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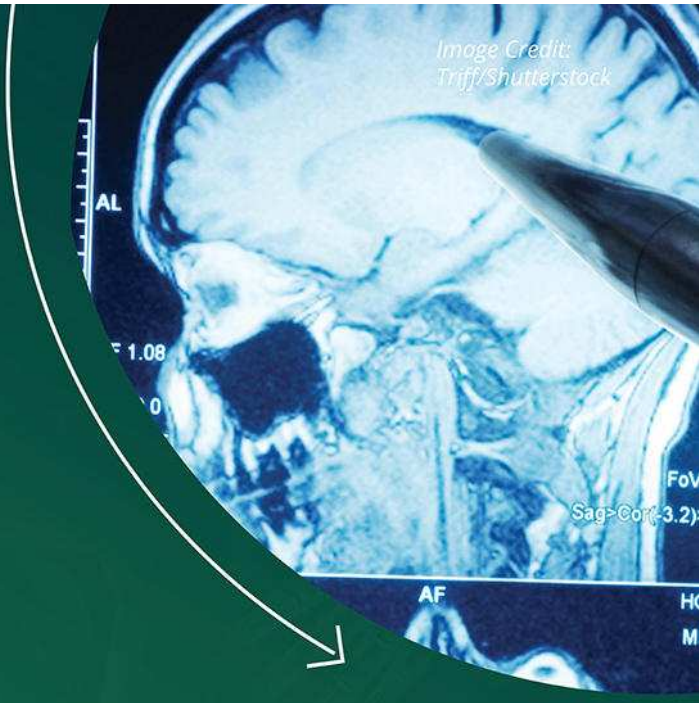

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Sideroblastic Anemia: Molecular Analysis of the *ALAS2* Gene in a Series of 29 Probands and Functional Studies of 10 Missense Mutations

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ABSTRACT: X-linked Sideroblastic Anemia (XLSA) is the most common genetic form of sideroblastic anemia, a heterogeneous group of disorders characterized by iron deposits in the mitochondria of erythroid precursors. XLSA is due to mutations in the erythroid-specific 5-aminolevulinate synthase (*ALAS2*) gene. Thirteen different *ALAS2* mutations were identified in 16 out of 29 probands with sideroblastic anemia. One third of the patients were females with a highly skewed X-chromosome inactivation. The identification of seven novel mutations in the *ALAS2* gene, six missense mutations, and one deletion in the proximal promoter extends the allelic heterogeneity of XSLA. Most of the missense mutations were predicted to be deleterious, and 10 of them, without any published functional characterization, were expressed in *Escherichia coli*. ALAS2 activities were assayed in vitro. Five missense mutations resulted in decreased enzymatic activity under standard conditions, and two other mutated proteins had decreased activity when assayed in the absence of exogenous pyridoxal phosphate and increased thermosensitivity. Although most amino acid substitutions result in a clearly decreased enzymatic activity in vitro, a few mutations have a more subtle effect on the protein that is only revealed by in vitro tests under specific conditions.
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KEY WORDS: X-linked sideroblastic anemia; ALAS2; heme synthesis; protoporphyrin

Introduction

Congenital sideroblastic anemia (CSA) comprises a group of heterogeneous disorders characterized by decreased heme synthesis and mitochondrial iron overload with ringed sideroblasts in the bone marrow (for a review, see [Camaschella, 2009]). The most common genetic form of CSA, X-linked sideroblastic anemia (XLSA; MIM♯ 300751), results from mutations in the specific erythroid gene encoding 5-aminolevulinate synthase (*ALAS2*, also known as *ALASE*; MIM♯ 301300; EC 2.3.1.37) [Fleming, 2002] localized on chromosome Xp11.21. ALAS is the first enzyme in the heme biosynthesis pathway and catalyzes the condensation of glycine and succinyl-coenzyme A into 5-aminolevulinic acid (ALA), the precursor of the tetrapyrroles [Gibson et al., 1958; Shemin and Kikuchi, 1958]. Pyridoxal 5′-phosphate (PLP) is the cofactor of the enzyme. Most of the 48 reported *ALAS2* mutations responsible for XLSA are missense mutations localized in exons 4 to 11 [Bergmann et al., 2010]. A variant in the proximal promoter was first reported as a mutation in an XLSA patient [Bekri et al., 2003], but this variant was subsequently found in unaffected individuals, leading to the conclusion that it is a low frequency polymorphism and not a causal mutation [May et al., 2005].

