Sideroblastic anemia: molecular analysis of ALAS2 gene in a series of 29 probands and functional studies of ten missense mutations.

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Title page

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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 ten of them, without any published functional characterization, were expressed in *E. 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.

Keys 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.). 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 A., 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 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 *E. coli* in order to study the activity of mutated protein (Cotter, et al., 1992; Cotter, et al., 1995; Cotter, et al., 1994; Cox, et al., 1994; Furuyama, et al., 1997; Furuyama, et al., 2006; Furuyama, et al., 1998; Harigae, et al., 1999a; Harigae, et al., 1999b; Prades, et al., 1995). In addition, in 2005, the crystallographic structure of ALAS2 from *Rhodobacter capsulatus (R.*

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 ten 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 and 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, CA, USA). 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 CA, USA), both strands were sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Sequencing products were purified (Sephadex G50, GE Healthcare, Piscataway,

NJ, USA), and analyzed using a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and the Seqscape analysis software (v2.6.0) (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). 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 Rhodobacter 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, USA) 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, Carlsbad, California, USA) were transformed with expression plasmids, and overnight cultures were grown in LB (Lennox L Broth Base, Invitrogen, Life Technologies, Carlsbad, California, USA) 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, USA), 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 2500 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, USA), and the samples were adjusted to a concentration of 0.2 mg of total protein/ml. Six hundred µl of samples were preincubated for 5 minutes 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, USA) were added to samples. The reaction was stopped by adding 60 µl of 100% trichloroacetic acid either immediately (T0) or after 20 minutes (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, St. Louis, MO, USA) (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 minutes instead of 5 minutes. The significance of the results was estimated using Student's t-test.

Immunoblotting

2.5 µg of total protein of the supernatant from bacteria lysates were taken up onto 1X Laëmmli buffer, and heated for 10 minutes at 100°C. Samples were analysed by SDS-PAGE using an 8% polyacrylamide gel followed by electro-transfer on a polyvinylidene fluoride (PVDF) membrane. Loading and transfer were confirmed by Ponceau red staining. After pre-incubation 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, USA) for 2H at room temperature. After washing three times with TBST for 10 minutes each time, the membrane was incubated with a secondary anti rabbit IgG, linked to Horseradish peroxidase (1/20 000, Amersham GE Healthcare, Piscataway, NJ, USA) for 2H at room temperature. The MBP fusion proteins were visualized (Immobilon Western, chemiluminescent HRP substrate, Millipore Corporation, Billerica, MA, USA) 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 and 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). Twelve of these 13 mutations caused amino acid substitutions (Table 1), and the thirteenth was a 48-bp deletion (c.-91_-44del) in the proximal promoter (Table 1 and Figure 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 9 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 µmoles/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 (ten missense mutations corresponding to six novel mutations and four 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. Figure S1). When the enzymatic activity was assayed from *E. coli* lysates immediately after cell disruption, five ALAS2 mutants (E242K, D263N, P339L, R375C, R411H) displayed significantly reduced ALAS2 activity, with residual activity ranging between 14 and 65 % of the normal construct (Figure 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 (Figure 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 pre-incubating them at 37°C for 30 or 60 minutes prior to the enzymatic assay (Figure 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 (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 9 patients; the characteristics available for these patients are reported in Suppl. table S3). This proportion is similar to that reported by Bergmann et al. (Bergmann, et al., 2010), suggesting that other loci must be involved.

Our data extend the allelic heterogeneity of XSLA, since 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 µmoles/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, since *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 (Table 3). 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, 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 PLP and increased thermosensitivity. This may be explained by reduced affinity for PLP and higher sensitivity to thermal denaturing of the apoenzyme as 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. Figure 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 Table1), to patients with mild microcytosis diagnosed at age 46 and 57 respectively (patients 14 and 15, see Table1). 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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Figure Legends

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), 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 Material and Methods. Enzyme activity was measured on at least 3 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.

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 Material and Methods. Specific activities are expressed in pmoles 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 minutes before the assay, as described in Material 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.

