Biochemical Defects in Retina-specific Human ATP Binding Cassette Transporter Nucleotide Binding Domain 1 Mutants Associated with Macular Degeneration*

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The retina-specific human ABC transporter (ABCR) functions in the retinal transport system and has been implicated in several inherited visual diseases, including Stargardt disease, fundus flavimaculatus, cone-rod dystrophy, and age-related macular degeneration. We have previously described a general ribonucleotidase activity of the first nucleotide binding domain (NBD1) of human ABCR (Biswas, E. E. (2001) Biochemistry 40, 8181–8187). In this communication, we present a quantitative study analyzing the effects of certain disease-associated mutations, Gly-863 → Ala, Pro-940 → Arg, and Arg-943 → Gln on the nucleotide binding, and general ribonucleotidase activities of this domain. NBD1 proteins, harboring these mutations, were created through in vitro site-specific mutagenesis and expressed in Escherichia coli. Results of the enzyme-kinetic studies indicated that these mutations altered the ATPase and CTPase activities of NBD1. The G863A and P940R mutations were found to have significant attenuation of the rates of nucleotide hydrolysis and binding affinities. On the other hand, the R943Q mutation had small, but detectable reduction in its nucleotide binding affinity and the rates of nucleotide hydrolysis. We have measured the nucleotide binding affinities of NBD1 protein and its mutants quantitatively by fluorescence anisotropy changes during protein binding to ethenoadenosine ATP (εATP), a fluorescent ATP analogue. We have correlated the dissociation constant (K_D) and the rates of nucleotide hydrolysis (V_max) of NBD1 and its mutants with the available genetic data for these mutations.

The retinal ATP binding cassette transporter (ABCR) protein acts as an outwardly directed flippase for the all-trans-retinal transport in rod (or cone) outer segments cells during the phototransduction cascade, and it was originally discovered in Xenopus and bovine retina as rim protein (1–3). Several inherited visual diseases such as Stargardt disease (STGD), fundus flavimaculatus, cone-rod dystrophy (also known as CORD4), retinitis pigmentosa, and age-related macular degeneration (AMD) have been linked to mutations in the ABCR gene, which encodes a photoreceptor-specific ATP-binding cassette (ABC) transporter (2–14). This transporter gene was identified in humans and localized to chromosomal position 1p22.1-p21 by fluorescense in situ hybridization, and has been fully characterized (2, 4, 15). This gene is expressed at high levels in the retina in rod photoreceptors, although it appears to be expressed also in cone photoreceptors (2, 16).

Typically, ABCR protein exhibits the characteristic features of an ABC transporter; it contains two conserved ATP binding cassettes and uses the energy of nucleotide hydrolysis to transport substrates through the membrane against a concentration gradient (18). This protein contains two highly hydrophobic transmembrane domains, each consisting of six α-helical segments that span the membrane (see Fig. 1A). These domains are thought to confer substrate specificity to the ABCR protein. The nature of the substrates of ABC proteins vary enormously and include sugars, peptides, drugs, lipids, steroids, amino acids, and polysaccharides (18–25). However, in vitro reconstitution studies carried out using purified bovine ABCR suggest that a retinoid, specifically trans-retinal, is the likely substrate of ABCR (26). In addition to the transmembrane domains, there are two hydrophilic ATP binding domains, one located at the N-terminal region (NBD1) and the other at the C-terminal region (NBD2). Both domains are peripherally located at the cytoplasmic face of the membrane (2, 18) (Fig. 1A). These domains are highly conserved, bind ATP, and couple ATP hydrolysis to the transport process (18). Each of these nucleotide binding domains (NBD) includes two short motifs associated with several nucleotide binding proteins and termed as Walker Motif A (GXXGXXGKT) and Walker Motif B (RXG<sub>ε</sub>-shyd4D) (27).

Advances in human molecular genetics have led to the discovery and identification of genes and genetic mutations that are unequivocally linked to various visual diseases (5, 9–15, 28, 29), as well as a detailed understanding of overall biology of the visual cycle (30). Many ABCR mutations related to recessive Stargardt disease, age-related macular degeneration, and fundus flavimaculatus map within nucleotide binding domain I or 2, pointing to a defect in the ATP-driven energy transduction process. Previous studies carried out in our laboratory on the first nucleotide binding domain (NBD1) demonstrated that this protein is active as a ribonucleotidase (17). Using competition binding assays, it was also found that this protein can function as a general nucleotide binding domain that is able to bind and hydrolyze ATP, CTP, GTP, and UTP, with a nucleotide preference CTP > GTP > ATP ➾ UTP.
In this report, we have focused our analysis on genetic mutations located within the NBD1 domain. We have explored the relationship between specific mutations in this domain with ABCR protein function using recombinant mutant proteins. We have chosen three mutations based on the previously published genetic phenotypes: Gly-863 → Ala, Pro-940 → Arg, and Arg-943 → Gln. The Gly-863 → Ala mutation was first reported as a disease-causing mutation and, subsequently, as one of the most frequently observed mutations in STGD patients (2, 6, 12, 31). The amino acid change, Pro-940 → Arg, has been reported as familial cosegregation in exudative AMD patients (14). The missense Arg-943 → Gln mutation was initially reported by Allikmets et al. (2) as a neutral polymorphism in STGD, because it was detected in control individuals but it has also been related to mild forms of AMD (2, 6, 14). We have specifically investigated the nucleotideid (ATPase and CTPase) activities of the mutant proteins by kinetic studies and ATP binding affinities (Kp) under equilibrium conditions using fluorescence anisotropy and the fluorescent ATP analogue, eATP.

