ALAD Inhibition by Porphobilinogen Rationalizes the Accumulation of δ-Aminolevulinate in Acute Porphyrias

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ABSTRACT: Patients with major forms of acute hepatic porphyria present acute neurological attacks with overproduction of porphobilinogen (PBG) and δ-aminolevulinic acid (ALA). Even if ALA is considered the most likely agent inducing the acute symptoms, the mechanism of its accumulation has not been experimentally demonstrated. In the most frequent form, acute intermittent porphyria (AIP), inherited gene mutations induce a deficiency in PBG deaminase; thus, accumulation of the substrate PBG is biochemically obligated but not that of ALA. A similar scenario is observed in other forms of acute hepatic porphyria (i.e., porphyria variegate, VP) in which PBG deaminase is inhibited by metabolic intermediates. Here, we have investigated the molecular basis of δ-aminolevulinate accumulation using in vitro fluxomics monitored by NMR spectroscopy and other biophysical techniques. Our results show that porphobilinogen, the natural product of δ-aminolevulinate deaminase, effectively inhibits its anabolic enzyme at abnormally low concentrations. Structurally, this high affinity can be explained by the interactions that porphobilinogen generates with the active site, most of them shared with the substrate. Enzymatically, our flux analysis of an altered heme pathway demonstrates that a minimum accumulation of porphobilinogen will immediately trigger the accumulation of δ-aminolevulinate, a long-lasting observation in patients suffering from acute porphyrias.

INTRODUCTION

Porphyrias are a family of disorders associated with a deficiency (inherited or acquired) in the enzymes of the heme metabolic pathway, leading to abnormally high levels of one or more of the intermediate metabolites and/or associated byproducts (Figure 1).1,2 Porphyrias are divided into erythropoietic, affecting the bone marrow, or hepatic when the enzyme deficiency mainly occurs in the liver.

Hepatic porphyrias tend to be autosomal dominant, and they include acute intermittent porphyria (AIP), variegate porphyria (VP), aminolevulinic acid dehydratase deficiency porphyria (ALA-dP), hereditary coproporphyria (HCP), and porphyria cutanea tarda (PCT).3 Hepatic porphyrias may be divided into acute and nonacute forms. Acute porphyrias include AIP (most frequent) VP, HCP, and ALA-dP. All of these forms of porphyria have in common that the carriers of corresponding gene defects may present acute life-threatening neurological attacks. With the exception of ultrarare ALA-dP, the acute neurological episodes always present a hepatic overproduction of both PBG and ALA.

Each porphyria is associated with a catalytic activity deficiency (or, rarely, hyperactivation) in a specific enzyme of the pathway,1 and they are diagnosed through biochemical analysis of blood, urine, and stool4 followed by genetic analysis.2,5 Figure 1B shows the excretion pattern in urine (green) and feces (brown) for the different porphyrias, which consistently shows a high accumulation in the metabolite that precedes the enzyme with deficient activity but also often accompanied by the accumulation of other metabolites of the pathway.6,7 This bar plot is a semiquantitative estimate based on the reported literature and, for the acute porphyrias, it represents the situation after a crisis. Importantly, such abnormal metabolite accumulation is behind the molecular pathophysiology of porphyrias and, for instance, accumulation of ALA likely exerts toxic effects on nerves, either directly by interacting with receptors for the structurally similar neurotransmitter γ-aminobutyric acid or by forming free radicals and reactive oxygen species.8 The observation that disorders associated with excess production of ALA but not PBG (hereditary tyrosinaemia or lead poisoning) have similar
clinical presentations to acute porphyria supports this hypothesis.

ALA accumulation is observed in ALA-dP and in AIP patients between 20- and 200-fold above normal levels (Figure 1B) and, to a minor extent, in VP. AIP, the most common form of porphyria due to its autosomal dominant character, results from the deficiency in the activity of porphobilinogen deaminase (PBGD, hydroxymethylbilane synthase or uroporphyrinogen I synthase; EC 4.3.1.8). Clinical expression of AIP is characterized by the intermittent occurrence of neurovisceral attacks, usually linked to environmental or acquired factors (nutritional status, steroid hormones or their metabolites, and drugs), which may intermittently induce acute exacerbations.