Title page

Title

Sideroblastic anemia: molecular analysis of the *ALAS2* gene in a series of 29 probands and functional studies of ten 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 ten of them, without any published functional characterization, were expressed in *E. 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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Keys 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.). 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 A., 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 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 *E. coli* in order to study the activity of mutated protein (Cotter, et al., 1992; Cotter, et al., 1995; Cotter, et al., 1994; Cox, et al., 1994; Furuyama, et al., 1997; Furuyama, et al., 2006; Furuyama, et al., 1998; Harigae, et al., 1999a; Harigae, et al., 1999b; Prades, et al., 1995). In addition, in 2005, the crystallographic structure of ALAS2 from *Rhodobacter capsulatus (R.*

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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 ten 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 and 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).

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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, CA, USA). 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 CA, USA), both strands were sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Sequencing products were purified (Sephadex G50, GE Healthcare, Piscataway,

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NJ, USA), and analyzed using a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and the Seqscape analysis software (v2.6.0) (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). 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.

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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* Rhodobacter 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, USA) 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, Carlsbad, California, USA) were transformed with expression plasmids, and overnight cultures were grown in LB (Lennox L Broth Base, Invitrogen, Life Technologies, Carlsbad, California, USA) 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, USA), 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 2500 rpm for 10 min, and then frozen at -80°C. The assay of ALAS2 activities of the control and mutant

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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, USA), and the samples were adjusted to a concentration of 0.2 mg of total protein/ml. Six hundred µl of samples were preincubated for 5 minutes 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, USA) were added to samples. The reaction was stopped by adding 60 µl of 100% trichloroacetic acid either immediately (T0) or after 20 minutes (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, St. Louis, MO, USA) (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.

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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 minutes instead of 5 minutes. The significance of the results was estimated using Student's t-test.

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Immunoblotting

2.5 µg of total protein of the supernatant from bacteria lysates were taken up onto 1X Laëmmli buffer, and heated for 10 minutes at 100°C. Samples were analysed by SDS-PAGE using an 8% polyacrylamide gel followed by electro-transfer on a polyvinylidene fluoride (PVDF) membrane. Loading and transfer were confirmed by Ponceau red staining. After pre-incubation 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, USA) for 2H at room temperature. After washing three times with TBST for 10 minutes each time, the membrane was incubated with a secondary anti rabbit IgG, linked to Horseradish peroxidase (1/20 000, Amersham GE Healthcare, Piscataway, NJ, USA) for 2H at room temperature. The MBP fusion proteins were visualized (Immobilon Western, chemiluminescent HRP substrate, Millipore Corporation, Billerica, MA, USA) 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 and 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). Twelve of these 13 mutations caused amino acid substitutions (Table 1), and the thirteenth was a 48-bp deletion (c.-91_-44del) in the proximal promoter (Table 1 and Figure 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 9 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.

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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 µmoles/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 (ten missense mutations corresponding to six novel mutations and four 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. Figure S1). When the enzymatic activity was assayed from *E. coli* lysates immediately after cell disruption, five ALAS2 mutants (E242K, D263N, P339L, R375C, R411H) displayed significantly reduced ALAS2 activity, with residual activity ranging between 14 and 65 % of the normal construct (Figure 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 (Figure 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 pre-incubating them at 37°C for 30 or 60 minutes prior to the enzymatic assay (Figure 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).

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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 (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 9 patients; the characteristics available for these patients are reported in Suppl. table S3). This proportion is similar to that reported by Bergmann et al. (Bergmann, et al., 2010), suggesting that other loci must be involved.

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Our data extend the allelic heterogeneity of XSLA, since 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 µmoles/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, since *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).

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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 (Table 3). 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, 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 PLP and increased thermosensitivity. This may be explained by reduced affinity for PLP and higher sensitivity to thermal denaturing of the apoenzyme as 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. Figure 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 Table1), to patients with mild microcytosis diagnosed at age 46 and 57 respectively (patients 14 and 15, see Table1). 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.

