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Overexpression of recombinant human antiquitin in *E. coli*: Partial enzyme activity in selected *ALDH7A1* missense mutations associated with pyridoxine-dependent epilepsy

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ABSTRACT

Pyridoxine-dependent epilepsy (PDE) is an autosomal recessive disorder characterized by early onset seizures responsive to pyridoxine and caused by a defect in the α-aminoadipic semialdehyde dehydrogenase (antiquitin) gene (*ALDH7A1*). We selected four PDE-associated missense *ALDH7A1* mutations, p.V367F, p.F410L, p.Q425R, and p.C450S, generated them in a recombinant human antiquitin cDNA with expression in *E. coli* at either 30 °C or 37 °C. One mutation, p.C450S, demonstrated substantial activity after expression at both temperatures, potentially contributing to milder biochemical and clinical phenotypes. The p.Q425R mutation yielded no activity at either temperature. The other two mutations yielded significant enzymatic activity at 30 °C and markedly reduced activity at 37 °C. For these latter three mutations, the markedly reduced or absent enzymatic activity resulting from expression at 37 °C may be consistent with pathogenicity.

1. Introduction

Pyridoxine dependent epilepsy (PDE) (OMIM: 266100) is a rare autosomal recessive metabolic disorder resulting from deficient activity of the enzyme α-aminoadipic semialdehyde dehydrogenase (ALDH7A1: E.C. 1.2.1.31) commonly known as antiquitin (ATQ) [1,2]. ATQ is a key enzyme in lysine catabolism in the brain, responsible for the conversion of α-aminoadipic acid semialdehyde (AASA) to α-aminoadipic acid. When ATQ activity is deficient, AASA accumulates and is in equilibrium with its cyclic form, Δ^1-piperideine-6-carboxylate (P6C). The latter complexes with pyridoxal phosphate causing a secondary pyridoxine (vitamin B6) deficiency and consequent seizures [2]. The seizures are resistant to standard antiepileptic medications but respond to treatment with pyridoxine. Diagnosis relies on clinical assessment of the response to pyridoxine, detection of elevated AASA in urine and sequence analysis of the ATQ gene [1,3]. There is currently no diagnostic enzyme assay available using patient-derived cells.

ATQ deficiency results from mutations in the ATQ gene (*ALDH7A1*) [2]. More than half of the known mutations are missense. The crystal structures of human ATQ (PDB ID: 2J6L) and the superimposable fish ATQ (PDB ID: 2JG7) have been solved [4–6] and may help to rationalize the consequences of individual missense mutations. In addition, clinical disease phenotype may be influenced by factors operating both in the affected child and prenatally, including genetic factors of pyridoxine metabolism and other enzymes in the lysine catabolic pathways, as well as dietary intake of pyridoxine or lysine [1–3,5,7,8].

The objective of this study was to examine the effects on ATQ enzymatic activity of 4 PDE-associated missense mutations, all associated with relatively mild biochemical and/or clinical phenotype. None of these had been previously tested for enzymatic activity. We used expression of recombinant human ATQ in *E. coli* and an automated assay of enzymatic activity using the natural substrate AASA, a simple and rapid approach we had previously established [9] for examining the effects of missense mutations on enzyme function. In this case, we expressed the mutant ATQ at 30 °C as well as at 37 °C to decrease losses to the insoluble fraction during expression and to determine whether there was any possibility of residual enzyme activity.

Abbreviations: AASA, α-aminoadipic semialdehyde; ATQ, antiquitin; HGMD, Human Genome Mutation Database; IPTG, isoproplythiogalactoside; P6C, Δ^1-piperideine-6-carboxylate; PDB, Protein Data Base; PDE, pyridoxine dependent epilepsy; PLP, pyridoxal phosphate; SDS-PAGE, SDS polyacrylamide gel electrophoresis.

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Table 1
Biochemical and clinical features of PDE patients with selected missense mutations.

Missense change	AASA level				Other features
	Sample type	Patient value	Affected range (N = study group size)	Normal	
p.V367G [10,11]	Plasma (μmol/l)	0.5	0.5–14 (N = 16)	<0.2	–Seizure onset 3 wks –Mild intellectual delay
	Urine (mmol/mol creatinine)	1.6	1.6–342 (N = 12)	<1	
p.F410L [12]	Urine (mmol/mol creatinine)	13		0–1	–seizure onset 9 h –normal psychomotor development at 2 yr
p.Q425R [13]	CSF (μmol/l)	4.9	1.9–14 (N = 7)	<0.1	–Seizure onset 2 days –Mild hypotonia –Folinic acid responsive
p.C450S [14–16]	Urine (mmol/mol creatinine)	4.7	4–75 (N = 10)	<1	–Seizure onset 4 days –Intellectual delay
	plasma (μmol/l)	0.9	1.1–8 (N = 10)	<0.2	

2. Methods

2.1. Selection of mutations

Four documented PDE-associated missense mutations were selected for study (Table 1): p.V367F, p.F410L, p.Q425R, and p.C450S, in exons 13–16 respectively. The mutations as described here and in their original reports are named relative to the most 3′ of the two translation initiator methionine codons (GenBank NM_001201377.1). All are located in the predicted catalytic domain of the protein sequence [4–6]. Table 1 summarizes the biochemical, genetic and clinical findings for the four individuals in whom the mutations were identified. Three of the four, p.V367G, p.Q425R, and p.C450S were each part of study groups of several affected patients. Each showed a relatively mild elevation of AASA compared to the other members of the group. The fourth mutation, p.F410L, was presented as a single case. It was noted that the affected child exhibited normal psychomotor development at 2 years of age.