Most of these mutations were described at the genomic level without further characterization of the mutated protein either in vitro or in vivo. In some patients, reduced ALAS enzymatic

Additional Supporting Information may be found in the online version of this article.

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activity has been reported in the bone marrow [Bottomley et al., 1992; Cotter et al., 1995; Cox et al., 1994; Harigae et al., 1999a], and in others the mutated cDNA has been expressed in *Escherichia coli* in order to study the activity of mutated protein [Cotter et al., 1992, 1994, 1995; Cox et al., 1994; Furuyama et al., 1997, 1998, 2006; Harigae et al., 1999a,b; Prades et al., 1995]. In addition, in 2005, the crystallographic structure of ALAS2 from *Rhodobacter capsulatus* was published, making it possible to map the XLSA causing mutations [Astner et al., 2005].

Here we report a series of 29 probands with SA. Thirteen different *ALAS2* mutations were found in 16 probands. Seven of the 13 mutations had never previously been described, including a 48-bp deletion in the proximal promoter region. The functional impact of the 12 remaining missense mutations was assessed in silico using bioinformatic tools. Moreover, cDNAs were expressed in *E. coli* in order to assess the functional consequences of 10 amino acid substitutions for six novel mutations and four previously reported but so far uncharacterized mutations. ALAS2 activity, enzyme thermosensitivity and pyridoxine responsiveness were studied.

Materials and Methods

Patients

We performed genetic analyses in 29 probands (17 males, 12 females) referred to our laboratory with a diagnosis of CSA based on the presence of sideroblasts in the bone marrow. Patients with syndromic or acquired forms of SA were excluded from this study. These patients originated from France, other Western European countries (Germany, Switzerland, Belgium), and Tunisia. Blood samples for genetic analysis were obtained from the patients or their parents after they had given signed informed consent in accordance with the requirements of the French Bioethics Committee "Agence of Biomedecine." Hematological parameters had been evaluated by standard methods in the respective referring hospitals, and erythrocyte protoporphyrin was measured as previously described [Deacon and Elder, 2001].

X-Inactivation Study

Analysis of X chromosome inactivation was performed as previously described [Chollet-Martin et al., 2007] using the androgen receptor polymorphism as a marker.

Molecular Analysis of the ALAS2 Gene

Genomic DNA was extracted from peripheral blood using the QIAamp DNA blood Mini Kit (Qiagen, Chatsworth, CA). Analysis of the *ALAS2* gene (GenBank genomic: NG_008983.1, GenBank mRNA: NM_000032.4, GenBank protein: NP_000023.2) was performed by bidirectional direct sequencing. The 11 exons of *ALAS2*, the proximal promoter (250 bp) and the exon–intron junctions were amplified by PCR (Supp. Table S1). After purification of PCR products (PCR purification kit, Qiagen), both strands were sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Sequencing products were purified (Sephadex G50, GE Healthcare, Piscataway, NJ), and analyzed using a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies) and the Seqscape analysis software (v2.6.0) (Applied Biosystems, Life Technologies). Identified mutations were confirmed on a second sample, when available, or by family study. Numbering of *ALAS2* mutations followed the international guidelines (www.hgvs.org/mutnomen). For the nucleotide numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence of the cDNA; for the amino acid numbering position 1 corresponds to the initiating methionine.

In Silico Prediction of the Functional Impact of ALAS2 Mutations

The Grantham score [Grantham, 1974] and six bioinformatic tools were used in silico to predict the impact of *ALAS2* mutations on protein structure or function, as previously described [Kannengiesser et al., 2009]. The bioinformatic tools consisted of polyphen [Sunyaev et al., 2001], SIFT [Ng and Henikoff, 2003], SNP3D [Yue et al., 2006], PANTHER [Brunham et al., 2005], UMD Predictor [Frederic et al., 2009], and GVGD [Tavtigian et al., 2006].

To get an overall estimate of the impact of each mutation, we calculated a "prediction score" from the number of programs that predicted that the alteration would be deleterious (Grantham score >100; GVGD: C25–C65; PANTHER: highly/probably deleterious; SIFT: affected; polyphen: damaging; UMD predictor: pathogenic; SNP3D: deleterious).

Localization of Amino Acids in *R. capsulatus*

The localization of the mutated amino acids in the 3D structure of ALAS2 was predicted by sequence homology using the crystal structure of ALAS from *R. capsulatus* (PDB 2BWN, 2BWO, and 2BWP) and Rasmol software [Sayle and Milner-White, 1995].

