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Nucleotide Binding Domain 1 of the Human Retinal ABC Transporter Functions as a General Ribonucleotidase†

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ABSTRACT: Members of the ATP binding cassette (ABC) superfamily are transmembrane proteins that are found in a variety of tissues which transport substances across cell membranes in an energy-dependent manner. The retina-specific ABC protein (ABCR) has been linked through genetic studies to a number of inherited visual disorders, including Stargardt macular degeneration and age-related macular degeneration (ARMD). Like other ABC transporters, ABCR is characterized by two nucleotide binding domains and two transmembrane domains. We have cloned and expressed the 522-amino acid (aa) N-terminal cytoplasmic region (aa 854−1375) of ABCR containing nucleotide binding domain 1 (NBD1) with a purification tag at its amino terminus. The expressed recombinant protein was found to be soluble and was purified using single-step affinity chromatography. The purified protein migrated as a 66 kDa protein on SDS−PAGE. Analysis of the ATP binding and hydrolysis properties of the NBD1 polypeptide demonstrated significant differences between NBD1 and NBD2 [Biswas, E. E., and Biswas, S. B. (2000) *Biochemistry 39*, 15879−15886]. NBD1 was active as an ATPase, and nucleotide inhibition studies suggested that nucleotide binding was not specific for ATP and all four ribonucleotides can compete for binding. Further analysis demonstrated that NBD1 is a general nucleotidase capable of hydrolysis of ATP, CTP, GTP, and UTP. In contrast, NBD2 is specific for adenosine nucleotides (ATP and dATP). NBD1 bound ATP with a higher affinity than NBD2 ($K_{mNBD1} = 200\ \mu M$ vs $K_{mNBD2} = 631\ \mu M$) but was less efficient as an ATPase ($V_{maxNBD1} = 28.9$ nmol min^{-1} mg^{-1} vs $V_{maxNBD2} = 144$ nmol min^{-1} mg^{-1}). The binding efficiencies for CTP and GTP were comparable to that observed for ATP ($K_{mCTP} = 155\ \mu M$ vs $K_{mGTP} = 183\ \mu M$), while that observed for UTP was decreased 2-fold ($K_{mUTP} = 436\ \mu M$). Thus, the nucleotide binding preference of NBD1 is as follows: CTP > GTP > ATP ≫ UTP. These studies demonstrate that NBD1 of ABCR is a general nucleotidase, whereas NBD2 is a specific ATPase.

The retina-specific member of the ATP binding cassette (ABC) transporter family (ABCR) has been linked through genetic studies to a number of inherited retinal diseases, including Stargardt macular dystrophy (*1*−*6*), fundus flavimaculatus (*2*, *5*, *7*, *8*), age-related macular degeneration (*3*, *6*, *8*, *9*), retinitis pigmentosa (*4*, *10*−*12*), and cone-rod dystrophy (*4*, *10*). Like other members of the ABC family, the ABCR protein is comprised of tandem transmembrane domains with two Walker (*13*) type A nucleotide binding motifs and one type B nucleotide binding motif. The identification of the specific mutations associated with

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various visual diseases (*14*, *15*) has helped the formulation of the analysis of their basis at a molecular level. Nearly all ABCR mutations identified in patients with recessive Stargardt disease, age-related macular degeneration, and fundus flavimaculatus map within nucleotide binding domain 1 or 2 (NBD1 or NBD2, respectively), pointing to a defect in the ATP-driven energy transduction process. As observed in several other visual pathologies (*16*, *17*), these patients display accumulation of lipofuscin in the retinal pigment epithelium, and how this relates to ABCR-mediated transport is unclear at the present time.

The ABCR protein is homologous to the bovine (*14*) and *Xenopus* (*15*) rim proteins previously identified in rod out segments. Several studies have localized ABCR to the disk membrane of rod outer segments (*14*, *16*−*18*). In vitro reconstitution studies carried out using purified bovine ABCR suggest that retinoids, specifically *all-trans*-retinal, is the substrate of ABCR (*19*). These findings were extended through ocular characterization of ABCR knockout mice (*20*) which displayed delayed dark adaptation and increased levels of *all-trans*-retinaldehyde and phosphatidylethanolamine in the outer segments following exposure to light.

