Late-onset X-Linked Sideroblastic Anemia

Missense Mutations in the Erythroid δ-Aminolevulinate Synthase (*ALAS2*) Gene in Two Pyridoxine-responsive Patients Initially Diagnosed with Acquired Refractory Anemia and Ringed Sideroblasts

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Abstract

X-linked sideroblastic anemia (XLSA) is caused by mutations of the erythroid-specific δ-aminolevulinate synthase gene (*ALAS2*) resulting in deficient heme synthesis. The characteristic hypochromic, microcytic anemia typically becomes manifest in the first three decades of life. Hematologic response to pyridoxine is variable and rarely complete. We report two unrelated cases of highly pyridoxine-responsive XLSA in geriatric patients previously diagnosed with refractory anemia and ringed sideroblasts. A previously unaffected 77-yr-old male and an 81-yr-old female were each found to have developed severe hypochromic, microcytic anemia with ringed sideroblasts in the bone marrow, which responded dramatically to pyridoxine with normalization of hemoglobin values. Sequence analysis identified an A to C transversion in exon 7 (K299Q) of the *ALAS2* gene in the male proband and his daughter. In the female proband a G to A transition was identified in exon 5 (A172T). This mutation resulted in decreased in vitro stability of bone marrow δ-aminolevulinate synthase activity. Each patient's recombinant mutant ALAS2 enzyme had marked thermolability. Addition of pyridoxal 5′-phosphate in vitro stabilized the mutant enzymes, consistent with the observed dramatic response to pyridoxine in vivo. This late-onset form of XLSA can be distinguished from refractory anemia and ringed sideroblasts by microcytosis, pyridoxine-responsiveness, and *ALAS2* mutations. These findings emphasize the need to consider all elderly patients with microcytic sideroblastic anemia as candidates for XLSA, especially if pyridoxine responsiveness is demonstrated. (*J. Clin. Invest.* 1995. 96:2090–2096.) Key words: pyridoxine • sideroblastic anemia • δ-aminolevulinate synthase • mutation • X chromosome

Introduction

The sideroblastic anemias are a heterogeneous group of disorders that may be either acquired, or less commonly, inherited. All are characterized by ineffective erythropoiesis resulting in anemia of varying severity, elevated serum iron, and the presence of ringed sideroblasts in the bone marrow (1). The etiology of the sideroblastic anemias is poorly understood. However, the biochemical and molecular basis of the best-documented inherited form, X-linked sideroblastic anemia (XLSA)[1] (McKusick No. 301300 [2]), has recently been characterized. XLSA has been shown to be due to mutations in the erythroid-specific δ-aminolevulinate synthase (*ALAS2*) gene at Xp11.21 encoding the first enzyme in the heme biosynthetic pathway (3–9). The disease is usually detectable in newborns but may not present clinically until midlife or, rarely, later (1, 10). Patients generally have severe hypochromic, microcytic anemia with increased iron storage that shows a partial clinical response to pyridoxine (1). A different sideroblastic anemia linked to Xq13 (McKusick No. 301310 [2]) is associated with ataxia in the two families described to date (11, 12). Other rare forms of sideroblastic anemia are clearly autosomal (13, 14).

The most prevalent form of sideroblastic anemia is an acquired disorder found primarily among the elderly, originally designated as primary acquired sideroblastic anemia or idiopathic acquired sideroblastic anemia (1). These disorders are now generally classified as refractory anemia with ringed sideroblasts (RARS), one of the myelodysplastic syndromes (15), and also a heterogeneous group of disorders. The majority of patients have a trilineage disorder involving erythroid, granulocyte, and megakaryocyte cells, and an increased risk of progression to leukemia (16). However, a "pure sideroblastic anemia" form has been identified with only erythroid dysplasia and a low risk of transformation to acute leukemia (17–20). Patients

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1. *Abbreviations used in this paper:* ALAS, aminolevulinate synthase; *ALAS2*, erythroid-specific δ-ALAS gene; Hb, hemoglobin; MBP, maltose-binding protein; MCV, mean corpuscular volume; nt, nucleotide; PLP, pyridoxal 5′-phosphate; RARS, refractory anemia with ringed sideroblasts; XLSA, X-linked sideroblastic anemia.