Expression of Normal and Mutant ALAS2 Enzymes in *E. coli*

To investigate the effect of the mutations on ALAS2 activity, mutant enzymes were expressed in *E. coli* starting from a construct with the normal cDNA (pMALc2-AE2) kindly provided by Dr. David Bishop [Cotter et al., 1994]. pMALc2-AE2 encodes a recombinant fusion protein consisting of a maltose binding protein (MBP, 387 AA) linked to the N-terminus of the mature human ALAS2 (509AA). We introduced the different mutations by site-directed mutagenesis using a Quick change kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The sequences of the mutagenesis primers are available upon request. The coding region of the resulting cDNAs was verified by sequencing.

Escherichia coli BL21 DE3 competent cells (Invitrogen, Life Technologies) were transformed with expression plasmids, and overnight cultures were grown in LB (Lennox L Broth Base, Invitrogen, Life Technologies) medium with 100 mg/ml ampicillin (PANPHARMA, Fougères, France). The next day, 20 ml of cultures in LB/ampicillin medium were initiated with the overnight cultures and grown to 1.2 A_{600} units. ALAS2 synthesis was induced by adding 0.1 mM isopropyl b-D-thiogalactopyranoside (IPTG) in the presence of 0.5 mM 4,6-dioxoheptanoic acid (Sigma-Aldrich, St. Louis, MO), also known as succinyl acetone, a strong inhibitor of the ALA dehydratase enzyme to prevent the transformation of ALA into porphyrins. Incubation was carried out in LB/ampicillin medium for 4 hr at 22°C. Cells were pelleted at 2,500 rpm for 10 min, and then frozen at −80°C. The assay of ALAS2 activities of the control and mutant enzymes under standard conditions was performed on crude bacterial lysates as previously described [Lien and Beattie, 1982] with modifications. Pellets were sonicated in 300 µl of HEPES 50 mM

pH 7.5 while maintained in ice. The total protein concentration was measured by means of a protein assay (Bio-Rad Laboratories GmBh, Hercules, CA), and the samples were adjusted to a concentration of 0.2 mg of total protein/ml. Six hundred microliters of samples were preincubated for 5 min at 37°C before the assay, then 100 µl of a mixture containing 1 mM Succinyl-CoA, 10 mM Glycine, 50 mM HEPES, and 0.5 mM PLP (all from Sigma-Aldrich, St. Louis, MO) were added to samples. The reaction was stopped by adding 60 µl of 100% trichloroacetic acid either immediately (T0) or after 20 min (T20) at 37°C. The reaction product 5-aminolevulinate (ALA) was quantified by colorimetry, after a reaction of ALA with acetyl acetone (Fluka Analytical, Sigma-Aldrich) [Mauzerall and Granick, 1956]. The ALA pyrroles were not separated from other pyrroles present in the bacterial lysate before adding Erlich's reagent. Instead, the ALA synthesized during the incubation was calculated from the OD obtained with Erlich's reaction after subtracting a blank corresponding to the OD obtained with the lysate from a bacterial clone expressing the ALAS2 C344X mutant. This mutant encodes a truncated protein shown to be devoid of enzymatic activity by the lack of difference in OD between T0 and T20 (data not shown). We checked that all bacterial lysates corresponding to wild-type and mutant recombinant ALAS2 (including the C344X mutant) yielded identical OD values at T0.

The specific activity (S_A) was expressed in pmol of ALA/mg total protein at 37°C. The residual activity (%) was determined by expressing the specific activity of mutants relative to that of the wild-type ALAS2.

In some cases, specific assay conditions were used consisting of either omitting PLP from the incubation mixture or preincubating the bacterial lysate at 37°C for 30 or 60 min instead of 5 min. The significance of the results was estimated using Student's *t*-test.

Immunoblotting

A total of 2.5 µg of total protein of the supernatant from bacteria lysates were taken up onto 1 × Laëmmli buffer, and heated for 10 min at 100°C. Samples were analyzed by SDS-PAGE using an 8% polyacrylamide gel followed by electrotransfer on a polyvinylidene fluoride (PVDF) membrane. Loading and transfer were confirmed by Ponceau red staining. After preincubation in blocking solution (7% skimmed milk in Tween 20 in Tris-buffered saline, TBST 0.15%) overnight at 4°C, the membrane was incubated with an anti-MBP antiserum (1/20 000, E8030S, New England Biolabs, Ozyme, Ipswich, MA) for 2 hr at room temperature. After washing three times with TBST for 10 min each time, the membrane was incubated with a secondary anti rabbit IgG, linked to Horseradish peroxidase (1/20,000, Amersham GE Healthcare) for 2 hr at room temperature. The MBP fusion proteins were visualized (Immobilon Western, chemiluminescent HRP substrate, Millipore Corporation, Billerica, MA) according to the manufacturer's instructions.