Recent studies in several laboratories have begun to explore the relationship between specific mutations and ABCR protein function using recombinant proteins (*21*) or polypeptides (*22*). Sun et al. (*21*) explored the influence of several mutations on azido-ATP binding in recombinant ABCR expressed in COS cells. These studies indicated that differences in ATP binding could be observed in several mutant proteins. However, limitations of the experimental system, such as the extreme lability of the recombinant polypeptides (H. Sun, personal communication), appear to preclude the possibility of extensive biochemical or structure−function analyses. In an effort to evaluate the contribution of the individual nucleotide binding domains in ABCR structure and function, our laboratory has taken an alternative approach by expressing the two nucleotide binding domains (NBD1 and NBD2) as individual polypeptides. The approach of molecular analysis of smaller fragments of a larger polypeptide has proven to be quite effective in the study of rhodopsin by Crouch and co-workers (*27*). Using this approach, the C-terminal domain of hABCR was found to be a functional ATPase in vitro (*26*). Site-directed mutagenesis was then used to introduce the Leu2027Phe mutation, which is genetically linked to Stargardt macular degeneration, into the recombinant polypeptide. Biochemical analysis of the recombinant polypeptide revealed a 14-fold decrease in K_m and a 9-fold decrease in the rate of ATP hydrolysis (V_{max}).

In this report, we describe the cloning, expression, and biochemical characterization of the first nucleotide binding domain (NBD1) of the human ABCR protein (hABCR). Expression of NBD1 as an individual polypeptide will allow (i) determination of whether the putative nucleotide binding domain is also an ATPase enzymatic site, (ii) analysis of the enzymatic activity and structure of this domain, (iii) comparison of the biochemical properties of NBD1 and NBD2 in an effort to better understand overall ABCR protein function, and (iv) the 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 retinal translocation process. Of equal importance is the fact that 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 pH85972 plasmid 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 in the pRK5 plasmid was obtained from J. 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, [α-^{32}P]GTP, [α-^{32}P]CTP, and [α-^{32}P]UTP were obtained from DuPont NEN (Boston, MA). Polyethyleneimine-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, Inc. (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 Nucleotidase Activity. The ATPase activity assays were carried out as previously described (*28*). The amount of NBD1 protein used in the assays was selected such that the rate of hydrolysis would be linear in the time range that was examined. A standard 10 μL reaction mixture contained 10 mM $MgCl_2$, 500 μM (or as indicated) [α-^{32}P]-ATP, and purified NBD1 protein (as indicated) in buffer C. Reaction mixtures were incubated at 37 °C for 30 min (unless stated otherwise), and reactions were terminated by addition of 2 μL of 200 mM EDTA followed by chilling on ice. Aliquots (2 μL) were applied to polyethyleniminecellulose strips which were prespotted with an ADP and ATP marker. The strips were developed with 1 M formic acid and 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 liquid scintillation counter. Reactions for kinetic analysis were carried out in a single tube and were initiated by the addition of NBD1 protein. At the indicated time points, aliquots (10 μL) were removed and transferred to tubes containing 2 μL of 200 mM EDTA. The remainder of the assay was carried out as described above. GTPase, CTPase, and UTPase assays were carried out in an analogous manner except that the appropriate ribonucleotides were substituted for ATP and ATP or ADP as required in the procedure.

Cloning and Expression of NBD1. NBD1 was amplified from the human retinal cDNA clone in pRK5. The DNA containing this domain was isolated by polymerase chain reaction (PCR) under high-fidelity reaction conditions using *Pfu* DNA polymerase. Oligonucleotide primers were de-