Table I. Hematologic Values for Patient 1 and Family Members

	Hb	RBC	MCV	RDW	WBC	Platelets
	g/dl	*× 10^{12}/liter*	*fl*	*Percent*	*× 10^{9}/liter*	*× 10^{9}/liter*
Proband: I-2						
Before pyridoxine treatment						
October, 1988	8.7		68		5.1	
December, 1988	4.9		69		5.1	497
February, 1989	6.9	2.79	78*		4.4	329
Weeks after pyridoxine treatment (200 mg/d)						
2‡	10.3	4.25	80		6.5	217
6‡	12.1	4.90	87	18.1	7.1	255
13‡	13.4	5.09	81	15.5	6.7	200
26§	13.0	5.08	83	14.3	5.7	213
100§	13.7		84		8.4	195
Other family members						
II-1, nephew	15.3	5.14	91.6	13.0	6.6	265
II-2, nephew	15.7	5.24	86.9	13.0	8.3	273
II-3, daughter	13.1	4.50	90.3	17.0	6.7	191
III-1, grandson	13.9	4.50	95.9	12.8	6.4	150
Normal range	13–16.5 (♂) 11.5–15.5 (♀)	3.6–5.4	80–99	11.6–13.9	4.0–10.5	150–400

RDW, red cell distribution width; * Transfused cells present; ‡ folic acid (5 mg 3 × per d) and 1 mg intramuscular vitamin B_{12} monthly were also taken; § reduced to 100 mg/d pyridoxine at 13 wk.

with RARS are typically unresponsive to pyridoxine and have a dimorphic erythrocyte population in which macrocytes predominate, resulting in an elevated mean corpuscular volume (MCV) (21, 22). However, some acquired sideroblastic anemia patients have been reported with a predominantly microcytic erythrocyte population and/or show a clinical response to pyridoxine (23–25).

In this communication, we describe two elderly patients who each presented after the seventh decade with a presumptive diagnosis of RARS. Both had predominantly microcytic erythrocyte populations and responded dramatically to pyridoxine. Molecular analysis revealed the occurrence in each patient of an inherited or constitutive point mutation in the *ALAS2* gene, indicating that some cases diagnosed as RARS actually have a late-onset form of XLSA.

Methods

Patient 1. The proband, I-2 (see Fig. 2 *A*), was a 77-yr-old male (date of birth 8/12/11) who presented with shortness of breath and fatigue of 6 wk duration. He did not smoke or drink alcohol. There was no history of anemia in the patient or in his family. On examination, he was pale but otherwise normal. He was found to have a severe hypochromic, microcytic anemia (Table I); hemoglobin (Hb) was 8.7 g/dl, MCV was 68 fl, serum ferritin was 400 μg/liter, transferrin saturation was 70%, serum vitamin B_{12} was 107 ng/liter, and serum folate was 3.2 μg/liter. The white cell count, differential, and platelet count were normal. Over the following 4 mo the proband received 11 U of blood and was treated unsuccessfully during the latter half of this time with 600 mg/d ferrous sulphate. Extensive investigations for blood loss were negative, and he was referred to the University Hospital of Wales for investigation of refractory anemia. On admission serum ferritin was 803 μg/liter, and serum bilirubin was normal (14 μmol/liter). Bone marrow aspiration was normocellular with normal megakaryocytes and normal granulopoiesis. There was moderate erythroid hyperplasia (49% erythroid cells), and some evidence of dyserythropoiesis with intercytoplasmic bridging, poor hemoglobinization, and 61% ringed sideroblasts. Cytogenetic studies of bone marrow showed a normal 46,XY karyotype. A presumptive diagnosis of RARS was made. The patient was treated with 200 mg/d oral pyridoxine, 15 mg/d oral folate, and 1 mg/mo intramuscular vitamin B_{12}. After 2 wk his hemoglobin rose to 10.3 g/dl and his MCV to 80 fl (Table I). For the next 2 yr, his hemoglobin was maintained at 12.1–13.7 g/dl and MCV at 81–87 fl on oral pyridoxine alone (100 mg/d). As his hemoglobin increased, the hypochromic microcytic population gradually decreased, his weight increased, and he felt much improved symptomatically. No further bone marrow sample was available, but he continued to show a very minor population of hypochromic microcytes in peripheral blood. He remained responsive to pyridoxine until his death from an unrelated illness at age 81.

Hematologic evaluation of his daughter showed normal serum ferritin (63 μg/liter), MCV (90.3 fl; Table I), and mean corpuscular hemoglobin (28.5 pg; reference range: 27–34 pg) with no indication of bimodality in cell size although there was a slightly increased red cell size distribution (red cell distribution width = 17%) encompassing a small excess of both microcytic and macrocytic erythrocytes. The mean corpuscular hemoglobin concentration (32.0 g/dl) was just below the normal range (reference range: 33–37 g/dl), and a small degree of hypochromia (4–8% cells with mean corpuscular hemoglobin concentration ≤ 28 g/dl) was noted. No abnormalities were detected in erythrocytes from his nephews or grandson (Table I).

