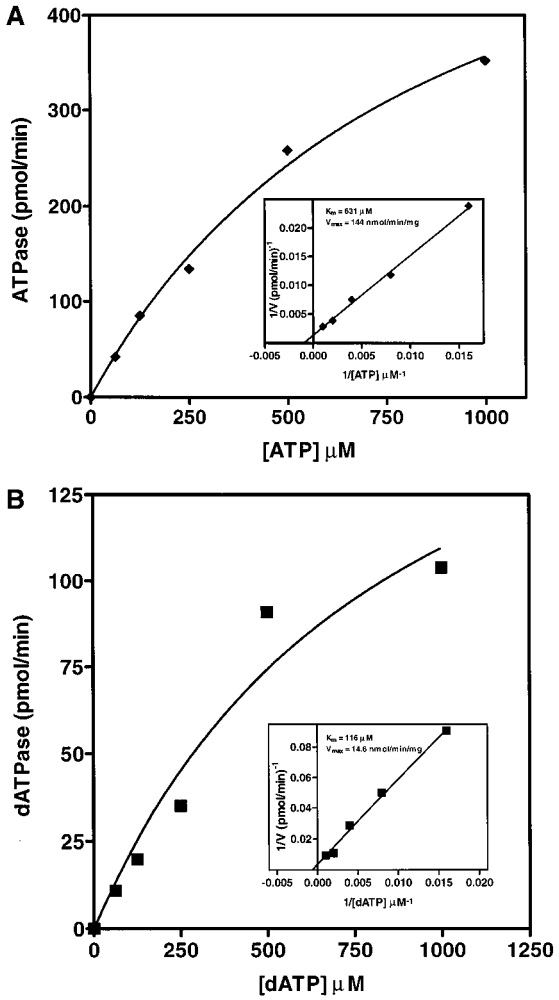

FIGURE 4: Kinetic analysis of ATP and dATP hydrolysis by NBD2. (A) ATPase. ATPase activity (*V*) vs ATP concentration ([S]). The curve was generated using a nonlinear regression analysis of the data. (Inset) Double-reciprocal plot (1/*V* vs 1/[S]) of the ATPase activity. The ATPase assay was carried out as described under Materials and Methods. The data points represent the mean of three separate experiments with SD ≤ ±4.0%. (B) dATPase. dATPase activity (*V*) vs ATP concentration ([S]). The curved was generated using a nonlinear regression analysis of the data. (Inset) Double-reciprocal plot (1/*V* vs 1/[S]) of the ATPase activity. The ATPase assay was carried out as described under Materials and Methods. The data points represent the mean of three separate experiments with SD ≤ ±4.0%.

DISCUSSION

In this article, we have described the expression and rapid purification of the second nucleotide binding domain of the human ABCR protein (NBD2). The expressed protein appeared to constitute approximately 30% of the total cellular protein (Figure 2), and was moderately soluble. The results we obtained appear comparable to those obtained for expression of a related ABC transporter, the MDR1 protein (*35*). In these studies, the investigators utilized the prokaryotic expression vector, pMal-c, and obtained comparable levels of solubility. We have developed a rapid purification scheme for the polypeptide which utilizes immobilized S-protein agarose affinity chromatography (Figure 2). Using this procedure, we were able to generate 0.5 mg of highly purified homogeneous protein from 2 L of induced cell culture.