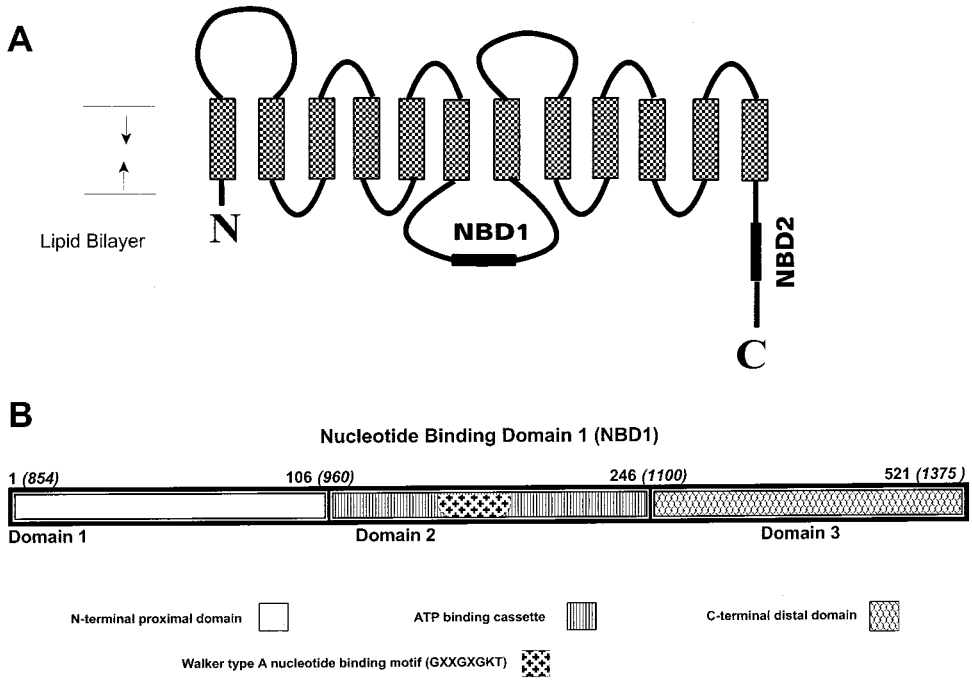

FIGURE 1: Schematic representation of the various domains of human ABCR. At the top is pictured the ABCR polypeptide with its 12 membrane-spanning helices (rectangles). The cytosolic domains mentioned in this paper are defined as follows: NBD1 (aa 854−1375) and NBD2 (aa 1898−2273). At the bottom is a linear representation of the NBD1 polypeptide, showing the location of the Walker type A nucleotide binding motif. The numbers outside the parentheses are the amino acid numbers of the NBD1 polypeptide; those italicized within parentheses are as they pertain to the full-length ABCR.

signed such that a *Bam*HI site with an in-frame ATG (Met) codon was present in the 5′-primer and a *Hin*dIII site after the stop codon in the 3′-primer. The amplified region spanned nucleotides 2641−4207, considering the ATG start codon as the first nucleotide of the ABCR gene. The PCR-amplified DNA was cloned into the pET29a expression vector (Novagen) in the *Bam*HI−*Hin*dIII sites. Putative recombinants were analyzed by restriction mapping. The absence of fortuitous mutations was confirmed by DNA sequencing. The resulting recombinant plasmid (pET29aNBD1) was used for expression of the polypeptide in *Escherichia coli* BL21DE3 cells following the procedure as described in the Results. The NBD1 polypeptide contained 522 amino acids (aa) spanning the entire N-terminal cytoplasmic domain of ABCR and has a deduced molecular mass of 57.8 kDa. The cloning was designed such that NBD1 was expressed as an S-tag fusion protein to facilitate purification, which added an additional 30 aa to the polypeptide. The calculated molecular mass of the NBD1 protein with the S-tag is 62 kDa.

Other Methods. Protein concentrations were determined by the method of Bradford (*29*) using bovine serum albumin as a standard. SDS−PAGE was carried out as described by Laemmli (*30*).

RESULTS

Design and Cloning of the Construct Containing NBD1. Two cytoplasmic domains (*1*) which are thought to play a role in ATP hydrolysis based on the presence of Walker (*13*) 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 854−1357 (2641−4207 bp), which we have defined as NBD1 (Figure 1) and is the focus of our current studies. Analysis of the amino acid sequence of the NBD1 region reveals the presence of a Walker type A nucleotide binding motif. The second cytoplasmic nucleotide binding domain, aa 1898−2273 (5793−6900), is defined as NBD2 (Figure 1). Located within NBD2 are Walker type A and type B nucleotide binding motifs.