**MATERIALS AND METHODS**

**Nucleic Acids, Enzymes, and Other Reagents**—The plasmid pET29a containing wild-type DNA corresponding to the N-terminal nucleotide binding domain (NBD1) of the human ABCR gene was obtained in our laboratory as previously described by E. E. Biswas (17). Ultrapure ribo- and deoxynucleotides were obtained from Amersham Biosciences, Inc. and were used without further purification. [α-32P]ATP and [α-32P]CTP were obtained from PerkinElmer Life Sciences (Boston, MA). Polyethylenimine-cellulose TLC strips were purchased from J. T. Baker Chemical Co. (Pittsburgh, PA). The oligonucleotides were synthesized by Integrated DNA Technologies (Corvalis, IA) and were of high purity (≥95%) as determined by autoradiography of the phosphorylated products. Oligonucleotides used in PCR were used without additional purification. The T7 expression system vector pET29a, BugBuster protein extraction reagent, and Bacteronase Nuclease were purchased from Novagen (Madison, WI). The Pfu DNA polymerase for PCR amplification was from Stratagene, Inc. (La Jolla, CA). The fluorescent ATP analogue, eATP, was obtained from Molecular Probes (Eugene, OR). Buffers—Buffer A was 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM dithiothreitol, and 15% (v/v) glycerol. Buffer B contained 50 mM Tris-HCl (pH 8.0) and 20 mM EDTA. Buffer C was 6 mM guanidine hydrochloride, 0.1 mM Tris-HCl (pH 8.0), and 0.5 mM l-arginine, and 2 mM EDTA. Buffer E contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 2 mM dithiothreitol. Buffer F was 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 50 mM NaCl, 5% glycerol, and 0.01% Nonidet P-40.

**Cloning of the Construct Containing NBD1 Wild-type Protein**—The wild-type construct pET29a/NBD1 was available in our laboratory. It was cloned from the human retinal cDNA clone in pRK5 as previously described by Biswas (17). Briefly, the DNA containing this domain was isolated by PCR under high fidelity conditions using Pfu DNA polymerase. Oligonucleotides primers were designed such that a BamHI site with an in-frame ATG (Met) codon was present in the 5′-primer and a HindIII site after the stop codon in the 3′-primer. The region amplified spanned nucleotides 2641–1407, 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 Corp., Milwaukee, WI) in the BamHI/HindIII sites. The absence of fortuitous mutations was confirmed by DNA sequencing carried out at the Nucleic Acid Core Facility of Thomas Jefferson University. The resulting recombinant plasmid (pET29a/NBD1) was used for expression of the wtNBD1 protein in Escherichia coli BL21(DE3) cells, following a procedure described earlier (32). This plasmid was also used as the parent of all the mutant constructs pET29aNBD1. The wild-type construct pET29aNBD1 was available in our laboratory. It was from Stratagene, Inc. (La Jolla, CA). The fluorescent ATP analogue, eATP, was obtained from Molecular Probes (Eugene, OR).

**Buffers**—Buffer A was 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM dithiothreitol, and 15% (v/v) glycerol. Buffer B contained 50 mM Tris-HCl (pH 8.0) and 20 mM EDTA. Buffer C was 6 mM guanidine hydrochloride, 0.1 mM Tris-HCl (pH 8.0), and 0.5 mM l-arginine, and 2 mM EDTA. Buffer E contained 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 2 mM dithiothreitol. Buffer F was 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 50 mM NaCl, 5% glycerol, and 0.01% Nonidet P-40.

