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Enzymatic defect in "X-linked" sideroblastic anemia: Molecular evidence for erythroid δ-aminolevulinate synthase deficiency

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ABSTRACT **Recently, the human gene encoding erythroid-specific δ-aminolevulinate synthase was localized to the chromosomal region Xp21–Xq21, identifying this gene as the logical candidate for the enzymatic defect causing "X-linked" sideroblastic anemia. To investigate this hypothesis, the 11 exonic coding regions of the δ-aminolevulinate synthase gene were amplified and sequenced from a 30-year-old Chinese male with a pyridoxine-responsive form of X-linked sideroblastic anemia. A single T → A transition was found in codon 471 in a highly conserved region of exon 9, resulting in an Ile → Asn substitution. This mutation interrupted contiguous hydrophobic residues and was predicted to transform a region of β-sheet structure to a random-coil structure. Prokaryotic expression of the normal and mutant cDNAs revealed that the mutant construct expressed low levels of enzymatic activity that required higher concentrations of pyridoxal 5′-phosphate to achieve maximal activation than did the normal enzyme. The amino acid substitution occurred in the exon containing the putative pyridoxal 5′-phosphate binding site and may account for the reduced ability of the cofactor to catalyze the formation of δ-aminolevulinic acid.**

The sideroblastic anemias are a clinically heterogeneous group of disorders resulting from inherited or acquired causes (1, 2). The inherited types are X chromosome-linked or autosomal, and the acquired forms are secondary to chemical induction or to inflammatory disease or are idiopathic. Patients in these classifications can be further subdivided into those who are responsive to pyridoxine and those who are refractory. Sideroblastic anemia is classified as a disorder of heme synthesis since the activities of certain heme biosynthetic enzymes were reported to be deficient (3), whereas globin synthesis was normal. Since many patients had reduced δ-aminolevulinate synthase [ALAS; succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating); EC 2.3.1.37] enzymatic activity in bone marrow (4, 5) and because pyridoxal 5′-phosphate (PLP), the pyridoxine metabolite, is a required cofactor, ALAS has been suggested as a candidate gene for mutations leading to some types of sideroblastic anemia (6).

The human genes encoding the housekeeping and erythroid forms of ALAS were isolated and characterized (7–9). Although the two genes predicted proteins with 59% amino acid identity, they were mapped to different chromosomes by somatic cell hybrid and *in situ* hybridization methods. The housekeeping gene mapped to chromosome 3p21, and the erythroid gene was localized to the X chromosomal region Xp21–Xq21 (9–13). The X-chromosomal assignment suggested that the erythroid form of ALAS (designated ALAS2) might be the enzymatic defect in "X-linked" sideroblastic anemia (XLSA).

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To investigate this hypothesis, the erythroid ALAS2 coding regions were amplified and sequenced from a male affected with pyridoxine-responsive XLSA. In this communication, an exonic point mutation in the ALAS2 gene that predicted the substitution of asparagine for a highly conserved isoleucine is characterized as evidence that the defect in XLSA is the deficient activity of the erythroid form of ALAS.

MATERIALS AND METHODS

Case Report. The proband was a recently immigrated 30-year-old Chinese male (63.5 kg, 168 cm) who was admitted to the Krankenhaus Spandau (Berlin) because of increasing weakness, nausea, and pallor. There was no history of recent trauma, blood loss, infection, drug or alcohol abuse, chemotherapy, or exposure to radiation, organic solvents, or heavy metals. Throughout his childhood he appeared more pale than his siblings and was easily fatigued. At age 16, he was hospitalized with severe anemia. Blood transfusions and administration of vitamin B complex preparations were therapeutic. Several subsequent crises were resolved with only vitamin B complex supplementation. Although his parents and two sisters were reported healthy and none of his relatives had similar symptoms, they were unavailable for clinical or biochemical studies.

Physical examination revealed pallor of the skin and conjunctiva, mild tachycardia (108 beats per min), and a grade I systolic murmur. Aside from sinus tachycardia, an electrocardiogram was normal as was his chest x-ray. Abdominal ultrasound revealed moderate splenomegaly (250 ml; normal, <155 ml).