Patient 2. The proband was an 81-yr-old female who complained of breathlessness. She was found to have a severe hypochromic, microcytic anemia with a hemoglobin of 4.3 g/dl and an MCV of 72 fl (Table II). Her white cell count was normal and her platelets were slightly elevated. Bone marrow examination showed gross erythroid hyperplasia with 30% ringed sideroblasts. Of note, she had presented 9 yr earlier with exacerbation of her chronic pulmonary obstructive airways disease, but no hematological abnormalities were found at that time (Table II). A peripheral blood film from the patient's daughter was normal. No other family members were available for study.

She was started on 600 mg/d oral pyridoxine and 5 mg/d folic acid. After 6 mo of treatment her hemoglobin was normal (Table II). The

Table II. Hematologic Values for Patient 2

Time of Evaluation	Hb	MCV	WBC
	g/dl	*fl*	*× 10⁹/liter*
9 yr before presentation	15.3	85	7.2
Presentation	4.3	72	8.9
After 6 mo treatment*	12.6	87	6.6
Treatment stopped			
After 12 wk	10.2		
After 15 wk	9.3		11.7
Treatment restarted*			
After 1 wk	11.2		7.0
After 2 wk	11.0		6.0
After 6 wk	13.0		5.7

* Pyridoxine, 600 mg/d.

ringed sideroblasts had disappeared from her bone marrow, her red cell indices normalized, and detailed examination of a peripheral blood film revealed no residual hypochromia or microcytosis. Cessation of treatment resulted in a relapse of the anemia after 3 mo with a definite microcytic, hypochromic population of red cells visible in peripheral films and ringed sideroblasts in the bone marrow. Both were reversed with 600 mg/d of pyridoxine. Once her hemoglobin had stabilized again the daily dose of pyridoxine was gradually reduced to 50 mg/d, which maintains normal hematologic values.

Bone marrow aminolevulinate synthase (ALAS) activity. ALAS activity was assayed in whole bone marrow from Patient 2 (taken at the time of relapse after pyridoxine withdrawal) by quantitation of [^{14}C]aminolevulinic acid using HPLC (26). The incubation medium contained 40 mM Tris-HCl, pH 7.4, 50 mM KH_2PO_4, 2 mM [2,3-^{14}C]succinic acid (sp act 3.0 Ci/mol) (Amersham International, Little Chalfont, UK), 50 mM glycine, 250 mM sucrose, and, unless otherwise indicated, 0.4 mM pyridoxal 5′-phosphate (PLP), (Sigma Chemical Co., St. Louis, MO). Aminolevulinic acid use was inhibited by 2 mM EDTA and 0.1 mM succinylacetone (4,6-dioxoheptanoic acid) (Sigma). Succinyl CoA generation was provided by 0.5 U of succinate thiokinase (Sigma), 5 mM GTP (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), 1.35 mM CoA (Sigma), and 2 mM $MgCl_2$. The reaction mixture (0.5 ml) was incubated for 1 h at 37°C in a shaking waterbath. 1 U of bone marrow ALAS activity is that amount of enzyme required to synthesize 1 pmol of aminolevulinic acid in 1 h/10^6 erythroblasts.

Enzyme stability analysis was carried out using lysed bone marrow samples from the patient and from six normal controls. The samples were preincubated at 37°C for 30 min in the assay reaction mixture minus the succinyl CoA generating system, with 0 or 5 mM PLP. After the preincubation period, the assay was initiated by the addition of the succinyl CoA-generating system, [2,3-^{14}C]succinic acid, and sufficient PLP to give a final concentration of at least 0.4 mM.

PCR, sequence, and restriction analysis. Genomic DNA was extracted by standard techniques (27) from lymphocytes of both patients and from fibroblasts of Patient 2 obtained with informed consent in accord with institutional guidelines. All exons, including the intron/exon boundaries, 1 kb of promoter region, and 350 nucleotides (nt) 3′ of the *ALAS2* gene from Patient 1 were PCR-amplified, subcloned, and sequenced as previously described (3). *ALAS2* exon 7 was amplified using oligonucleotides; sense, 5′GCCGCCGAATTCTTTGCCAGGTCAAACC3′, and antisense, 5′GCCGCCGAATTCGACCAACACTAGTAAACAT3′, with an annealing temperature of 60°C. Exon 5 from Patient 2 was amplified, subcloned, and sequenced as previously described (8). The remaining exons from the *ALAS2* gene of Patient 2 were sequenced directly from PCR products using solid-phase direct sequencing as described (28). The cDNA nucleotide numbering was modified from our original sequence (29) to account for an additional 67 nucleotides in exons 1 and 2 identified in the genomic sequence (Bishop, unpublished observations) and not present in the reported cDNA clone.