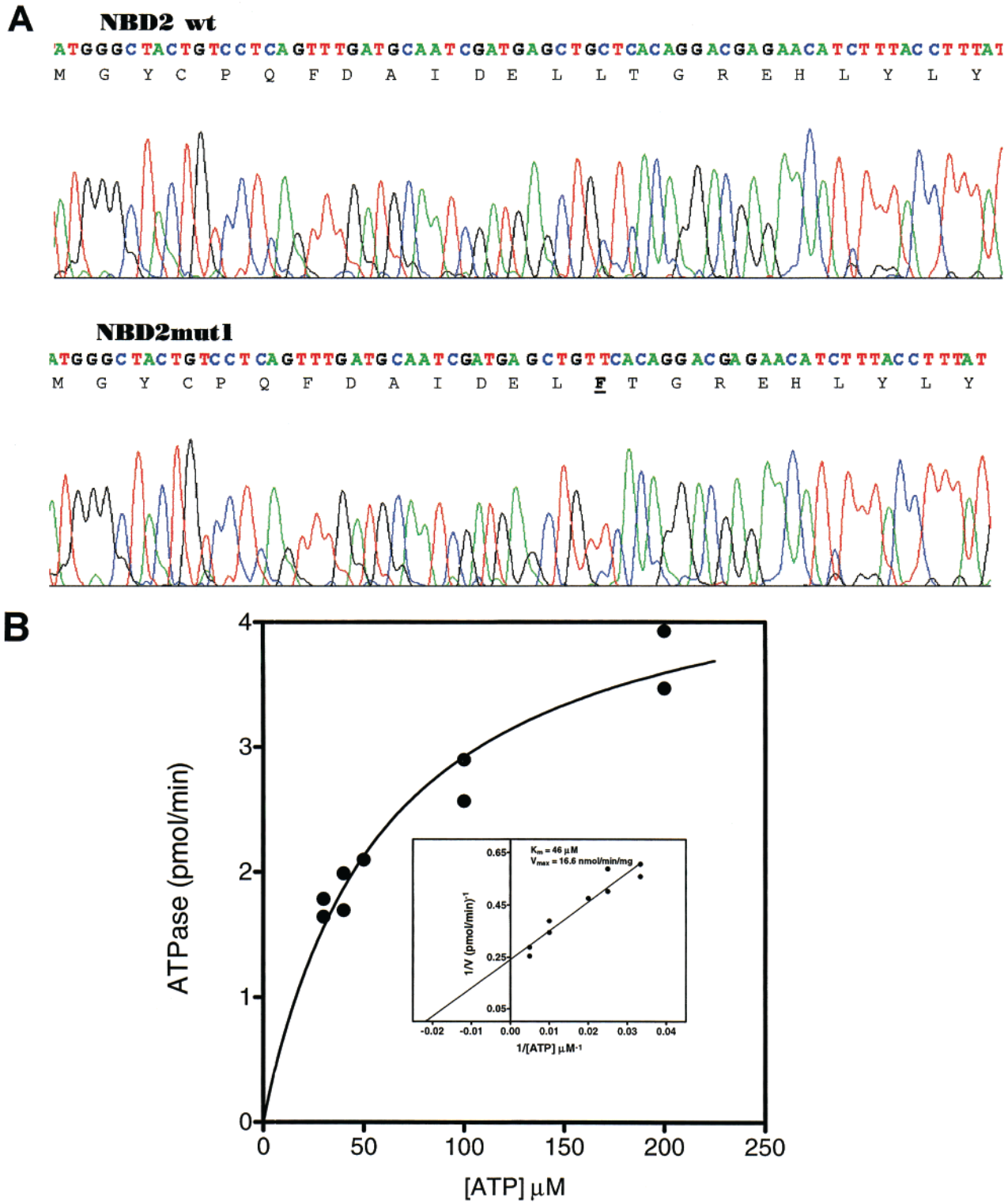

FIGURE 5: (A) DNA sequence analysis of NBD2mut1 (L2027F). Chromatogram of DNA sequence analysis surrounding the region of the L2027F mutation of NBD2. The wild-type and mutant nucleotide sequences along with their corresponding amino acid translation are shown. (B) ATPase activity of the Leu2027Phe NBD2 mutant protein. Kinetic analysis of the ATPase activity demonstrating the difference in the initial rate of ATP hydrolysis. The corresponding Lineweaver−Burk plot is shown as an inset. The ATPase assays were carried out as described under Materials and Methods. The data points represent the mean of three separate experiments with SD ≤ ±4.0%.

The NBD2 Domain Is a Fully Functional ATPase. With the availability of highly purified preparations of NBD2, it was possible to investigate the question of whether this domain alone was enzymatically active as an ATPase. Several possibilities exist including (i) NBD2 could act as a functional ATPase, (ii) NBD2 may function as only a "regulatory/cooperative" nucleotide binding site, and not as a hydrolysis site, and (iii) no enzymatic activity would be observed as a consequence of the experimental system (e.g., if physical interaction of the two binding sites is required for ATP hydrolysis to occur). The results presented in Figure 3 clearly demonstrate that the NBD2 polypeptide itself is fully capable of ATP hydrolysis. This protein was also able to utilize dATP as a substrate, but appeared to do so with

less efficiency (Figures 3 and 4). These data also demonstrated that interaction between nucleotide binding domains NBD1 and NBD2 is not required for ATPase activity.