Acknowledgments

We would like to thank David Bishop for the gift of the *ALAS2* wild type plasmid, Jérome Lamoril and Vasco Da Silva for their helpful discussions, Yolande Kroviarski, Nathalie Clément, Anne Marie Robréau, Gilles Hetet, Claire Oudin, Dominique Henry, Sylvie Simonin for their technical assistance, and Vincent Oustric for his help with the Rasmol software. The research was funded by Agence Nationale de la Recherche (ANR 07-MRAR-008-03).

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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 (µmoles/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	1448	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.	1172	1.40	exon 8	c.1016C>T	p.Pro339Leu	present study
6	M	47	n.a.	6.3	<70	63	2537	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	2284	n.a.	exon 9	c. 1354C>G	p.Arg452Gly	present study
11	M	20	+	11.3	62	100	1172	n.a.	exon 9	c.1354C>T	p.Arg452Cys	(Bottomley, et al., 1995)[f]
12	M	n.a.	+	10	<70	n.a.	1000	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	1000	0.64	exon 11	c.1715G>A	p.Arg572His	present study
15	M	57	+	12.2	73	28	1000	n.a.	exon 11	c.1715G>A	p.Arg572His	present study
16	M	0.75	+/-	6.3	53	n.a.	1200	n.a.	Promoter	c.-91_-44 del	-	present study

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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.

Expression data have been studied for the following mutations: [e] pR411C (Furuyama, et al., 1998), and [f] p.R452C (Furuyama, et al., 2006).

Normal values: Hb: 12-15.5 g/dL (anemia: Females<12g/dL, Males <13g/dL), MCV: 80-90 fL (microcytosis: <80fL), Tr Sat: 12-45% (Females) 15-50% (Males), Serum Ferritin: Females 20-150 µg/L, Males 30-300 µg/L. PP <1.9µmoles/L RBC.

TABLE 2: Summary of results for missense mutations

ALAS2 Mutation	Structural impact[a]		*In silico* protein score	ALAS2 activity	
	AA RC	Localization		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 % ‡	ND
p.Asp263Asn	Asp120	Internal α5-helix	Deleterious	14 % ‡	ND
p.Pro339Leu	Pro196	Surface 1aa before α8-helix	Deleterious	65 % ‡	ND

p.Arg375Cys	Arg232	Surface α8-helix	Deleterious	53 % ‡	ND
p.Arg411His	Arg268	Internal α1-helix	Deleterious	23 % ‡	ND
p.Arg411Cys	Arg268		Deleterious	25 % [f]	ND
p.Arg452Gly[d]	Met309	Surface α14-helix	Deleterious	109 %	Normal
p.Arg452Cys[d]	Met309		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; ‡ Student's t-test, p value < 0.05

[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).

Figure Legends

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), 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 Material and Methods. Enzyme activity was measured on at least 3 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.

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 Material and Methods. Specific activities are expressed in pmoles 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 minutes before the assay, as described in Material 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.

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14	M	46	-	13.2	68	63	1000	0.64	exon 11	c.1715G>A	p.Arg572His	present study
15	M	57	+	12.2	73	28	1000	n.a.	exon 11	c.1715G>A	p.Arg572His	present study
16	M	0.75	+/-	6.3	53	n.a.	1200	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.

Expression data have been studied for the following mutations: [e] pR411C (Furuyama, et al., 1998), and [f] p.R452C (Furuyama, et al., 2006).

Normal values: Hb: 12-15.5 g/dL (anemia: Females<12g/dL, Males <13g/dL), MCV: 80-90 fL (microcytosis: <80fL), Tr Sat: 12-45% (Females) 15-50% (Males), Serum Ferritin: Females 20-150 µg/L, Males 30-300 µg/L. PP <1.9µmoles/L RBC.