Confirmation of the K299Q mutation in Patient 1 and screening of other family members was accomplished by BsrI (New England Biolabs Inc., Beverly, MA) restriction of amplified exon 7. In Patient 2, mismatch PCR for confirmation of the A172T mutation was performed with oligonucleotides; sense, 5′AGACTAGCCAGGGAGAGACT3′, and antisense, 5′GAAATGTTGGGCAAAGGGATATGCATCGG3′, with an annealing temperature of 60°C. The underlined nucleotide differed from the normal sequence (5′ . . . TCAG 3′), such that an AvaII restriction site was introduced only into the product amplified from a template containing the G566A mutation. BsrI-restricted exon 7 and AvaII-restricted exon 5 PCR products were resolved by electrophoresis in 2% agarose gels.

Expression of recombinant normal and mutant ALAS2. Recombinant normal and mutant ALAS2 were generated using the pMAL fusion protein expression system (New England Biolabs Inc.), resulting in the maltose-binding protein (MBP) attached to the NH_2 terminus of ALAS2 with a Factor Xa–cleavable linkage. The A172T and K299Q mutations were introduced into the pMALc2-AE2 expression clone (8), by site-directed mutagenesis using the unique site-elimination method (30). The resulting clones, pMALc2-AE10 (K299Q) and pMALc2-AE13 (A172T), were sequence confirmed for the entire coding region.

The expression constructs were transfected into *Escherichia coli* BL21, pLysS (Novagen Inc., Madison, WI) and overnight cultures grown in Luria broth (GIBCO BRL, Gaithersburg, MD) medium with 50 μg/ml ampicillin (Sigma) and 34 μg/ml chloramphenicol (Sigma). The next day, 200-ml cultures in Luria broth/ampicillin medium were initiated with a 1:40 dilution of the overnight cultures and grown to 0.6–0.8 A_{600} U. Induction with 0.4 mM isopropyl β-D-thiogalactopyranoside was carried out in Luria broth/ampicillin media for 4 h at 37°C. Cells were pelleted at 4,000 *g* for 15 min. The pellet was resuspended in 5 ml of column buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, 5 mM DTT, 0.2 mM PMSF, and 10 μM PLP), and frozen at −70°C. RNase A (Sigma), DNase I (Sigma), and $MgCl_2$ were added to the thawed lysates to final concentrations of 200 μg/ml, 20 μg/ml, and 1 mM, respectively. The lysates were incubated for 20 min at room temperature and then centrifuged at 4°C for 30 min at 10,000 *g*. The supernatants were retained and filtered through a 0.45-μm filter (Gelman Sciences, Inc., Ann Arbor, MI) to yield the crude *E. coli* extract. This was then diluted with 3 vol of column buffer and applied to an amylose resin affinity column (New England Biolabs). MBP-ALAS2 fusion proteins were eluted from the column with column buffer containing 10 mM maltose, and stored at −20°C in 50 mM Hepes, pH 7.5, 50% glycerol, 1 mM EDTA, 5 mM DTT, 0.2 mM PMSF, 10 μM PLP. Crude extract and affinity-purified normal and mutant ALAS2 fusion proteins were analyzed by SDS-PAGE in 10% gels with a 3% stacking gel according to the method of Laemmli and Favre (31).

Effect of PLP on recombinant normal and mutant ALAS2 activity. The incubation conditions of the ALAS2 assay were as previously described (32) with detection by Ehrlich's reagent after reaction with ethyl acetoacetate (33). 1 U of recombinant ALAS2 activity is that amount of enzyme required to catalyze the production of 1 nmol of δ-aminolevulinate per h under the conditions of the assay. Protein concentration was determined by a modification of the fluorescamine method (34).

Normal and mutant recombinant MBP-ALAS2 fusion proteins were assayed in 0 or 0.4 mM PLP for 15 min. The activities of the recombinant MBP-ALAS2 fusion proteins were also assessed by preincubating in assay medium minus succinate, plus 0 or 0.2 mM PLP for 30 min before assay. The assay (15 min) was initiated by addition of 1 mM succinate and the PLP concentration was adjusted to 0.4 mM.

Recombinant MBP-ALAS2 fusion proteins eluted from the affinity resin were assessed for protease contamination using a casein-resorufin (Boehringer Mannheim Biochemicals, Indianapolis, IN) assay as described (35), with modifications as detailed in the manufacturer's instructions. Samples were incubated for 24 h, followed by spectrophotometric assay at A_{574}.