**In Vitro Site-directed Mutagenesis of the NBD1 Gene**—Site-directed mutagenesis was carried out using a mutagenesis kit (Stratagene, La Jolla, CA) as previously described (33). Using the NBD1 expression vector pET29aNBD1 as the template, 18 cycles of PCR were performed (each cycle was 50 s at 95 °C, 50 s at 60 °C, and 15 min at 68 °C) using complementary oligonucleotides as mutagenic primers to generate the mutant NBD1 proteins as follows: Gly-863 → Ala (5′-GAT CAT GTG TTT CCA GCA GAC TAT GGA ACC CCC-3′ and 5′-GTC GGG TTC CAT AGT CTG CTG GAA ACA CCT GAT C-3′), Pro-940 → Arg (5′-GCT AAA GAT TTT TGA GCG TGT TCG GCG ACC AGC TG-3′ and 5′-CAG CAC GAC ACC AAA TAC TTA CCA-3′), Arg-943 → Gln (5′-GAT TTA TGG GCA TGG CCA GCC AGC TGT GGA CC-3′ and 5′-GGT CCA CAG CTT GCT GGC CAC AGG GCT CAA AAA TC-3′). The authenticity of the mutations 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.

**Expression of Wild-type and Mutant Constructs of NBD1**—Following verification of the desired mutations, the pET29a constructs were used to transform E. coli strain BL21(DE3) cells, in which the expression of the recombinant proteins is under control of the lacUV5 promoter. A typical induction was: cultures of BL21(DE3) harboring the desired construct were grown with shaking to OD600 = 0.4 at 37 °C, at which time the induction of protein was initiated by adding of IPTG to 0.4 mM, grown, and shaken for 2 h more. The cells were harvested by centrifugation at 4 °C, and the level of expression was analyzed by SDS-PAGE.

**Extraction and Purification of Recombinant NBD1 Proteins**—The wild-type and mutant NBD1 proteins were extracted from inclusion bodies using a protocol, which combines the use of BugBuster protein extraction reagent (Novagen, Madison, WI) to process the insoluble fraction and yield purified inclusion bodies, with a method described by Booth (34) for solubilization and renaturation of inclusion-body protein. After harvesting the expressed proteins, the cell pellets were resuspended in room temperature BugBuster reagent, and protease inhibitors were added. After incubation, the cell suspension was centrifuged to collect purified inclusion bodies. Following cell lysis, the pellet of inclusion bodies was resuspended in buffer B and centrifuged once more. The inclusion body proteins were solubilized in Buffer C containing 6 M guanidine hydrochloride. Protein refolding was achieved by dilution in Buffer D. The renatured protein was sequentially dialyzed in Buffer E. After overnight dialysis, the conductivity of the protein was checked to adjust to that of dialysis buffer, and 15% glycerol (final concentration) was added. The dialyzed protein was concentrated to ~1 mg/ml approximately using Amicon Ultrafiltration and stored at −80 °C. This protein is essentially homogenous as analyzed by SDS-PAGE.

**Assay for Nucleotidase Activity**—The ATPase and CTPase activity assays were carried out as previously described (17, 33, 35). The amount of NBD1 protein (wild-type and mutants) 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 MgCl2, 10 μM [32P]ATP, and 50 μM unlabeled ATP and ADP. The wild-type or mutant proteins (as indicated) in buffer A, each reaction in these assays was performed in duplicate. All reaction mixtures were incubated at 37 °C for 60 min (unless stated otherwise) and terminated by adding 1 μl of 500 mM EDTA followed by chilling on ice. Aliquots (1 μl) of each reaction mixture were applied to the polyethylenimine-cellulose strips, which were pre-spotted with an ADP and ATP marker. The strips were developed with 5% 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 Beckman LS500 liquid scintillation counter using a tolue-ene-based scintillator. In the kinetic analyses, reactions were carried out in a single tube and were initiated with the addition of each NBD1 protein studied. At the indicated time points, 10-μl aliquots were removed and transferred to tubes containing 1 μl of 500 mM EDTA, and the tubes were held on ice until completion of the last time point. The remainder of the assay was carried out as described above. CTPase assays were carried out in an analogous manner except that the appropriated ribonucleotides were substituted for ATP and ADP or ADP as required in the procedure.

**Fluorescence Anisotropy Assay**—ATP binding to NBD1 proteins was studied using the fluorescent substrate ATP analogue eATP (1′N′-[ethenoadenosine 5′-triphosphate]). The fluorescence anisotropy studies were performed using the back titration method earlier described by Boyer et al. (36). The fluorescence anisotropy (R) is defined by,

\[ R = (I_v - G \times I_h) / (I_v + 2 \times G \times I_h) \]  

(1)

where \(G\) is the instrumental correction factor for the fluorometer, defined by,

\[ G = I_{hh} / I_{lo} \]  

