Crystal Structure of Human Pyrroline-5-carboxylate Reductase

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Pyrroline-5-carboxylate reductase (P5CR) is a universal housekeeping enzyme that catalyzes the reduction of Δ1-pyrroline-5-carboxylate (P5C) to proline using NAD(P)H as the cofactor. The enzymatic cycle between P5C and proline is very important for the regulation of amino acid metabolism, intracellular redox potential, and apoptosis. Here, we present the 2.8 Å resolution structure of the P5CR apo enzyme, its 3.1 Å resolution ternary complex with NAD(P)H and substrate-analog. The refined structures demonstrate a decameric architecture with five homodimer subunits and ten catalytic sites arranged around a peripheral circular groove. Mutagenesis and kinetic studies reveal the pivotal roles of the dinucleotide-binding Rossmann motif and residue Glu221 in the human enzyme. Human P5CR is thermostable and the crystals were grown at 37 °C. The enzyme is implicated in oxidation of the anti-tumor drug thioproline.

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linked to the oxidation of NADPH rather than production of proline. The preference for NADPH and insensitivity to proline implies that the essential function of P5CR from erythrocytes is regulation of the NADP+/NADPH ratios and the intercellular redox potential. Third, P5CR from bacteria is involved in metabolism of proline-analog drugs such as thioproline and 3,4-dehydro-l-proline.\(^{18,26}\) It is worth noting that the multimer architecture appears to be essential for the function of P5CRs from different organisms. The biological unit of the native enzyme is found to be a dimer in Neisseria meningitidis,\(^{26}\) a tetramer in yeast,\(^{27}\) an octomer in rat lens,\(^{28}\) a decamer in Streptococcus pyogenes\(^{26}\) and a 10–12-mer in human.\(^{23}\)

Although there are tissue-specific differences in enzyme kinetics and inhibitor sensitivity, only a single-copy gene located to human chromosome 17 has been identified to encode P5CR,\(^{29}\) which has two transcript variants termed PYCR1 and PYCR2. Recently, the crystal structures of P5CR from N. meningitides MC58 and S. pyogenes have been reported.\(^{26}\)

Results and Discussion

Overall structure

The full-length P5CR polypeptide fold is comprised of 13 α-helices and eight β-strands, which together form two distinct domains (Figure 1(c)). The N-terminal domain (domain A) is formed by seven α-helices and eight β-strands, and has a modified NAD(P)-binding Rossmann-fold. The extended eight-stranded β-sheet, rather than the classic six-stranded β-sheet, and anti-parallel strands \(β^7\) and \(β^8\) indicate that the fold belongs to the 6-phosphogluconate dehydrogenase N-terminal domain-like family. The relatively parallel \(β^1–β^6\) strands curl around to form a hemi-β-barrel structure. Helices \(α^1–α^7\) lie on both sides of the hemi-β-barrel and form a helix-strand-helix sandwich. The C-terminal domain (domain B) is formed by six relatively longer α-helices (\(α^8–α^13\)) and has a 6-phosphogluconate dehydrogenase C-terminal domain-like fold. Helices \(α^6, α^9\) and \(α^{11}\), which are relatively parallel with each other, pack against \(α^{10}\) to form an independent hydrophobic α-helical domain. These four α-helices will twist with their counterparts in the other monomer and constitute the basis of dimerization. Helix \(α^{10}\), which is close to domain A of the same monomer with a minimum distance of 11 Å, forms the circular groove with domain A that constitutes the binding site for cofactor and substrate analog. The C-terminal helices \(α^{12}\) and \(α^{13}\), which extend from residue 238 to residue 275, form the wall of the central, negatively charged channel together with their counterparts in the other nine molecules.

The monomer

The full-length P5CR polypeptide chain is encoded by PYCR1 (NCBI accession number NM_006907) was expressed in Escherichia coli with a hexahistidine tag at its N terminus. The purified P5CR resulted in a single band on SDS–PAGE and has an apparent molecular mass of \(~370\) kDa as determined by gel-filtration chromatography, suggesting a decamer. However, the aggregative precipitation occurred rapidly at low temperature (<4 °C) and low salt concentration (<0.3 M NaCl). Crystals of human P5CR suitable for X-ray diffraction analysis could be obtained only at 37 °C.

