Molecular and functional analysis of the C-terminal region of human erythroid-specific 5-aminolevulinic synthase associated with X-linked dominant protoporphyria (XLDPP)

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Received October 24, 2012; Revised and Accepted December 12, 2012

Frameshift mutations in the last coding exon of the 5-aminolevulinic synthase (ALAS) 2 gene were described to activate the enzyme causing increased levels of zinc- and metal-free protoporphyrin in patients with X-linked dominant protoporphyria (XLDPP). Only two such so-called gain-of-function mutations have been reported since the description of XLDPP in 2008. In this study of four newly identified XLDPP families, we identified two novel ALAS2 gene mutations, a nonsense p.Q548X and a frameshift c.1651–1677del26bp, along with a known mutation (delAGTG) found in two unrelated families. Of relevance, a de novo somatic and germinal mosaicism was present in a delAGTG family. Such a phenomenon may explain the high proportion of this mutation in XLDPP worldwide. Enhancements of over 3- and 14-fold in the catalytic rate and specificity constant of purified recombinant XLDDPP variants in relation to those of wild-type ALAS2 confirmed the gain of function ascribed to these enzymes. The fact that both p.Q548X and c.1651–1677del26bp are located in close proximity and upstream from the two previously described mutations led us to propose the presence of a large gain-of-function domain within the C-terminus of ALAS2. To test this hypothesis, we generated four additional nonsense mutants (p.A539X, p.G544X, p.G576X and p.V583X) surrounding the human XLDDPP mutations and defined an ALAS2 gain-of-function domain with a minimal size of 33 amino acids. The identification of this gain-of-function domain provides important information on the enzymatic activity of ALAS2, which was proposed to be constitutively inhibited, either directly or indirectly, through its own C-terminus.

INTRODUCTION

Porphyrias are a group of inherited metabolic disorders of haem biosynthesis; for review see (1). Erythropoietic protoporphoria (EPP, [MIM 177000]) is caused by the accumulation of the last intermediate in the haem biosynthetic pathway, metal-free protoporphyrin IX (PP) as a result of a
partial deficiency of the enzyme ferrochelatase (FECH, EC 4.99.1.1) due to mutations in the FECH gene. A clinical presentation of cutaneous photosensitivity is characteristic for all protoporphyria patients. However, a small number of them show a distinct biochemical feature of a relatively high level of zinc-protoporphyrin (ZnPp) compared with the rest of the patients (2,3). Neither mutations nor the low-expressed allele were found in the FECH gene of these patients and ferrochelatase activity measured in lymphoblasts was found to be normal (2,3). These observations led to the description of a novel disease identity, named X-linked dominant protoporphyria (XLDPP, MIM 300752). Among the affected individuals, two different mutations have so far been identified in the ALAS2 gene, which is located in the X-chromosome (a third mutation, c.1734delG, was reported by Doheny et al. at the annual meeting of the American Society of Human Genetics in 2009 (4)). This gene is one of the two encoding 5-aminolevulinic synthase (ALAS, EC 2.3.1.37), the first regulatory enzyme of the haem biosynthetic pathway (2).

ALAS, a homodimer, catalyzes the condensation of glycine (Gly) and succinyl CoA (sCoA) to generate 5-aminolevulinic phosphate (PLP) as a cofactor. The enzymatic reaction requires pyridoxal 5'-phosphate (PLP) as a cofactor. The ubiquitously expressed isofrom of ALAS, ALAS1, is responsible for the ubiquitous production of haem for the synthesis of cytochromes and other haemoproteins, whereas the erythroid-specific isoenzyme ALAS2, expressed at high levels in erythroid cells, is essential for the synthesis of haem in haemoglobin (7).

Many deleterious mutations in the ALAS2 gene are known to cause X-linked sideroblastic anaemia (XLSA) (8,9). However, unlike those found in XLSA, two known mutations causing XLDPP share a common feature of enhancing the enzyme activity. These so-called ‘gain-of-function’ mutations c.1706–1709delAGTG (p.E569GfsX24) named delAGTG and c.1699–1700delAT (p.M567EfsX2) named delAT were found in the last coding exon of the gene and therefore, affect the C-terminal (C-ter) domain of the enzyme (2). An increased ALAS2 enzymatic activity leads to the overproduction of ALA and subsequently, the accumulation of both metal-free and ZnPp. As a consequence, the ferrochelatase-catalyzed reaction becomes rate-limiting in the erythroid haem biosynthetic pathway. Thus, the ‘metal-free PP to ZnPp ratio’ allows the clinicians to differentiate between EPP and XLDPP.

