Disease variants of human Δ¹-pyrroline-5-carboxylate reductase 2 (PYCR2)

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ABSTRACT

Pyrroline-5-carboxylate reductase (PYCR in humans) catalyzes the final step of l-proline biosynthesis by catalyzing the reduction of L-Δ¹-pyrroline-5-carboxylate (L-P5C) to l-proline using NAD(P)H as the hydride donor. In humans, three isoforms PYCR1, PYCR2, and PYCR3 are known. Recent genome-wide association and clinical studies have revealed that homozygous mutations in human PYCR2 lead to postnatal microcephaly and hypomyelination, including hypomyelinating leukodystrophy type 10. To uncover biochemical and structural insights into human PYCR2, we characterized the steady-state kinetics of the wild-type enzyme along with two protein variants, Arg119Cys and Arg251Cys, that were previously identified in patients with microcephaly and hypomyelination. Kinetic measurements with PYCR2 suggest a sequential binding mechanism with L-P5C binding before NAD(P)H and NAD(P)⁺ releasing before L-Pro. Both disease-related variants are catalytically impaired. Depending on whether NADPH or NADH was used, the catalytic efficiency of the R119C protein variant was 40 or 366 times lower than that of the wild-type enzyme, while the catalytic efficiency of the R251C protein variant was 7 or 26 times lower than that of the wild-type enzyme. In addition, thermostability and circular dichroism measurements suggest that the R251C protein variant has a pronounced folding defect. These results are consistent with the involvement of Arg119Cys and Arg251Cys in disease pathology.

1. Introduction

Δ¹-pyrroline-5-carboxylate reductases (PYCRs or P5CRs) have been identified as important housekeeping enzymes of l-proline biosynthesis and are broadly distributed across all three domains of life [1]. l-Proline is synthesized from l-glutamate by the sequential action of Δ¹-pyrroline-5-carboxylate (L-P5C) synthase (a.k.a. aldehyde dehydrogenase 18A1, ALDH18A1) and PYCR (Scheme 1). l-Proline is converted to l-glutamate via proline dehydrogenase (PRODH) and L-glutamic semi-aldehyde (L-GSAL) dehydrogenase (GSALDH) (a.k.a. aldehyde dehydrogenase 4A1, ALDH4A1) (Scheme 1). Both pathways share the intervening L-P5C/L-GSAL equilibrium step.

In terms of the current state of knowledge for human PYCR (EC 1.5.1.2), a 2012 study on proline biosynthesis from melanoma tissue extracts characterized three separate human genes encoding isozymes PYCR1, PYCR2, and PYCR3 [2]. Subcellular localization studies identified PYCR3 as a cytosolic enzyme whereas PYCR1 and PYCR2 were identified as mitochondrial enzymes [2]. Pairwise global sequence alignment of PYCR1 (NCBI RefSeq NP_008838.2, UniProtKB P32322) and PYCR2 (NCBI RefSeq NP_037460.2, UniProtKB Q96C36) indicates 84.4% amino acid sequence identity between the two proteins [3]. To date, structural knowledge of P5CR consists of X-ray crystal structures from seven organisms (Homo sapiens, Bacillus cereus, Coxiiella burnetii, Streptococcus pyogenes, Neisseria meningitides, Plasmodium falciparum, and Medicago truncatula) deposited in the Protein Data Bank [4]. Among the human enzymes, X-ray crystal structures of PYCR1 and PYCR2 have been reported [5–7]. A 1.85 Å structure of the ternary complex of human PYCR1 with NADPH and L-tetrahydro-2-furoic acid (L-THFA), an analog of l-proline (PDB code 5UAV), provides insights into the active site and catalytic mechanism [5]. This structure resolved a question about the location of the active site, showing unequivocally that NADPH binds in the Rao-Rossmann dinucleotide binding domain, which is consistent
with structures of P5CR from plants and bacteria [8]. Although progress has been made on the structural and functional characterization of human PYCR1, considerably less is known about human PYCR2, which is the focus of this work.