Albeit their clinical significance, the biochemical mechanism behind the accumulation of metabolites associated with acute porphyrinas has not been experimentally demonstrated. It has been shown that some porphyrins are able to inhibit ALAD, with the subsequent ALA accumulation observed in AIP and other acute porphyrinas, offering a plausible explanation for the pathogenic metabotype.

**Materials and Methods**

**Computational Analysis.** Ligand docking simulation was accomplished using the open-source programs for AutoDock Vina and AutoDock4.2 in combination with AutoDock Tools (ADT). The human aminolevulinate dehydratase structure (PDB: 1E51) was used as a protein receptor. The prediction, optimization, and refinement of ALA and PBG ligand structures were achieved through minimization by applying coarse-grained simulations with a united force field by employing 25 × 106 steps using the Open Babel package (v 2.4.0). Protein–ligand docking was performed at both binding sites of the enzyme where side chains of Tyr196, Lys 199, Tyr205, Phe208, Arg209, Arg221, Lys 252, and Tyr276 residues were flexible. Docking results were analyzed using in-house Matlab scripts and visualized using PyMOL.

**Protein Sample Preparation.** PBGD and UROIIIS expression and purification were described by Fortian et al., and ALAD and UROD expression also followed previously described protocols. Briefly, freshly transformed Escherichia coli BL21 (DE3) cells (Invitrogen) were used for protein expression. Overexpression was induced by the addition of 0.5 mM IPTG overnight at 20 °C. After centrifugation of the cultures, the cell pellets were thoroughly sonicated, and the proteins were purified from the cellular extracts by nickel affinity chromatography, followed by size-exclusion chromatography (Superdex 75 26/60; GE Healthcare) under isocratic conditions, in 20 mM Tris, 150 mM NaCl, pH 8.0. All of the purification steps proceeded at 4 °C to minimize proteolysis, and the resulting proteins were concentrated and stored at 4 °C.

**Enzymatic Assays.** ALAD activity was monitored at 298 K by NMR spectroscopy. All of the samples contained 30 μM ALA and 0.11 mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d₆ (deuterated at all positions other than the methyl groups), used as a reference, and variable amounts of ALA (range: 1–50 mM) and PBG.
The reaction was kept for a period of 400 min and monitored by independent spectra every 20 min (vide infra). The signals for the two methylenes of ALA (at 2.50 and 2.78 ppm) were used as reporters of the reaction progression. The integrals that are normalized to the reference intensity were fitted to an exponential decay, and the initial reaction rate was extracted from a linear extrapolation of the initial decay.

PBGD activity was achieved by a tandem assay with UROS to render UROIII, which was quantified by high-performance liquid chromatography (HPLC). PBGD activity was tested using 10 μM enzyme in 20 mM Tris, 150 mM NaCl, pH 8.0, and a variable amount of PBG (range 0−1.8 mM). The reaction was left for 5 min at 37 °C and then immediately stopped by fast-freezing in liquid nitrogen. After de-icing, UROS was added in the dark (15 μM) to convert the HMB into uroporphyrin III, followed by oxidation with sodium metabisulfite and trichloro acetic acid. Porphyrins were then quantified by HPLC analysis (vide infra).

Fluxomic experiments were performed by tandem enzymatic assays of the cytosolic enzymes of the heme biosynthesis pathway. ALAD, PBGD, UROIIIS, and UROD were at 30, 1, 3, and 8 μM, respectively. The buffer conditions were the same as in the ALAD experiment and PBG. The experiments with a reduction in PBGD activity were obtained by 1/200 dilution (50 nM), 1/500 dilution (2 nM), and 1/1000 dilution (1 nM). For the PBG-doped flux reactions, PBG was added to a 250 μM concentration, and the enzyme concentrations were 8, 1, 1, and 7.6 μM for ALAD, PBGD, UROIIIS, and UROD respectively. The experiments were measured by NMR, with the same conditions as the ALAD experiment, and HPLC to quantify all of the porphyrins.