TABLE 2: Summary of results for missense mutations

ALAS2 Mutation	Structural impact[a]		*In silico* protein score	ALAS2 activity	
	AA RC	Localization		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 % ‡	ND
p.Asp263Asn	Asp120	Internal α5-helix	Deleterious	14 % ‡	ND
p.Pro339Leu	Pro196	Surface 1aa before α8-helix	Deleterious	65 % ‡	ND

p.Arg375Cys	Arg232	Surface α8-helix	Deleterious	53 % ‡	ND
p.Arg411His	Arg268	Internal α1-helix	Deleterious	23 % ‡	ND
p.Arg411Cys	Arg268		Deleterious	25 % [f]	ND
p.Arg452Gly[d]	Met309	Surface α14-helix	Deleterious	109 %	Normal
p.Arg452Cys[d]	Met309		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; ‡ Student's t-test, p value < 0.05

[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).

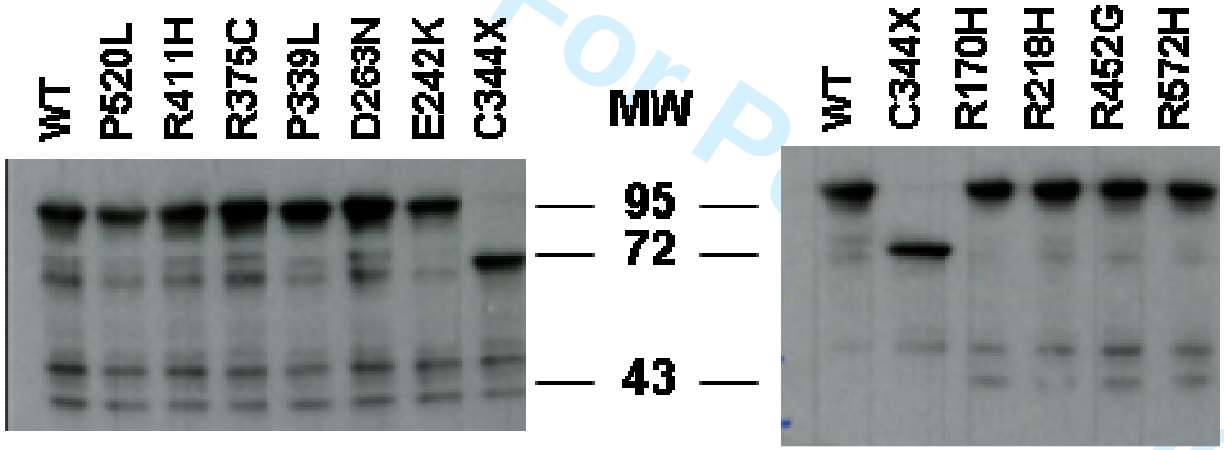
[b] or preincubated at 37°C.

[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*.

