Supplementary Data

The data supplied in the following is a continuation of the discussion of mutations in the enzyme ALAS that are known to result in XLSA. Discussed here are mutations of eALAS that involve residues not directly involved in binding PLP or either substrate, and that would hence not be expected to directly influence the catalytic activity of eALAS.

Buried XLSA mutations physically distant from the active site (green in Figures 5A and 5B)

R170C/S/H/L: Four substitutions of Arg170 (Arg28) have been identified to date, the largest number for any one position. Arg170 participates in a buried and hence particularly stable salt bridge to Asp198 (Asp55). Mutation of Arg170 to cysteine, serine, histidine or leucine forfeits this stabilizing effect, significantly destabilizing the enzyme. Arg170, furthermore, physically stacks upon the carbonyl group of Ala223 (Ser80) of the glycine rich stretch. Loss of this interaction would destabilize this loop, indirectly affecting PLP binding.

A172T: Ala172 (Arg30) is located at the interface of the N-terminal domain and both catalytic domains, and in particular opposite Gly220 (Gly77) of the glycine-rich stretch. Located near the molecular surface, alanine may be replaced by linear residues such as arginine in ALAS_Rc. Replacing alanine by threonine will, however, destabilize the interface and through the glycine-rich stretch possibly affect PLP binding. This interpretation is supported by this mutation being reported as showing significant responsiveness to PLP.

D263N: Asp263 (Asp120) is located near the twofold symmetry axis of the eALAS dimer and hence close to the symmetrically placed Asp263*. As both lack a positively charged neighbor and a change to the uncharged, isosteric asparagine induces XLSA leads us to speculate that they interact and stabilize the dimer by coordinating a divalent cation.

M426V: Replacing methionine by valine in the hydrophobic core of the catalytic domain constitutes a conservative exchange as both are hydrophobic. Valine is, however, branched at C_3 potentially disrupting the neighboring Gly220 (Gly77) and Ala221 (Ala78) of the glycine-rich stretch and indirectly affecting PLP binding. This could explain the reported responsiveness to PLP.

H524D: His524 (His381) occupies a pocket that is generally hydrophobic. It stacks upon Trp194 (Trp51) thereby significantly stabilizing the interface of N-terminal and C-terminal domain. Substituting histidine for negatively charged aspartate will severely disrupt this interaction as the aspartate will presumably flip out of the hydrophobic pocket. The neighboring conserved residues
Pro520 (Pro377) - Ser521 (Ser378) - Pro522 (Pro379) furthermore interact with the catalytic domain and catalysis may thus also be affected.

**XLSA mutations on the protein surface** (highlighted in blue in Figures 5A and 5B)

**M154I:** Met154 (Ile11) forms a hydrophobic contact to C of Asp408 (Asp265). Replacing it by an isoleucine would normally be considered tolerable. However this position is at the interface of the N-terminal domain with the catalytic domain of the second monomer. Located one helical turn away from Asp159 (Glu17, see below) this may subtly affect the active site closure.

**D159N/Y:** Asp159 (Glu17) binds Arg511 (Arg368) in a salt bridge and presumably stabilizes the closed conformation. Replacing this by any non-negative residue will destabilize this interaction between N- and C-terminal domain, and may slow enzymatic catalysis considerably.

**T161A:** Thr161 (Arg19) is located at the same interface as Asp159 (previous mutation). Replacement by alanine may incur the loss of a stabilizing hydrogen bond, destabilizing the enzyme.

**D190V:** Replacing Asp190 (Asp47) by valine places an unfavorable, hydrophobic residue onto the surface of eALAS, destabilizing the enzyme. This may also affect the association of ALAS with the -subunit of ATP-specific succinyl-CoA synthetase, the enzyme generating succinyl-CoA in erythrocyte mitochondria (Furuyama & Sassa, 2000).

**R204Q:** Arg204 (Gln61) forms a salt bridge to Asp173 (Gly31), linking the N-terminal -helix 1 to the catalytic domain. Loss of this interaction may affect the glycine-rich stretch of the second monomer located immediately below, explaining the partial responsiveness to PLP.

**S251P:** Hydrophilic Ser251 (A108) is located in a pocket on the surface of eALAS. Replacing this with larger hydrophobic and conformationally restrictive proline will serve to destabilize the catalytic domain of ALAS.

**G351R:** In ALAS, Gly208 (corresponding to Gly351 in eALAS) is surface-exposed and far removed from both PLP (~25Å) and succinyl-CoA (~21Å). It does, however, lie opposite the N-terminus of the second monomer in ALAS. As eALAS is N-terminally extended by 130 residues, Gly351 would be buried at the interface of the N-terminal and catalytic domain in human eALAS, an interaction that would be disrupted by replacing glycine by arginine. Why this mutant is PLP responsive eludes a structural explanation.

**R411C/H:** Arg411 (Arg268) in -helix 9 forms a partly solvent-exposed salt bridge to Asp408 (Asp265) on the next helical turn. Replacing this with hydrophobic cysteine or histidine may...
destabilize the loop C-terminal of _9 that includes Thr420 (Ser277) and Thr421 (Thr278), involved in PLP-phosphate binding. Appropriately, this mutation is mildly responsive towards PLP.

R436W, R448Q, R452C/H, R458H: Arg436 (Ala293) in _11 as well as Arg448 (Asp305), Arg452 (Met309) and Arg458 (Lys315) in _12 are all surface-exposed, positively charged residues, poorly conserved in other ALAS. Nevertheless, their replacement (by hydrophobic or hydrophilic residues) induces XLSA. Conspicuously, these residues cluster together on the surface of ALAS, suggesting that they may have a common function possibly to interact with another protein. eALAS associates with the _-subunit of ATP-specific succinyl-CoA synthetase, the enzyme generating succinyl-CoA for eALAS (Furuyama & Sassa, 2000). Interestingly, the mutation D190V (see above), also located on the surface distal to the active site channel, induces the complete loss of this interaction (Furuyama & Sassa, 2000), indicating that the positively charged cluster may be involved in eALAS / succinyl-CoA synthetase interaction.
Legend to supplementary stereo figures:

These figures reproduce the individual panels of Figure 3 but in stereo, for additional clarity:

Figure 1

Electron density of the internal aldimine consisting of PLP (green) covalently bound to Lys248. Only residues in contact with PLP are shown. Residues of the second subunit are marked by an asterisk.

Figure 2

Electron density of PLP-glycine intermediate (cyan). The view is rotated compared to Figure 1 to indicate the residues involved in glycine binding. Arg374 appears vital for glycine recognition. The interaction is, however, weakened by covalent bond formation - possibly aiding later decarboxylation.

Figure 3

In three of four monomers of the succinyl-CoA/ALAS complex, PLP is not covalently bound to Lys248. This is documented by positive difference electron density in the absence of PLP and side chain of Lys248 (green, contoured at 3σ), the negative difference density in “enforced” Schiff-base bond (red, contoured at -3σ, structure in narrow bonds), and the refined electron density of unbound PLP (blue, contoured at 1σ, thick bonds).

Figure 4

The adenine and ribose moieties of succinyl-CoA bind within a hydrophobic (white) pocket with positively charged rim (blue) adjacent to the active site channel.

Figure 5

The electron density is well defined for the 3’-phosphate ADP moiety and for the succinyl-carboxylate group of succinyl-CoA, indicating that the central portion is not rigidly bound in the absence of the second substrate glycine.