Laboratory studies revealed a hemoglobin content of 5.7 g/dl, a hematocrit of 19%, and an erythrocyte count of 3.0×10^6 cells per μl. The mean corpuscular volume was 63 fl per cell (normal, 85–95 fl per cell), the mean corpuscular hemoglobin concentration was 19 pg per cell (normal, 26–32 pg per cell), the reticulocyte count was 0.5%, the leukocyte count was 6800 cells per μl, and the platelet count was 600,000 cells per μl. A peripheral blood smear showed a hypochromic microcytic anemia with marked anisocytosis, poikilocytosis, and polychromasia; a normal leukocyte differential; and no pathologic cells. The serum iron concentration of 247 μg/dl (normal, 39–167 μg/dl) was above normal while the severely elevated serum ferritin concentration of 784 ng/ml (normal, 30–220 ng/ml) indicated iron overload. Otherwise, the serum chemistries were within normal limits including: lactate dehydrogenase, creatine phosphokinase, total bilirubin (0.4 mg/dl), haptoglobin, transferrin (235 μg/dl), lead, vitamin B_6 (2.1 ng/ml), vitamin B_{12} (498 pmol/l), aspartate aminotransferase, alanine aminotransferase, folic acid, and alkaline

Abbreviations: ALAS, δ-aminolevulinate synthase; ASO, allele-specific oligonucleotide; PLP, pyridoxal 5′-phosphate; nt, nucleotide(s); t_m, melting temperature; KBL, 2-keto-3-aminobutyrate coenzyme A ligase; XLSA, X-linked sideroblastic anemia.

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phosphatase. The protein electrophoresis and complement measurements were normal and the Coombs tests were negative. Hemoglobin electrophoresis was normal and a sucrose lysis test and an acidified serum lysis test (Ham test) were negative. Erythrocyte osmotic fragility studies showed a flattened and broadened curve.

Bone marrow examination showed a hypercellular marrow with a left-shift and slightly megaloblastoid erythropoiesis with severely disturbed maturation. Myelopoiesis and the number of megakaryocytes appeared grossly normal. There was a population of medium-sized abnormal cells with round pale nuclei surrounded by a narrow rim of cytoplasm, which in retrospect were most likely partially lysed sideroblasts. Prussian blue staining revealed iron overloading with a large number of ringed sideroblasts (≈60%), sideroblasts (≈10%), and only a few intermediate sideroblasts (<2%).

Pyridoxine therapy (300 mg daily, subcutaneously) was started; after 2 weeks the hemoglobin increased to 9.0 g/dl, the hematocrit increased to 31%, and the serum iron decreased to 56 μg/dl. At this time the therapy regimen was changed to 300 mg orally twice daily with reduction to 100 mg daily after 1 week. The hemoglobin was 12.8 g/dl after 4 weeks, the hematocrit was 45%, erythrocytes were 5.6×10^6 cells per μl, and the serum iron was 98 μg/dl, but the erythrocytes remained microcytic and hypochromic. Porphyrin intermediates were within normal limits including: urinary δ-aminolevulinic acid and porphobilinogen, serum uroporphyrin and coproporphyrin, erythrocyte coproporphyrin, free erythrocyte protoporphyrin, and serum protoporphyrin. The marrow showed an increased number of erythroid cells, partly megablastoid, but most with normal maturation and differentiation. The iron content of the marrow stroma cells was reduced and few sideroblasts (12–15%) or ringed sideroblasts (2–5%) were seen. The previously noted abnormal cells were absent. The patient was discharged on 100 mg of pyridoxine daily. Blood analysis 2.5 months later revealed the following values: hemoglobin, 14.1 g/dl; hematocrit, 43%; 6.1×10^6 erythrocytes per μl; mean corpuscular volume, 74 fl; mean corpuscular hemoglobin, 23 pg; 1.1% reticulocytes; 230,000 platelets per μl; serum iron, 180 μg/dl; transferrin, 274 μg/dl. At this time the bone marrow appeared normal except for an increased iron content in the stroma cells and an increase of sideroblasts (≈55%) but with few ringed forms (≈5%). Pyridoxine therapy was then discontinued. Blood values 4 months later were as follows: hemoglobin, 12.9 g/dl; hematocrit, 44%; 5.8×10^6 erythrocytes per μl; mean corpuscular volume, 75 fl per cell; mean corpuscular hemoglobin, 22 pg per cell; reticulocyte count, 0.7%; leukocyte count, 8400 cells per μl; platelet count, 250,000 cells per μl; transferrin, 295 μg/dl; serum ferritin, 1210 μg/liter; serum iron, 163 μg/dl; and vitamin B_6, 12.6 μg/liter. A repeat abdominal ultrasound examination showed only a slight splenomegaly (180 ml). Pyridoxine therapy was then reinstated at 100 mg daily.