Results

Twenty-nine patients with a diagnosis of CSA were referred to our laboratory for molecular exploration. All of them had ringed sideroblasts in the bone marrow, and most of them had mild to severe anemia at the time of referral. The *ALAS2* gene was sequenced. Thirteen different mutations, including 12 substitutions and 1 deletion, were identified in 16 probands (10 males, 6 females). Table 1 summarizes clinical data for patients carrying an *ALAS2* mutation.

Five of the six affected females had highly skewed X inactivation consistent with a diagnosis of XLSA (data not shown). The remaining affected female (proband 8) was not informative for the androgen receptor polymorphism.

Six of these mutations had already been reported [Bottomley, 2004; Bottomley et al., 1995; Goncalves et al., 2004; May and Bishop, 1998], whereas the other seven had not been described before [Harigae and Furuyama, 2010]. Twelve of these 13 mutations caused amino acid substitutions (Table 1), and the 13th was a 48-bp deletion (c.-91_-44del) in the proximal promoter (Table 1 and Fig. 1). This deletion led to the removal of the TATA-like box localized between −82 and −76 bp upstream of the translational initiating codon [Cox et al., 1991] and of the first nine nucleotides of exon 1. A rare P520L variant, previously reported as a putative modifier of iron overload with an allelic frequency of 0.0013 in Caucasians [Lee et al., 2006], was found in one female proband (number 13, Table 1) in the absence of any other sequence variation.

Erythrocyte protoporphyrin concentration was measured in six patients carrying an *ALAS2* mutation (Table 1). In all XLSA cases, the protoporphyrin concentration was within the normal range of values (less than 1.9 µmol/l of red blood cells).

We used various different in silico software products to predict the functional consequences of the *ALAS2* missense mutations. All the substitutions were predicted to be deleterious, apart from R218H and E242K (Supp. Table S2).

To assess in vitro the functional consequences of ten amino acid substitutions that had not been studied before (10 missense mutations corresponding to 6 novel mutations and 4 previously reported mutations without functional data) we expressed the mutant cDNAs in *E. coli*. Immunoblotting was performed to confirm that the expression level of recombinant normal and mutant proteins were similar (Supp. Fig. S1). When the enzymatic activity was assayed from *E. coli* lysates immediately after cell disruption, five ALAS2 mutants (E242K, D263N, P339L, R375C, and R411H) displayed significantly reduced ALAS2 activity, with residual activity ranging between 14 and 65% of the normal construct (Fig. 2 and Table 2).

Expression of the remaining five mutated cDNAs (R170H, R218H, R452G, P520L, and R572H) resulted in the production of a protein with an enzymatic activity that was no different from that of the normal construct.

For four mutants, we also measured the activity of the protein in the absence of added exogenous PLP (Fig. 3A). R170H and R218H mutants displayed significantly reduced activity in this situation, whereas neither R452H nor R572H did. We also tested the thermal stability of these four mutated enzymes by preincubating them at 37°C for 30 or 60 min prior to the enzymatic assay (Fig. 3B). Once again, the R170H and R218H mutants displayed significantly increased thermosensitivity, while the R452H and R572H mutants did not significantly differ from the wild type. Adding exogenous PLP to the preincubation medium prevented the loss of activity induced by preincubating at 37°C for all the mutants as well as for the normal enzyme (data not shown).

Discussion

The results reported here for a series of 29 probands with CSA highlights a number of interesting points regarding the genetic heterogeneity of the disease and the functional consequences of *ALAS2* mutations. *ALAS2* variants were found in 16 out of the 29 probands, including a P520L variant previously reported as being a rare polymorphism [Lee et al., 2006]. Recently, other gene defects have been identified in autosomal recessive forms of CSA

Table 1. Hematological and Biochemical Parameters of Patients Presenting *ALAS2* Mutations