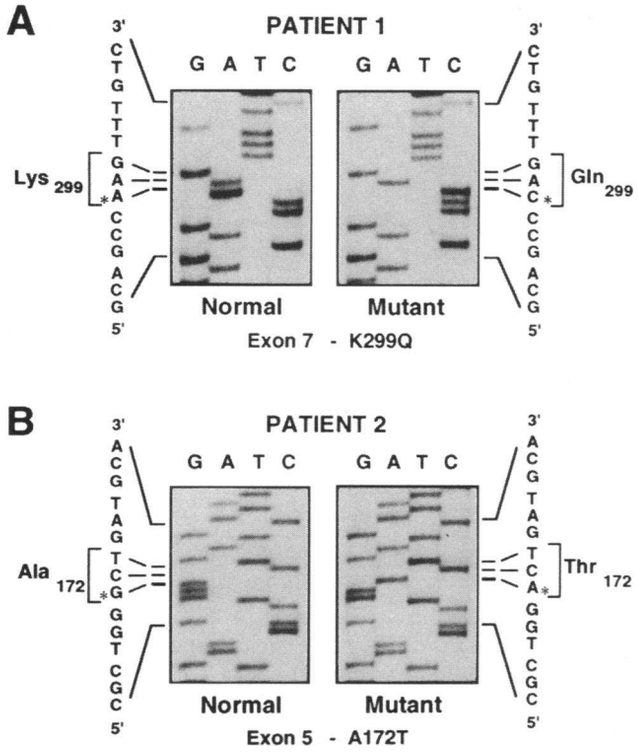

Figure 1. Sequence analysis of the *ALAS2* gene. (*A*) Exon 7 from Patient 1, demonstrating the A to C transversion at nt 947, predicting the substitution of glutamine for lysine at residue 299 (K299Q). (*B*) Exon 5 from Patient 2, demonstrating the G to A transition at nt 566 predicting the substitution of threonine for alanine at residue 172, (A172T).

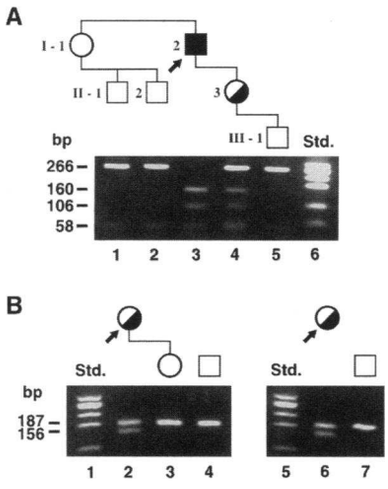

Figure 2. (*A*) BsrI restriction of exon 7 PCR products from Patient 1 and other family members. The proband is hemizygous for the K299Q mutation (lane *3*), his daughter is a heterozygote (lane *4*), whereas the other family members tested were normal (lanes *1, 2,* and *5*). (*B*) Mismatch PCR and AvaII digestion of exon 5 from Patient 2 (lane *2*), her daughter (lane *3*), and a normal control (lane *4*). PCR-amplified DNA from fibroblasts of Patient 2, demonstrating the presence of the A172T mutation (lane *6*) and indicating that the mutation was inherited and not a somatic change in the hematopoietic tissue. Lane *7* contains PCR-amplified DNA from fibroblasts of a normal control. In all gels the *Std.* lane was the size standard (HaeIII digestion of ϕX 174).

Results

Patient 1. Sequence analysis of all 11 exons, including the intron/exon boundaries, 1 kb upstream of exon 1 and 350 bp downstream from exon 11, identified only a single nucleotide difference from the normal *ALAS2* cDNA sequence (8) of Patient 1. This A to C transversion in exon 7, corresponding to nt 947 in the cDNA, predicted a substitution of glutamine for lysine at residue 299 (K299Q), (Fig. 1 *A*). The mutation introduced a BsrI restriction site and digestion of the 324-bp PCR product from Patient 1 resulted in fragments of 160, 106, and 58 bp, compared to fragments of 266 and 58 bp in digested PCR products from normal individuals, confirming the mutation in Patient 1. Restriction with BsrI identified the same mutation in his daughter but not in his grandson or two nephews (Fig. 2 *A*). The K299Q mutation was not found in 100 alleles from unrelated Caucasian females by BsrI restriction of amplified *ALAS2* exon 7 from genomic DNA (data not shown), indicating the mutation was not a common polymorphism.