**HPLC Analysis.** The offspring of an enzymatic assay was separated by HPLC analysis on a ODS Hypersil C18 column (5 μm, 3 × 200 mm; Thermo Scientific, MA, USA) in a Shimadzu HPLC chromatograph (Kyoto, Japan), as previously described.19 Porphyrins were separated with a 60 min gradient elution and a two-component mobile phase consisting of ammonium acetate (1 M, pH 5.16, solvent A) and 100% acetonitrile (solvent B). Gradient elution commenced upon injection at 0% B, which was increased to 65% B for 30 min, held for 5 min, returned to 0% B in 15 min, and held for 10 min to re-equilibrate the column at 0% B before the next injection. The flow rate was 1 mL/min. All analyses were performed at 20 °C and were detected by fluorescence with an excitation wavelength of 405 nm and emission wavelength of 610 nm. A standard commercial lyophilized porphyrin kit containing UROI dihydrochloride, Hepta I heptamethyl ester, Hexa I hexamethyl ester, Penta I pentamethyl ester, and COPRO I tetramethyl ester (Frontier Scientific, Logan, UT) was used to calibrate the porphyrins in the chromatogram.

**NMR Spectroscopy.** All NMR experiments were recorded at 298 K on a Bruker 600 MHz (12 T) Avance III spectrometer or a Bruker 400 MHz (9.4 T), both equipped with a QXI (1H,13C,15N,31P) probehead. Kinetic reactions were followed using a 1D 1H p3919gp with water signal suppression using a binomial 3-9-19 pulse with echo gradient pair (20 min). 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 was used as a reference and for the metabolite quantification.
Data Analysis of the Metabolic Fluxes. The normalized intensities (in equivalents of ALA) from the different metabolites were fitted to a single exponential (ALA, COPROIII, COPROI, and total porphyrin content in the NMR experiment), to a linear function (UROI), or to a double exponential (all of the other metabolites) using in-house built Matlab scripts. The same strategy was used for the integration of the area under the curve, used in the cylinder plots of Figure 3C.

Statistical Analyses. Error bars were obtained from triplicated data and correspond to the standard deviation.

RESULTS

PBG Is a Competitive Inhibitor of ALAD. ALAD is an octamer in which each monomeric subunit catalyzes the condensation of two ALA molecules to render one molecule of PBG.17 X-ray structures of ALAD from different species (including humans) are available as a complex with inhibitors that resemble the substrate, allowing the identification of the active site that stabilizes the substrate via hydrogen bonds and salt bridges.17,20 The enzyme–product complex also belongs to the reaction coordinate, and a high-resolution structure of the E. coli ALAD enzyme cocrystallized with PBG reveals that the pyrrole also occupies the active site and forms similar interactions.21

First, we docked the substrate (ALA) and the product (PBG) into the high-resolution structure of human erythrocyte ALAD (PDB code: 1ES1). Consistent with the above-mentioned structural data, our computational analysis reveals interactions mainly with Tyr196, Lys 199, Tyr205, Phe208, Arg209, Lys 252, Tyr276, and the zinc ion (Figure 2A). Interestingly, according to docking analysis, both ALA and PBG would interact with ALAD with similar affinities, which contrast with the usually low affinities found in the enzyme–product complexes, designed in a way that the product can effectively dissociate.

To investigate the relative affinities of ALA and PBG for ALAD, we have set up an assay to determine its catalytic activity, using NMR spectroscopy. Freshly purified ALAD (30 μM) was incubated with variable amounts of ALA, and the reaction was monitored in real time by 1H NMR, quantifying the disappearance of the ALA signals corresponding to the protons from methylenes (Figure 3B). The dependence of the initial rate with the substrate composition, shown in Figure 2B (solid line), can be fitted to a Michaelis–Menten model to estimate the kinetic parameters of the enzyme ($K_{M} = 695 \mu M$ and $k_{cat} = 31 \text{ s}^{-1}$).
We then investigated the potential effect of PBG on the catalytic activity of human ALAD. As mentioned, structural data\textsuperscript{21} and our docking model of the complex (Figure 2A) support the idea that PBG association to ALAD should compete with the incorporation of the substrate. Consistently, in the presence of 5 mM PBG, the reaction rate of ALAD as a function of the substrate concentration (Figure 2B, dashed line) shows a displacement in the $K_M$ which is characteristic of a competitive inhibition. Yet, it is also clear that $V_{\text{max}}$ (and consequently $k_{\text{cat}}$) is also lowered in the presence of an excess of PBG, a situation that best fits with a mixed inhibition mechanism.