Patient	Gender	Age at onset (years)	Pyridoxine response	Hb (g/dL)	MCV (fL)	Tf Sat (%)	Ferritin (μg/L)	PP (μmol/L RBC)	ALAS2 Mutation[a]			Genetic report
									Localization	cDNA[b]	Protein	
1	F	43	—	11.2	96	100	371	n.a.	exon 5	c.509G>A	p.Arg170His	[May and Bishop, 1998]
2	F	0.5	—	8	84	100	1,448	n.a.	exon 6	c.653G>A	p.Arg218His	Present study
3	M	0.5	+	1.5	69	n.a.	256	n.a.	exon 6	c.724G>A	p.Glu242Lys	Present study
4	F	20	+	9.2	41[c]	68	899	n.a.	exon 6	c.787G>A	p.Asp263Asn	[Bottomley, 2004]
5	M	31	+	3.5	76	n.a.	1,172	1.40	exon 8	c.1016C>T	p.Pro339Leu	Present study
6	M	47	n.a.	6.3	<70	63	2,537	1.70	exon 8	c.1123C>T	p.Arg375Cys	Present study
7	M	7	+	7.2	60	86	780	n.a.	exon 9	c.1231C>T	p.Arg411Cys	[Bottomley, 2004][e]
8	F[d]	n.a.	n.a.	9.1	41[c]	71	600	1.80	exon 9	c.1232G>A	p.Arg411His	[Goncalves et al., 2004]
9	F	63	+	10.4	73	70	428	1.65	exon 9	c.1232G>A	p.Arg411His	[Goncalves et al., 2004]
10	M	38	+	10.4	70	93	2,284	n.a.	exon 9	c.1354C>G	p.Arg452Gly	Present study
11	M	20	+	11.3	62	100	1,172	n.a.	exon 9	c.1354C>T	p.Arg452Cys	[Bottomley et al., 1995][f]
12	M	n.a.	+	10	<70	n.a.	1,000	0.56	exon 9	c.1354C>T	p.Arg452Cys	[Bottomley et al., 1995][f]
13	F	45	—	8.5	56	n.a.	n.a.	n.a.	exon 9	c.1559C>T	p.Pro520Leu	[Lee et al., 2006]
14	M	46	—	13.2	68	63	1,000	0.64	exon 11	c.1715G>A	p.Arg572His	Present study
15	M	57	+	12.2	73	28	1,000	n.a.	exon 11	c.1715G>A	p.Arg572His	Present study
16	M	0.75	+/−	6.3	53	n.a.	1,200	n.a.	Promoter	c.-91_-44 del	—	Present study

M, male; F, female; Hb, hemoglobin; MCV, mean corpuscular volume; Tf Sat, transferrin saturation; PP, erythrocyte protoporphyrin; RBC, red blood cells; n.a., not available.

[a]The GenBank reference sequence for *ALAS2* gene are: GenBank genomic: NG_008983.1, GenBank mRNA: NM_000032.4 and GenBank protein: NP_000023.2.

[b]Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to HGVS guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

[c]Values of MCV were indicated for the microcytic population of RBCs in female patients in whom two populations of RBCs were detected.

[d]Not informative for X inactivation.

[e]Expression data have been studied for the following mutations: pR411C [Furuyama et al., 1998].

[f]Expression data have been studied for the following mutations: p.R452C [Furuyama et al., 2006].

Normal values: Hb: 12–15.5 g/dL (anemia: females <12 g/dl, males <13 g/dl), MCV: 80–90 fL (microcytosis: <80 fL), Tr Sat: 12–45% (females) 15–50% (males), serum ferritin: females 20–150 μg/L, males 30–300 μg/l. PP <1.9 μmol/l RBC.

(MIM# 205950): a homozygous mutation was found in *GLRX5* in a consanguineous proband [Camaschella et al., 2007], and *SLC25A38*, which encodes a putative glycine transporter, was found to be mutated in the affected members of different families [Guernsey et al., 2009]. Thus, for the 13 probands without any *ALAS2* mutation, and for one patient with the P520L variant, these two genes were explored and mutations were identified in *SLC25A38* for four probands (manuscript submitted). No variant was identified in any of the three genes explored in the other nine patients; the characteristics available for these patients are reported in Supp. Table S3. This proportion is similar to that reported by Bergmann et al. [2010], suggesting that other loci must be involved.

Our data extend the allelic heterogeneity of XSLA, because we identified seven novel mutations including a deletion in the proximal promoter region, in addition to five mutations already known. As expected, XLSA symptomatic heterozygous females displayed highly skewed X-chromosome inactivation [Aivado et al., 2006; Cazzola et al., 2000].