L-proline biosynthesis has been implicated in different types of cancers. Besides supporting protein synthesis, L-proline biosynthesis has been shown to promote tumorigenesis by providing NAD(P)H-derived reducing equivalents to support aerobic glycolysis and the oxidative branch of the pentose phosphate pathway [9]. Upregulation of PYCR2 has been found in different cancers such as melanoma, breast, prostate, esophageal tumors, lung metastasis, Kaposi’s sarcoma and primary effusion lymphoma, and cervical cancer [2,10–13]. In a mass spectrometry-based screening of metabolites in tissue sections from 256 esophageal squamous cell carcinoma (ESCC) patients, Sun et al. (2019) found a significant upregulation of L-proline metabolism in the cancer region compared to the normal epithelium and muscle regions [11]. Immunohistochemistry staining of the ESCC tissue sections identified PYCR2 as the most enriched metabolic enzyme in the cancer region, consistent with increased L-proline found in the same tissue region [11]. In two separate human melanoma cell lines, tumor cell death was induced by silencing PYCR2 expression, indicating a critical role for PYCR2 in cancer cells [14]. In Kaposi’s sarcoma cells, Choi et al. (2020) verified that Kaposi’s sarcoma-associated herpesvirus K1 oncoprotein binds endogenous PYCR2 to commandeer L-proline biosynthesis for increased intracellular L-proline production that promotes in vitro 3D spheroid culture growth and tumorigenesis [12]. The viral K1 protein enhanced PYCR2 enzymatic activity by lowering the \( K_M \) for L-P5C by 4-fold and diminishing product inhibition by L-proline [12].

Autosomal recessive disorders of PYCR1 and PYCR2 have been specifically linked to hypoprolinemia, cutis laxa and progeroid syndrome, microcephaly, and neurological disorders. Mutations in PYCR1 generally lead to cutis laxa type IIB (OMIM # 612940) [15] whereas mutations in PYCR2 are uniquely linked to genetic hypomyelinating leukodystrophy type 10 with microcephaly (OMIM # 616420) [16–18]. Thus, it has been suggested that PYCR1 mutations are most strongly associated with skin disorders while PYCR2 mutations appear more prominent in neurological disorders [18]. In a 2016 study of PYCR2 mutations in patients with lethal microcephaly, loss of PYCR2 was proposed to impair mitochondria function and disrupt oxidative balance, namely \( \text{NAD(P)}^+ \) levels [18]. Thus, PYCR2 not only drives cellular supply of L-proline but it is also important for redox cycling of \( \text{NAD(P)}^+ \).

The motivation to investigate PYCR2 and disease variants stems in part from a study by Nakayama et al. (2015), which showed that two families were affected by the same allelic autosomal-recessive condition in the PYCR2 gene [17]. Two PYCR2 variants, R119C and R251C, were

**Scheme 1. Enzymatic reactions of proline metabolism in the mitochondrion.** Structural protein images are PyMol renderings of the following X-ray crystal structures: dimeric view of TIPRODH (PDB code 2EKG), dimeric view of human PYCR1 (PDB code 5UAV), tetrameric view of human ALDH4A1 (PDB code 4OE5), and monomeric view of the L-glutamate-5-phosphate reductase domain of human ALDH18A1 (PDB code 2HSG).
each identified by a genome-wide association study to have a statistically significant linkage to the observed neurological traits of hypomyelination and microcephaly in the patients. These findings, along with the growing recognition of PYCR2 being implicated in different cancers, motivated our investigation of the kinetic mechanism of PYCR2 and the biochemical penalties of the clinically relevant R251C and R119C protein variants on PYCR2 function. Here we report the steady-state kinetic parameters of PYCR2 wild-type and protein variants R251C and R119C along with thermostability and secondary structure analysis. Product(s) inhibition studies of PYCR2 wild-type indicate a sequential-order binding mechanism with L-P5C binding first.

2. Materials and methods

2.1. Materials and reagents

Milli-Q Ultrapure water was used for all experiments. The following reagents were purchased from Millipore-Sigma: DL-5-hydroxylysine hydrochloride, sodium metaperiodate, Dowex 50WX4 hydrogen form (200–400 mesh) cation exchange resin, sodium phosphate buffer, ε-amino-N-caproic acid, N-tosyl-l-phenylalanine chloromethyl ketone, bovine serum albumin, NADPH, NADH, and ammonium fluoride. The following reagents were from Fisher Scientific: LB Agar Miller, ampicillin, yeast extract, NaOH, NaCl, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and ethylenediamine tetraacetic acid (EDTA) disodium salt. Glyceraldehyde and tryptophan were purchased from Research Products International. Reagents β-ocetyl-o-glucopyranoside (β-OG) and β-proline were purchased from Combi-Blocks. Ethanol (190- or 200-proof) was purchased from Decon Labs. Tris(hydroxymethyl)aminoethane (Tris), glycine, isopropropyl β-thiogalactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), leupeptin hemisulfate, and NAD+ were purchased from Gold Biotechnology.imidazole and (Oε2)aminobenzaldehyde (o-AB) were purchased from Acros Organics. Hydrochloric acid (HCl) 34–37% Omnitrace was purchased from EMD-Millipore.