Induction of Expression of pET29bNBD1 in E. coli Strain BL21DE3. E. coli cells (strain BL21DE3) harboring the pET29bNBD1 plasmid were grown with shaking at 37 °C to OD_{600} of 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 polypeptide appeared to be of the anticipated size (∼62 kDa), as determined by SDS−PAGE (Figure 2). The cells were harvested by centrifugation at 5000*g* for 10 min, 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 the 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, the protease inhibitors leupeptin, pepstatin A, antipain, and chymostatin (1.0 μg/mL each), 0.1 mM benzamidine-HCl, 0.1 mM $NaHSO_3$, 2.5 μg/mL TPCK, and 2.5 μg/mL 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. Freezing and thawing resulted in a degree of cell lysis greater 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 centrifugation at 30000*g* for 60 min at 4 °C. A second extraction of the cell pellet was carried out to

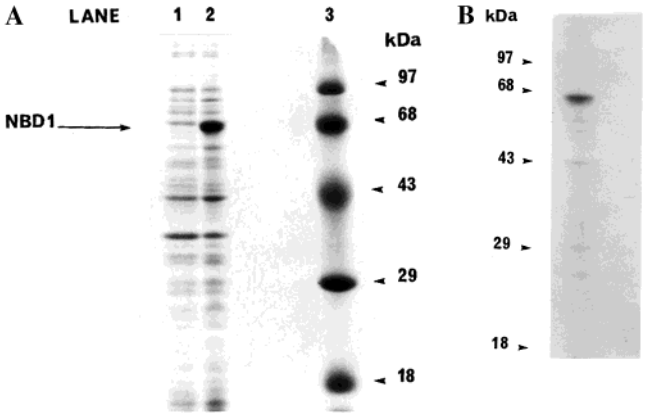

FIGURE 2: (A) SDS−PAGE analysis of the expression of NBD1 polypeptide in *E. coli.* Expression of the recombinant protein was carried out as described in the Results in the presence of 0.5 mM IPTG. Equal amounts of cells before and after induction were analyzed by 5 to 18% SDS−PAGE followed by Coomassie Blue R250 staining: lane 1, BL21(DE3)/pET29aNDB1 (clone 10-1) cells before induction; lane 2, BL21(DE3)/pET29aNDB cells (clone 10-1) after induction; and lane 3, protein molecular mass standards. (B) SDS−PAGE of S-protein agarose purification of NBD1. Aliquots (25 μL) of the indicated fractions were analyzed on a 10 to 15% SDS−PAGE gel which was stained with Coomassie Blue R250: NBD1 polypeptide following purification by S-protein agarose chromatography.

remove any residual protein, and the two supernatants were combined. The NBD1 polypeptide appeared to be moderately soluble. The extract (fraction I) was further purified as described below.

Purification of S-Tag NBD1 by Immobilized S-Protein Agarose Chromatography. The NBD1 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 $MgCl_2$.

All procedures were carried out at 4 °C unless otherwise indicated. When we started 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 S-tag NBD1 was eluted isocratically with buffer B containing 3 M $MgCl_2$. Fractions (1 mL) were collected. The fractions were assayed for protein and analyzed by SDS−PAGE (Figure 2). The protein is essentially homogeneous following elution from the S-protein column. Minor impurities ($\leq$5%) present in the affinity-purified protein were determined to be protease-degraded fragments of NBD1 containing the S-tag, as determined by Western blot analysis. This limited proteolysis persisted despite the use of protease inhibitors during purification. The peak fractions were pooled, and $MgCl_2$ was removed by dialysis against buffer B containing 20% glycerol, and finally concentrated using ultrafiltration. The yield of NBD1 from this single-step purification was 0.5 mg from 2 L of induced culture.

ATPase Activity of NBD1. Previous studies (*26*) carried out with the recombinant NBD2 polypeptide demonstrated that it is a functional ATPase and that its rate of nucleotide hydrolysis, when normalized to account for the difference in molecular mass, is comparable to that observed with reconstituted preparations of bovine ABCR. The availability of highly purified homogeneous preparations of NBD1 has now allowed us to examine the role of this domain in the ATPase activity of the ABCR protein. We focused our attention on determining (i) whether NBD1 has ATPase activity or whether other functional domains of the ABCR protein are required for its ATPase activity, (ii) the relative contribution of the multiple nucleotide binding motifs to the overall nucleotide hydrolysis of ABCR, and (iii) the differences, if any, in nucleotide binding and hydrolysis properties between NBD2 and NBD1. It was hoped that purified NBD1 could be used to help resolve these questions, provided that the recombinant protein was enzymatically active. Protein titrations of ATPase activity versus NBD1 and NBD2 are shown in Figure 3. NBD1 was clearly able to carry out ATP hydrolysis; however, NBD2 hydrolyzed approximately 5-fold more ATP than NBD1 over the concentration range that was examined.