We clearly show that a defect in the first step of the heme biosynthetic pathway prevents the abnormal accumulation of erythrocyte protoporphyrin both in patients with *ALAS2* mutations (Table 1) as well as in one patient with the *SLC25A38* mutation (data not shown). Therefore, this simple measurement may provide a useful screening test before sequencing *ALAS2* and *SLC25A38*. Indeed, elevated erythrocyte protoporphyrin levels have been described in other anemic syndromes, including many types of CSA linked to *ABCB7*, *SLC19A2*, or mitochondrial defects [Camaschella, 2009]. Furthermore, erythrocyte samples were available for three unexplained cases of CSA (without any identified mutations of *ALAS2* or *SLC25A38*) out of the 14 in our series. In contrast to the XLSA patients, they had an elevated level of erythrocyte protoporphyrin (>1.9 µmol/l red blood cells, data not shown).

Finally, we addressed the functional consequences of *ALAS2* mutations in order to evaluate their deleterious impact. The deletion in the proximal promoter of the *ALAS2* gene led to the elimination of the TATA-like motif and the first 9 bp of exon 1. The TATA-like motif has been shown to be functionally important by mutagenesis studies [Surinya et al., 1997]. It is likely that some other sequence upstream of the deletion may act as a weak promoter, because *ALAS2* mRNA was detected at a lower concentration in the bone marrow of the male patient 15 than in unrelated controls (data not shown). The complete loss of ALAS2 expression is probably lethal, as shown by the absence of ALAS2-null embryos following specific disruption of *ALAS2* gene in mice [Nakajima et al., 1999].

The finding of a previously reported variant P520L as the only sequence variation in a proband female with a highly skewed pattern of X-inactivation raises the question of the possible relevance of this mutation. However, expression studies of the P520L cDNA failed to reveal any functional impact.

The functional consequence of the six novel missense mutations was evaluated by studying the enzymatic activity of the recombinant mutant protein expressed in *E. coli*. Three previously reported mutants, for which such data were not available, were also studied (Table 1). A significant decrease in the enzymatic activity measured in vitro was evidenced for five mutants: E242K, D263N, P339L, R375C, and R411H (Fig. 2 and Table 2). The histidine substitution at position 411 (R411H mutation) decreased ALAS2 activity to the same extent as the cysteine substitution previously reported [Furuyama et al., 1998].

For four other mutations (R170H, R218H, R452H, and R572H), in vitro activity was not different from the wild-type control under standard conditions. Two of these mutants (R170H and R218H) showed reduced activity in the absence of exogenous

Figure 1. Hemizygous deletion of 48 base pairs in the proximal promoter of *ALAS2* gene. **A:** PCR amplification of the promoter region from a normal control (lane 1; expected size 627 bp); male proband 15 (lane 2; shorter PCR product of 579 bp) and his mother (lane 3; healthy carrier); M: DNA molecular weight markers (Fermentas, 100 bp ladder). **B:** Sequence of the proximal promoter of the *ALAS2* gene (Adapted from [Cox et al., 1991]) showing the TATA-like box (in bold), the beginning of exon 1 (underlined in black), and the deletion (underlined in gray).

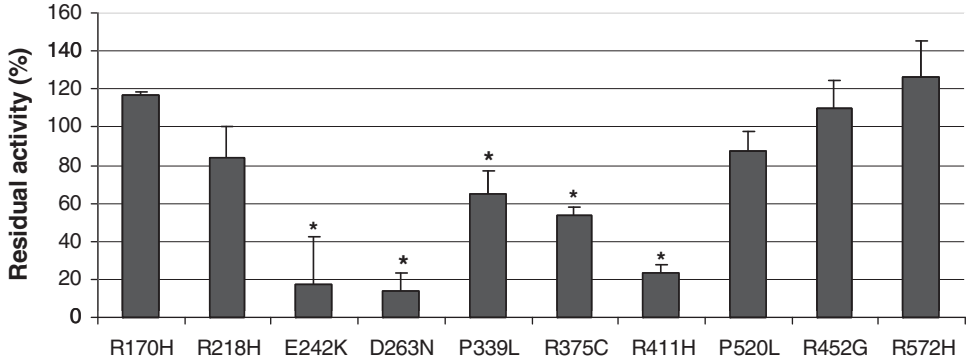

Figure 2. Residual activities of the various different recombinant ALAS2 mutant enzymes assayed under standard conditions. The residual activity of each mutant is expressed as a percentage of the normal enzyme activity as described in Materials and Methods. Enzyme activity was measured on at least three independent bacterial cultures. The statistical significance of comparisons of the specific activities between each mutant and the normal enzyme was established using Student's *t*-test (*P value <0.05). Five mutants (E242K, D263N P339L, R375C, and R411H) display significant loss of function.