(2)

where \(I_{hh}\), \(I_{lo}\), and \(I_v\) are the fluorescence intensities with horizontal-horizontal, horizontal-vertical, vertical-vertical, and vertical-horizontal orientations of the excitation and emission polarizers.
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The fluorescent ATP analogue, eATP, was added to buffer F to 0.05 mM. Each point in the titration curve was obtained by starting with 1.5 ml of a solution of 100 μg/ml (1.73 μM) protein. Aliquots of 100 μl were successively removed from the starter solution containing the protein-eATP complex and replaced by 100 μl of fresh buffer F containing eATP. After incubation of samples at room temperature with constant stirring for 2–3 min in quartz cuvettes, fluorescence anisotropy was measured for each dilution using a custom made Photon Counting Spectrofluorometer equipped with a Glan Thomson polarizer in both excitation and emission channels. The excitation wavelength was set at 300 nm, and fluorescence anisotropy was recorded at an emission wavelength of 412 nm. Global analysis of the data was conducted using BIOEQS and/or PRISM (GraphPad Software Inc.) programs, using a monomer-ligand binding model (37–39). BIOEQS calculates the concentration of various species in the equilibrium-binding curve numerically using a constrained optimization algorithm in which the mass balance constraints are incorporated as Lagrange multipliers (37). The program then relates this species concentration vector to the anisotropy observed at each point in the titration and fits the free energy and plateau values by adjusting these floating parameters using a Marquardt-Levenberg algorithm (40). The simple binding model was used to determine ΔG for the binding of an NBD1 monomer unit to the eATP. This model includes free eATP, free NBD1, and NBD1-eATP complex (Equation 3),

\[ \text{NBD1} + \text{eATP} \rightarrow (\text{NBD1}+\text{eATP}) \]  

(Eq. 3)

Initial values of NBD1, eATP, and NBD1-eATP were 0, 12, and 130 milli-anisotropy (mA), respectively. From the ΔG values, the equilibrium dissociation constant (K_d) was calculated by the relationship,

\[ \Delta G^\circ = -RT\ln K_d \]  

(Eq. 4)

where R represents the gas constant, and T is the temperature in kelvin.

Homology-based Modeling—Homology-based modeling of NBD1 polypeptide was carried out in several discreet steps. First, we aligned

the NBD1 sequence with ATP binding domains of ABC transporters with known structures and available crystallographic coordinates. Preliminary structure was generated using the Swiss-PDB program. This structure was refined by using SYBYL6.7 software (Tripos Inc., St. Louis, MO).

Other Methods—Protein concentrations were determined by two methods. The Bradford assay (41) using bovine serum albumin as a standard and the extinction coefficient method where: \(\varepsilon_{280} = 4.1 \times 10^4 \) M⁻¹ cm⁻¹ in Tris-HCl (pH 7.0) and 4.35 × 10⁴ M⁻¹ cm⁻¹ in 0.1 M KOH for wild-type NBD1 protein. Protein analysis was performed using SDS-PAGE as described by Laemmli (42).

RESULTS

Design and Cloning of the NBD1 Mutant Constructs—Analysis of the amino acid sequence of human ABCR protein indicates the presence of two cytoplasmic nucleotide binding motifs (4). The first cytoplasmic nucleotide binding domain (NBD1) comprises amino acids (aa) 854-1375 (2641–4207 bp) (17), is defined as NBD1, and is the focus of our current studies (Fig. 1). The NBD1 domain contains both Walker type A and B nucleotide binding motifs (Fig. 1B) (27). To define this domain more critically we have divided NBD1 in three subdomains α (aa 854–960), β (aa 961–1100), central domain containing the Walker A and B motifs), and γ (aa 1101–1375) as illustrated in Fig. 1A. We have analyzed the nucleotide binding and hydrolysis of three structurally important and disease related mutations: Gly-863 → Ala, Pro-940 → Arg, and Arg-943 → Gln (Table I). All of these mutations are located within the α-domain (Fig. 1C) (17).

Purification of NBD1 Mutant Proteins from the E. coli Whole Cell Extracts—Introduction of mutations into wild-type NBD1 polypeptide appears to decrease the solubility of the expressed...
proteins as determined by SDS-PAGE and Western blot analyses (data not shown). Therefore, we explored the extraction of recombinant proteins (wild-type and mutants) from the inclusion bodies following the procedure of Booth et al. (34) with minor modifications as described under "Materials and Methods." Similar to that observed by Booth et al. (34), the use of this procedure allowed us to obtain refolded proteins in high purity (Fig. 2). Minor impurities present in the proteins were determined to be protease-degraded fragments of NBD1, as described previously by Biswas (17) for the affinity-purified NBD1 protein. The inclusion body protein purification methodology described here yielded highly concentrated, purified, and homogeneous preparations of protein. The yield of NBD1 proteins was >10 mg from 2 liters of induced cell culture.