The crystal structure of P5CR was solved using single-wavelength anomalous diffraction (SAD) phasing method from a SeMet derivative and has been determined to 2.8 Å resolution with an \(R\)-factor of 23.6% and \(R_{\text{free}}\) of 27.3%. The last 44 residues (276–319) of each monomer are missing due to the lack of electron density. Data collection and refinement statistics are given in Table 1.

The crystal structure demonstrates a decameric architecture: two monomers form the basic catalytic subunit of one dimer, and five homodimers are related by a non-crystallographic 5-fold axis to form the decamer (Figure 1(a) and (b)). The P5CR decamer resembles a yo-yo in shape, with overall dimensions of about 110 Å×110 Å×87 Å, and has a circular groove around the periphery of the decamer and large bulges at either end. The structure of the ternary complexes indicates that this circular groove is the binding site for the cofactor and substrate analog.

The dimer

From the crystal structure, two P5CR monomers related by a crystallographic 2-fold axis twist around each other to form an intimate dimer, which acts as the basic functional unit. The hydrophobic portion (\(α^8–α^{11}\)) of domain B is essential for dimerization (Figure 1(d)). The basic biological unit is formed when one molecule packs with the equivalent part of another molecule via hydrophobic interactions. Helices \(α^8\) and \(α^9\), and their relatively parallel counterparts in another
molecule, form the nesting core. Helix α10, which plays an important role in formation of the binding site, surrounds this core with helices α11–α13, making a nesting shell. This nested part forms a 2965 Å² contact area, compared with the 13,615 Å² area of one monomer, indicating that this region plays a key role to stabilize the dimer.

Dimer–dimer interaction, architecture and stability of the decamer

Domain B is important for dimerization, and in dimer–dimer interactions for decamer formation. Five homodimers are related by a non-crystallographic 5-fold axis and contact each other via residues of α9–α12 in domain B. Besides hydrophobic packing between dimers, an elaborate hydrogen bond network between the loop/turns of α9–α10, α11–α12, and α12–α13 contributes significantly to decamer formation.

We identified three parts that mediate the interaction between two successive dimers (Figure 1(e)). Part I, which is formed by the α9–α10 loop in molecule A and the α11–α12 loop in molecule E, has five inter-molecular hydrogen bonds and is a source of structural rigidity in decamer formation. Part II has 12 ideal hydrogen bonds between α12 and α13 of molecules H and E, including two elaborate water-mediated hydrogen bonds. Part III is formed by the α11–α12 loop of molecule H and the α9–α10 loop of molecule I. There are only three hydrogen bonds in this part, between Asp229(H), Pro234(H) and Val193(I), Gly196(I), Arg199(I) (where the subunit is identified in parentheses after the residue number), that have only a limited contribution to decamer formation. Besides these hydrogen bonds, we found another interesting interaction for decamer formation: the pyrrolidine rings of Pro198(A) and Pro234(E), and of Pro234(H) and Pro198(I), are parallel with each other at respective distances of about 6 Å. The conjugate effects of these two pairs of proline residues, which are distributed at both ends of the dimer contact area, also help to stabilize the decamer. To investigate the stability of the decamer, the P5CR apo enzyme was incubated with urea, and the oligomeric state was analyzed using gel-filtration chromatography (Figure 1(f)). The intact decamer was stable in buffer containing 0.5 M urea and began to dissociate into homodimers as the concentration of urea was increased gradually from 1 M to 4 M, with no monomer species observed during this process. Interestingly, P5CR could be refolded from 6 M urea-treated protein on Superdex200. Furthermore, our evidence indicates that human P5CR is thermostable. P5CR does not significantly lose its enzyme activity after 1 h of incubation at 40 °C, indicating the relatively high stability of P5CR. The half-life of human P5CR at 68 °C is 140 min (Figure 1(g)). These results indicate that the decamer form of human P5CR should be the basic stable oligomeric configuration under physiological buffer conditions.