Oligonucleotide Synthesis. Oligonucleotides (Table 1) were synthesized with phosphoramidite chemistry on a model 380B DNA synthesizer (Applied Biosystems), deprotected with ammonia at 55°C for 5 hr, lyophilized from water, and used without further purification. Oligonucleotides for genomic amplification each contained a 5′-GCCGCC clamp (14) to facilitate digestion, an *Eco*RI site, and 17–22 nucleotides (nt) of sense or antisense ALAS2 intronic sequence.

Genomic DNA Amplification and Sequencing. Amplification of each ALAS2 exon and 10–100 nt of flanking intronic sequences was accomplished by a modification of the method of Saiki *et al.* (15). The 100-μl PCR mixture contained 1 μg of genomic DNA, all four dNTPs (each at 50 μM), 100 pmol each of the sense and antisense oligonucleotides, 10 μl of 10× *Taq* polymerase buffer, and 2.5 units of *Taq* polymerase (Promega). Incubation at 94°C for 6 min was followed by 30 cycles on a model PTC-100 programmable thermal cycler (MJ Research) with denaturation at 94°C for 1 min, primer annealing at the melting temperature (t_m) of the hybridizing region of the respective oligonucleotide for 1 min (see Table 1), and extension at 72°C for 2 min.

For sequence analysis, the amplified products were digested with *Eco*RI (Promega), purified by agarose gel electrophoresis, electroeluted onto DE-85 paper (Whatman), subcloned into pGEM9Z (Promega), and then sequenced by the dideoxynucleotide method (16) using Sequenase Version 2.0 (United States Biochemical).

Allele-Specific Oligonucleotide Hybridization. PCR-amplified genomic DNA was applied to duplicate nylon membranes (Zeta-Probe, Bio-Rad) for hybridization with normal and mutant oligonucleotides. Allele-specific oligonucleotides (ASOs) were 5′-labeled with [γ-^{32}P]ATP using T4 kinase (New England Biolabs) according to standard procedures (17). The membranes were soaked in 2× SSC (1× SSC = 0.15 M sodium chloride/15 mM sodium citrate, pH 7.0) (18) for 20 min and mounted in a dot-blot apparatus (Schleicher & Schuell), and each well was washed with 200 μl of 10× SSC. A 50-μl sample of each amplification product was mixed with 350 μl of denaturation reagent (0.4 M NaOH/25 mM EDTA) and divided into two 200-μl portions for application to the duplicate membranes. Subsequently, each well was washed with 200 μl of 10× SSC, and the membranes were baked for 2 hr in a vacuum oven at 80°C and were prehybridized in 6× SSPE/10× Denhardt's solution/0.5% SDS at t_m −5°C for 1 hr (18) (1× SSPE = 0.15 M NaCl/10 mM sodium phosphate, pH 7.0/1 mM EDTA). Hybridization was carried out overnight in the same solution with the normal or mutant ASOs at 1×10^6 cpm/ml (Table 1). After hybridization, the membranes were washed for two 30-min periods in 6× SSC/0.1% SDS at 5°C below the t_m. Additional washes at t_m plus 1°C resulted in improved specificity. Membranes were then au-

Table 1. Synthetic oligonucleotides used for ALAS2 gene analysis

Primer	Description	Sequence	t_m, °C	
			Calculated	Employed
P115	Amplification, 3′ intron 8	5′-GCCGCCGAATTCATGATCCTGTTGCTCT-3′	47	54
P116	Amplification, 5′ intron 9	5′-GCCGCCGAATTCAGCGTGAGGCTCCCAGA-3′	53	54
T1424	ASO, exon 9 normal	5′-CCACATCATCCCCATCC-3′	55	50
A1424	ASO, exon 9 mutant	5′-CCACATCAACCCCATCC-3′	55	50
PT7	Sequencing, T7 promoter	5′-TAATACGACTCACTATAGGG-3′	—	—
PSp6	Sequencing, Sp6 promoter	5′-GATTTAGGTGACACTATAG-3′	—	—
P46	Sequencing, exon 9	5′-ATGCTGCAGGCTTCATC-3′	—	—
P197	Sequencing, exon 9	5′-CTAGAATCTGTGCGGCTGCT-3′	—	—