To better characterize such inhibition, we have expanded the experiment and measured ALAD activity within a range of substrate and PBG concentrations. Figure 2C shows ALAD catalytic activity as a function of the PBG concentration (and normalized to the catalytic rate in the absence of an inhibitor). Here, we used reaction equivalents to account for the fact that the enzyme binds two molecules of ALA. Specifically, 1 equiv would correspond to 0.125 mol of any of the porphyrin species, 0.5 mol of PBG, and 1 mol of ALA. This representation is convenient because it becomes independent of the substrate characteristics, displaying the effect of a given excess of inhibitor on the catalytic activity. Inspection of Figure 2C reveals that PBG inhibition of ALAD is very efficient and an excess of 2.5 equiv of PBG already halves ALAD enzymatic activity. Considering all data sets, we obtained a $K_i$ value of 1.8 mM for the inhibition of ALAD by PBG.

**Excess of PBG Does Not Inhibit PBGD.** PBGD catalyzes the condensation of four PBG units to render the linear tetrapyrrole hydroxymethylbilane (HMB) (Figure 1A). In the AIP condition, this enzyme is deficient, and PBG is expected to accumulate. In theory, PBGD inhibition by an excess of the substrate could also partially explain the metabolite accumulation found in this condition and, thus, we decided to evaluate the enzyme activity in a broad range of substrate concentrations. To that end, a tandem assay where the unstable HMB is converted into UROgenIII by the action of UROS (used here as a reagent) and, to less extent, spontaneously to UROgenI is used,\textsuperscript{22} where after the reaction the produced porphyrinogens are subsequently oxidized to porphyrins and quantified by HPLC.

Figure 2D shows the enzyme activity of PBG as a function of the concentration of the substrate. The solid line is the fitting to a Michaelis–Menten model, which agrees well with the experimental data reflecting that no inhibition by an excess of the substrate is observed for this enzyme. The kinetic parameters of the enzyme are $K_M = 125 \mu$M and $k_{\text{cat}} = 120 \text{s}^{-1}$.

**In Vitro Flux Model of the Cytosolic Moiety of the Pathway.** To understand the putative effect of the accumulation of PBG in the heme biosynthetic pathway, we have in vitro tested the cytosolic moiety of the pathway, using purified enzymes and monitoring the metabolite accumulation over time. Specifically, we coupled the reactions of the four cytosolic enzymes: $\delta$-aminolevulinic acid deaminase (ALAD), porphobilinogen synthase (PBGD), uroporphyrinogen III synthase (UROS), and uroporphyrinogen decarboxylase (UROD) (Figure 3A). The reaction was monitored for about 4 h, kept under reducing conditions and in the absence of molecular oxygen. The flow reaction was simultaneously monitored by NMR spectroscopy and HPLC analysis.\textsuperscript{4}$^1$H NMR spectroscopy was used to quantify ALA (methylene peaks at $\delta = 2.77$ and 2.5 ppm), PBG (methylene peak at $\delta = 2.4$ ppm), and the total porphyrin content (complex signal at 2.25 ppm) (Figure 2B). The peaks at $\delta = 2.65$ ppm (porphyrins) and $\delta = 2.57$ ppm (PBG) (Figure 2B) were not used in the integration due to signal overlap. In turn, HPLC can quantify oxidized porphyrins, which are assumed to be the porphyrinogens produced by the reaction: UROgenIII/I and COPROgenIII/I as well as the decarboxylation.

**Figure 4.** Flux analysis as investigated by NMR spectroscopy (A, C, E, G) or HPLC analysis (B, D, F, H). (A, B) Experiment with full PBGD concentration (1 $\mu$M). (C–H) Experiments with a reduction in PBGD activity were obtained by 1/200 (50 nM) (C, D), 1/500 (2 nM) (E, F), and 1/1000 (1 nM) (G, H). The NMR data is represented as molar fraction as a function of time for the ALA consumption (blue circles), PBG formation (green squares), and total porphyrin formation (black squares). The HPLC data shows the absolute porphyrin formation over time in nmol for UROgenI (circles), UROgenIII (squares), COPROgenI (diamonds), and COPROgenIII (stars). The red line corresponds to the best fitting to the corresponding function (linear, exponential, or biexponential, see text).
intermediates Hepta III/I, HexaIII/I, and PentalIII/I (Figure S1).