A variety of structural features may contribute to the thermostability of human P5CR and its resistance to denaturing agents. These include a remarkably high number of proline residues (16, or 5% of the
amino acid content); a total of 20 intra-molecular hydrogen bonds and the conjugate effects of two pairs of proline residues; hydrophobic packing between dimers; and a decreased surface area of the oligomeric quaternary structure. However, no disulfide bond was found in the crystal structure.

There is a negatively charged channel through the center of the decamer, extending by about 87 Å from top to bottom, and with a minimum radius of 25 Å (Figure 1(b)). The ten long helices in the C terminus of this decamer form the walls of the channel. Although the C-terminal 40 residues unique to the human enzyme are missing from the crystal structure due to lack of electron density, they may lie freely in this channel after the polypeptide chain is folded. Evidence shows that their absence is not due to proteolysis (data not shown). Further work is underway to investigate the function of this channel.

**Active center**

The ternary complexes of the P5CR enzyme with NAD(P)H and substrate analog have been crystalized and their structures determined in order to investigate the catalytic mechanism of P5CR (Table 1). In these complex structures, although B-factors for NAD(P)H atoms are quite high (82–93 Å²), the entire cofactor and substrate analog molecules could be placed accurately from unambiguous electron density (Figure 2(a) and (b)). The ternary complexes of NADH and NADPH appear to have the cofactor and substrate analog bound in a similar conformation around the circular groove near the Rossmann motif. Therefore, the ternary complex of P5CR with NADH and substrate analog is used to characterize the active center.

The binding site is formed mainly by residues located on loops between β6–β7, α7–α8, and α10–α11 of one monomer, and by residues located on helix α10 of the second monomer in one homodimer. All ten bound small molecules are distributed around the circular groove like string circled around a yo-yo (Figure 1(b)). This decamer configuration with ten catalytic sites in one biological unit may provide a structural basis to explain why the turnover rate of P5CR is at least ten times higher than most house-keeping enzymes.²³
There are two main parts that constitute the binding site: the hydrophobic wall and the charged center (Figure 2(c)). The hydrophobic wall is formed by Phe158(A), Arg200(H) and Arg204(H). The phenyl ring of Phe158(A) is parallel with the ribose, followed by adenine at a distance of 3.6 Å, which induces conjugate effects between these two rings to stabilize NADH binding. Moreover, the phenyl ring of Phe158(A) and the long side-chains of Arg200(H) and Arg204(H) essentially form three pillars in the hydrophobic wall. The contact area between this wall and NADH is nearly 50%, corresponding to the total area of adenine and the following ribose (93.5 Å² over 198 Å²). The charged pocket, which stabilizes NADH with hydrogen bonds, is formed by residues in α7, α10, α11, and β6–β8, and the loops/turns between them. Arg129(A) forms the ceiling of this pocket; His219(A) and Glu130(A), Ser154(A), Gly157(A) and Gln208(H) form the right-hand and the left-hand wall of this pocket, respectively. As these residues contact the binding cofactor largely via hydrogen bonds, this pocket contributes significantly to cofactor binding.