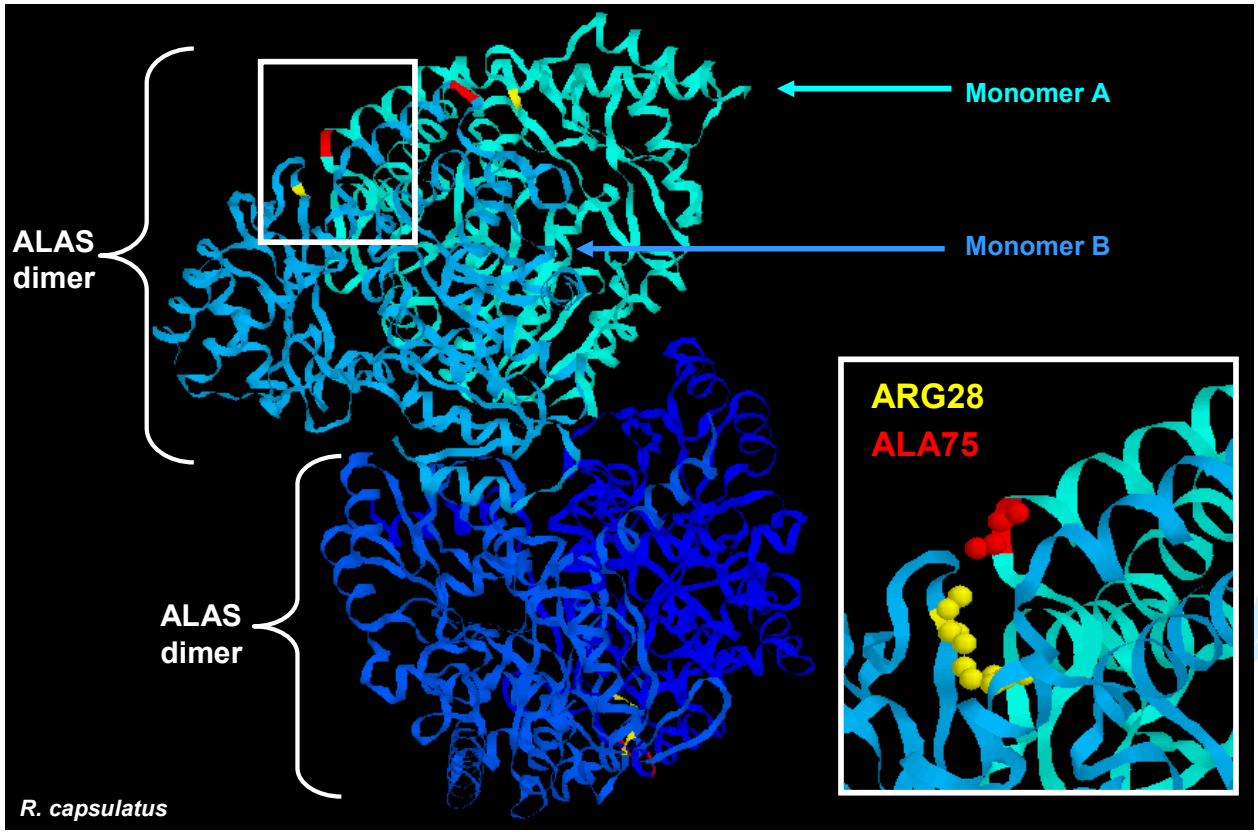
[f] previously described (Furuyama, et al., 2006)

Supp. figure S1: Immublotting
Western blot analysis of bacterial cell lysates from *E. coli* transformed with normal and mutant ALAS2 cDNAs. After the transfer, the membranes were probed with a primary antibody directed against MBP.
MW: reported molecular weight of the ladder (PageRulerTM prestained protein ladder, Fermentas, Thermo Fischer Scientific).



Suppl. figure S2: Localization of Arg28 (e.g. R170 in human) and Ala75 (e.g. R218 in human) on ALAS of *Rhodobacter capsulatus*.

The structure of ALAS of *R. capsulatus* (PDB 2BWO, Rasmol software, 2 ALAS dimers) is represented. The blue ribbon and the cyan ribbon correspond to two monomers. Arg28 (in yellow) on one monomer and Ala75 (in red) on the other monomer are quite close to each other in the quaternary structure.