2.2. Recombinant cDNAs and site-directed mutagenesis

Mutations were generated using the Quick-Change method (Stratagene) in a pET15b-ATQ plasmid as previously described [9]. Oligonucleotide primers designed for mutagenesis are shown in Table 2 along with PCR primer/restriction enzyme combinations used for the initial confirmation of successful mutagenesis. Mutant plasmids were purified using a BioRad Quantum Prep kit. DNA sequencing was applied to verify the accuracy of the entire recombinant sequence.

2.3. Overexpression of plasmids

Expression of recombinant ATQ in *E. coli* BL21(DE3) was induced with 1 mM IPTG at either 30 °C or 37 °C. IPTG induction at 30 °C was used to minimize nonspecific losses to the insoluble fraction that occur during overexpression in *E.* coli and to optimize conditions for productive protein folding. After 3 h of induction cells were harvested by centrifugation. Pellets were extracted directly with Bug Buster Master Mix (BBMR) (Novagen) following the supplier's protocol. We had previously determined that BBMR did not significantly interfere with the ATQ enzymatic assay and provided more consistent extraction sample to sample compared to sonication. A plasmid lacking the ATQ insert, the 'empty vector', was subjected to the same induction and extraction process to provide a negative control for the enzyme assay.

2.4. ATQ enzymatic assay

The AASA substrate was generated using a recombinant lysine aminotransferase gene cloned from *Streptomyces clavuligerus* as described previously [9]. ATQ activity, expressed as nmol NADH produced/minute/mg protein, was determined spectrophotometrically using an Abbott Alcyon 300 analyzer as previously described [9]. The routine assay included 11 ng/μl total protein and 0.56 mM AASA in the assay mixture. We had previously determined that this assay gave a linear response up to a total protein concentration of 15 ng/μl [9]. The baseline for sensitivity of the assay was previously estimated as equivalent to 3% of the wild-type (WT) enzyme activity [9].

Table 2
ATQ mutations, mutagenesis and mutation verification.

Mutation	Oligonucleotide primers	
cDNA/amino acid missense	Mutagenesis[a,b]	PCR primers[b,c] & restriction enzyme confirmation
c.1100T>G	F: 5′ GAAAGAAGGTGGCACAG**GC**GTCTATGG**T**GGCAAGGTTATGG 3′	Primers 9F/8R
p.V367G	R: 5′ CCATAACCTTGCC**A**CCATAGAC**GC**CTGTGCCACCTTCTTTC 3′	+MspI
c.1230C>A	F: 5′ GCTCCGATTCTCTATGTCTT**C**AAA**CTG**AAGAATGA**G**GAAGAGGTC 3′	Primers 3F/10R
p.F410L	R: 5′ GACCTCTTC**C**TCATTCTT**CAG**TTT**G**AAGACATAGAGAATCGGAGC 3′	+PstI
c.1274A>G	F: 5′ GCATGGAATAATGAAGTAAA**GC**GCGGACTTTCAAGTAGCATC 3′	Primers 6F/8R
p.Q425R	R: 5′ GATGCTACTTGAAAGTCC**GC**G**C**TTTACTTCATTATTCCATGC 3′	+HhaI
c.1346T>A	F: 5′ GGACCTAAAGGATCAGACT**CC**GGCATTGTAAATGTCAAC 3′	Primers 8F/8R
p.C450S	R: 5′ GTTGACATTTACAATGCC**GG**AGTCTGATCCTTTAGGTCC 3′	+MspI

[a] The mutated codon is underlined. In some cases a synonymous codon was used to replace a rarely used codon.
[b] Base changes are indicated in bold.
[c] Primer sequences: 3F 5′ CCACTCCACACCAAGCAGGCAGTGAGCATG 3′;6F 5′ GCCACGATGCGTCCATTG**GC**CACAC 3′; 8F 5′ GAAGTAA**G**ACAGGGACTTTCAAG**A**AGC 3′; 8R 5′ CCAGGCATCA CTGCCAGACTCCCT**TC** 3′; 9F 5′ GCAAAGAAAGAAGGTGGCAC**C**G 3′; 10R 5′ CCATGCAAAGACCTCTTC**C**TCATTCT**GC** 3′.