ABCR NBD1 Is a Ribonucleotidase and Not a Specific ATPase or GTPase. To investigate the specificity of nucleotide binding, we carried out nucleotide inhibition studies of ATPase activity. Results are presented in Figure 4A. These studies showed, that in contrast to NBD2, NBD1 is capable of binding all four ribonucleotides. Previous nucleotide inhibition studies using native bovine ABCR demonstrated that both GTP and ATP could inhibit ATP photoaffinity labeling (*18*). This suggests that GTP binding may be mediated through the first nucleotide binding domain (NDB1) as previous studies carried out in this laboratory showed that binding at NBD2 was specific for ATP. Following this, the ability of NBD1 to hydrolyze other ribonuclotide triphosphates was examined. As shown in Figure 4B, the observed relative preference of NBD1 for a substrate was as follows: CTP > GTP > ATP. NBD1 was least effective as a UTPase, with a 5-fold lower observed extent of nucleotide hydrolysis.

Kinetic Analysis of rNTP Hydrolysis. Kinetic analyses of ATP, CTP, GTP, and UTP hydrolysis by NBD1 are shown in panels A−D of Figure 4, respectively. Analysis of the rNTPase activity of NBD1 in the presence of varying concentrations of respective substrates demonstrates simple Michaelis−Menten kinetics. The kinetic parameters of NBD1 for ATP hydrolysis are as follows: $K_m = 200$ μM and $V_{max} = 28.9$ nmol min^{-1} mg^{-1}. This is significantly different than that observed in previous studies with NBD2 (*26*), where $K_m = 631$ μM and $V_{max} = 144$ nmol min^{-1} mg^{-1}. Kinetic analysis carried out with the remaining rNTPs indicated that the binding efficiencies for GTP and CTP were of the same order of magnitude as that observed for ATP, 183 and 165 μM, respectively. The K_m for UTP was 436 μM, which is nearly twice that observed for ATP, CTP, and GTP. The rates of nucleotide hydrolysis, V_{max}, for CTP, GTP, and UTP were comparable: 6.3, 8.2, and 12.3 nmol min^{-1} mg^{-1}, respectively.

DISCUSSION

The human retinal ABC transporter protein is a large complex molecule with two nucleotide binding domains and 12 membrane-spanning helices, and consequently, the mechanism of energy transduction in the transport of 11-*cis*-retinal to ROD cells is very complex. Analysis of its mechanism of nucleotide binding and hydrolysis in studies that examine