### Table I

| Mutation    | Base change | Mutant sequence                                                                 | Related disease       |
|-------------|-------------|---------------------------------------------------------------------------------|-----------------------|
| Gly-863     | G → C       | 5′-GATCAGGTTGTTTCCAGCAGACTATGGAACCCAC-3′                                      | STGD, AMD, FFM, RP    |
| Pro-940     | C → G       | 5′-GGTAAAGATTTTTGAGCGCTTGGGCCGGCCAGCTG-3′                                      | Exudative AMD         |
| Arg-943     | G → A       | 5′-GATTTTTGAGCGCTTGGGCCGGCCAGCTG-3′                                            | Polymorphism          |

a Refs. 2, 5, 8–12, 31, 45.

b Ref. 14.

c Refs. 2, 5, 9–11, 14, 45.

d Ref. 6.

![Fig. 2. Purification of NBD1 and mutant proteins.](image)

**A**. SDS-PAGE analysis of the expression of NBD1 wild-type and mutant proteins in E. coli. Equal amounts of cells before and after induction were analyzed by 5–18% SDS-PAGE followed by Coomassie Blue R-250 staining: lanes 1 and 2, BL21(DE3)/pET29aNBD1 wild-type cells before and after induction, respectively; lane 3, BL21(DE3)/pET29aNBD1/G863A cells before induction, and after induction in lane 4; lanes 5 and 6, BL21(DE3)/pET29aNBD1/P940R cells before induction and after induction, respectively; lane 7, BL21(DE3)/pET29aNBD1/R943Q cells before induction and lane 8 after induction. B, SDS-PAGE of inclusion body-protein purification of NBD1. Lane 1, wild-type NBD1 protein; lane 2, NBD1/G863A mutant protein; lane 3, NBD1/P940R mutant protein; and lane 4, NBD1/R943Q mutant protein. 4.5 μg of each protein was loaded onto a 5–18% SDS-PAGE, which was stained with Coomassie Blue R-250. Protein molecular mass markers are as indicated.

![Fig. 3. ATPase and CTPase activities of NBD1 wild-type and NBD1/G863A mutant proteins.](image)

**A**. Comparison of ATP hydrolysis by NBD1wt and NBD1/G863A polypeptides. Protein titration of purified NBD1 wild-type and NBD1/G863A proteins in a standard ATPase assay was carried out as described under "Materials and Methods" at 37 °C for 60 min using the indicate amounts of protein and ATP concentration of 500 μM. B, CTP hydrolysis by wtNBD1 and NBD1/G863A. Protein titration of purified NBD1 wild-type and NBD1/G863A proteins in a standard CTPase assay was carried out as described under "Materials and Methods" at 37 °C for 60 min using the indicate amounts of protein and CTP concentration of 500 μM. C, time-course analysis of ATP hydrolysis. Standard ATP assays were carried out as described under "Materials and Methods" at 37 °C for the times indicated using [α-32P]ATP and 2.5 μg of purified NBD1 wild-type and NBD1/G863A proteins. NBD1 wild-type (□) and G863A mutant (○).
We have investigated (i) the effects of three inherited mutations on the nucleotidase activity of NDB1 protein, (ii) the relation of pathogenicity of these mutations with the alterations in ABCR. Protein titrations of ATPase and CTPase activities, as well as kinetic analysis of NBD1wt and NBD1-P940R proteins in a standard ATPase assay were carried out as described under “Materials and Methods.” The assays were carried out at 37 °C for 60 min using the indicate amounts of protein and ATP concentration of 500 μM. B. CTP hydrolysis by wtNBD1 and the mutant P940R. Protein titration of purified NBD1 wild-type and NBD1/P940R proteins in a standard CTPase assay was carried out as described under “Materials and Methods” at 37 °C for 60 min using the indicate amounts of protein and CTP concentration of 500 μM. C. time-course analysis of ATP hydrolysis by P940R mutant and wild-type protein. Standard ATP assays were carried out as described under “Materials and Methods” at 37 °C for the times indicated using [α-32P]ATP and 2.5 μg of purified NBD1 wild-type and NBD1/P940R proteins. NBD1 wild-type (●) and P940R mutant (△).