Hybridization temperatures (t_ms) were calculated (37) for the nucleotides involved in the initial hybridization under the specified monovalent cation and primer concentrations. The sequence coordinate of the normal (T1424) and mutant (A1424) ASOs is from the cDNA sequence of ALAS2 (8). All sequencing primers were annealed with template by heating at 65°C and cooling to room temperature before extension in the sequencing reaction at 37°C.

toradiographed using X-Omat AR film (Eastman Kodak) with two Cronex Lightning Plus intensifying screens (DuPont) for 1–2 hr at −70°C.

Expression of Normal and Mutant ALAS2 in *Escherichia coli*. The full-length cDNA clone pGEM4ZAE214 (8) was found to differ from the genomic sequence by a single base change (C → T) at nt 542, a Phe → Ser substitution. The genomic ALAS2 sequence included a *Dde* I site due to C542, which was confirmed in 100 alleles by restriction analysis of PCR-amplified DNA from unrelated Caucasians (data not shown). The T542C transition in clone pGEM4ZAE214 was made by site-directed PCR mutagenesis using the overlap extension method (19). The sequence of the corrected clone was PCR modified at the 5′ end to initiate with the coding sequence for mature mitochondrial ALAS2 (Q45) as predicted by homology with the chicken ALAS1 peptide sequence (20). The 5′ PCR primer contained an *Nde* I site to allow cloning into the pET5a expression vector (ref. 21; Novagen, Madison, WI) adding a methionine residue to the N-terminal Q45. Overlap extension PCR was used to introduce the I471N mutation (reported here for a pyridoxine-responsive patient) by conversion of the ATC codon 471 to AAC. The entire normal and mutant ALAS2 coding sequences and restriction junctions were sequenced in the pET expression vector constructs. The pET5a expression constructs were transfected into *E. coli* BL21(DE3)pLysS (Novagen) and grown in LB medium plus ampicillan and chloramphenicol to an A_{600} of 1 unit and then induced by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 hr. Cells were harvested from 5-ml samples, suspended in 1 ml of 50 mM Hepes, pH 7.5/2 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/2 mM EDTA/aprotinin (3 μg/ml), and frozen. The thawed lysate was centrifuged at 10,000 × *g* for 30 min, and the supernatant was assayed for ALAS activity as described (22) and with detection by Ehrlich's reagent after reaction with ethyl acetoacetate (23). One unit of activity is that amount of enzyme required to catalyze the production of 1 nmol of δ-aminolevulinate per hour under the conditions of the assay. Protein concentration was determined by a modification of the fluorescamine procedure (24).

Computer Analysis. Analysis of restriction sites, protein secondary structure predictions and database comparisons were conducted with MacVector Version 3.5 (International Biotechnologies) and the GCG Package (25) from the Genetics Computer Group, Madison, WI.

RESULTS

Patient Diagnosis. Although it has not been possible to study additional family members of this Chinese proband, he clearly exhibited the classic phenotype of XLSA, including microcytic, hypochromic anemia with abundant ringed sideroblasts in the marrow, striking anisocytosis and poikilocytosis, broadened and flattened osmotic curve, iron overloading with beginning hemosiderosis, and moderate hypersplenism. The possible diagnosis of myelodysplastic syndrome type 2 (refractory anemia with ringed sideroblasts) (26) was not compatible with the observed early age of onset, lack of known exposures to mutagenic agents, absence of progression, and pyridoxine responsiveness leading to nearly normal hemoglobin levels. The pathologic marrow cells described in the case report completely disappeared after pyridoxine therapy, consistent with their being partially lysed sideroblasts as a consequence of the observed increase in erythrocyte osmotic fragility. All other differential diagnoses were ruled out as the cause of the disease by the laboratory tests, bone marrow examinations, and imaging procedures.