2.2. DL-P5C synthesis

DL-P5C was synthesized according to a modified form of the protocol by Williams and Frank [19], which involved the metaperiodation of DL-5-hydroxylysine hydrochloride at 4 °C with the reaction product purified by Dowex 50WX4 cation exchange column chromatography using 1 M HCl to elute the fractions of DL-P5C. Synthesized DL-P5C was quantified using the o-AB assay in which DL-P5C and o-AB react to form a yellow conjugate that is measured at 443 nm (ε443 = 2590 M−1 cm−1) [20]. DL-P5C stock solutions were stored in 1 M HCl at 4 °C. DL-P5C as the main reaction product was confirmed by liquid chromatography-electrospray ionization-multiple reaction monitoring (LC-ESI-MRM) mass spectrometry. Additional details concerning the synthesis and mass spectrometry characterization of DL-P5C are provided in the Supplementary Materials (Sup. Fig. 1).

2.3. Site-directed mutagenesis of PYCR2

The DNA sequence encoding human (Homo sapiens) PYCR2 wild-type transcript 1 (NCBI RefSeq NM_013328.4) was cloned into the pKAbH vector at the BamHI and NdeI sites with an N-terminal (8x)His-tagged sequence (pKAbH/PYCR2). The pKAbH/PYCR2 construct includes an ampicillin resistance marker gene and was used as a template for site-directed mutagenesis. We used a modified protocol from the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Life Technologies) to introduce mutations via overlapping mutagenic and non-mutagenic sequen

Expression and purification of PYCR2 wild-type and protein variants

The pKAbH/PYCR2 constructs for wild-type and the R251C and R119C protein variants were transformed into E. coli BL21 (DE3) pLysS competent cells (Novagen-EMD Millipore Sigma) for recombinant protein expression. Cells were grown on LB Agar Miller plates containing 50 pg/ml ampicillin and 34 μg/ml chloramphenicol (Chem-Impex International). Starter cultures from the growth plates were then made in LB broth, grown overnight and used to inoculate six 1 L-cultures of Terrific Broth media (prepared from recipe in Sambrook et al. [1990] consisting of tryptone, yeast extract, glycerol, and water) [21], which also contained 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The 1 L cultures were grown at 37 °C (180 rpm) until reaching an optical density at 600 nm (OD600) ≈ 0.8–1.0. The temperature was then lowered to 18 °C and IPTG (0.4 mM final concentration) was added to the cultures to induce recombinant protein expression. After induction with IPTG for 10 h (18 °C, 180 rpm), cultures were then treated with 100 μg/ml chloramphenicol with continued shaking at 180 rpm at 18 °C for an additional 2 h. The chloramphenicol treatment was found to improve the solubility of the PYCR2 proteins, as chloramphenicol was previously reported to enhance chaperone-assisted folding of target proteins [22]. Cells were then harvested by centrifugation (5400×g) (Avanti J-E High-Speed Centrifuge, Beckman Coulter) for 20 min at 4 °C. Cell pellets were stored at −80 °C. Upon thawing, the cell pellets were resuspended in NPI-5 cell lysis buffer (5 mM imidazole, 500 mM NaCl, 50 mM sodium phosphate buffer, pH 7.5) containing 500 μM Tris(3-hydroxypropyl)phosphine reducing agent (Strem Chemicals), 0.01% TritonX-100 detergent (Amresco), 0.01% Brij-35 polyoxyethylene detergent (Santa-Cruz Biotechnology), 10 mM β-OG, and five protease inhibitors (β-naphtho-N-caprylic acid, PMSF, leupeptin hemisulfate, N-tosyl-l-phenylalanine chloromethyl ketone, and β-naphtyl-l-lysine chloromethyl ketone). The cell suspension was then sonicated for 5 min with 5 s on and 15 s off pulse settings using a Sonic Dismembrator (Model F 550, Fisher Scientific). The resulting cell lysates were centrifuged (30960×g) for 1.5 h at 4 °C. PYCR2 (wild-type and protein variants) was observed in both the insoluble and soluble fractions. Recovery of enzyme from the insoluble fraction was unsuccessful; therefore, only the protein in the soluble fraction was purified and analyzed.