Patient 2. Single strand conformation polymorphism analysis (36) of amplified exon 5 from Patient 2 identified a mobility shift relative to that of the normal sequence while single strand conformation polymorphism of the other 10 exons and the 5′ and 3′ regions of the *ALAS2* gene did not identify any other mobility shifts (data not shown). Exon 5 from Patient 2 was amplified and subcloned into EcoRI-restricted pGEM4Z and multiple clones were sequenced. A single point mutation, a G to A transition at nt 566 (Fig. 1 *B*) was identified in 5 of 20 clones sequenced from this heterozygote. The G to A base change predicted the substitution of threonine for alanine at residue 172 (A172T). All other exons were further analyzed by direct sequencing of PCR products using solid-phase sequencing, and the base change in exon 5 was the only difference from the normal sequence. Mismatch PCR was used to introduce an AvaII site into PCR products from Patient 2. Restriction with AvaII resulted in fragments of 156 and 31 bp from the mutant allele, whereas the PCR product derived from the normal allele of Patient 2 (187 bp) remained uncut (Fig. 2 *B*). Mismatch PCR and restriction of DNA from fibroblasts of Patient 2 also demonstrated the presence of the base change (Fig. 2 *B*), indicating that this was a constitutive change rather than a somatic mutation. Analysis of genomic DNA from the patient's daughter revealed that she was not a carrier of the A172T mutation (Fig. 2 *B*). The A172T mutation was not a common polymorphism as indicated by its absence in 100 normal alleles by mismatch PCR and restriction analysis (data not shown).

ALAS activity in bone marrow from Patient 2 was in the normal range and was inactivated (43%), as was the normal enzyme (40%), when assayed in the absence of PLP (Table III). Similarly, when the bone marrow erythroblast lysates were preincubated without PLP for 30 min at 37°C before assaying, the normal enzyme lost 55% of its activity relative to preincubation in the presence of PLP. In contrast, even in the presence of 5 mM PLP, the preincubated mutant enzyme from Patient 2 lysate lost 68% of its activity (Table III).

Expression and characterization of recombinant normal and mutant ALAS2. Recombinant normal, K299Q, and A172T ALAS2 were expressed in *E. coli* as fusion proteins with MBP using the pMAL expression system, as described in the Methods. Expression resulted in > 10% of the ALAS2 fusion protein remaining soluble in the supernatant by SDS-PAGE gel electrophoresis (data not shown). Affinity purification of soluble MBP-ALAS2 fusion proteins resulted in > 90% purity (Fig. 3).

Recombinant normal and mutant MBP-ALAS2 fusion proteins, purified in the presence of 10 μM PLP, were assayed in 0 and 0.4 mM PLP (Table IV). In contrast to the bone marrow

Table III. Effect of PLP on Bone Marrow ALAS Activities from Normal Individuals and from Patient 2

Source	ALAS activity*						
	No preincubation			30-min preincubation			
	0.4 mM PLP	*0 mM PLP*	*Decrease‡*	*5 mM PLP*	*Decrease‡*	*0 mM PLP§*	*Decrease‡*
Normal	900±140	540±85	40%	900±140	0%	405±57	55%
Patient 2	800	432	43%	256	68%	ND‖	ND

* Assayed as described in Methods. Units are pmol per 10^6 erythroblasts per h, mean±SD, $n = 6$; ‡ relative to 0.4 mM PLP, no preincubation; § adjusted to 0.4 mM PLP in the assay; ‖ ND, not determined.

assay, the recombinant normal and mutant enzymes were both stable without added PLP in the assay. Interestingly, the specific activities of the affinity-purified mutant enzymes were higher than the normal enzyme, consistent with the bone marrow data from Patient 2 that showed normal ALAS activity.

As was the case in the bone marrow assay, preincubation of the normal recombinant enzyme for 30 min at 37°C in the absence of added PLP resulted in a 56% loss of activity, with 0.2 mM PLP preventing inactivation (Table IV). In contrast to the bone marrow result, addition of only 0.2 mM PLP substantially protected the mutant recombinant enzymes from inactivation during preincubation. In vitro, the Patient 2 mutant recombinant enzyme was significantly more unstable than the normal enzyme to preincubation minus PLP, losing 94% of its activity. Patient 1 recombinant enzyme was also less stable than the normal enzyme under these conditions. To evaluate whether the observed loss of activity during preincubation in the absence of PLP was due to contaminating protease activity, each of the enzyme solutions eluted from the affinity column was incubated for 24 h with a casein-resorufin proteolytically chromogenic substrate as described in the Methods. No protease activity was detected in any sample. The assay was sensitive enough to detect 1 pg/ml trypsin (Sigma).