Identification of an Exonic Point Mutation in the ALAS2 Gene. Genomic DNA was isolated from the proband and each of the 11 ALAS2 exons, including their intron–exon junctions (D.F.B. and Minxu Lu, unpublished data), was PCR-amplified, subcloned into pGEM9Z, and completely sequenced. Only one nucleotide difference from the normal human sequence (8, 9) was identified. This transition (T → A) at nt 1424 of the cDNA (8) predicted an Ile → Asn substitution at codon 471, designated I471N (Fig. 1). This mutation in exon 9 was confirmed by allele-specific oligonucleotide hybridization of the mutant probe (A1424) to PCR-amplified genomic DNA from the hemizygote and by the absence of hybridization of this probe to the DNAs of two unrelated normal females (Fig. 2). Conversely, the normal probe (T1424) hybridized to the control DNAs but not to the DNA from the patient. The I471N mutation did not occur in 100 ALAS2 alleles from unrelated normal Caucasian females in a survey by dot-blot analysis (data not shown).

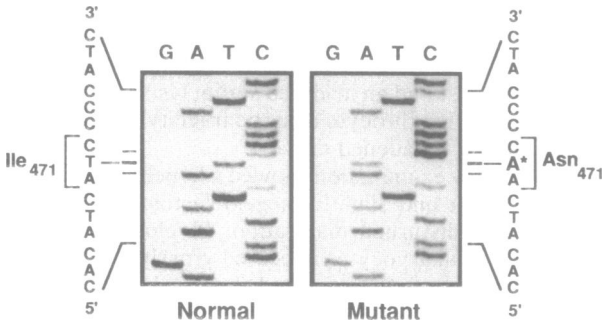

FIG. 1. Exon 9 nucleotide sequence in the region of the I471N mutation in a patient with XLSA. Dideoxynucleotide sequencing was conducted with ^{35}S-labeled dATP and exon 9 DNA was amplified and subcloned from a normal individual and from the proband. The normal and mutant reaction products were electrophoresed on 0.4-mm-thick 8% polyacrylamide/7 M urea sequencing gels and autoradiographed using Kodak X-Omat AR film.

The I471N Mutation Disrupts the Predicted Secondary Structure of a Highly Conserved Region of the ALAS2 Polypeptide. To date, nine homologous ALAS cDNA sequences from various organisms have been reported to the GenBank data library (Release 67). Multiple sequence alignment of the nine ALAS peptide sequences and the homologous 2-keto-3-aminobutyrate coenzyme A ligase (KBL) peptide sequence from *E. coli* (27) was facilitated with the PILEUP program of the GCG Package. Alignment of the 51-residue region (amino acids 447–497) surrounding the ALAS2 mutation in this patient with other ALAS peptides revealed that the mutation was in the middle of a highly conserved hydrophobic region found in all 10 sequences (Fig. 3). The core conserved sequence for eukaryotic organisms was SHI(I/V)P(I/V/L), designated a "SHIP" box.

Secondary structure analysis, based on the algorithm of Chou and Fasman, predicted a β-sheet region from residues 468 to 474 encompassing the mutation (Fig. 4*B*). This structure was conserved in all 10 sequences. The I471N mutation markedly changed the secondary structure prediction by converting the entire region to a random-coil structure (Fig. 4*A*).

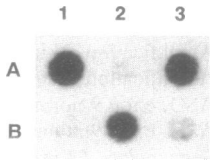

FIG. 2. Allele-specific oligonucleotide hybridization to PCR-amplified DNA from the ALAS2 exon 9 genomic region in normal females (lanes 1 and 3) and in the proband with XLSA (lane 2). Amplified DNA was dot-blotted onto nylon membranes, hybridized with 17-mer radiolabeled oligonucleotides to the normal (T1424; row A) or mutant (A1424; row B) sequences (Table 1, ASO primers), and washed at 56°C. Exposure was for 2 hr with two intensifying screens.