In this report, we describe five new XLDPP patients from four unrelated families. Three of the patients were shown to carry the delAGTG mutation including one with a somatic and germlinal mosaicism—the first such case. Two other patients carried novel mutations, a 26 bp deletion and a nonsense mutation in the ALAS2 gene, respectively. The two novel mutations were located in the last exon further upstream from the two previously known XLDPP mutations, which inspired us to test a hypothesis of the existence of a ‘gain-of-function domain’ within the C-ter region of ALAS2. By site-directed mutagenesis of all four known human XLDPP mutations and four putative neighbouring mutations in the ALAS2 gene, we were able to define the boundary of the ‘gain-of-function domain’.

RESULTS

Four patients from four unrelated families were referred to specialized porphyria centres in Switzerland, Australia, the Netherlands and France, because of a history of skin photosensitivity associated with an increased level of PP in erythrocytes (Table 1). Both Patients 2 and 3 presented with abnormal liver function at the initial visit. The percentage of ZnPp was characteristic for XLDPP as described in the previous study (2). No mutations were found in the FECH gene and all patients were homozygous for the wild-type IVS3–48T allele, (i.e. absence of the low-expressed IVS3–48C allele; data not shown). The biochemical and genetic evidence suggested XLDPP and ruled out EPP.

The ALAS2 gene was, therefore, sequenced in all patients, which led to the identification of three separate mutations (Fig. 1A). A thus far unknown c.1642C>T, p.Q548X substitution was found in the Australian patient (Patient 2). The Dutch patient (Patient 3), whose parents originally came from Afghanistan, presented with a novel 26 bp deletion in exon 11 of the ALAS2 gene, c.1651–1677delTCTGTGCCC TGCCTGCAATTTCGTGTC (p.S551PfsX5; designated as del26bp). Both the substitution and the novel deletion were absent in 100 Caucasian chromosomes and in 1000 genomes database (data not shown). They were predicted to generate truncated proteins shorter than those from both the delAT and delAGTG mutations (Fig. 1B).

Patients 1 and 4, both females, carried the previously described delAGTG mutation (Fig. 1A). Interestingly, Patient 4 presented with a severe painful photosensitivity since childhood (onset at the age of 3 years), whereas her mother showed only mild symptoms beginning in adulthood (Table 1; Fig. 2A). At the initial analysis, the mutation which was found in the daughter, was not observed in a blood DNA sample of the mother. An additional DNA analysis performed on a second blood DNA sample using a new set of primers was able to exclude specific allele amplification in the mother (data not shown). Furthermore, a careful examination of the electropherogram identified a weak signal which was almost indistinguishable from the background noise. The sequence pattern of that signal matched with that from her daughter, suggesting the presence of the delAGTG mutation, but to a much lesser degree in the mother (Fig. 2B). The suspicion of a somatic mosaicism was confirmed by capillary electrophoresis-laser-induced fluorescence (CE-LIF) quantification. Around 13% of the mother’s genomic DNA from both whole blood (Fig. 2C) and buccal mucosa (data not shown) contained the delAGTG mutation. The germlinal mosaicism in the mother was confirmed by allele segregation analysis. The proband’s half-sister, who neither shows any XLDPP phenotype nor carries the delAGTG mutation (Fig. 2C), inherited the same X chromosome as the proband from their mother (Fig. 2A; Supplementary Material, Table S1). The presence of a somatic mosaicism for delAGTG and the wild-type sequence combined with the result of allele segregation analysis suggest that both a germline and a somatic mosaicism were present in the mother.

All four different human mutants of ALAS2 associated with XLDPP reported so far, Q548X, del26bp, delAT and delAGTG, were generated in vitro by site-directed
mutagenesis and expressed in *Escherichia coli*. Enzymatic assays in crude bacterial extracts showed significantly higher ALAS2 activities in the mutants compared with that of the wild-type enzyme (Fig. 1C and D). The degree of increase in ALAS activity among the mutants was in the order: p.Q548X ≥ del26bp. delAT. delAGTG, which coincided with the sequence of location of these mutants along the C-ter of the ALAS2 enzyme (Fig. 1B and C). In this study, the specific activities of all XLDPP variants measured in bacterial lysates were found to be 2- to 3.5-fold higher than that of the wild-type ALAS2 (Fig. 1C) compared with the 20- to 40-fold increases initially reported (2). These discrepancies could be the results from an improvement in the expression level of the wild-type construct.