**Consequences of the Gly-863 → Ala Mutation on the ATPase / CTPase Activity of NDB1 Protein**—The ABCR mutation G863A is a commonly encountered mutation and has been reported in patients suffering from Stargardt disease (2, 9–11), age-related macular dystrophy (11), fundus flavimaculatus (5), and retinitis pigmentosa (10). Analysis of the rates of hydrolysis of ATP and CTP in the mutant protein demonstrated that they were significantly reduced (Fig. 3). The results presented in Fig. 3A indicated that the ATPase function of G863A mutant protein was reduced ~3-fold as compared with NDB1wt, indicating ~70% of inhibition of the ATPase activity. The V_{max} (ATPase) for G863A mutant was 128 pmol/min/mg and that of the wild-type NDB1 was 584 pmol/min/mg (Table II). A time-course analysis of ATP hydrolysis using 2.5 μg of protein (Fig. 3C) suggested the actual rates of ATP hydrolysis were attenuated 3-fold as a consequence of the mutation. We have earlier reported that the NDB1wt has significantly higher CTPase than ATPase activity (17). However, in the mutant G863A protein the CTPase activity was reduced (Fig. 3B). In this case, the CTP hydrolysis of G863A was reduced to ~30% of the activity of NDB1wt. The V_{max} for G863A mutant was 104 pmol/min/mg and that of the wild-type NDB1 was 376 pmol/min/mg (Table II).

**Pro-940 → Arg Mutation Abolishes the CTPase Function of Wild-type NDB1 Protein**—The missense mutation P940R has been reported in patients with exudative age-related macular degeneration (14), which is the least frequent but most severe
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The NBD1 P940R mutant protein had defects in both ATP and CTP hydrolysis (Fig. 4). We observed significant alterations of the nucleotidase activities. The CTPase activity in the P940R mutant protein was reduced to 84 pmol/min/mg whereas the ATPase activity was diminished to 129 pmol/min/mg (Table II). Therefore, the diminution of the nucleotidase activities was 55% for ATPase and 85% for CTPase, respectively. The time-course analysis of ATP hydrolysis presented in Fig. 4C demonstrates that the rates of hydrolysis of both NBD1wt and P940R proteins were linear over 60 min before reaching equilibrium. The P940R mutation severely affected the NBD1 function, although unlike G863A, the degree of inhibition was different for ATP and CTP.

Arg-943 → Gln Is the Least Influential on ATP and CTP Hydrolysis of NBD1wt Protein—The amino acid change R943Q has been described as a polymorphism, because it has been found in control populations (5, 9–11, 14). This mutation was introduced into wild-type pET29aNBD1 plasmid using the site-directed mutagenesis protocol. After expression and purification, its ability to hydrolyze ATP and CTP were assessed.

Results presented in Fig. 5A demonstrate that the ATPase activity was somewhat reduced (~12% less than NBD1wt protein). The $V_{\text{max}}$ for R943Q mutant was 392 pmol/min/mg, and that of the wild-type NBD1 was 584 pmol/min/mg (Table II). The time-course analysis of ATPase activity (Fig. 5C), suggested that the ATPase activity of R943Q protein function was about 40% reduced with respect to that observed for NBD1wt. The $V_{\text{max}}$ for R943Q mutant was 172 pmol/min/mg and that of the wild-type NBD1 was 376 pmol/min/mg (Table II). Therefore, the CTPase activity of R943Q was reduced 2-fold compared with that observed with wild-type NBD1 (Fig. 5), indicating a higher inhibitory effect of this mutation on the CTPase (~57%) than ATPase (~12%) activity. Overall, the inhibition of the ribonucleotidase activity generated for this mutation was far less severe than that observed with Gly-863 → Ala or Pro-940 → Arg mutations.

Kinetic Analysis of Nucleotide Hydrolysis—Kinetic analysis of ATP and CTP hydrolysis by NBD1wt and NBD1 mutants are shown in Fig. 6. The kinetic parameters of NBD1wt for ATP hydrolysis were as follows: $K_m = 169 \mu M$ and $V_{\text{max}} = 584$ pmol/min/mg. The results obtained for mutant proteins indicate a major change in the ATPase activity (Table II). In general, the G863A and P940R point mutations increased the $K_m$ values as follows: 80, 66 $\mu M$, respectively. The rate of ATP hydrolysis was also altered and the $V_{\text{max}}$ values were 392 (R943Q), 129 (G863A), and 128 (P940R) pmol/min/mg. This represented a 78% decrease in $V_{\text{max}}$ for the G863A and P940R mutants, whereas the $V_{\text{max}}$ for R943Q mutant was diminished by only 33%. It is evident from the results presented above that ATP hydrolysis was significantly impaired in these mutants. As a result, the $K_m$ values presented here are not reliable estimates of the binding affinities or dissociation constants. Therefore, we chose to determine dissociation constants ($K_m$) of nucleotide binding by direct equilibrium measurements in solution.