At the top of the nicotinamide ring in NADH, the binding substrate analog l-glutamate, Glu301(A), was built into the structure on the basis of the reliable F₀−Fₐ difference electron density. Glu301(A) is relatively parallel with the nicotinamide ring, and the nitrogen atom of l-glutamate is close to the C5 atom in the nicotinamide ring. As NADH should act as a proton donor to transfer one proton to the amide of the substrate analog during the reaction cycle, this position is important for the P5CR catalytic mechanism. There are two hydrogen atoms belonging to atom C5 in the nicotinamide ring, one forming hydrogen bonds with the oxygen atom of Gly157(A) and helping to stabilize NADH in the binding site, while the second one is transferred from NADH to the nitrogen atom of Glu301(A) for substrate reduction. The electron...
density for the NADPH complex indicates residues that may play an important role in substrate specificity. In the NADH complex shown in Figure 2(a), the conformation of Phe158(A) suggests space in the expected binding site for the 2'-phosphate group of NADPH is limited. However, in the NADPH complex shown in Figure 2(b), the NADPH cofactor changes orientation in the pocket (relative to the NADH in Figure 2(a)) and the 2'-phosphate group interacts with Leu218(A), His219(A) and Ser220(A). It is interesting to note that the conformation of Arg204 varies with substrate binding. In the NADH complex, Arg204(H) is directed towards Ser220(A) and occupies the 2'-phosphate-binding site. In the NADPH complex, by contrast, Arg204(H) switches conformation to allow the 2'-phosphate group to enter its binding site and interact with Leu218(A), His219(A) and Ser220(A). The role of Arg204 and Ser220 may be analogous to that of Arg35 and Ser31 in the bacterial P5CR.

Figure 1 (legend on next page)
complexes, which are considered to be critical determinants for the specificity of binding NADPH over NADH.\textsuperscript{26} The crystal structures also suggest an interesting conformational regulator of enzyme activity. When comparing the structures of the P5CR apo enzyme and its complex with NADH (Figure 2(d)), relatively large side-chain r.m.s. deviations of 3.9 Å and 4.2 Å are observed for residues Glu221(A) and Arg200(H), respectively, compared with the average r.m.s. deviation of 0.3 Å for all C\textsubscript{\alpha} atoms between the two molecules. By carefully comparing the apo and complex structures, we propose that Glu221(A) may play a role of switch for cofactor binding. In the P5CR apo enzyme structure, the conformation of the Glu221(A) side-chain allows for two ideal hydrogen bonds with Arg200(H) and Arg204(H), respectively. In the complex structure, however, the side-chain of Glu221(A) flips conformation and hydrogen bonds with Arg200(H) and Arg204(H) are broken. With the conformation change of Glu221(A), the side-chain of Arg200(H) also changes to accommodate the adenine group of NADH and acts as one hydrophobic pillar for this pocket.

**Comparison with S. pyogenes and N. meningitides P5CR**

The structures of two bacterial P5CR enzymes have been determined recently. *N. meningitides* P5CR is a homodimer; while *S. pyogenes* P5CR has a decameric architecture, suggesting that oligomerization varies according to species.\textsuperscript{26} From superposition of the crystal structures of human, *S. pyogenes* (PDB ID codes 2AHR and 2AMF) and *N. meningitides* Mc58 (PDB ID codes 1YQG and 2AG8) P5CR, they share a generally similar monomer structure and dimer architecture, although their

![Figure 1](image-url). The P5CR structure. (a) Overall view of the P5CR crystal structure. A ribbon representation of only one homodimer is shown, with each monomer presented in magenta and gold, respectively. The other four homodimers, which form the decamer, are shown in molecular surface representation. (b) The potential surface of the decamer ternary complex. The binding cofactor and substrate analog are shown as magenta spheres around the circular groove. The three dimensions are labeled and the negatively charged central channel with 25 Å diameter is shown. (c) Two views related by 90° of the monomer structure of P5CR, showing domains A and B with their secondary structure units in detail. (d) Two views related by 90° of the dimer structure of P5CR, showing how two monomers coil around each other to form one dimer. Monomers are shown in cartoon representation in magenta and gold, respectively. Secondary structure units of molecule 1 are named as α\textsubscript{x-1}; and in molecule 2 are named as α\textsubscript{x-2}. (e) Stereo view of the inter-homodimer interactions. Only two homodimers are shown at the interface. Molecules A and H, which belong to one dimer, are shown in magenta and yellow, respectively; molecules E and I, which belong to the other dimer, are drawn in green and red, respectively. There are 11 inter-homodimer, five water-mediated inter-molecular and four intra-molecular hydrogen bonds, which form an interaction web between two homodimers. Hydrogen bonds are shown as broken cyan lines. (f) Investigation of the decamer stability with different concentrations of urea. In the Superdex200 profiles, P5CR eluted at the decamer peak when treated with no urea (black) or with 0.5 M urea (magenta). The decamer began to dissociate into dimers from 1 M urea (yellow) and 2 M urea (blue) and dissociated completely into dimers at 4 M urea (red). P5CR was refolded into a decamer from 6 M urea in Superdex200 (shown as a green curve). No monomer was observed for any concentration of urea. (g) Thermal inactivation of P5CR: (1) after incubation at various temperatures (20–75 °C) for 10 min, the relative activities of P5CR were measured at 25 °C. (2) The time-dependent thermal inactivation of P5CR was investigated at 40 °C and at 68 °C. The relative activities were measured at 25 °C and plotted against time.
levels of primary structure similarities are as low as 22–26%.