Table 2. Summary of Results for Missense Mutations

	Structural impact[a]			ALAS2 activity	
ALAS2 Mutation	AA RC	Localization	In silico protein score	Standard: with added PLP	Specific: without added PLP[b]
p.Arg170His[c]	Arg28	Internal β1-sheet strand 1	Deleterious	117%	Defective
p.Arg218His	Ala75	Internal α3-helix	Neutral	83%	Defective
p.Glu242Lys	Glu99	Surface α4-helix	Neutral	17%[g]	ND
p.Asp263Asn	Asp120	Internal α5-helix	Deleterious	14%[g]	ND
p.Pro339Leu	Pro196	Surface 1aa before α8-helix	Deleterious	65%[g]	ND
p.Arg375Cys	Arg232	Surface α8-helix	Deleterious	53%[g]	ND
p.Arg411His	Arg268	Internal α1-helix	Deleterious	23%[g]	ND
p.Arg411Cys	Arg268	Internal α1-helix	Deleterious	25%[f]	ND
p.Arg452Gly[d]	Met309	Surface α14-helix	Deleterious	109%	Normal
p.Arg452Cys[d]	Met309	Surface α14-helix	Deleterious	100%[f]	ND
p.Pro520Leu	Pro377	Internal	Deleterious	87%	ND
p.Arg572His	ND[e]	ND[e]	Deleterious	126%	Normal

AA RC: amino acid, *Rhodobacter capsulatus*; ND, not determined.
[a]Crystallography of ALAS2 from *R. capsulatus* permitted molecular modeling to predict the localizations of numerous mutations on the protein (Rasmol, PDP 2BWN, 2BWO, and 2BW).
[b]Or preincubated at 37°C.
[c,d]These amino acids are hot spots of mutation with numerous substitutions: R170L/S/C [Edgar et al., 1998; Furuyama and Sassa, 2002; May and Bishop, 1998] and R452H/C/S/G [Bottomley et al., 1995] and this study.
[e]Structural prediction for R572H is not possible because the C-terminal part of human enzyme is longer than enzyme of *R. capsulatus*.
[f]Previously described [Furuyama et al., 2006].
[g]Student's *t*-test, P value < 0.05.