First, we tuned up the enzyme concentrations not to reflect the intracellular enzyme concentrations but to in vitro produce a metabolic flux that qualitatively approaches the relative metabolite concentrations observed in the cell. Specifically, a combination of 8 mM ALA added to a mixture of the four enzymes (30 μM ALAD, 1 μM PBGD, 3 μM UROS, 8 μM UROD) produced an exponential reduction in the ALA concentration, which, according to NMR analysis, was converted mostly into porphyrins (Figure 4A, represented as a fraction of the ALA conversion into PBG and subsequently into porphyrins). In turn, HPLC analysis shows that COPROgenIII is the major species, with little accumulation of other intermediates (Figure 4B, showing the absolute concentration of the different porphyrin species). Type I byproducts (UROgen1 and COPROgen1) were observed but accumulated at very low concentrations, as typically observed in healthy individuals. Under these experimental conditions, all of the different metabolites in the pathway are created and consumed (all but the end products). ALA decreases monotonically, while PBG is immediately consumed to produce porphyrinogens within the reaction time frame. No accumulation of any other porphyrin or precursor is observed over time, indicating that all of the reactions reach a steady state with no abnormal metabolite accumulation that would preclude the flow progression.

Experimental data was subsequently fitted to a kinetic model. The time evolution of ALA (flux origin), total porphyrin content (as determined by NMR), and COPROgenI/III (end point of the flux, as determined by HPLC) were fitted to a single exponential decay, while the intermediate metabolites in the flow (PBG, UROgenIII, UROgenI, and the decarboxylation intermediates) were fitted to a biexponential model to account for the formation and elimination of the metabolite. UROI formation was adequately fitted to a linear model of accumulation, consistent with its nonenzymatic unimolecular synthesis. Altogether, this situation qualitatively mimics the condition of normal enzymatic flux, also present in healthy individuals. Under these experimental conditions, all of the different metabolites in the pathway are created and consumed (all but the end products). ALA decreases monotonically, while PBG is immediately consumed to produce porphyrinogens within the reaction time frame. No accumulation of any other porphyrin or precursor is observed over time, indicating that all of the reactions reach a steady state with no abnormal metabolite accumulation that would preclude the flow progression.

Inhibition of ALAD by PBG Explains the Accumulation of ALA in a Situation of Deficient PBGD Activity. As mentioned, AIP patients inherit a catalytically deficient PBGD enzyme. To qualitatively reproduce this situation, we progressively reduced the PBGD concentration in our flux experiment (1/200, 1/500, and 1/1000 dilutions), leaving the concentrations of ALA and the other enzymes unperturbed (Figure 4C–H). As expected, a reduction in PBGD activity results in the accumulation of PBG (Figure 4C,E,G, green squares) and also a reduced porphyrin production, due to the limited capacity of the pathway to generate linear tetrapyrroles. Yet, Figure 4 also shows that the accumulation of PBG and that of ALA seem to be coupled, an observation that we attribute to the ALAD inhibition by PBG. Of note, the limited production of porphyrins does not result in the accumulation of any porphyrin intermediate (Figure 4D,F,H), consistent with the absence of accumulation of porphyrins in AIP (Figure 1).

Finally, to in vitro reproduce the situation of a crisis, we repeated the same flux analysis but now leaving the PBGD concentration at the normal value and seeding the reaction with a small amount of PBG (250 μM) (Figure S2). Under these conditions, ALA is still in very large excess with respect to PBG (16-fold excess in the number of equivalents), but now the small amount of PBG is large enough to convert the transient PBG accumulation into a steady-state accumulation, with a plateau of ALA and PBG reached at about 6 h (Figure S2, blue circles). Again, no change in the kinetic profiles nor accumulation of any other porphyrins is observed. Finally, the spontaneous formation of UROI also seems to be unaffected by the ALA/PBG accumulation.

The metabolic accumulation can be better appreciated in the flow diagram (Figure 3C), where the cylinder size is proportional to the accumulation of metabolites in the seeding conditions with respect to the “unseeded” reaction flux (integral value for the 6 h of reaction). Once more, such accumulation is restricted to ALA and PBG at similar sizes. Thus, a small transient accumulation of PBG can alter the delicate equilibria between ALA and PBG, impeding the optimal function of ALAD and resulting in the accumulation of both metabolites. This scenario qualitatively resembles the situation observed during an acute attack in AIP.