The structural similarities include the Rossmann motif in the N-terminal domain and the \( \alpha \)-helical dimerization domain at the C terminus. An eight-stranded \( \beta \)-sheet is surrounded by \( \alpha \)-helices on either side to form a helix-strand-helix sandwich around the active-site cleft (Figures 1(c) and 3(b)). This helix-strand-helix sandwich is strictly conserved at the primary structure level when sequences are aligned (Figure 3(a)). This may support the hypothesis that bacterial and human P5CR share the same evolutionary origin. The alignment indicates also that only 21 amino acid residues out of about 319 are strictly conserved in the above-mentioned P5CRs, which originate from two different domains of living organisms ranging from bacteria to human.

The superposition of bacterial and human P5CR structures show that the main differences occur in the surface loops surrounding the active-site cleft (Figure 3(b)). The bacterial P5CR structures have six \( \alpha \)-helices in the N-terminal domain, the N-terminal domain of the human enzyme has seven \( \alpha \)-helices, including an additional helix from residues Lys57 to Ser63. The difference exhibits great changes in the orientation of \( \alpha_2 \) and the angle of twist between the N and C-terminal domains (Figure 3(b)). For this reason, we could superimpose these structures in only one domain. After superimposing well the N-terminal domains, we found that the outside \( \alpha_2 \) regions are progressively closer to the N-terminal domain from \( S. \) pyogenes P5CR to \( N. \) meningitides Mc58 P5CR to human P5CR. As very clear density is observed for bound NADP\(^+\) in the \( S. \) pyogenes P5CR crystal structure and only partial density is seen for bound NADP\(^+\) in the \( N. \) meningitides Mc58 structure,\(^{26}\) we speculate that the \( \alpha_2 \) region may play a very important role in NADP\(^+\) binding from species to species.

**Mutagenesis**

To characterize the functional roles of the key residues in the active site, we constructed the point mutants E221A, E221G, which is responsible for the recognition of the NADH moiety in our structure. N-truncated and C-truncated P5CR mutants were