To overcome the potential ambiguities in the interpretation of the enzymatic activity results obtained with bacterial cell lysates, expression vectors and overexpression systems were engineered for the production of human ALAS2 (hALAS2) and XLDPP variants in *E. coli* and subsequent purification of the recombinant enzymes. Steady-state kinetic parameters for purified wild-type hALAS2 and XLDPP variants are reported in Table 2. The catalytic rate constants ($k_{cat}$) for the XLDPP variants were from 3- to 3.4-fold greater than that of wild-type hALAS2. The Michaelis constants for succinyl-CoA were consistently lowered, while those for glycine were largely unaffected in the variants, with the exception of the delAGTG variant, in which the Michaelis constant for glycine was reduced by 50%. Consequently, the specificity constants ($k_{cat}/K_m$) of the XLDPP variants, particularly towards succinyll-CoA, were increased by up to 14-fold. These results indicate unequivocally that the XLDPP, gain-of-function, mutations cause an increase in the catalytic rate and specificity constants of hALAS2. Thus, these findings confirm and extend the results obtained with lysates of bacterial cells harbouring XLDPP variants.

To determine the boundary of an alleged domain within the C-ter of ALAS2 which harbours the gain-of-function feature, we generated four additional nonsense mutants p.A539X, p.G544X, p.G576X and p.V583X that are located in close proximity to the four known XLDPP mutations. These nonsense mutations are predicted to generate a series of truncated proteins that are 4–48 amino acid residues shorter than the full-length enzyme. Whilst the upstream p.A539X showed obviously a loss-of-function with an activity (in crude bacterial extracts) being approximately equal to that of the negative control p.C344X, both p.G544X and p.G576X clearly exhibited a gain-of-function. The downstream p.V583X mutant that was only four amino acid residues shorter than the full-

### Table 1. Clinical and biological data from patients with X-linked protoporphyria

<table>
<thead>
<tr>
<th>Clinical</th>
<th>Patient 1 (Switzerland)</th>
<th>Patient 2 (Australia)</th>
<th>Patient 3 (the Netherlands)</th>
<th>Patient 4 (France)</th>
<th>Mother of P4 (France)</th>
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</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Age of onset</td>
<td>2 years</td>
<td>in childhood</td>
<td>&lt;1 year</td>
<td>3 years</td>
<td>22 years</td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>Severe</td>
<td>Severe</td>
<td>Severe</td>
<td>Severe</td>
<td>Mild</td>
</tr>
<tr>
<td>Gallstone</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<table>
<thead>
<tr>
<th>Biochemical</th>
<th>Haematological</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>13.6</td>
<td>8.9</td>
<td>11.7</td>
<td>12.1</td>
<td>10.9</td>
</tr>
<tr>
<td>MCV (Fl)</td>
<td>93</td>
<td>83</td>
<td>94</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Haptoglobin (g/l, 0.3–2.0)</td>
<td>n.a.</td>
<td>0.13</td>
<td>n.a.</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>LDH (IU/L, &lt;288)</td>
<td>168</td>
<td>1270</td>
<td>257</td>
<td>145</td>
<td>201</td>
</tr>
<tr>
<td>Total bilirubin (μmol/l, &lt;20)</td>
<td>8</td>
<td>91</td>
<td>73</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Iron (μmol/l, 10–26)</td>
<td>5.1</td>
<td>5.0</td>
<td>10</td>
<td>9.0</td>
<td>11.5</td>
</tr>
<tr>
<td>Transferrin (g/l, 2.0–4.0)</td>
<td>38</td>
<td>4.3</td>
<td>2.75</td>
<td>3.41</td>
<td>3.25</td>
</tr>
<tr>
<td>Sat. Transferrin (%), 20–45</td>
<td>38</td>
<td>4</td>
<td>15</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Ferritin (μg/l, 3–105)</td>
<td>8</td>
<td>13</td>
<td>21</td>
<td>24</td>
<td>34</td>
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<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAT (IU/I, &lt;31)</td>
<td>14</td>
<td>166</td>
<td>325</td>
<td>19</td>
<td>40</td>
</tr>
<tr>
<td>ASAT (IU/I, &lt;31)</td>
<td>20</td>
<td>291</td>
<td>333</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/I, &lt;104)</td>
<td>40</td>
<td>67</td>
<td>115</td>
<td>60</td>
<td>86</td>
</tr>
<tr>
<td>γ-GT (IU/I, &lt;55)</td>
<td>n.a.</td>
<td>146</td>
<td>277</td>
<td>32</td>
<td>42</td>
</tr>
<tr>
<td>Porphyrins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total erythrocyte PP (μmol/l RBC, 1.9)</td>
<td>13.9</td>
<td>57.9</td>
<td>17.2</td>
<td>46</td>
<td>17.7</td>
</tr>
<tr>
<td>% ZnPP (≥72)</td>
<td>66</td>
<td>19</td>
<td>35</td>
<td>68</td>
<td>58</td>
</tr>
<tr>
<td>FECH activity (nmol mesozinc/h/mg of protein at 37°C, ≥3.5)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>5.7</td>
<td>4.3</td>
<td>3.8</td>
</tr>
<tr>
<td>FECH mutation</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>FECH IVS3–48 genotype</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
<td>T/T</td>
</tr>
<tr>
<td>ALAS2 mutation</td>
<td>delAGTG</td>
<td>c.1642C &gt; T, del26</td>
<td>delAGTG</td>
<td>delAGTG</td>
<td>delAGTG</td>
</tr>
</tbody>
</table>