PYCR2 proteins were purified at 4 °C by immobilized metal affinity column chromatography (IMAC) using a Ni2+-NTA Superflow resin (Qiagen). A stepwise gradient of 5 mM, 60 mM, and 500 mM imidazole in 50 mM sodium phosphate (pH 8) buffer was used to elute the PYCR2. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% β-mercaptoethanol (Acros Organics) as a reducing agent. Fractions containing PYCR2 were pooled and dialyzed (10 kDa molecular weight cutoff (MWCO) Dialysis Snakeskin Tubing, ThermoFisher Scientific-Pierce Biotech) into 50 mM HEPEs (pH 7.5) buffer containing 250 mM NaCl, 0.5 mM EDTA, 500 μM THPP, and 10% (v/v) glycerol. PYCR2 proteins were concentrated using an ultrafiltration stirrer cell (Model 8050, Amicon, EMD Millipore-Sigma) and with a 5 kDa PLC low bind 44.5 mm diameter ultrafiltration disc (EMD Millipore-Sigma) at 4 °C until the protein solution volume was reduced to approximately 5 ml. Yields for each purified PYCR2 protein were typically 0.3–4 mg per 1 g of wet cell pellet. Aliquots of 1 ml were then placed into 2 ml microtubes and flash-
frozen in liquid nitrogen. The tube lids were subsequently pierced with a fine needle and the frozen samples were then further concentrated by freeze-dry vacuum lyophilization (FreeZone Bulk Tray Dryer, Labconco) for 3–5 h until the volume was reduced to 250 μl. The resulting concentrated protein was next exchanged into fresh 50 mM HEPES (pH 7.5) buffer as described above by microdialysis using inverted 15 kDa MWCO Tube-O-Dialyzer tubes (G Biosciences-Geno Technology). Protein concentration was quantified by the 660 nm Pierce Protein Dye Assay (ThermoFisher Scientific-Pierce Biotech) using bovine serum albumin standards as per manufacturer’s instructions. Finally, all protein samples were flash-frozen in liquid nitrogen in 500 μl aliquots and stored at –80 °C. Proteins were analyzed by SDS-PAGE under reducing conditions on a 4–20% gradient gel and visualized using Bio-Safe Coomassie Blue G250 stain (Sup. Fig. 2). The N-terminal (8x)His tag was retained in all the purified PYCR2 proteins.

2.5. Kinetic characterization of PYCR2 enzymes

Kinetic assays of PYCR2 were performed at 37 °C in 0.1 M Tris-HCl (pH 7.5) reaction buffer containing 0.01% Brij-35 detergent and 1 mM EDTA using a protocol modified from previous studies [6,23-25]. The catalytic activity was monitored by measuring the L-PSC-dependent oxidation of NAD(P)H at 340 nm and at 380 nm, using millimolar extinction coefficients 6.22 mm−1 cm−1 and 1.314 mm−1 cm−1, respectively [26,27]. The concentrations of NADPH and NADH along with NADP+ (VWR International) and NAD+ in 10 mM Tris-HCl pH 8 buffer, were spectrophotometrically determined and stock solutions were stored at –80 °C. DL-PSC substrate solution stored at 4 °C in 1 M HCl, was neutralized to pH 7.5 just prior to enzyme assays by adding 1 M Tris-HCl (pH 9.0) buffer. We note that at pH 7.5, the equilibrium between L-PSC and L-GSL strongly favors the cyclic imine form L-PSC [27]. NAD(P)H was varied (0–580 μM) while holding DL-PSC fixed at 5000 μM and, DL-PSC was varied (0–5500 μM) while holding NAD(P)H fixed at 500 μM. NAD+ product inhibition assays were performed by holding DL-PSC constant (5000 μM) while varying NADH (0–500 μM) at different NAD+ concentrations (0–500 μM). Assays testing l-proline inhibition were conducted by holding NADH constant (500 μM) and varying DL-PSC (0–5500 μM) at different l-proline concentrations (0–500 μM). For assays in which DL-PSC was varied, the ionic strength was maintained at approximately 450–480 mM Tris+·Cl− at each DL-PSC concentration by adding a balance of 1 M Tris-HCl (pH 7.5) buffer into the reaction mixture. The final assay volume was 600 μl in 1.5 ml rectangular polystyrene spectrophotometer cuvettes (1 cm pathlength) (USA Scientific). Reactions were prewarmed at 37 °C for 5 min and initiated by adding the enzyme last. The absorbance decrease due to oxidation of NAD(P)H was monitored at 340 and 380 nm in a Varian Cary Bio 50 UV-Vis Spectrophotometer (Agilent Technologies) against blanks from which the varied substrate had been omitted. Final concentrations of PYCR2 enzyme used in the assays, [E], were 0.06 μM wild-type, 0.6 μM R251C variant, and 3 μM R119C variant.