Influence of Gly-863 → Ala, Pro-940 → Arg, and Arg-943 → Gln Mutations on ATP Binding Capacity of NBD1 Polypeptide—We have utilized fluorescence anisotropy analysis to evaluate changes in ATP binding. Fluorescence anisotropy is normally used for direct measurements of ligand binding by determining the concentrations of bound and free ligand in solution due to differences in anisotropy values between bound and free ligand (37–39). Therefore, true equilibrium measurements are possible in this procedure without needing to isolate the protein-ligand complex from the free ligand. A number of fluorescent nucleotide analogues are commercially available for use in the analysis of nucleotide binding to NBD1 protein and its mutants. We have utilized etheno-adenosine triphosphate, αATP, in our studies because of its close structural similarity to ATP. Anisotropy was measured using 50 nM αATP. The wavelengths were 300 nm for excitation and 412 nm for emission. The fluorescence anisotropy changes with protein concentrations are shown in Fig. 7. For wild-type and mutant NBD1 proteins, sigmoidal semilog plots were obtained in each case indicating equilibrium saturation binding of αATP by these proteins. The plots, shown in Fig. 7, were generated by

| Parameter          | Wild-type | R943Q | P940R | G863A |
|--------------------|-----------|-------|-------|-------|
| Enzyme kinetics    |           |       |       |       |
| $V_{\text{max}}$ (pmol/min/mg) |           |       |       |       |
| ATP                | 584       | 392   | 129   | 128   |
| CTP                | 376       | 172   | 84    | 104   |
| ATP binding        |           |       |       |       |
| $\Delta G^*$ (kcal/mol) |           |       |       |       |
| $K_p$ (m)          |           |       |       |       |
| ATP                | $9.9 \times 10^{-7}$ | $5 \times 10^{-7}$ | $1.2 \times 10^{-6}$ | $2.7 \times 10^{-6}$ |
| CTP                |           |       |       |       |
| Inhibition (%)     |           |       |       |       |
| ATP                | 0         | 33    | 78    | 78    |
| CTP                | 0         | 54    | 78    | 72    |

| Disease severity   | –         | ++     | +     | +     |

FIG. 6. Kinetic analysis of ATP and CTP hydrolysis by NBD1 wild-type and mutant proteins. A, plot of ATPase activity (pmol/min) versus ATP concentration (μM). B, plot of CTPase activity (pmol/min) versus CTP concentration (μM). The ATPase and CTPase assays were carried out as described under “Materials and Methods,” using 2.5 μM of each protein and 30 min of reaction time. The curves were generated using a nonlinear regression analysis of the data. Each set, the data points represent the mean of three separate experiments with S.D. ± 4%. Results of analysis for double-reciprocal plots ($1/V$ versus $1/[S]$) of the ATPase and CTPase activities were tabulated in Table II. NBD1 wild-type (□), mutant G863A (○), mutant P940R (△), and mutant R943Q (●).
nonlinear regression analysis of the data using a commercial graphing software (PRISM, GraphPad Inc.). The binding parameters were determined from the fluorescence anisotropy data by equilibrium binding analysis and fitted to Equation 3, using the BIOEQS program (Table II). This nonlinear regression analysis gave dissociation constants \(K_D\) for each of these proteins as follows: 9.9 \(\times\) 10\(^{-7}\), 2.7 \(\times\) 10\(^{-6}\), 1.2 \(\times\) 10\(^{-5}\), and 5 \(\times\) 10\(^{-7}\) M, and \(\Delta G = -8.2, -7.6, -8.1,\) and \(-8.6\) kcal/mol for wild-type protein, G863A, P940R, and R943Q, respectively. Thus, the ATP binding affinity was impaired in the G863A mutant and to a lesser extent in the P940R mutant. The binding capacity of R943Q was comparable to wild-type.

Homology-based Modeling of NBD1—NBD1 polypeptide sequence was aligned with ATP binding domains of known ABC family proteins with available crystallographic structures. The two proteins that were used here are: ATP binding domain of histidine permease from Salmonella typhimurium (Hisp) Walker A and B motifs are illustrated in these text boxes. The mutated amino acids are circled.

FIG. 7. Fluorescence anisotropy titrations of NBD1 proteins with the fluorescent ATP analogue, 6ATP. A, NBD1 wild-type protein (●). B, NBD1/G863A mutant (○). C, NBD1/P940R mutant (▲). D, NBD1/R943Q mutant (▼). The titrations were carried out as described under “Materials and Methods.” All samples were incubated at room for 2–3 min with constant stirring before taking anisotropy measures. Each fluorescence anisotropy measure was collected for 6ATP:NBD1 protein samples serially diluted in Buffer F containing 0.05 M 6ATP. The concentration of starter and maximum protein was 100 μg. The data were fitted with BIOEQS using a simple binding model (monomer) for 6ATP binding to wild-type, G863A, P940R, and R943Q NBD1 proteins.