*Normal range in females; n.a., not available.
length enzyme, had a normal activity (Fig. 3). Thus, the 'gain-of-function domain' spans over a minimum of 33 amino acids, i.e. between residues 544 and 576 of ALAS2.

**DISCUSSION**

In this second XLDPP study, we report five new patients. In agreement with the study of Whatley et al. (2), all five XLDPP patients of our study showed a relatively high proportion of ZnPP in total erythrocyte PPs. Importantly, the percentage of ZnPP in erythrocytes is a unique feature which can distinguish XLDPP from EPP of which the patients normally show, 13% of ZnPP (2). So far, the two known frameshift mutations (delAGTG and delAT) have been identified in 18 unrelated families (2,10,11). These frameshifts predict disruptions of the 19–20 C-ter amino acids of ALAS2. This study extended the total number of XLDPP families to 28%, i.e. 16 out of 20, of the families carry the delAGTG mutation. Although the delAGTG families are spread over three continents, they are all of European origin (Fig. 4). However, no evidence of a founder effect has been found among eight unrelated delAGTG-families from France, UK, South Africa and Switzerland based on haplotype analysis (data not shown). Therefore, delAGTG may represent a hotspot mutation in ALAS2. The repeated occurrence of delAGTG in the exon 11 ALAS2 normal sequence may be the result of a usual DNA replication error. The finding of a de novo mutation in connection with a somatic and germinal mosaicism in the mother of Patient 4 strongly supports this notion of delAGTG being a mutational hot spot. The transmission of this mutation to her daughter as a constitutive inherited mutation may be a common genetic mechanism involved in the delAGTG-associated XLDPP world-wide expansion. In numerous X-linked or autosomal dominant diseases such as Duchenne muscular dystrophy, haemophilia A, lethal osteogenesis imperfecta, neurofibromatosis type I, and androgen insensitivity syndrome (12), somatic and/or germ-line mosaicism for de novo mutations have been found. However, in analogy to these diseases, our data suggested that a single molecular mechanism is a plausible explanation for the emergence of a recurrent mutation responsible for 80% of the XLDPP cases. This observation of a somatic and germ-line mosaicism also points to a methodological, as well as a counselling problem in XLDPP. In this respect, in families with a single affected child, one must consider the possibility of a parental somatic/germ-line mosaicism. Then, a careful interpretation of the ALAS2 electropherogram and a subsequent CE/LIF quantification should be carried out in order to detect somatic mosaicism, especially among 'mutation-negative' parents of the XLDPP cases. This finding necessitates the use of this second
unrelated mutation-detection method. However, mutation detection in mosaic cases will never be 100% sensitive, either because the ratio of the mutant to the wild-type may be too low to be detected or because the mutant DNA may be totally absent in the somatic cells.