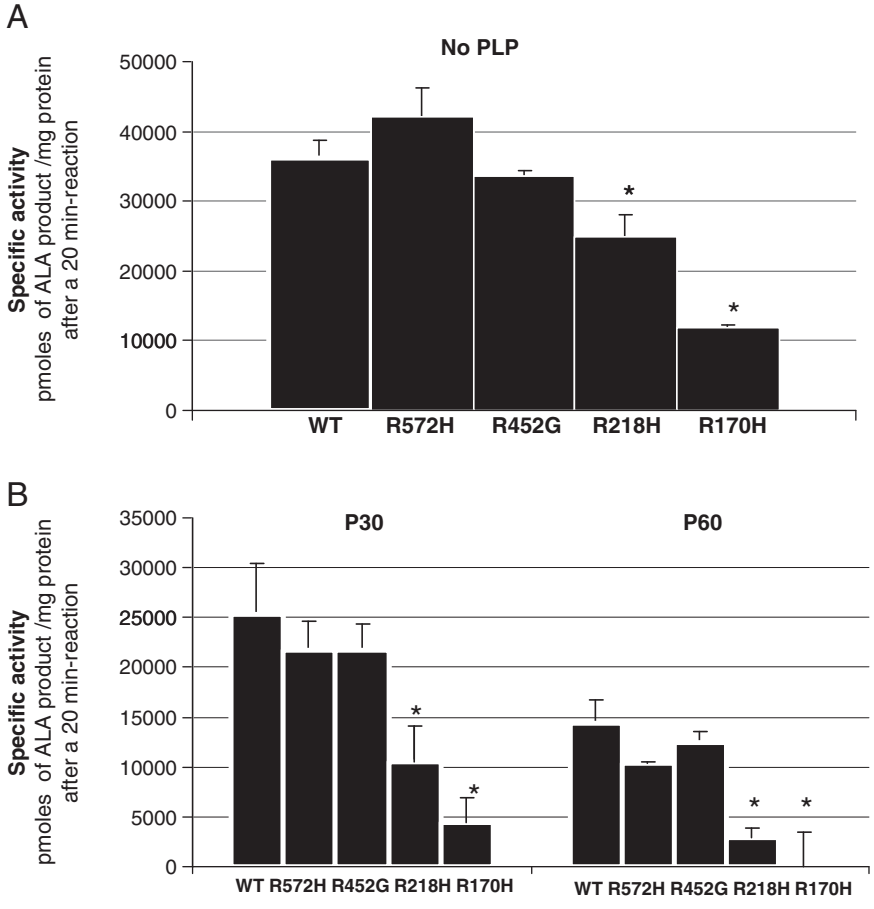

Figure 3. PLP-dependence and thermosensitivity of the recombinant normal and mutant ALAS2. **A:** The specific activity of wild type enzyme and four mutant enzymes (R170H, R218H, R452G, and R572H, showing no loss of function under standard conditions) was measured without adding PLP to the assay, as described in Materials and Methods. Specific activities are expressed in pmol of ALA produced by mg protein after subtracting the specific activity of C344X (negative control). The statistical significance of the comparisons between each mutant and the normal enzyme was established using Student's *t*-test (*P value < 0.05). Two mutants (R170H and R218H) display PLP sensitivity. **B:** The specific activity of wild type enzyme and four mutant enzymes (R170H, R218H, R452G, and R572H) was measured after preincubating for 30 or 60 min before the assay, as described in Materials and Methods. The statistical significance of comparisons between each mutant and the normal enzyme was established using Student's *t*-test (*P value < 0.05). Two mutants (R170H and R218H) are thermosensitive.

PLP and increased thermosensitivity. This may be explained by reduced affinity for PLP and higher sensitivity to thermal denaturing of the apoenzyme compared to the holoenzyme, the proportion of the two forms being modified by the presence of added PLP. These results are consistent with the structural analysis of ALAS from *R. capsulatus* leading to the prediction that the substitution of the R170 would affect the binding of PLP [Astner et al., 2005]. However, the patient with the R170H mutation did not respond to PLP therapy. Because in silico tools did not predict any deleterious impact for R218H, and because both this mutant and the R170H mutant behaved similarly in our functional studies, we localized the corresponding positions on the quaternary structure of *R. capsulatus* (see Supp. Fig. S2). Interestingly, A75 (at a position homologous to that of R218 in humans) in one monomer is quite close to R28 (at homologous position to human R170) on the other monomer, suggesting the possibility that these two amino acids may be important for the dimerization of ALAS2.

Finally, two mutated proteins, R452G and R572H, did not differ from the wild type in the in vitro system despite damaging in silico predictions. These two mutants were as active and as thermostable as the normal enzyme. Similar findings had previously been reported for other mutations, including R452C and R452H [Furuyama et al., 2006]. Although the R452G and R572H mutants did not display loss of function in *E. coli*, several lines of evidence support their implication in XLSA. The CGC codon encoding R452 is a hot spot for mutations. This arginine R452 has been found to be substituted in approximately one-quarter of patients with XLSA [Furuyama et al., 2006]. In our series, R452C occurred in two independent probands. Two independent probands were also carrier of the R572H mutation and one of them, for whom blood sample was available, showed an erythrocyte protoporphyrin concentration at the lower end of the normal range. These observations suggest that R452G and R572H are causal mutations. We can hypothesize the implication of additional factors in the bone marrow or a defective enzyme processing as has been suggested for K299Q and D190V, previously reported to display normal activities [Cotter et al., 1995; Furuyama et al., 1997].

As previously reported, iron overload may be a major complication of XLSA, and the iron depletion by either iron chelators or phlebotomy [Camaschella, 2008] not only effectively prevents the deleterious effects of iron overload, but also improves erythropoiesis.

A correlation between these "milder" defects and the phenotype of the patients remains speculative, given the known intrafamilial variability of XSLA. It is noteworthy that the patient with R452G has a mild anemia, and the two patients with the R572H mutations have near-to-normal hemoglobin levels, microcytosis, and a relatively late onset of the disease. It is interesting to note the wide variability in the degree of anemia in patients with *ALAS2* mutations, ranging from a baby boy with a very severe form of the disease requiring repeated blood transfusions (patient 3, see Table 1), to patients with mild microcytosis diagnosed at age 46 and 57 respectively (patients 14 and 15, see Table 1). In these last two cases, the diagnosis was suspected because of iron overload in absence of transfusion, and confirmed by bone marrow examination revealing the presence of ringed sideroblasts.

In conclusion, we confirm in a large cohort of patients that about half of the cases of non syndromic sideroblastic anemia are accounted for by *ALAS2* mutations, and that a high degree of clinical heterogeneity parallels the diversity of the mutations and of their functional consequences.

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