Enzyme activity expressed as initial reaction velocity (v0), in units μM s−1, was calculated using a modified equation of the modern formulation of the Bouguer-Beer-Lambert law of optical spectroscopy [28] from the linear portion of the initial absorbance decrease per unit time. Assays were performed in triplicate and data were plotted as mean ± standard deviation. Data were fit by nonlinear regression to the Henri-Michaelis-Menten equation using SigmaPlot 12.0 (version 12.0.0.182, Systat Software) to determine the theoretical maximal reaction velocity limit (Vlim) and the apparent Michaelis-Menten constant (Kapp) parameters (value ± standard error). The Kapp for L-PSC was assumed to be half the Kapp value for the racemic DL-PSC mixture. The apparent catalytic turnover rate (kcatapp) and substrate specificity constant or catalytic efficiency values (kcatapp/Kapp) were calculated from Vlim and Kapp.

Product(s) inhibition kinetics were analyzed by global fitting of the data to different inhibition models using Enzyme Kinetics Wizard Add-in on SigmaPlot 12.0 and by Hanes-Woolf plot analysis. Additional details pertaining to the equations used for global fitting models of steady-state kinetics and of product(s) inhibition kinetics are provided in the Experimental Procedures section of the Supplementary Materials.

2.6. Thermal stability assays

Thermofluor assays were used to determine the thermal stabilities of PYCR2 wild-type and protein variants. Each protein was microdialyzed into 50 mM HEPES (pH 8.0) buffer containing 150 mM NaCl, 0.5 mM EDTA, 500 μM THPP reducing agent, and 10% (v/v) glycerol. Assays were performed in a 50 μl reaction volume using white 8-strap 0.2 ml PCR tubes with individual flat optically clear caps (Phexin Research Products). The reaction mixtures included 6.4 μM of enzyme alone, and addition of 1 mM NAD+ and/or 1 mM l-proline (pH 8.0) with the reaction volume balanced with 100 mM potassium phosphate (pH 7.0) buffer. Sypro Orange fluorescent dye (λex = 470 nm, λem = 570 nm, Molecular Probes) was added last at (25X), and then the PCR tubes were covered with aluminum foil to protect from light during mild agitation using a rocking platform for 4 h at 4 °C. Next, a mini plate spinner (Model MPS C1000, LabNet) was used for 60 s at 2500 rpm to force liquid contents towards the bottom of the PCR tubes. Sample tubes were then loaded into an iCycler chassis (Bio-Rad) to initiate data collection using the MyIQ Single-Color Real-Time PCR Detection System with optics module (CCD camera bandpass optical Filter 2 position at (λex = 485 ± 20 nm, λem = 530 ± 30 nm)) using iQ5 Optical System software (Bio-Rad, version 2.1). Thermal denaturation curves were recorded using a 10 min thermal equilibration period at 20 °C followed by increasing temperature increments at 0.2 °C per 15 s from 20-90 °C. The instrument’s software provided overlay plots of relative fluorescence units (RFU) vs. temperature (T) and of -d(RFU)/dT vs. T. From the raw numerical data, mean values of triplicate control blank curves without the enzyme were subtracted from corresponding mean values of quadruplicate curves with the enzyme for data analysis using Microsoft Excel 2017 and SigmaPlot 12.0. The local trough minima from the plots of -d(RFU)/dT vs. T for each protein are the reported melting point temperature (Tm) values.

2.7. Circular dichroism (CD) spectroscopy

PYCR2 wild-type and protein variants were microdialyzed into 10 mM sodium phosphate buffer (pH 8.0) containing 250 mM ammonium fluoride for 16 h at 4 °C. Then, 1200 μl of each protein sample (final concentration 4 μM) was loaded individually into a 0.01 cm-path length micro-cylindrical Suprasil cell (Starna Cells) and spectral data in the far UV wavelength range of 180–260 nm were collected at 22 °C using a CD spectrophotometer (Jasco, Model J-815) with Spectra Manager software (Jasco, version 2.10.01). Averaged baseline scans of buffer alone were measured before averaged sample scans of each protein sample. Instrument scanning parameters were 0.5 nm data pitch interval, standard sensitivity, 4-sec data integration averaging time, bandwidth = 1.00, continuous scan mode with scanning speed of 100 nm/min, baseline correction = baseline measured, and accumulation scans = 16. The CD spectra of these protein samples were initially collected in ellipticity (θ, in units mdeg), which were then converted into mean residue ellipticity [(θ)MR, in units deg cm2 dmol−1] using

\[
[\theta]_{MR} = \frac{\theta}{(100N_{C}l)}
\]

where N is the number of amino acid residues in the protein, ε is the protein concentration in molality, and l is the path length in cm [29]. The CD spectra and corresponding high tension (HT) voltage spectra of PYCR2 wild-type and protein variants R119C and R251C have been deposited in the publicly accessible Protein Circular Dichroism Data Bank (http://pcdcb.crysr.bbk.ac.uk). Further discussion on estimated
secondary structure elements (8 individual components) for each protein determined from a mathematical fit to the experimental CD spectra (190–250 nm) using the online BeStSel algorithm [30], can be found in the doctoral thesis dissertation by Patel (2020) [31].