Supp. table S1: Primers Table

Exon		**Sequence**	Amplicon length (bp)
Prom -1	F R	TGG TCT GAT TCC AAA GCC CAA ATG CGA GGA AAG AGA CAA AAA GGG GGA G	627
2	F R	TTG CCA GGC CCT CAT GAT GGA A AGG CAA AGA TGG CCA GTA TAA CTT GGA	325
3	F R	GGC TTT AGG GGT ACA TTA GAT CTC AGC A ATC ATG GGA TGT GTA CTG GCT GCT TTT	324
4	F R	TGT GTT GCA TTG AGC TTA AAG AGG ACA A AAG GCC CTT CTG TAC TGT TTC CCC TAC T	295
5	F R R	GAG TTG GAG AAC TCA AAA TCA GCA ACA T CCC AAG TTT CCA CTG CCA GCT CT Internal sequence Primer TTC CAT GTG TGG TTT TTC ATC TCC TCT	418 380
6	F R	GAG AAA CCT ACC CAG TTC CTC GAT GC ACT GGA TGC TGT ATT GCA GGA TAC CA	316
7	F R	GGT TGA AGT GGG AGT ACT GGG ACA GA TGA TCA TCA TGG TTT TTG TGA GAC CAA	296
8	F R	GAG ATG GAG CTG GGG AAG GGT TAT ATT TTG TAA GGG CCT CCT CTC TGG A	280
9	F R	TGT TGC TCT GGG ACT GAT TAT GGG A GGA GCG TGA GGC TCC CAG AAT AA	387
10	F R	AGC TAG TGA TGG TGG AGC CAG AGT G TCT TTC AGA TCC TGG GGC TGA GG	282
11	F R	GGC TCA TCT GTA CTG TGA CAG ATT TGG A GGC ACA CAA CAA AGC AGA AGA CAG G	324

Supp. Table S2: *in silico* evaluations of 12 *ALAS2* mutations

ALAS2 Mutation (protein)	**Grantham Score**	**GVGD** [£]	**PANTHER**	**SIFT**	**Polyphen**	**UMD predictor**	**SNP3D**	***In silico* Score** [$]
p.Arg170His	29	Moderate risk *C25*	Highly deleterious *-4.1649*	Affected *0.00*	probably damaging *PSIC: 2.626*	Probable polymorphism *59*	Deleterious *-1.29*	Deleterious 5/7
p.Arg218His	29	Weak risk *C0*	Unlikely deleterious *-2.31925*	Tolerated *0.11*	Benign *PSIC: 0.241*	Polymorphism *47*	Non deleterious *1.44*	Neutral 0/7
p.Glu242Lys	56	High risk *C55*	Unlikely deleterious *-1.99823*	Tolerated *0.44*	Benign *PSIC: 1.163*	Polymorphism *41*	Non deleterious *1.41*	Neutral 1/7
p.Asp263Asn	23	Weak risk *C15*	Probably deleterious *-2.78604*	Affected *0.00*	possibly damaging *PSIC: 1.976*	Probable polymorphism *53*	Deleterious *-0.60*	Deleterious 4/7
p.Pro339Leu	98	High risk *C65*	Highly deleterious *-4.73676*	Affected *0.00*	probably damaging *PSIC: 3.181*	Pathogenic *82*	Deleterious *-2.34*	Deleterious 6/7
p.Arg375Cys	180	High risk *C65*	Highly deleterious *-4.79784*	Affected *0.00*	probably damaging *PSIC: 2.734*	Pathogenic *100*	Deleterious *-1.12*	Deleterious 7/7
p.Arg411His	29	Moderate risk *C25*	*error**	Affected *0.00*	probably damaging *PSIC: 2.686*	Pathogenic *100*	Deleterious *-2.63*	Deleterious 5/6
p.Arg411Cys	180	High risk *C65*	*error **	Affected *0.00*	probably damaging *PSIC: 3.361*	Probably pathogenic *71*	Deleterious *-3.31*	Deleterious 6/6

p.Arg452Gly	125	High risk *C65*	Possibly deleterious *-2.75867*	Tolerated *0.024*	probably damaging *PSIC: 2.119*	pathogenic *82*	Non Deleterious *1.16*	Deleterious 6/7
p.Arg452Cys	180	High risk *C65*	Highly deleterious *-4.26738*	Affected *0.03*	probably damaging *PSIC: 2.174*	Pathogenic *100*	Non Deleterious *0.47*	Deleterious 6/7
p.Pro520Leu	98	High risk *C65*	Highly deleterious *-4.7838*	Affected 0.01	probably damaging PSIC: 2.899	Pathogenic 82	Non Deleterious 0.46	Deleterious 5/7
p.Arg572His	29	Weak risk *C0*	Unlikely deleterious *-2.25602*	Affected *0.01*	probably damaging *PSIC: 2.103*	Pathogenic *76*	*error*	Deleterious 3/6

* failure of the software to give a prediction

£ For GVGD we used the ALAS2 sequences for *Homo sapiens* (isoform 1), *Mus musculus*, *Rattus norvegicus*, *Bos taurus* and *Danio rerio*.