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**Figure 2.** The active site. (a) A stereo view of the NADH-binding site. The key residues interacting with NADH are labeled and shown in magenta stick representation; the bound NADH and substrate analog are shown in yellow stick representation. Hydrogen bonds are shown as broken black lines; the \( H^+ \) transfer path is shown as a broken red line between the C5 atom of the nicotinamide ring and the nitrogen atom of L-glutamate. The binding cofactor and substrate are covered by the \( F_o-F_c \) electron density map (green) contoured at 1.7\( \sigma \). (b) A stereo view of the NADPH-binding site. The key residues interacting with NADPH are labeled and are shown in magenta stick representation; the bound NADPH and substrate analog are shown in yellow stick representation. Binding cofactor and substrate are covered by the \( F_o-F_c \) electron density map (blue) contoured at 1.7\( \sigma \). (c) The molecular surface covering the binding site. NADH is shown in yellow stick representation; L-glutamate is shown in white stick representation. Some important residues for cofactor and substrate analog binding are labeled; six perfect hydrogen bonds to stabilize NADH are drawn as broken blue lines; the C5 atom of the nicotinamide ring is shown with larger atomic radius; the proton transfer path between the C5 atom and the nitrogen atom of L-glutamate is shown by the broken red line. (d) The unique conformational switch in P5CR for cofactor and substrate analog binding. On the left, key residues are labeled and shown as yellow sticks; on the right, the equivalent region of the ternary complex is shown with the bound NADH, which is drawn as white sticks. The conformational switch Glu221(A) is highlighted by a red circle.
constructed, and the kinetic parameters for the point and truncation mutants were determined for the reverse reaction. Residues Glu221 and the C-terminal residues are specific to human P5CR. The E221A mutant shows a decrease of $K_M$ and $K_{cat}$ ($K_M = 0.73(\pm 0.023) \text{ mM;} K_{cat} = 13 \text{ s}^{-1}$, fixed 1 mM NADP⁺) compared with the wild-type ($K_M = 1.26 (\pm 0.032) \text{ mM;} K_{cat} = 55 \text{ s}^{-1}$, fixed 1 mM NADP⁺),

Figure 2 (continued)
which means the affinity of E221A for substrates is increased. Earlier reports on the distinctive preference of P5CR from different tissue extracts for NADPH or NADH²,³ led us to compare affinities of recombinant P5CR for NADP⁺ or NAD⁺ in this thioproline dehydrogenase activity assay. The affinity of P5CR for NAD⁺ (apparent $K_M = 0.151 \pm 0.023$ mM) is 20-fold higher than for NADP⁺ (apparent $K_M = 3.06 \pm 0.042$ mM) with 1 mM fixed thioproline, which suggests P5CR has a strong preference for NAD⁺ over NADP⁺ in this reaction. However, the affinity of the E221A mutant for NAD⁺ (apparent $K_M = 0.235 \pm 0.032$ mM) was only twofold higher than for NADP⁺ (apparent $K_M = 0.480 \pm 0.021$ mM). Comparison of the apo enzyme and ternary complex structures reveals

**Figure 3** (legend on next page)
Figure 3. (a) Structure-based sequence alignment between P5CR in human and in different organisms as indicated. Arrows indicate β-strands; cylinders denote α-helices. Background-red residues indicate those that are conserved; background-yellow denotes residues identified to be more than 80% conserved. Residues that are important for cofactor and substrate analog binding are framed in black. (b) Superposition of P5CR from human (yellow), Neisseria meningitides Mc58 (green) and Streptococcus pyogenes (red). The α2 helix of all three structures is shown in ribbon representation and the other parts are shown as smooth lines. The NADP cofactor bound in the structure of P5CR from Streptococcus pyogenes is shown in magenta stick representation.

some differences in the conformation of Glu221 surrounding the NADH-binding hydrophobic pocket. The fine changes in conformation would result in an opening and closing of the binding groove to facilitate the cofactor to enter and leave the active site. The switch between the observed “resting” state of Glu221, Arg200 and Arg204, and an increased Km for NAD+ and a decreased Km for NADP+ reflected by the E221A mutant, confirm the essential role of Glu221 as a conformational switch for cofactor selectivity for thrioproline dehydrogenase activity. Interestingly, when Glu221 was mutated to glycine, which is highly conserved in all P5CR members with the exception of the human enzyme, the protein was expressed as inclusion bodies in E. coli. Furthermore, the N-truncated P5CR mutant resulted in inclusion bodies in E. coli even after the protein was induced at 14 °C without IPTG, and ligated into the restricted sites of the pET28a(+) vector (Novagen Inc.) with a His, tag at the N terminus. Over-expression of the protein was induced with 0.25 mM IPTG when the absorbance at 600 nm (A600) was ~0.5, and the cells were harvested after 3 h incubation at 37 °C. The selenomethionyl derivative protein was produced by expression in methionine-deficient E. coli strain B834 (DE3). After precipitation in 20% saturated ammonium sulfate, the soluble protein was incubated with 1 mg/ml of RNaseA (Sigma) and 15 units/ml of DNaseI (Takara) at room temperature in 20 mM Caps (pH 9.4), 0.5 M NaCl overnight. His-tagged P5CR was purified using Ni²⁺-nitrilotriacetic acid agarose (Qiagen), chromatography on Superdex200 and Resource Q (Pharmacia).