Discussion

The two patients reported here initially were diagnosed as having the acquired disorder RARS. However, these patients were unusual in that they had hypochromic, microcytic erythrocytes and their anemia was corrected by pyridoxine therapy. Neither patient had any family history of anemia nor had either previously suffered from anemia or any other hematologic problem. The slightly abnormal red cells of the daughter of Patient 1 were insufficient to classify her as a carrier of XLSA. Therefore, even though the blood picture was unusual, RARS was considered the most probable diagnosis in these elderly patients. Treatment of Patient 1 with 200 mg/d, followed by 100 mg/d of pyridoxine resulted in normalization of his erythrocyte MCV (Table I), but a few hypochromic, microcytic erythrocytes remained. In Patient 2, treatment with 600 mg/d of pyridoxine and subsequent maintenance on 50 mg/d completely normalized her erythrocyte values (Table II). Notably, the bone marrow became free of ringed sideroblasts and detailed examination of peripheral blood films failed to find any evidence of hypochromic, microcytic erythrocytes. Complete remission of anemia in pyridoxine-responsive sideroblastic anemia patients is most unusual.

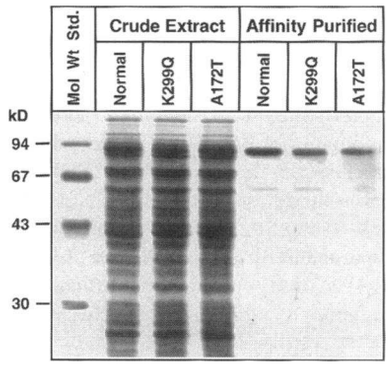

Figure 3. SDS-PAGE gel of recombinant normal, K299Q, and A172T MBP-ALAS2 fusion proteins. (Lane *1*) 14–94 kD-protein molecular weight standards (Pharmacia LKB Biotechnology, Inc.). (Lanes *2*, *3*, and *4*) total crude lysate supernatants (~ 75 μg) after induction of *E. coli* transformed with the normal, K299Q, and A172T expression constructs, respectively. (Lanes *5*, *6*, and *7*) the purified normal, K299Q, and A172T fusion proteins (~ 3 μg) after amylose affinity chromatography.

Pyridoxine-responsiveness and erythrocyte microcytosis suggested that the underlying defect might be XLSA. Thus, genomic DNA from each patient was isolated and the *ALAS2* coding region and adjacent intron/exon boundary sequences were analyzed. Patient 1 had a single nucleotide change in exon 7, an A to C transversion that predicted a lysine to glutamine substitution at codon 299 (K299Q). This is the first instance of an exon 7 mutation in XLSA. That the K299Q mutation also was found in his daughter indicated the inherited rather than acquired nature of this lesion. In Patient 2, a single point mutation was identified in exon 5; a G to A transition that predicted the substitution of threonine for alanine at codon 172 (A172T). The A172T mutation was not a somatic mutation of the bone marrow as it was present in DNA extracted from both the patient's lymphocytes and skin fibroblasts. Thus, the remarkable presentation of XLSA in the eighth and ninth decades of life expands the clinical phenotype and defines a variant late-onset form of XLSA due to mutations of the *ALAS2* gene, a diagnosis which should be considered in the evaluation of patients with microcytic erythrocytes thought to have RARS.

The dramatic normalization of hemoglobin levels with pyridoxine therapy in these patients suggested that both the K299Q and A172T mutations resulted in particularly B_6-responsive forms of ALAS2 deficiency. Evidence in support of this hypothesis was obtained from stability studies using highly purified recombinant normal and mutant ALAS2 (Table IV). Both recombinant mutant enzymes preincubated for 30 min at 37°C in the absence of PLP were much less stable than normal enzyme preincubated without PLP. Preincubation with 0.2 mM PLP substantially protected these enzymes from inactivation suggesting a mechanism for the observed dramatic pyridoxine re-

Table IV. Effect of PLP on ALAS2 Activities of Normal and Mutant Recombinant Enzyme

Enzyme source	ALAS2 activity (U/mg)*						
	No preincubation			30-min preincubation			
	0.4 mM PLP	*0 mM PLP*	*Percent decrease‡*	*0.2 mM PLP§*	*Percent decrease‡*	*0 mM PLP§*	*Percent decrease‡*
Normal	36,200±1,700	38,500±2,300	0	34,600±1,100	4	15,800±2,700	56
K299Q	54,700±480‖	52,400±2,200	4	50,600±2,000	7	13,800±2,300	75
A172T	50,000±1,300	48,300±3,400	3	44,700±1,100	11	3,200±690	94

* Assayed as described in Methods. Units are nanomoles δ-aminolevulinate per hour, mean±SD, $n = 4$; ‡ relative to 0.4 mM PLP, no preincubation; § adjusted to 0.4 mM PLP in the assay; ‖ $n = 3$.

sponsiveness of the patients in vivo. Similarly, PLP protected the housekeeping ALAS1 isozyme from thermal inactivation (37).