$ To get an overall impression of the likely 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: pathogenous; SNP3D: deleterious).

Supp. table S3: Proposed grouping of Congenital Sideroblastic Anemia patients

Congenital Sideroblastic Anemia form (Gene)	**No. of mutations**	**No. of probands**	**Females / Males**	**Range of ages at diagnosis** $	**Characteristics of anemia** (MCV)	**Severity of Anemia** £ (Lowest Hb)
X-linked sideroblastic anemia (*ALAS2*)	13	16	6/10	6m-63y	Microcytic in most cases	Mild to severe
Autosomal recessive (*SLC25A38*)	4	4	2/2	2d-3y	microcytic	Severe
	-	2	0/2	3m-7m	Microcytic	Severe
Unexplained CSA	-	6	4/2	early-62y	Non microcytic	Mild (2) Severe (3) NA (1)
	-	1*	0/1	NA	NA	NA

$ d: day; m: month; y: years

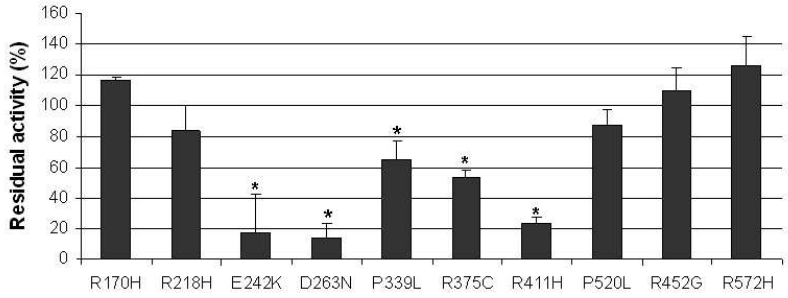
£ Classication of anemia: Mild, Hb >10 g/dl; moderate, Hb 7–10 g/dl; severe Hb <7 g/dl; NA: not available

* Familial case (another brother affected)

Figure 1

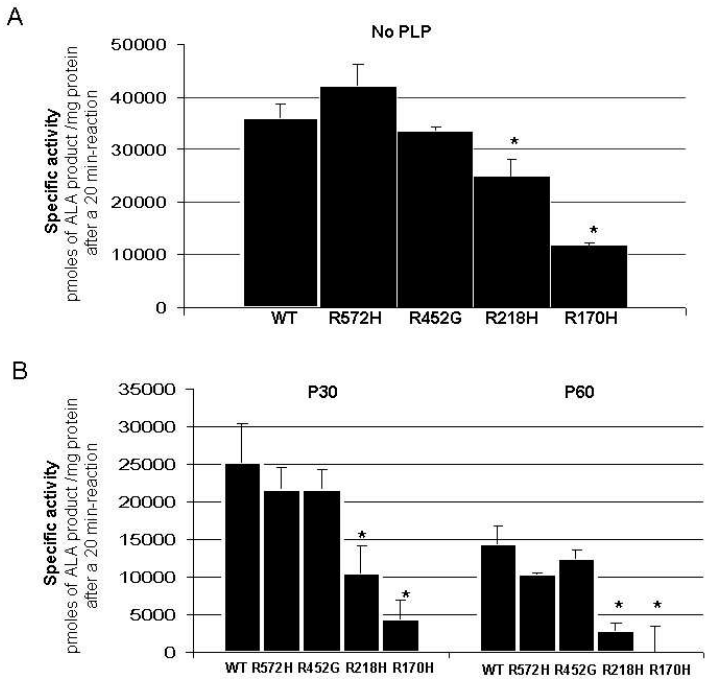
254x190mm (96 x 96 DPI)

Figure 2



254x190mm (96 x 96 DPI)

Figure 3



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