Limitations of the Study. The approach we employed is reductionist, and all of the experiments reported in this study are in vitro. Other intracellular factors that may affect the porphyrin accumulation in AIP patients were not considered. The concentrations of the enzymes in the flow analyses may not reflect the intracellular concentrations of the heme pathway.

■ DISCUSSION

The pathophysiology of porphyrias is directly associated with an abnormal accumulation of heme-pathway-associated metabolites in the body and biofluids. Plasma and urinary concentrations of ALA and PBG are always markedly increased during acute symptomatic crises in AIP, and ALA is also found elevated in the other forms of acute porphyria.5,11 ALA is supposed to be responsible for the neurotoxic effect observed in AIP,23–25 reduced ALAD is associated with an elevated risk of lead poisoning,25 and ALA accumulation in the brain might be related to an oxidative damage of different components, including the glutamate transporter.26 Yet, albeit its clinical significance, the molecular mechanism of ALA accumulation during a crisis episode in an acute porphyria has not been investigated at the biophysical level.

Here, we have employed a biophysical approach to demonstrate that, in vitro, PBG is a mixed inhibitor of ALAD, reducing the ability of the substrate to reach the active site, which ultimately results in the accumulation of ALA. Such inhibition is totally consistent with the observation that the product release is the limiting step in the ALAD reaction.14 The relatively high affinity of PBG for ALAD as compared to the substrate implies that an excess of a bit more than two-fold in the product/substrate ratio may already inhibit more than half of the enzyme. The similar affinities of ALA and PBG for the enzyme can be structurally explained by the hydrogen bonds and electrostatic interactions that both metabolites share with the active site residues in human ALAD. In any case, a deficient activity of PBGD due to an inherited deleterious mutation will produce an enrichment of the two precursors, ALA and PBG, via PBG inhibition of ALAD, as observed in AIP patients. This contrasts with the alternative mechanism where an excess of PBG would block the pathway by directly inhibiting PBGD.
To investigate ALA and PBG accumulation in conditions closer to the ones observed in the cell, we have used heme fluxomics. Our flow analysis does not pretend to quantitatively reproduce the heme pathway in the erythrocytes nor in the liver, but it has proven useful to gain qualitative insight into the effect of a deficient PBGD activity and a situation that reproduces the crisis occurring in AIP patients and other acute porphyrias patients. In AIP, a genetic defect in PBGD producing a significant reduction in PBGD activity in conjunction with impaired heme-mediated repression of hepatic ALAS (ALAS1) will lead to a marked overproduction and accumulation of ALA and PBG. In our flux experiments, the impaired heme repression of the pathway was simulated by the large initial ALA concentration, while the defect in PBGD was simulated either by reducing the enzyme concentration or by supplementing the reaction flux with an initial concentration of PBG. Under these conditions, our in vitro flux analysis shows that the pathway has certain tolerability to PBG accumulation and, below a certain PBG concentration, the flux is able to assimilate the PBG excess, fueling it upstream of the pathway. This situation offers a plausible explanation for a normal pathway or for the condition found in AIP patients in the absence of a crisis. On the other hand, above a certain threshold, the excess of PBG will significantly inhibit ALAD, modulating the flow and triggering the accumulation of both metabolites, as observed in a crisis in AIP patients. That said, it is important to emphasize the in vitro character of the experiment that does not contemplate many other factors occurring in the cell.

Interestingly, the in vitro metabolic flow still maintains the metabolite accumulation after 7 h. This situation is also comparable to AIP patients who, after the recovery from an acute attack, may continue with increased levels of the precursors during the asymptomatic phase, a condition that may persist for a long period of time.

In summary, we have found that inhibition of ALAD by PBG provides a plausible explanation for the accumulation of ALA in AIP and other acute porphyrias. Based on this mechanism, we hypothesize a model where, to trigger a crisis, the activation of ALAS is required due to a transitory heme shortage and a minimum accumulation of PBG. This mechanism also provides a molecular rationale for the existing therapeutic intervention lines such as the hemin infusion (palliative or preventive), carbohydrate loading, and targeting of hepatic ALAS1 by small interfering RNA vectors (Givosiran), also supporting the plethora of novel therapies under development.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00434.