3. Results

3.1. Steady-state kinetic parameters of PYCR2 wild-type and protein variants

In previous studies, the enzymatic activities of the PYCR2 protein variants associated with microcephaly and hypomyelination in human patients were not reported. Therefore, to provide insight into the biochemical penalties of the R119C and R251C protein variants, we determined the steady-state kinetic parameters with respect to DL-P5C and NAD(P)H substrates.

To provide a comparison, we first determined the steady-state kinetic parameters of PYCR2 wild-type (Fig. 1, Table 1). PYCR2 did not show a strong preference for NADH or NADPH when keeping DL-P5C fixed as indicated by similar apparent catalytic efficiencies ($k_{cat}/K_{M}$) of 161,000 M$^{-1}$ s$^{-1}$ and 111,000 M$^{-1}$ s$^{-1}$, respectively. Also, PYCR2 displayed similar apparent catalytic efficiency values (within a factor of 2) when varying DL-P5C and keeping NADH or NADPH constant.

Both R251C and R119C protein variants exhibited substantially

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Fig. 1. Steady-state kinetics of PYCR2. Overlay of individual Henri-Michaelis-Menten curve fits to traces of 0.06 μM PYCR2 wild-type (dark blue), 0.6 μM R251C variant (dark gray), and 3 μM R119C variant (dark orange) enzymes. Assays were performed in 0.1 M Tris-HCl (pH 7.5) reaction buffer at 37 °C with varying DL-P5C (0–5500 μM) concentration and holding NADH fixed at 500 μM. Each data trace is plotted as (mean ± SD) of three technical replicates with non-linear least-squares fit to the Henri-Michaelis-Menten equation using SigmaPlot 12.0. Note that the vertical axes for the wild-type and variants differ. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
R119C variant appears to be the more compromised of the two protein variants. The thermal denaturation traces for PYCR2 wild-type and protein variants showed a more drastic decrease in thermal stability for R119C compared to the wild-type enzyme. The CD spectra of PYCR2 wild-type and protein variants are shown in Table 1. The CD spectra of PYCR2 wild-type and protein variants exhibited a positive amplitude around 194 nm and a pronounced negative amplitude around 207 nm, indicating a loss of α-helical character showing a decrease in α-helical content. The thermal unfolding of PYCR2 proteins was monitored using the Sypro Orange reporter dye which increases in fluorescence emission at 340 nm upon binding to surface-exposed hydrophobic patches as the protein unfolds. The thermal unfolding of PYCR2 proteins was monitored using the Sypro Orange reporter dye which increases in fluorescence emission at 340 nm upon binding to surface-exposed hydrophobic patches as the protein unfolds. The thermal unfolding of PYCR2 proteins was monitored using the Sypro Orange reporter dye which increases in fluorescence emission at 340 nm upon binding to surface-exposed hydrophobic patches as the protein unfolds.
the CD spectrum of PYCR2 wild-type indicated a more distorted α-helical character showing similar features but with reduced amplitudes [40]. Most notably, the R251C variant displayed the least overall regular α-helical character of the PYCR2 proteins, yet the R251C variant also displayed strong overall regular β-strand or β-sheet character showing modest positive amplitude around 196 nm with a fairly flat CD trace having minor negative amplitude around 223 nm [40].

To provide more insight into the observed differences in the CD spectra of each protein, secondary structure analysis was performed using the β-structure selection (BeStSel) method algorithm that takes into account the twist of β-structures [30]. The overlay traces of the BeStSel algorithm fits to the experimental CD spectra (190–250 nm) of PYCR2 wild-type, R251C variant, and R119C variant (Sup. Fig. 6) exhibited high Pearson correlation coefficients of 0.9928, 0.9790, and 0.9975, respectively, suggesting a strong reliability of the secondary structure estimations for each protein. This analysis further confirmed significantly less α-helical character in R251C relative to the other PYCR2 proteins.
4. Discussion

In this work, we biochemically characterized human PYCR2 wild-type and the disease-linked protein variants R119C and R251C to gain insight into how these variants may contribute to hypomyelinating leukodystrophy type 10, an autosomal recessive neurological disorder. Different clinical studies have found that patients with inborn genetic errors in the PYCR2 gene initially possess normal growth parameters and brain anatomy at birth, but then progressively develop microcephaly and hypomyelination that lead to cognitive and motor deficits that end life later in childhood [41]. A recent 2016 study of 14 affected individuals reported that patients either harbor a stop codon mutation resulting in depletion of PYCR2 or harbor one of five missense mutations in the dimerization domain of PYCR2, such as Arg199Trp and Cys232Gly [18]. Expression levels of missense PYCR2 proteins were not found to be significantly decreased relative to PYCR2 wild-type, however, based on coimmunoprecipitation experiments of PYCR2 in 293T cells, there was impaired protein multimerization of the patient protein variants [18], indicating the protein variants may not form a proper oligomeric structure, which for human PYCR1 is a pentamer-of-dimers [5].