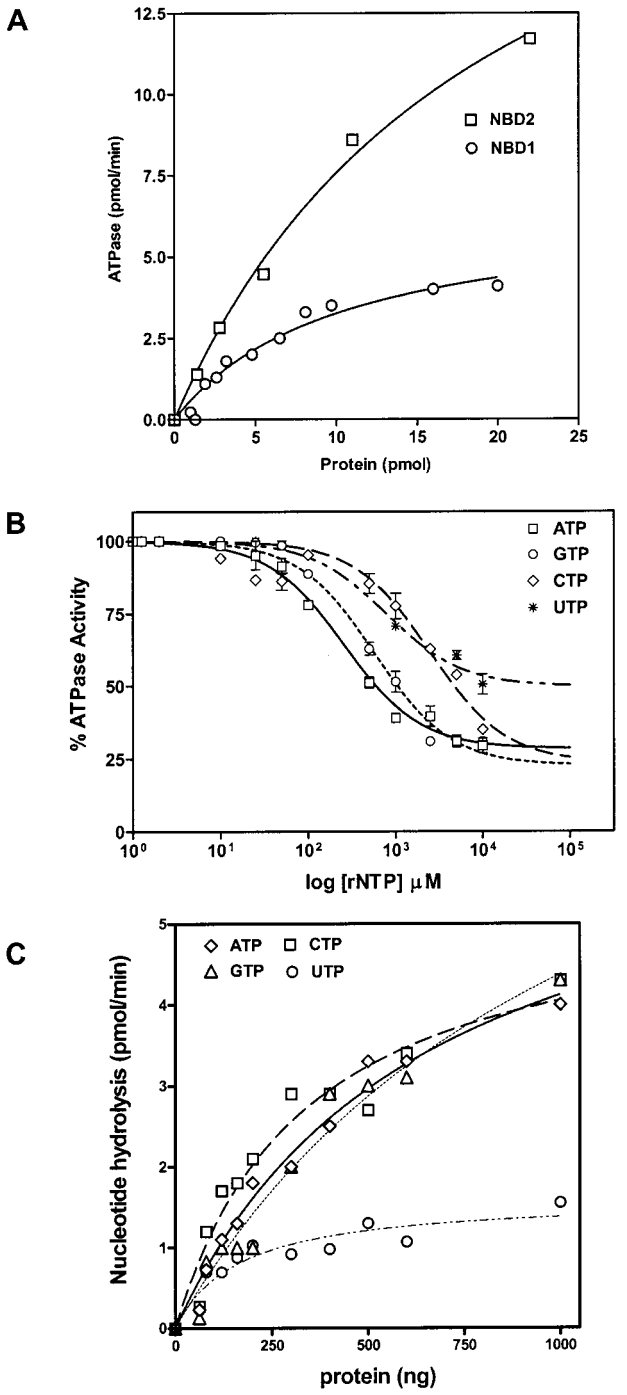

FIGURE 3: ATPase activity of NBD1. (A) Comparison of ATP hydrolysis by NBD1 and NBD2 polypeptides. Protein titration of purified NBD1 and NBD2 polypeptides in a standard ATPase assay. The assays were carried out as described in Materials and Methods at 37 °C for 30 min using the indicated amounts of protein and an ATP concentration of 500 μM. (B) Nucleotide inhibition of ATP hydrolysis. Inhibition of ATP hydrolysis with varying concentrations of unlabeled ATP (□), GTP (○), CTP (◇), and UTP (∗). The ATPase assay was carried out as described in Materials and Methods using 250 ng of the purified NBD1 protein. (C) Nucleotidase activity of NBD1. Protein titration of the purified NBD1 polypeptide in a standard ATPase assay. The assays were carried out as described in Materials and Methods at 37 °C for 30 min using the indicated amounts of protein and the following labeled ribonucleotides: ATP (◇), GTP (△), CTP (□), and UTP (○).

only the whole molecule may prove to be difficult and misleading. We have taken the approach of analyzing the structure and function of the two nucleotide binding domains as individual polypeptides (NBD1 and NBD2). Here, we have cloned, expressed, and purified the first nucleotide binding domain of the human ABCR protein (NBD1). The expressed protein was soluble (Figure 2). Approximately 400 μg of homogeneous and stable NBD1 was purified using single-step S-protein agarose affinity chromatography (Figure 2), allowing us to carry out a complete structure−function analysis of this domain.

NBD1 Is a General Ribonucleotidase. Using highly purified homogeneous preparations of NBD1, we have examined the nucleotidase activity of this domain. Previously, we have shown that the second nucleotide binding domain of hABCR, NBD2, is a fully functional ATPase and that its level of activity, when normalized to account for differences in molecular mass, is comparable to that of reconstituted preparations of bovine ABCR. As shown in Figure 3, NBD1 is also capable of ATP hydrolysis, although the level of its activity is about 5-fold lower than that of NBD2. Nucleotide challenge experiments demonstrated that nucleotide binding to NBD1 is not specific for ATP. All ribonucleotides can bind and be hydrolyzed; however, UTP was least effective as a substrate. Previous studies with purified preparations of bovine ABCR have shown that GTP can compete for labeling with azido-ATP (*18*). It is not uncommon for proteins with multiple nucleotide binding domains to vary in their binding affinity and specificity, for example, the SecA protein from *E. coli* (*31*). Other ABC transporters, such as the cystic fibrosis transmembrane conductance regulator (CFTR), have been shown to utilize GTP as a substrate (*32*). Studies carried out with the MalK subunits of several bacterial systems (*34*, *35*) have shown that these members of the ABC family can hydrolyze CTP and GTP as well as ATP. However, hABCR NBD1 appears to be the first report of a nucleotide binding domain from a mammalian ABC protein functioning as a general nucleotidase.