The two novel mutations (p.Q548X and del26bp) are both located in the last exon of the ALAS2 gene as are the two known mutations delAT and delAGTG. The predicted disruptions in the C-ter caused by p.Q548X and del26bp are 39 and 36 amino acids, respectively. These truncated proteins are therefore shorter than those generated by both delAT and delAGTG (19 and 20 amino acids, respectively). These observations led us to speculate that the ‘gain-of-function domain’ may be larger than initially thought. Unlike XLDPP, mutations causing XLSA are spread over the exons 4 – 11 (9,13,14). The majority of the XLSA mutations are missenses. A small number of nonsense and frameshift mutations leading to truncated enzymes have been identified only in female XLSA patients (9). Among all known null mutations, p.Y506fsX22, generates a premature stop codon at amino acid residue 528 and thus, the longest truncated protein. This truncated protein presented with a severe loss-of-function in agreement with the XLSA phenotype (15). Based on the data from both XLSA and XLDPP, it is evident that the switch from ‘loss-of-function’ to ‘gain-of-function’ lies between amino acid residues 528 and 548. Thus, the upper boundary of the ‘gain-of-function domain’ is situated within a stretch of 21 amino acid residues.

In an attempt to map more precisely the location of both upper and lower boundaries, we generated a series of variants and defined a ‘gain-of-function domain’ with a minimal length of 33 amino acid residues ranging from amino acids 544 to 576. In a recent in vitro study, deletion of amino acids 554–587 at the C-ter end of the enzyme (p.A554X) resulted in a higher catalytic activity (16), which is in agreement with our data. Although the ‘gain-of-function domain’ has been identified in the C-ter of human ALAS2, one should be aware of the possibility of existing of other such domains elsewhere in the enzyme. Indeed in the mouse enzyme, ‘hyperactive variants’ of ALAS2 can be generated by substituting very few residues within the active site loop (17). However, so far, no such evidences exist in the human enzyme.

The exact molecular mechanisms of ALAS2 missense mutations causing enzyme deficiency and of deletion
Table 2. Kinetic parameters for human ALAS2 wild-type and variant enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{m}^{\text{Oe}}$ (μM)</th>
<th>$k_{cat}/K_{m}^{\text{Oe}}$ (s$^{-1}$ μM$^{-1}$)</th>
<th>$K_{m}^{\text{Oe}}$ (mm)</th>
<th>$k_{cat}/K_{m}^{\text{Oe}}$ (s$^{-1}$ mm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.030 ± 0.002</td>
<td>4.3 ± 0.6</td>
<td>0.007 ± 0.001</td>
<td>15 ± 3</td>
<td>0.0020 ± 0.0005</td>
</tr>
<tr>
<td>DelAGTG</td>
<td>0.11 ± 0.01</td>
<td>1.2 ± 0.2</td>
<td>0.09 ± 0.02</td>
<td>8.0 ± 0.9</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>Q548X</td>
<td>0.09 ± 0.01</td>
<td>1.3 ± 0.2</td>
<td>0.07 ± 0.01</td>
<td>11 ± 1.5</td>
<td>0.008 ± 0.002</td>
</tr>
<tr>
<td>Del26</td>
<td>0.09 ± 0.01</td>
<td>1.2 ± 0.1</td>
<td>0.08 ± 0.02</td>
<td>17 ± 3</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>DelAT</td>
<td>0.10 ± 0.01</td>
<td>1.0 ± 0.1</td>
<td>0.10 ± 0.02</td>
<td>10 ± 1</td>
<td>0.010 ± 0.002</td>
</tr>
</tbody>
</table>

mutations increasing enzyme activity are not fully understood. The molecular pathophysiology of XLSA has been a subject of research in the past. In an early study, Furuyama and Sassa (18) demonstrated that the interaction between ALAS2 and the β subunit of human ATP-specific succinyl CoA synthetase (SUCLA2) promotes efficient use of succinyl CoA and facilitates translocation of ALAS2 into mitochondria. An ALAS2 mutant (D190V) from a patient with pyridoxine-refractory XLSA, but not those (R441C and M426V) from patients with pyridoxine-responsive XLSA, failed to interact with SUCLA2 (18). In a recent study, Bishop et al. (19) further explored the role of the ALAS2-SUCLA2 interaction in the regulation of erythroid heme biosynthesis. A purified delAT mutant enzyme could bind strongly to a SUCLA2 affinity column, whereas a number of XLSA mutants could not (19). It is also interesting to note that missense mutation Y586F leads to decreased activity (20). This C-terminal missense mutation is located further downstream from the gain-of-function domain we defined for the deletion mutations. This suggests that different and non-exclusive molecular mechanisms, including SUCLA2 affinity, ALAS2 translocation and succinyl-CoA availability, may be involved in the action of ALAS2 mutants.