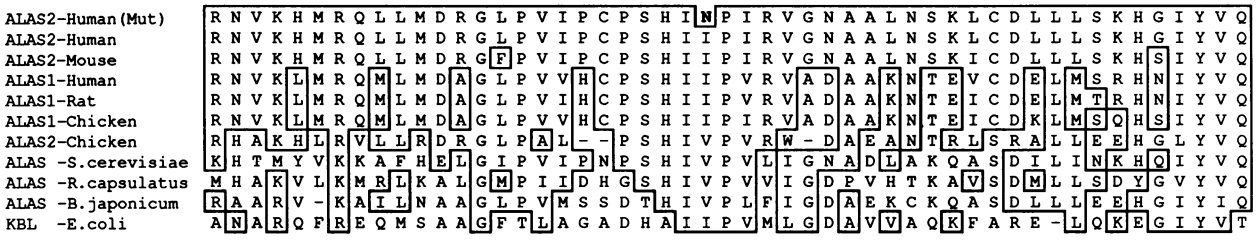

FIG. 3. Conserved amino acid sequences in the region of the mutation in the proband ALAS2 gene product. The first sequence is that of the proband (Mut) in this study, showing the replacement of the conserved isoleucine by asparagine. The remaining sequences are the normal human ALAS2 (8, 9); mouse ALAS2 (28); human ALAS1 (8, 9); rat ALAS1 (29, 30); chicken ALAS1 (20); chicken ALAS2 (31); the ALAS genes from *Saccharomyces cerevisiae* (32), *Rhodobactor capsulatus* (33), and *Bradyrhizobium japonicum* (34); and the KBL gene of *E. coli* (27). The boxed residues are identical or very similar in structure and function to those of ALAS2.

Expression of Normal and Mutant ALAS2 Enzymes. Normal and mutant ALAS2 enzymes were expressed in *E. coli* and the specific activities of the soluble enzymes were determined. When the enzymes were extracted from the bacteria in the presence of protease inhibitors and assayed in the presence of 0, 10, 100, and 1000 μM added PLP, the specific activities of the normal enzyme were 128, 150, 184 and 195 units/mg, respectively. In contrast, for the same PLP concentrations, the activities of the mutant enzyme were 0, 0, 1.21 and 4.79 units/mg, respectively. These results indicate that the mutant enzyme is optimally activated at PLP concentrations at least 10-fold greater than required for the normal enzyme.

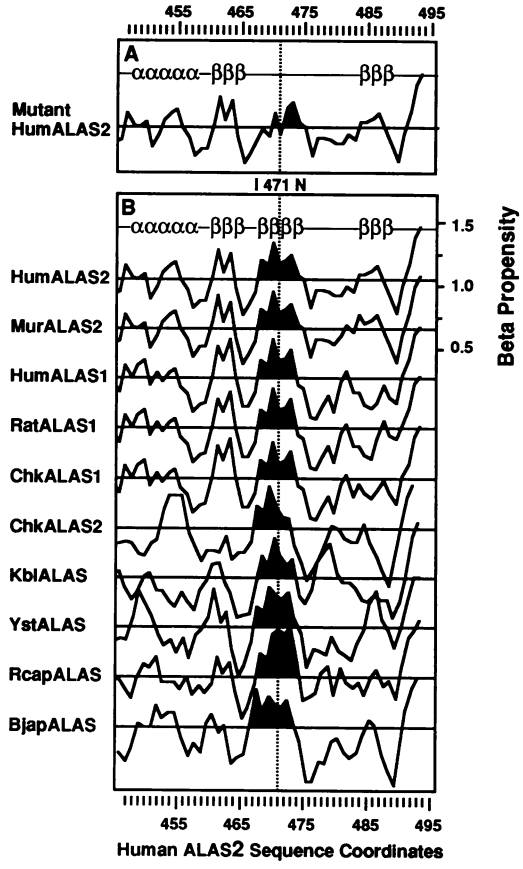

FIG. 4. Predicted secondary structure in the region of the I471N mutation. (*A*) Mutant human ALAS2. (*B*) Normal ALAS. The graph(s) in the lower part of *A* and *B* indicate(s) the propensity for β-sheet formation in a sliding window of four residues with the horizontal line indicating the threshold for propagation of the structure as determined by the program PEPPLOT in the GCG Package. At the top of *A* and *B*, the symbols indicate the overall predicted structure for mutant and normal human ALAS2 peptides for the same region and sequences as shown in Fig. 3. α, α-helix; β, β-sheet; –, random coil; Hum, human; Mur, murine; Chk, chicken; KbL, KBL; Yst, yeast; Rcap, *R. capsulatus*; Bjap, *B. japonicum*.