Kinetics of ATP and dATP Hydrolysis by NBD2. Nucleotide challenge experiments, shown in Figure 3C, clearly demonstrated that the binding is specific for adenosine nucleotides (ATP or dATP), as none of the other ribonucleotides were able to inhibit ATP hydrolysis. Earlier photoaffinity labeling studies carried out by Illing and co-workers (*10*) indicated that the full-length native form of bovine ABCR can also bind GTP. One may speculate, given the results presented here, that the full-length hABCR may also be capable of binding guanosine ribonucleotides; however, this binding may be mediated through NBD1 and not NBD2. In reports analyzing the contributions of multiple nucleotide binding motifs, for example, the SecA protein of *E. coli* (*36*), it is not uncommon for them to vary with respect to their binding affinity and specificity.

Analysis of the kinetic parameters of NBD2 determined that the K_m for ATP was 631 μM and that the rate of hydrolysis was $V_{max} = 144$ nmol of ATP min^{-1} mg^{-1}. If the V_{max} value is normalized for the difference in size between NBD2 (43 kDa) and full-length ABCR (220 kDa), then the rate is 29 nmol of ATP min^{-1} mg^{-1}, which is in good agreement with that observed for reconstituted preparations of native bovine ABCR (27 nmol of ATP min^{-1} mg^{-1}). The data presented in Figure 3 demonstrate that, although NBD2 can hydrolyze dATP, it does so with much less efficiency. Determination of the K_m (116 μM) and V_{max} (14.6 and 2.92 nmol min^{-1} mg^{-1}) normalized for dATP hydrolysis indicates that the NBD2 binds dATP with higher efficiency than the ribo form of the nucleotide. Therefore, our studies suggest that overall adenine base recognition is significant, but that the enzyme can bind both ribo and deoxy forms. In fact, the deoxy form appears to be bound more tightly, yet is hydrolyzed with less efficiency. As we indicated under Results, this increase in binding affinity (also observed with the Leu2027Phe mutant) was accompanied by a decrease in the rate of hydrolysis (V_{max}).

Functional Consequence of L2027F Mutation Associated with Stargardt Disease. The *L2027F* mutation in the full-length ABCR gene is associated with Stargardt disease (SGD1) (*9*, *21*). Site-directed mutagenesis has proved to be an important tool for investigating the structure and function of important visual proteins (*37*). Introduction of the point mutation *L2027F* in the NBD2 polypeptide was observed to increase the affinity of ATP binding 14-fold ($K_m = 46$ μM) while decreasing the rate of ATP hydrolysis ($V_{max} = 16.6$ nmol of ATP min^{-1} mg^{-1}). This represents a 9-fold decrease in V_{max}, and a 14-fold decrease in K_m from that observed with the wild-type NBD2 polypeptide. This decrease in V_{max} to 11% of the wild-type molecule is fairly significant because it implies that 89% of the original activity was lost due to the mutation. The observed defects in retinal transport associated with the *L2027F* mutation may be related, at least in part, to the decreased rate of ATP hydrolysis. The observed increased affinity of binding should lead to an increase in the half-life of the ADP-bound form of the molecule which would reduce the ATP turnover rate. In addition, it is possible that protein conformations of the ADP- versus ATP-bound ABCR are important for retinal transport. If this were the case, a decrease in the rate of ADP release following ATP hydrolysis would have an effect on overall transport. The role of ABCR in the pathology of Stargardt disease is complex and perhaps multi-factorial and will no doubt require numerous lines of investigation to completely delineate. Several distinct ABCR mutations have been associated with this disease. The disease itself is progressive, and onset is later in childhood rather than at birth. As many of the mutations map to the ATP binding cassette region, it is possible that these mutations will affect the ATPase activity of ABCR and hence its overall transport function. What we have demonstrated in this report is that the Leu2027Phe mutation does indeed lead to a significant decrease in the basal level of ATPase of the NBD2 domain of ABCR.

These results demonstrate that the C-terminal domain of the human ABCR transporter protein is a functional ATPase and an attenuated dATPase. Further studies are required to understand the structure and function of this domain. Our ongoing studies on the mutational analysis of this domain would likely shed light on the mechanism of the energy transduction involving ATP and/or dATP hydrolysis.

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