In conclusion, since the description of XLDPP in 2008, the rapid increase in the number of families diagnosed suggests that XLDDP may be under-diagnosed in children and adults with photosensitivity. The delAGTG mutation is found in 80% of the XLDPP cases without an apparent founder effect. A unique sporadic somatic and germlinal mosaicism mode of expansion of this mutation is demonstrated in one family. This mechanism could be responsible for the worldwide over-representation of the delAGTG mutation and the ubiquitous occurrence of DNA replication errors at this position also justifies general consideration of the delAGTG mutation in de novo XLDDP cases. We have also demonstrated that the 'gain-of-function domain' spans a minimum of 33 amino acids between residues 544 and 576 of human ALAS2. The results of this study further verified the molecular mechanism of XLDDP, and provided a basis for characterization of future XLDDP phenotypes as well as an insight into possible therapeutic targeting.

MATERIALS AND METHODS

Subjects

Patients were identified in specialist porphyria centers in Australia, France, the Netherlands and Switzerland. Blood samples for genetic analysis were obtained from the patients and their relatives after they had given signed informed consent in accordance with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments. Haematological and biochemical parameters had been evaluated by standard methods in the respective referring hospitals. Erythrocyte protoporphyrin was measured as previously described (21) according to the European Porphyria Network guidelines and quality control schemes (www.porphyria-europe.org). The percentage of ZnPP was calculated from fluorescence emission spectra of ethanol or acetone extracts of erythrocyte haemolysates (20).

DNA analysis

Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Mini kit (Qiagen, Chatsworth, CA). Analysis of FECH and ALAS2 genes were performed by bidirectional direct sequencing. All exons, their flanking sequences and promoter were PCR-amplified (primers and conditions are available from the authors on request). PCR products were purified using ExoSAP-IT (USB Products Affymetrics, Cleveland, USA). After purification, 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 on a 3130XL Genetic Analyzer using the Seqscape analysis software (v2.6.0) (Applied Biosystems).

Capillary electrophoresis-laser-induced fluorescence (CE-LIF)

CE-LIF is based on PCR amplification of genomic DNA using fluorescence-labelled primers and separation of the PCR products on a capillary sequencer. The sequencer can precisely separate DNA fragments that differ in size by only 1 nt and quantify each DNA fragment by estimation of the peak area (PA) (22). Genomic DNAs of Patient 4, her mother, a normal female control and a male patient carrying the delAGTG mutation (male II-7, family B, previously described (2)) were diluted at 30 ng/μl. PCR was carried out using an 5′ [JOE]-labelled forward primer to generate a normal product of 108 bp and a deleted one of 104 bp, respectively. PCR conditions and primer sequences can be required from the authors. One microliter of the PCR product was then mixed with 10 μl of Hi-Di Formamide (Applied Biosystems) and 1 μl of ILS600 (Internal Lane Standard 600, Promega). The mixture was denatured for 2 min at 90°C and cooled on ice. Denatured products were then separated on an ABI 3130XL capillary sequencer and results were analysed with GeneMapper v4.0 software (Applied Biosystems). The
normal and the deletion products were expressed as percentage of the PAs in the electropherogram.

Allele segregation analysis using microsatellite markers on chromosome X

A total of 13 microsatellite markers on the X chromosome were selected for the allele segregation analysis among five members of family 4 (Supplementary Material, Table S1). The analysis was performed on an ABI 3130XL capillary sequencer and the results were analysed with GeneMapper v4.0 software (Applied Biosystems). Nine of the 13 microsatellites were informative. They were, therefore, used to construct haplotypes of these individuals.

Site-directed mutagenesis and protein purification

To investigate the effect of the mutation on ALAS2 activity and determine the area of the C-ter domain implicated in gain of function, different mutant enzymes were expressed in E. coli. ALAS2 del26bp, Q548X, A539X, G544X, G576X and V583X mutations were introduced into maltose-binding protein (MBP) fusion pMALc2-AE2 (MalE-ALAS2 WT (8)) by site-directed mutagenesis using the Quick-Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Note that del26bp correspond to an insertion of a TAG codon in place of the 26 bp deletion. Primers can be request from the authors. The sequences of resulting clones were verified to ensure that only desired mutations have been introduced and that the remainder of the sequence was correct.