DISCUSSION

Previous studies of mammalian ALAS, the first enzyme of the heme biosynthetic pathway, revealed that hepatic, but not erythroid ALAS, was induced in response to exogenously delivered porphyrinogenic drugs such as allylisopropylacetamide or 3,5-dicarbethoxy-1,4-dihydrocollidine (35, 38). In addition, hemin administration blocked transport of ALAS to the mitochondria in adult rat liver, but hemin had no effect on ALAS activity or transport in erythroid tissue (39–41). Insight into these differences was gained by the demonstration that erythroid and nonerythroid forms of ALAS had different physical and kinetic properties (42). Subsequently, the existence of an erythroid form of ALAS was further supported by demonstration of immunologic and molecular size differences between erythroid and nonerythroid ALAS in chicken and rat (43, 44). The recent isolation and sequencing of cDNAs encoding the erythroid form of ALAS in chickens (31, 45) verified these biochemical and immunological findings. Generalization of these findings was provided by the identification of erythroid genes for ALAS in mice (46) and humans (8–10, 47).

Thus, in higher eukaryotes two homologous, but different, ALAS isozymes are synthesized that exhibit tissue-specific regulation. ALAS1, the housekeeping enzyme, is responsible for regulating heme production primarily for cytochromes and other hemoproteins; this enzyme is tightly regulated both by feedback inhibition of transcription (29, 48) and of transport to the mitochondria (39, 49). In contrast, ALAS2 is an erythroid-specific isozyme expressed at high levels only in fetal liver and in adult bone marrow. The erythroid isozyme, which is required for hemoglobin synthesis, is highly active (31) and is not repressed or inhibited by heme. Of note, the housekeeping gene (ALAS1) mapped to chromosome 3p21 (10, 12, 50) and the erythroid gene (ALAS2) was localized to the X chromosome (11–13). The latter findings supported the suggestions (11, 13) that mutations in ALAS2 caused ALAS deficiency and XLSA.

In the proband with pyridoxine-responsive XLSA reported here, a single amino acid residue was altered in the ALAS2 polypeptide. The I471N mutation occurred at the 3′ end of exon 9 in the ALAS2 gene and was in the highly conserved SHIP box region that is present in all eukaryotic ALAS sequences reported to date (Fig. 3). Since the sideroblastic anemia in the proband was pyridoxine-responsive, it is possible that the structural alteration resulting from the I471N mutation reduced the binding of PLP in the active site, resulting in deficient ALAS2 activity unless the vitamin was supplemented at pharmacologic concentrations.

Notably, the putative PLP binding site of the enzyme also is in exon 9 (36). Previously, the PLP covalent binding site in

E. coli KBL was identified by isolation and sequencing of the KBL peptide containing the N^{ϵ}-pyridoxyllysine (36). At this time it was noted that this peptide had remarkable sequence identity with a region of the chicken hepatic ALAS1 amino acid sequence (36). Since KBL (27) appears to be homologous (34% amino acid identity and 59% amino acid similarity as determined by the BESTFIT program of the GCG package) to the human erythroid ALAS2 enzyme, it is extremely likely that the corresponding lysine (residue K386) in ALAS2 is the PLP attachment site in this peptide. This lysine is the first residue of exon 9. Thus, it is possible that the exon 9 domain plays an important role in cofactor binding and that the I471N mutation may reduce the ability of PLP to catalyze the formation of δ-aminolevulinic acid.

The severe reduction of enzymatic activity for the I471N construct expressed in *E. coli* and the mutant enzyme's responsiveness to high concentrations of PLP demonstrates that the I471N mutation is consistent with the phenotype observed in the patient. The apparent reduced affinity for PLP by the mutant enzyme may be a consequence of improper folding of the mutant enzyme during prokaryotic synthesis due to the local aberration in structure predicted for this mutation and in the absence of sufficient PLP to overcome a reduced affinity for the cofactor. Clearly, further expression and site-specific mutagenesis studies are required to delineate the function of the SHIP domain.

The identification of mutations in additional patients with X chromosome-linked sideroblastic anemia will potentially reveal genetic heterogeneity leading to both pyridoxine-responsive and pyridoxine-refractory forms of sideroblastic anemia. It is also reasonable to suggest that some forms of acquired sideroblastic anemia could be due to somatic ALAS2 deficiency. Since other defects, for example, in pyridoxine metabolism, have been implicated in the sideroblastic anemias, further studies will be required to determine the extent of involvement of ALAS2 in these disorders.

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