We showed the R119C and R251C variants are catalytically impaired enzymes, consistent with a role in disease pathology. Depending on the cofactor used, the catalytic efficiency of the R119C variant is 40 or 366 times lower than that of wild-type enzyme, while the catalytic efficiency

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**Table 4**

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<th>Ligand(s)</th>
<th>( T_m (\degree C) )</th>
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<tr>
<td></td>
<td>Wild-type</td>
</tr>
<tr>
<td>None</td>
<td>69.2</td>
</tr>
<tr>
<td>NAD(^+)</td>
<td>70.0</td>
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<tr>
<td>L-Pro</td>
<td>69.4</td>
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<td>NAD(^+) + L-Pro</td>
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\( T_m \) values determined as the local minimum value from data in the plot of \(-d(RFU)/dT\) vs. \( T \) (SigmaPlot 12.0, version 12.0.0.182, Systat Software) with data plotted as (mean ± SD) of \( n = 4 \) technical replicates.

\( \) Each ligand concentration was 1 mM in the assays.

\( \) Enzyme concentration was 6.4 \( \mu M \) in all assays.

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**Scheme 2.** Cleland diagram of the proposed sequential-ordered binding mechanism of human PYCR2.

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**Fig. 3.** Thermal stability assays. Overlay of individual Thermofluor assay traces of \(-d(RFU)/dT\) vs. \( T \) (\( \degree C \)) for PYCR2 wild-type (dark blue), R251C variant (dark gray), and R119C variant (dark orange) in the presence of 1 mM L-Pro. The left-sided and right-sided vertical axes are numerically scaled differently to reflect values for PYCR2 wild-type and both protein variants, respectively. The local trough minimum indicates the melting point temperature (\( T_m \)) for each enzyme. All Thermofluor assays were performed with 6.4 \( \mu M \) enzyme. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
of the R251C variant is 7 or 26 times lower than that of the wild-type enzyme. In addition, the thermostability and CD measurements suggest that the R251C variant has a pronounced folding defect. The biochemical properties of the R119C and R251C variants may be rationalized by examining the structural context at those two residues. Arg119 and Arg251 are conserved in PYCR1 and PYCR2, allowing the rationalization by examining the structural context at those two residues. Arg119 and Arg251 are conserved in PYCR1 and PYCR2, allowing the rationalized by examining the structural context at those two residues. Arg119 and Arg251 are conserved in PYCR1 and PYCR2, allowing the rationalized by examining the structural context at those two residues. Arg119 and Arg251 are conserved in PYCR1 and PYCR2, allowing the rationalized by examining the structural context at those two residues.

Fig. 4. CD spectra of PYCR2 Overlay of CD spectra (186.5–260 nm) of PYCR2 wild-type (dark blue), and R251C variant (dark gray) and R119C variant (dark orange). All CD spectra were collected with 4 μM enzyme in 10 mM sodium phosphate buffer (pH 8.0) containing 250 mM ammonium fluoride buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Product(s) inhibition kinetic studies revealed L-proline is a competitive inhibitor with respect to L-P5C and, NAD$^+$ is a mixed inhibitor with respect to NADH, all for PYCR2 wild-type enzyme. Taken together, these inhibition patterns indicate a sequential-ordered binding mechanism with L-P5C binding first to the enzyme followed by NADH (Scheme 2). This binding order is atypical of the general family of dehydrogenase enzymes, i.e. lactate dehydrogenase and human GSALDH, which first bind NAD$^+/\text{NADH}$ before the second substrate. In this case the proposed binding order is supported by X-ray crystal structures of PYCR1 in complex with L-THFA and NADPH (Sup. Fig. 8). Sup. Fig. 8 shows that L-THFA, which is an analog of both L-proline and L-P5C, is buried within the active site. The binding site for L-THFA would be obstructed if NADPH were to bind first, thus supporting the sequential binding order of the smaller L-P5C molecule followed by binding of the larger NAD(P)H molecule. Specifically, Sup. Fig. 8 panels E and F show that NADPH is more solvent exposed which would facilitate the sequential release of NADP$^+$ followed by L-proline. These findings are consistent with previous studies in which bacterial (S. pyogenes) and plant (A. thaliana) P5CRs were also proposed to follow a sequential-ordered binding mechanism with L-P5C binding prior to NAD(P)H. Having the sequential-ordered binding mechanism of PYCR2 established will help guide development of new inhibitors and/or substrate analogs as molecular probes for modulating PYCR2 activity.