Expression constructs were transfected into E. coli BL21 DE3 according to the manufacturer’s protocol (Invitrogen, Saint Aubin, France). Different controls were used for assays: ALAS2 WT, ALAS2 C344X, ALAS2 delAT and ALAS2 delAGTG (2). ALAS2 C344X encodes a truncated enzyme shown to be devoid of enzymatic activity (13).

Towards the determination of the steady-state kinetic parameters of the XLDPP variants and wild-type hALAS2, new expression plasmids were constructed, using pGF23 as the vector, such that the encoded proteins were not fused to the MBP (23). The XLDPP variants and wild-type hALAS2

Figure 3. Study of the C-terminal domain of the ALAS2 enzyme. The upper panel is a schematic drawing of the ALAS2 gene: dark boxes represent the untranslated regions and grey boxes the coding exons. All known mutations causing XLSA are listed above the drawing (promoter mutations are not included; * Ducamp et al. 2011 (13); ** Percy et al. 2006 (26); *** Goncalves et al. 2004 (27); **** Pereira et al. 2009 (28); ***** May et al. 2005 (29); others reviewed by Harigae and Furuyama 2010 (9)), whereas all known mutations causing XLDPP are below the drawing. In the lower panel, ALAS2 activity for normal enzyme, negative control (C344X), XLDPP mutations (in black) and different artificial truncated recombinant proteins (in grey) are shown after a 20-minute reaction at both 33°C (empty, grey and black bars) and 37°C (diagonally stripped bars); means and ranges for five experiments are shown.
enzymes were overproduced in E. coli BL21 bacterial cells and purified as previously described by Ferreira and Dailey with some modifications (23). Briefly, BL21 cells harbouring the expression plasmids were grown in a low phosphate medium containing 100 μg/ml ampicillin at 37°C for 16 h. After centrifugation, the bacterial pellet was suspended in buffer A (20 mM potassium phosphate, 1 mM EDTA, 5 mM β-mercaptoethanol, 10% glycerol, 1 mg/ml protamine sulphate and 20 mM PLP, at pH 8.0). The cells were then lysed using a French press cell, and the wild-type and variant enzymes were precipitated with 45% ammonium sulphate. Following the 45% ammonium sulphate precipitation, the pellet was dissolved in buffer A containing 5% dimethyl sulfoxide and loaded onto an Ultrogel Aca-44 gel filtration column equilibrated with buffer A. Following elution, the sample was loaded onto a DEAE-Sephacel column equilibrated with buffer A. The resin was sequentially washed with buffer A, buffer A containing 20 mM KCl and buffer A containing 50 mM KCl. hALAS2 was then eluted with buffer A containing 100 mM KCl. Protein purity was assessed by Sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and the enzyme concentration determined using the bicinchoninic acid assay with bovine serum albumin as standard (24).

ALAS2 assays

ALAS2 activities of both negative and wild-type controls, as well as the mutant enzymes, were determined in bacteria crude lysates, as previously described (13).

Steady-state kinetics

Steady-state kinetics of the reactions of the wild-type hALAS2 and XLDP variants were measured at 30°C using an established coupled enzyme assay (25). In all cases, a 2 μM enzyme was reacted with five different glycine concentrations ranging from 5 to 100 mM, each of which was tested at five different succinyl-CoA concentrations ranging from 1 to 40 mM. This resulted in five-by-five matrices containing 25 data points for each enzyme variant. These data were used to construct primary plots from which the apparent maximal velocities were in turn used to construct secondary plots defining $K_m$ and $k_{cat}$ values.

Immunoblot analysis of recombinant enzymes

To investigate the expression level of the recombinant proteins, we performed western blots using an antibody directed against MBP as previously described (13). Loading and transfer were confirmed by Ponceau red staining.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We would like to thank the patients and their family members for their participation in the study; David Bishop, Mount Sinai School of Medicine, New York, for kindly providing the bacterial expression vector containing the normal ALAS2 cDNA.

Figure 4. Worldwide distribution of ALAS2 mutations in XLDP disease. Since the first characterization of XLDP 4 years ago, delAGTG has been found in 16 unrelated XLDP families from Italy (four families), France (four families), UK (two families), USA (two families), the Netherlands (two families), South Africa (one family) and Switzerland (one family). No evidences of a founder effect have been found among eight unrelated delAGTG families from France, UK, South Africa and Switzerland based on haplotype analysis.
REFERENCES