Two Figures with additional experiments (PDF)

Access Codes
Human porphobilinogen deaminase (PBGD). NCBI accession code: AAA60029
Human δ-aminolevulinate deaminase (ALAD). NCBI accession code: AAH00977
Human uroporphyrinogen III synthase (UROS). NCBI accession code: AAG36795
Human uroporphyrinogen decarboxylase (UROD). NCBI accession code: AAC50482

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Notes
The authors declare no competing financial interest.

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REFERENCES

(1) Balwani, M.; Desnick, R. J. The porphyrias: advances in diagnosis and treatment. Blood 2012, 120, 4496–4504.
(2) Elder, G.; Harper, P.; Badminton, M.; Sandberg, S.; Deybach, J. C. The incidence of inherited porphyrias in Europe. J. Inherited Metab. Dis. 2013, 36, 849–857.
(3) Stein, P. E.; Badminton, M. N.; Rees, D. C. Update review of the acute porphyrias. Br. J. Haematol. 2017, 176, 527–538.
(4) Aarsand, A. K.; Petersen, P. H.; Sandberg, S. Estimation and application of biological variation of urinary delta-aminolevulinic acid and porphobilinogen in healthy individuals and in patients with acute intermittent porphyria. Clin. Chem. 2006, 52, 650–656.
(5) Baumann, K.; Kauppinen, R. Penetrance and predictive value of genetic screening in acute porphyria. Mol. Genet. Metab. 2020, 130, 87–99.
(6) Marsden, J. T.; Rees, D. C. Urinary excretion of porphyrins, porphobilinogen and delta-aminolevulinic acid following an attack of acute intermittent porphyria. J. Clin. Pathol. 2014, 67, 60–67.
(7) Anderson, K. E.; Bloomer, J. R.; Bonkovsky, H. L.; Kushner, J. P.; Pierach, C. A.; Pimstone, N. R.; Desnick, R. J. Recommendations for the diagnosis and treatment of the acute porphyrias. Ann. Intern. Med. 2005, 142, 439–450.
(8) Ricci, A.; Di Pierro, E.; Maracci, M.; Ventura, P. Mechanisms of Neural Damage in Acute Hepatic Porphyrias. Diagnostics 2021, 11, 2205.
(9) Jordan, P. M.; Warren, M. J.; Mgbeje, B. I. A.; Wood, S. P.; Cooper, J. B.; Louie, G.; Brownlie, P.; Lambert, R.; Blundell, T. L. Crystallization and preliminary X-ray investigation of Escherichia coli porphobilinogen deaminase. J. Mol. Biol. 1992, 224, 269–271.
(10) Pluta, P.; Roversi, P.; Bernardo-Seisdedos, G.; Rojas, A. L.; Cooper, J. B.; Gu, S.; Pickersgill, R. W.; Millet, O. Structural basis of pyrrole polymerization in human porphobilinogen deaminase. Biochim. Biophys. Acta, Gene. Subj. 2018, 1882, 1948–1955.
(11) Schmitt, C.; Lenglet, H.; Yu, A.; Delaby, C.; Benecke, A.; Lefebvre, T.; Letteron, P.; Paradis, V.; Wahlin, S.; Sandberg, S.; Harper, P.; Sardh, E.; Sandvik, A. K.; Hof, J. R.; Aarsand, A. K.; Chiche, L.; Bazille, C.; Scoazec, J. Y.; To-Figueras, J.; Carrascal, M.; Abian, J.; Mirmiran, A.; Karim, Z.; Deybach, J. C.; Puy, H.; Peoc’h, K.; Manceau, H.; Gouya, L. Recurrent attacks of acute hepatic porphyria: major role of the chronic inflammatory response in the liver. J. Intern. Med. 2018, 284, 78–91.
(12) Afonso, S. G.; Enríquez De Salamanca, R.; Batlle, A. Folding and unfolding of delta-aminolevulinic acid dehydratase and porphobilinogen deaminase induced by uro- and protoporphyrin. Int. J. Biochem. Cell Biol. 1997, 29, 493–503.