Mechanistic Significance of the Difference in Properties of NBD1 and NBD2. Current and recent studies carried out in this laboratory indicate that the enzymatic properties of NBD1 and NBD2 are significantly different with respect to (i) the specificity of their ribonucleotide substrates, with NBD2 being strictly specific for adenosine nucleotides while NBD1 is a general nucleotidase; and (ii) ATP hydrolysis, with the two nucleotide binding domains differing in their kinetic properties and binding affinities. The ability of NBD1 to utilize any available ribonucleotide combined with its K_m of 200 μM allows this domain to provide energy for transport under a variety of cellular conditions. Unlike NBD2, NBD1 is not strictly dependent on ATP for hydrolysis and will operate at high efficiency, even under conditions of low ribonucleotide concentrations. A review of the literature indicates that thorough analyses aimed at determining the individual ribonucleotide concentrations in photoreceptor cells have yet to be carried out. A general study carried out by Salceda et al. (*35*) using bovine rod outer segments suggests that the concentration is less than 500 μM. Work carried out by Griffiths et al. (*36*) indicates that natural light-induced DNA damage in the mitochondria of aging photoreceptor cells leads to defects in the activity of enzymes associated with oxidative metabolism. The mitochondrial damage could lead to decreased levels of ATP in photore-

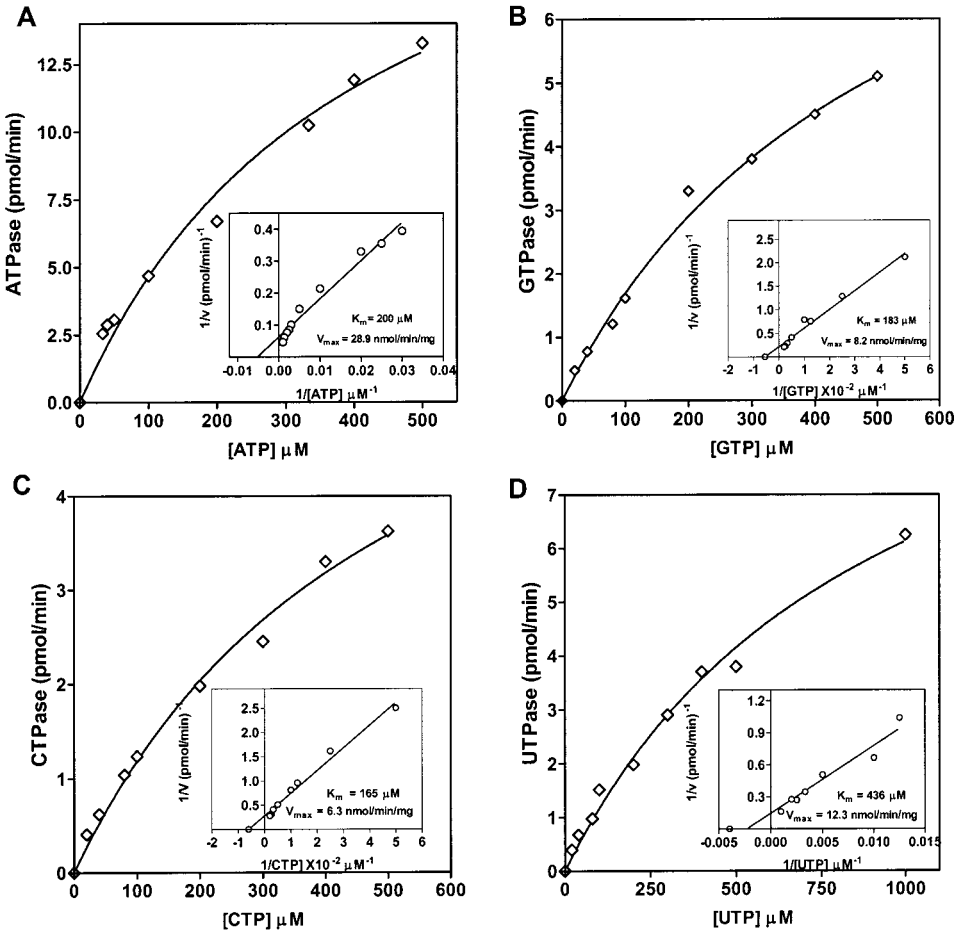

FIGURE 4: Kinetic analysis of nucleotide hydrolysis by NBD1. Nucleotidase activity (V) vs substrate concentration ([S]). The curve was generated using a nonlinear regression analysis of the data. The insets show double-reciprocal plots ($1/V$ vs $1/[S]$) of the ATPase activity: (A) ATPase, (B) GTPase, (C) CTPase, and (D) UTPase. The assays were carried out as described in Materials and Methods. In each set, the data points represent the mean of three separate experiments with a standard deviation less than or equal to ±4.0%.