Activity assays and thermostability of P5CR

Activity assays of the P5CR and mutant P5CR enzymes were determined by measuring the change in absorbance at 340 nm, using a HITACHI UV2800 spectrophotometer as described. In brief, the reaction was initialized by adding 0.5 mM P5CR into 200 μl of reaction buffer
containing 300 mM Tris–HCl (pH 9.0) (in which P5CR achieved maximized activity), various concentrations of NAD(P)* (0.1–8 mM), and thiopropyl (0.2–10 mM). The l-proline dehydrogenase activity assays of P5CR were performed in a 200 mM diethanolamine buffer (pH 10), containing 5 mM NAD* and 20 mM l-proline. Using the protein extinction coefficient of NAD(P)H (6.22), initial rates of the product formation were calculated as the increase of absorbance at 340 nm/min from the first 100 s of a 5 min interval. After incubation at various temperatures (20–75 °C) for 10 min, the relative activities of P5CR were measured at 25 °C. The time-dependent thermal inactivation of P5CR was investigated at 40 °C and at 68 °C. The relative activities were measured at 25 °C. Reproducibility of the results was confirmed by taking each measurement at least twice with a fluctuation of less than 8%. A sample with both substrates in the absence of P5CR was employed as a negative control.

**Mutagenesis of human P5CR cDNA**

Site-directed mutagenesis was used to introduce single-point mutations into the P5CR cDNA by two consecutive steps of PCR comprising four different primers (Table 2). Overlapping oligonucleotides containing internal point mutations (underlined) were used in the first round of PCR to amplify template plasmid pET28a(+), which contains the 0.95 kb EcoRI-Xhol fragment of P5CR, then using non-mutagenic primers:

PYCRI-F: 5′-GAAAGAATTCATGACGTGGGCTTCAATCGGCG-3′
PYCRI-R: 5′-GAAACTCAAGCTAATCTTCTGCCGCTGG-3′

Oligonucleotides to amplify again. The N-truncated (deletion of N-terminal 11 amino acid residues) and C-truncated (deletion of C-terminal 24 amino acid residues) P5CR was amplified with the primers (Table 2). The resulting products were gel-purified and digested for cloning into pET28a(+). The target sequences were confirmed by DNA sequencing.

**Crystallization**

The purified protein was concentrated to 15 mg/ml in storage buffer (20 mM Caps (pH 9.4), 0.5 M NaCl, 1 mM DTT). Crystallization was performed using the hanging-drop, vapor-diffusion technique with reservoir solutions containing 50–60 mM Tris–HCl (pH7.5), 0.8–1 M sodium acetate, 30–40 mM imidazole (pH 6.5) for the apo enzyme, and 0.1–0.2 M Bicine (pH 9.0), 0.8–1 M sodium acetate, 0.1–0.2 M imidazole (pH 6.5) and 0.1–0.2% (w/v) agarose for the selenomethionyl derivative protein. Drops were prepared by mixing 1 μl of protein solution, 1 μl of reservoir solution for the selenomethionyl derivative protein and another 1 μl of 100% paraffin oil for the apo enzyme in a 37 °C incubator. Crystals appeared after one to three days, and reached a maximum size of 300 mm × 200 mm × 80 mm. Crystals of the ternary complexes and inhibitor were obtained by soaking crystals of the apo enzyme in a solution of 2 μl of reservoir solution for the apo enzyme containing 0.3 mM NAD(P)H and 0.3 mM l-glutamate in a 37 °C incubator overnight.