The bone marrow studies also showed a decreased stability (relative to normal) of Patient 2 ALAS2 after preincubation even in the presence of 5 mM PLP. Unfortunately, there was insufficient mutant enzyme sample for analysis of the effect of PLP on preincubation activity. The instability of the preincubated bone marrow mutant enzyme as compared to the PLP-stabilized preincubated recombinant mutant enzyme could be due to other factors in crude marrow extracts affecting availability of PLP. In addition, the mutant enzyme may have been more susceptible to proteolysis in these extracts. Aoki et al. (38) described the increased susceptibility to a specific mitochondrial protease of apo-ALAS from bone marrow of patients with pyridoxine-responsive XLSA. On the other hand, the maltose-binding protein moiety could be a stabilizing influence on the recombinant enzymes. Nonetheless, the presence of the maltose-binding protein moiety of the fusion protein did not exert a major effect on the ALAS2 enzymatic activity as the normal ALAS2 fusion protein had unchanged activity after cleavage with Factor Xa (data not shown). No detectable proteolytic activity was found in the affinity-purified enzyme samples, suggesting that the loss of activity during preincubation was due to inherent thermolability and not due to protease activity.

Interestingly, without preincubation, the mutant enzyme activity was normal in the bone marrow assay, even though the patient presented with a Hb of 4.2 g/dl. Bottomley et al. (39) reported a higher than normal level of ALAS activity in bone marrow from some sideroblastic anemia patients. Since the bone marrow assay measures both cytoplasmic and mitochondrial ALAS2 activity, in Patient 2 the cytoplasmic activity could be high relative to normal individuals but still result in normal total activity if the mitochondrial activity was low. Thus it is possible that Patient 2 (A172T) was producing increased amounts of enzyme, as might be expected by release of translation inhibition with high iron levels (40), and/or mutant enzyme with higher specific activity (Table IV) while the mutant enzyme was markedly unstable and subject to rapid inactivation before or in the course of mitochondrial import. Since only the mitochondrial enzyme has access to substrates in vivo, this would result in reduced heme biosynthesis and anemia.

A decline in PLP availability or metabolism may have precipitated the late-onset of XLSA in these patients. An age-related decline in pyridoxine metabolism in combination with a reduced vitamin intake has been described in elderly populations (41, 42). Thus, it is possible that reduced PLP metabolism in addition to an already unstable ALAS2 enzyme due to the inherited mutation resulted in a decrease in functional mitochondrial ALAS2 activity and precipitated the anemia in the male patient. Subsequent pyridoxine therapy rapidly and dramatically reversed the anemia. Similarly, an age-related decline in PLP metabolism may have contributed to the onset of disease in Patient 2. Since she is a heterozygote, skewed Lyonization or an age-related selection of hemopoietic clones expressing the mutant allele, or loss of clones expressing the normal allele, could also have contributed to the onset of XLSA. An increase in skewed Lyonization has recently been reported in aging females (43, 44).

While RARS patients are generally unresponsive to pyridoxine, some show a partial response and, rarely, a more complete response. These reports may in some cases reflect a missed diagnosis of late-onset XLSA. MacGibbon and Mollin (23) reported a 52-yr-old male with severe, hypochromic anemia, (serum iron 196 μg/dl, total iron-binding capacity 210 μg/dl, Hb 6 g/dl, and normal serum B_{12} and folate levels) who demonstrated an increase of hemoglobin to 12 g/dl over 60 d with pyridoxine treatment of 1 mg/d. Despite this improvement, the hypochromic erythrocytes and ringed sideroblasts persisted (23). Meier et al. (24) reported a 70-yr-old patient who was also quite responsive to pyridoxine therapy (200 mg/d) resulting in the complete remission of all hematologic abnormalities (no values given) within 4 wk. Subsequent withdrawal of pyridoxine resulted in the reappearance of the sideroblastic anemia, which was again reversed with pyridoxine therapy (24). In addition, several other cases of middle-aged/elderly individuals with pyridoxine responsive RARS have been reported (10, 39). These cases had a clinical course suggestive of late-onset XLSA due to mutations of *ALAS2*. The identification of inherited *ALAS2* mutations in the two late-onset patients reported here resulted in a clear rationale for pyridoxine therapy and a reduction of their anxiety since XLSA does not progress to leukemia.

These cases emphasize the importance of evaluating all acquired sideroblastic anemia patients for pyridoxine responsiveness, irrespective of sex or age, particularly if they have microcytic red cells. Careful studies of erythrocyte size distribution in family members may be helpful. A diagnosis of sideroblastic anemia with microcytic, hypochromic erythrocytes and especially a positive response to pyridoxine warrants further investigation for *ALAS2* mutations. Once a specific lesion is identified, genetic studies are indicated for family members with no obvious hematologic abnormality but who nevertheless may be latent carriers of XLSA.

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