Serum levels of L-proline in healthy adult humans are reported to range from 125-633 μM with mean levels around 270 μM [46, 47]. The competitive inhibition constant determined here for L-proline ($K_{IC}^{app} = 145 \pm 8 \mu M$) is within physiological L-proline concentrations suggesting that PYCR2 is susceptible to product feedback inhibition by L-proline. Because concentrations of normal human fasting plasma L-P5C and of L-P5C released into extracellular medium by cultured human skin fibroblasts have been observed to be below 100 μM [48, 49] and the $K_{IC}^{app}$ determined here for L-P5C is 1.5 mM ± 0.069 mM, PYCR2 is likely operating under $\nu_0/K_{IC}^{app}$ conditions and thereby sensitive to feedback regulation by L-proline.

On a more clinical note, previous studies which have profiled blood and plasma metabolites of PYCR2-deficient patients have reported no significant decreases in L-proline [17, 18]. However, it remains unknown what the L-proline concentrations are in the relevant affected neurological and cerebrospinal fluid (CSF) of these patients. Recently, Escande-Beillard et al. (2020) developed a Pycr2$^{-/-}$ mouse model in which the Pycr2 knockout mice were observed to have neurological symptoms similar to human hypomyelinating leukodystrophy type 10 [6]. PYCR1 protein expression was unchanged in the Pycr2 knockout
mice, indicating loss of PYCR2 is not compensated by increased PYCR1 activity [6]. Changes in brain amino acid levels from Pycr2 knockout mice were evaluated and determined to be unremarkable, except for the excitatory neurotransmitter, L-glycine, which was significantly increased in all brain tissues examined indicating cerebral hyperglycinemia in the Pycr2 knockout mice [6]. In both human primary fibroblasts isolated from PYCR2-deficient patients and in Pycr2 knockout mouse tissue, serine hydroxymethyltransferase 2, the rate-limiting mitochondrial enzyme responsible for L-glycine synthesis, was found to be significantly upregulated [6]. Thus, although depletion of PYCR2 does not appear to impact L-proline levels, a significant change in L-glycine levels is observed suggesting an important link between L-proline and L-glycine.

Progressive neurological deterioration is also associated with P5CS deficiency, which is marked by hypoprolinemia and lower urea cycle intermediates (e.g., ornithine, citrulline, arginine). Unfortunately, P5CS deficiency cannot be treated by fortified amino acid supplements [50, 51]. Unsuccessful supplementation therapies may be due to a number of complications such as low bioavailability, high clearance rates, different intestinal absorption and plasma kinetics between amino acids derived from amino acid supplements and whole protein, as well as inadequate delivery to affected tissues [52]. Thus, L-proline serum levels in PYCR2-deficient patients may not be an adequate biochemical marker to determine whether PYCR2 deficiency causes abnormal L-proline bioavailability in patients, especially in the CSF. Escande-Beillard et al. (2020) proposed cerebral hyperglycinemia in hypomyelinating leukodystrophy type 10 patients could trigger excitotoxicity by activation of N-methyl-D-aspartate (NMDA) receptors leading to hypomyelination and axonal damage. Thus, therapeutic NMDA receptor antagonists or other treatments aimed at lowering cerebral L-glycine levels might be of benefit to PYCR2-deficient patients rather than supplement therapies [6].

In conclusion, we have shown that human PYCR2 protein variants R119C and R251C result in impaired enzyme function and structural stability, thus potentially leading to disrupted L-proline biosynthesis and metabolic dysfunctions that contribute to hypomyelinating leukodystrophy type 10 with microcephaly [41].

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Databases

CD spectral data (180–260 nm) were deposited in the Protein Circular Dichroism Data Base (PCDDB). PCDDB ID codes are CD0006228000 (PYCR2 wild-type), CD0006229000 (PYCR2 R119C variant), and CD0006230000 (PYCR2 R251C variant).

Author contributions

S.M.P. and D.F.B. designed the experiments. X.L. made the PYCR2 expression construct. S.M.P conducted the experiments and analyzed the
data. J.S. helped design the CD spectroscopy experiments and performed the LC-ESI-MS/MS mass spectrometry of DL-PSC. J.J.T. helped with the structural analysis of PYCR2 mutants. All authors contributed to data analysis and interpretation, writing of the manuscript, and have approved the final version of the manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

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References