DISCUSSION

The rod outer segment in human retina plays an important role in the phototransduction process in eye (43). The rod outer segment ATP binding cassette transporter protein (ABCR) is involved in the transport of retinoids. It uses the energy of nucleotide triphosphate hydrolysis to carry out trans-retinal transport. To understand the energy transduction process involved in retinal transport, our approach was to delineate the structure and function of individual nucleotide binding domains and to analyze genetic mutations localized to these regions. Recently, we cloned and expressed the regions of ABCR genes encoding NBD1 (17) and NBD2 (33) domains and performed molecular and biochemical studies on these expressed proteins. The studies demonstrated that NBD2 functions as a specific ATPase that hydrolyzes only ATP or dATP. In contrast, NBD1 was found to act as a general ribonucleotidase, capable of binding and hydrolyzing ATP, CTP, GTP, and UTP.

Alterations of Nucleotidase Activity of NBD1 Due to Genetic Mutations Leading to Inherited Retinal Dystrophies—We have analyzed three genetic mutations in the NBD1 domain of ABCR that are also related to Stargardt disease, AMD, fundus flavimaculatus, and retinitis pigmentosa. These mutations were introduced in the wild-type-NBD1 polypeptide, and their respective recombinant proteins were expressed. Using re-folded and highly purified and homogeneous preparations of wild-type, G863A, P940R, and R943Q NBD1 proteins (Fig. 2), we were able to examine the biochemical consequences of these mutations on the nucleotide binding and hydrolysis activity of
Effects of G863A, P940R, and R943Q Mutations on the eATP Binding to NBD1 Protein—The biochemical effects in eATP binding could be due to defects in nucleotide binding or hydrolysis or both. It is difficult to measure equilibrium binding constant or $K_D$ in solution, and thus, the Michaelis-Menten constant ($K_m$) determined from enzyme kinetic studies is often used for assessing binding affinity. However, $K_m$ is not a true measure of binding affinity especially in cases where the enzymatic activity is very low. The availability of purified, homogenous, stable, and abundant NBD1 proteins allowed us to measure the true dissociation constants ($K_D$) for eATP binding using fluorescence anisotropy (37). Fluorescence anisotropy has been frequently used to examine protein-ligand interactions under equilibrium conditions (40). The results indicated that dissociation constants for both wild-type and R943Q nonpathogenic mutation were comparable, and the values were $9.9 \times 10^{-7}$ M and $5 \times 10^{-7}$ M, respectively. In fact, the dissociation constant for R943Q was lower than that of the wild-type. On the other hand, the dissociation constants for P940R and G863A pathogenic mutations were $1.2 \times 10^{-6}$ M and $2.7 \times 10^{-6}$ M, respectively. The overall order of nucleotide binding affinity was: R943Q > wild-type > P940R > G863A. The values for the free energy change involved in nucleotide binding ($\Delta G^*$) were as follows: $-8.2$ (wild-type), $-8.6$ (R943Q), $-8.1$ (P940R), and $-7.6$ (G863A) kcal/mole, respectively. Interestingly, these results demonstrate the effects of genetic mutants on the nucleotide binding ability in thermodynamic and energetic terms.
Biochemical Defects in Mutants Appear to Be Related to the Disease Severity—According to our enzyme kinetic and fluorescence anisotropy results, the mutations that most severely affected both the ATPase activity and eATP binding of NBD1 were G863A and P940R. The G863A mutation has been reported as one of the most frequently observed mutations in STGD patients in North America (2) and Netherlands (12). The results of enzyme–kinetic studies presented here demonstrated a significant inhibition of the ATPase activity (Fig. 3A). The P940R appeared to have a significant defect in nucleotide hydrolysis similar to that observed with G863A mutation. The presence of the mutation P940R has been correlated with exu-
deteriorate the NBD1 function for binding and hydrolysis of the phosphates.

**Structural Model of NBD1**—Homology alignment indicated that the NBD1 polypeptide is homologous to the ATP binding domains of two known ABC transporters (Fig. 8): histidine permease (Hisp) and maltose transporter protein (Malk) (46, 47). The model is based on these two x-ray crystal structures. The model illustrates the spatial distribution of the amino acid changes of Pro-940 for arginine, and Arg-943 for glutamine, as described in this report on the ribonucleotidase activity of ABCR NBD1 mutants associated with macular degeneration. The biochemical defects described in this report on the ribonucleotidase activity allow us to speculate that this mutation can in some way deteriorate the NBD1 function for binding and hydrolysis of the ribonucleotides. The R943Q mutation displayed the minimal defects in nucleotide binding. Incidentally, this mutation appears to be associated with only milder forms of AMD (Table I). It has also been reported to be associated with neutral polymorphism (Table I). These results correlate well with the results presented here. The R943Q mutation has also been shown to occur in conjunction with G863A leading to a more severe pathogenic state in humans (45).

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