(13) Afonso, S. G.; Chimarro, S.; de Salamanca, R. E.; Del Carmen Batlle, A. M. delta-Aminolevulinic acid dehydratase inactivation by uroporphyrin I in light and darkness. Int. J. Biochem. 1994, 26, 255–258.
(14) Jaffe, E. K. The porphobilinogen synthase catalyzed reaction mechanism. Bioorg. Chem. 2004, 32, 316–325.
(15) Trott, O.; Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 2010, 31, 455–461.
(16) Fortian, A.; Castaño, D.; Ortega, G.; Lain, A.; Pons, M.; Millet, O. Uroporphyrinogen III Synthase Mutations Related to Congenital Erythropoietic Porphyria Identify a Key Helix for Protein Stability. Biochemistry 2009, 48, 454–461.
(17) Erskine, P. T.; Norton, E.; Cooper, J. B.; Lambert, R.; Coker, A.; Lewis, G.; Spencer, P.; Sarwar, M.; Wood, S. P.; Warren, M. J.; Shoeling-Jordan, P. M. X-ray structure of S-aminolevulinic acid dehydratase from Escherichia coli complexed with the inhibitor levulinic acid at 2.0 A resolution. Biochemistry 1999, 38, 4266–4276.
(18) Phillips, J. D.; Parker, T. L.; Schubert, H. L.; Whitby, F. G.; Hill, C. P.; Kushner, J. P. Functional consequences of naturally occurring mutations in human uroporphyrinogen decarboxylase. Blood 2001, 98, 3179–3185.
(19) Urquiza, P.; Lain, A.; Sanz-Parras, A.; Moreno, J.; Bernardo-Seisdedos, G.; Dubus, P.; González, E.; Gutiérrez-de-Juan, V.; García, S.; Eraña, H.; San Juan, I.; Macías, I.; Ben Bdira, F.; Pluta, P.; Ortega, G.; Oyarzábal, J.; González-Muñoz, R.; Rodríguez-Cuesta, J.; Anguita, J.; Díez, E.; Blouin, J.-M.; de Verneuil, H.; Mato, J. M.; Richard, E.; Falcón-Pérez, J. M.; Castilla, J.; Millet, O. Repurposing ciclopirox as a pharmacological chaperone in a model of congenital erythropoietic porphyria. Sci. Transl. Med. 2018, 10, eaat7467.
(20) Coates, L.; Beaven, G.; Erskine, P. T.; Beale, S. I.; Avissar, Y. J.; Gill, R.; Mohammed, F.; Wood, S. P.; Shoeling-Jordan, P.; Cooper, J. B. The X-ray structure of the plant like S-aminolevulinic acid dehydratase from Chlorobium vibrioforme complexed with the inhibitor levulinic acid at 2.6 A resolution. J. Mol. Biol. 2004, 342, 563–570.
(21) Mills-Davies, N.; Butler, D.; Norton, E.; Thompson, D.; Sarwar, M.; Guo, J.; Gill, R.; Azim, N.; Coker, A.; Wood, S. P.; Erskine, P. T.; Coates, L.; Cooper, J. B.; Rashid, N.; Akhtar, M.; Shoeling-Jordan, P.; M. M. Structural studies of substrate and product complexes of S-aminolevulinic acid dehydratase from humans, Escherichia coli and the hyperthermophile Pyrococcus furiosus. Acta Crystallogr. Sect. D, Struct. Biol. 2017, 73, 9–21.
(22) Shoeling-Jordan, P. M.; Leadbeater, R. Coupled assay for uroporphyrinogen III synthase. Methods Enzymol. 1997, 281, 327–336.
(23) Bonkowsky, H. L.; Schady, W. Neurologic manifestations of acute porphyria. Semin. Liver Dis. 1982, 2, 108–124.
(24) Becker, D. M.; Kramer, S. The neurological manifestations of porphyria: A review. Medicine 1977, 56, 411–423.
(25) Kelada, S. N.; Shelton, E.; Kaufmann, R. B.; Khoury, M. J. Delta-Aminolevulinic Acid Dehydratase Genotype and Lead Toxicity: A HuGE Review. Am. J. Epidemiol. 2001, 154, 1–15.
(26) Emanuelli, T.; Pagel, F. W.; Porciúncula, L. O.; Souza, D. O. Effects of S-aminolevulinic acid on the glutamatergic neurotransmission. Neurochem. Int. 2003, 42, 115–121.