**Data collection and processing**

Single-wavelength anomalous dispersion (SAD) data for Se-P5CR were collected at 100K using an SBC2 3 k × 3 k CCD detector on beamline BL19-ID of APS (Argonne National Laboratory, USA). The selenomethionated P5CR crystal was transferred to a cryoprotectant solution for data collection and diffraction to 3.2 Å. The X-ray diffraction data for P5CR apo-enzyme crystals were collected at 100 K using a Rigaku R-AXIS IV+ image plate with a Rigaku FR-E rotating CuKα anode in-house X-ray generator at 40 kV and 50 mA (λ = 1.5418 Å) and diffraction to 2.8 Å resolution. Diffraction data for the ternary complex were collected at 100 K with a Rigaku rotating CuKα anode MM-007 in-house X-ray generator at 40 kV and 20 mA (λ = 1.5418 Å) and diffraction to 2.8–3.0 Å resolution. All intensity data were processed and scaled using the software HKL2000. Data collection statistics are summarized in Table 1.

**Phasing, model building and refinement**

Five molecules in one asymmetric unit were identified by MOLREP. Heavy-atom searching and initial phasing were performed by SOLVE. Positions for the 40 selenium atoms anticipated from the number of methionine residues in the amino acid sequence were located. There are 22 positions with occupancy greater than 50% and the remainders are greater than 30%. Density modification and NCS phase restraints were implemented in RESOLVE.

**Table 2. Mutagenesis of P5CR, effects on enzyme activity and kinetic constants**

<table>
<thead>
<tr>
<th>Mutation site and primers</th>
<th>Catalytic activity</th>
<th>Soluble protein</th>
<th>Location in structure</th>
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<tr>
<td>E221A-F: 5′-GCGCAAGATGCTGCTGACTCACCGGCCAGCACCCGAGCCAG-3′</td>
<td>Km for NADP* decreased</td>
<td>Soluble</td>
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<tr>
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<tr>
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<td>C terminal Free in channel</td>
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<td>E221G-R: 5′-GTCCTTGACCTGCGCTGCTGATGCTGACGCCG-3′</td>
<td>Not detected</td>
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<td>N truncated-F: 5′-GAAAGAATTCATGCTTGGCCGCAAGGGCTT-3′</td>
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**Phasing, model building and refinement**

Five molecules in one asymmetric unit were identified by MOLREP. Heavy-atom searching and initial phasing were performed by SOLVE. Positions for the 40 selenium atoms anticipated from the number of methionine residues in the amino acid sequence were located. There are 22 positions with occupancy greater than 50% and the remainders are greater than 30%. Density modification and NCS phase restraints were implemented in RESOLVE.
Although electron density for 75% of the residues was unambiguous, RESOLVE could not automatically trace the structure at 3.2 Å resolution. Initial tracing of the monomer polypeptide chain was performed manually using the program O,35 and an initial pentamer model was generated using NCS relations determined with CNS.36 The initial refinement was performed using simulated annealing in CNS initially with very tight NCS restraints. At later stages of positional refinement, restraints were relaxed and a bulk solvent correction was applied under the guidance of RHSE. Model geometry was verified using the program PROCHECK.37 Solvent molecules were located from stereochemically reasonable peaks in the ωA-weighted Fo−Fc difference electron density map.

Denaturation and refolding of P5CR

In order to study the quaternary conformational stability of the decamer of P5CR in solution, urea was used to denature proteins. The protein was incubated in 20 mM Caps (pH 9.4), 0.5 M NaCl containing 0.0 M, 0.5 M, 1.0 M, 2.0 M, and 4.0 M urea, respectively, for 40 min at 25 °C. The samples were then injected into a Superdex 200 column (10/300 mm) and separated by AKTA purifier equilibrated with the incubation buffer. To test the refolding ability of P5CR, the protein was incubated in the above buffer containing 6 M urea under the same conditions and the sample was then loaded onto the column equilibrated by the buffer but containing no urea. The molecular weight was measured by chromatography where the elution volume (ml) of the peak is compared to the elution of a standard in the same column.

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