ceptor cells. In that case, NBD1, by virtue of its lower K_m, could function as a "workhorse" of the ABCR molecule. Further studies which determine the physiological concentrations of the various ribonucleotide rod photoreceptor cells will help in understanding the significance of the relative affinities of these two domains. Sun et al. (*25*) have postulated that NBD2 interacts in an allosteric manner with NBD1. With regard to interaction between NBD1 and NBD2, we did not observe any direct interaction via reconstitution of the two subunits in vitro *(*data not shown). However, an allosteric interaction is still possible without direct physical contact, perhaps mediated by another part of the molecule. The possibility of whether together the two domains interact to produce a unique functional behavior cannot be ruled out with the knowledge available at this time. However, this and previous work carried out on NBD2 of hABCR (*26*) are consistent with data obtained with full-length ABCR molecules.

NBD2 has a V_{max} which is 10-fold higher than that of NBD1. However, it is the 5-fold difference in relative binding affinity between NBD1 and NBD2 that may be most significant. Figure 5A illustrates the possible functional implication of these differences at various substrate concentrations. At a substrate concentration of 50 μM, NBD2 will operate at an efficiency of ~5% of its V_{max}, while at that concentration, NBD1 will be operating at ~25% efficiency, and hence would be the major contributor to rNTP hydrolysis. One may speculate that having two nucleotide binding domains with differing substrate specificities allows the ABCR to be more generally responsive to wide variations in rNTP concentration. For example, at ATP concentrations of >500 μM, NBD2 would predominate as the active site (Figure 5B). On the other hand, when the ATP concentration is low, NBD1 allows ABCR to continue to function by utilizing any available ribonucleotide triphosphates (Figure 5B). Very little work has been done regarding the cellular nucleotide in which ABCR functions; however, one report suggests that the maximal concentrations of GTP and ATP in isolated bovine ROS are less than 500 μM (*35*). As stated in the previous paragraph, some studies (*36*) suggest that ATP concentrations in photoreceptor cells may decrease with aging. If this is the case, then the ability to function at a lower concentration range may be significant.

The ABCR is a complex molecule that will require additional study for the full delineation of its mechanism of action. This report demonstrates the usefulness of utilizing recombinant polypeptides to examine separately the nucleotidase activities of individual nucleotide binding domains in proteins that contain more than one of these domains. In addition, this system will prove to be useful for detailed structure−function analysis of NBD1 mutations associated with various retinopathies, such as age-related macular degeneration and Stargardt disease.

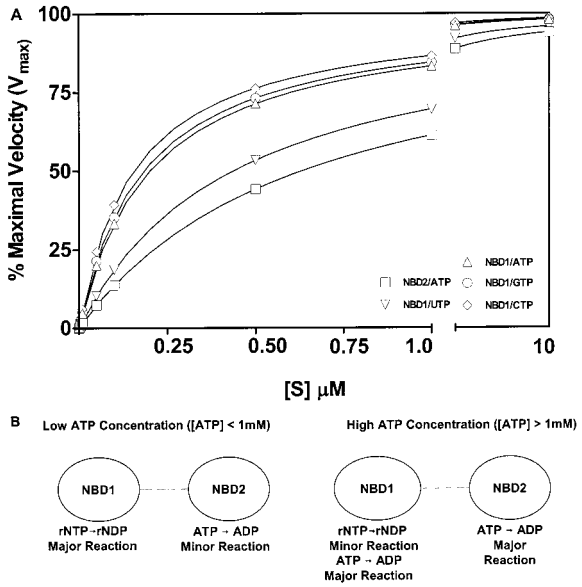

FIGURE 5: Mechanistic implications of the two nucleotide binding domains. (A) Theoretical plot of the enzymatic activity of the two nucleotide binding domains as a function of substrate concentration. The experimentally derived K_m and V_{max} were used to generate a theoretical plot of NBD1 and NBD2 as a percentage of their maximal velocity at various substrate concentrations using the Michaelis−Menten equation. (B) Model of the dynamics of nucleotide hydrolysis by ABCR. Schematic diagram depicting the relative contribution to overall nucleotide hydrolysis by the two nucleotide binding domains of ABCR depending upon the local rNTP concentration.

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