The Active Site of an Algal Prolyl 4-Hydroxylase Has a Large Structural Plasticity*

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Prolyl 4-hydroxylases (P4Hs) are 2-oxoglutarate dioxygenases that catalyze the hydroxylation of proline residues in peptides. They play an important role in collagen synthesis, oxygen homeostasis, and plant cell wall formation. We describe four structures of a P4H from the green alga Chlamydomonas reinhardtii, two of the apoenzyme at 1.93 and 2.90 Å resolution, one complexed with the competitive inhibitor Zn$^{2+}$, and one with Zn$^{2+}$ and pyridine 2,4-dicarboxylate (which is an analogue of 2-oxoglutarate) at 1.85 Å resolution. The structures reveal the double-stranded β-helix core fold (jellyroll motif), typical for 2-oxoglutarate dioxygenases. The catalytic site is at the center of an extended shallow groove lined by two flexible loops. Mutagenesis studies together with the crystallographic data indicate that this groove participates in the binding of the proline-rich peptide-substrates. It is discussed that the algal P4H and the catalytic domain of collagen P4Hs have notable structural similarities, suggesting that these enzymes form a separate structural subgroup of P4Hs different from the hypoxia-inducible factor P4Hs. Key structural differences between these two subgroups are described. These studies provide first insight into the structure-function relationships of the collagen P4Hs, which unlike the hypoxia-inducible factor P4Hs use proline-rich peptides as their substrates.

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2 The abbreviations used are: P4H, prolyl 4-hydroxylase; C-P4H, collagen P4H; HIF, hypoxia-inducible transcription factor; Cr-P4H-1, C. reinhardtii P4H type 1; His$_{6}$-Cr-P4H-1-V29, Cr-P4H-1 construct starting from Val-29 and containing the N-terminal His tag; Cr-P4H-1-G30, Cr-P4H-1 construct starting from Gly-30; SeMet, selenomethionine; SeMet(apo), apo crystal form of Cr-P4H-1 obtained with SeMet-labeled His$_{6}$-Cr-P4H-1-V29 construct; needle(apo), apo crystal form of Cr-P4H-1 obtained with His$_{6}$-Cr-P4H-1-V29 construct; (Z)ncomplex, liganded crystal form of Cr-P4H-1 obtained with Cr-P4H-1-G30 construct; CAS, clavaminic synthase; FIH, factor-inhibiting hypoxia-inducible factor 1; SUMO, small ubiquitin-like modifier.

The catalytic domains of all P4Hs contain four conserved residues; a histidine and an aspartate in a His-X-Asp motif, which together with a second, distal histidine bind the Fe$^{2+}$ ion, and a basic residue that binds the 5-carboxylate group of the 2-oxoglutarate (21). The structure of the catalytic domain of HIF-P4H-2 complexed with Fe$^{2+}$ and a 2-oxoglutarate analogue, [[[4-(hydroxy-8-iodoisouquinolin-3-yl)carbonyl]amino]acetic acid, was solved recently (15) and shown to contain an 8-stranded β-helix core fold (jellyroll motif, also referred to as the double-stranded β-helix fold) typical of the 2-oxoglutarate dioxygenases (3, 22). The structure of the C-P4H tetramer is currently unknown, but...
that of the peptide-substrate binding domain of the human C-P4H-I α subunit has been solved by NMR and x-ray crystallography (23, 24). It consists of 2.5 tetratricopeptide repeats and contains solvent-exposed tyrosines that are likely involved in peptide-substrate binding. The catalytic domain is insoluble and, therefore, not suitable for crystallographic analysis.

Detailed knowledge of the structure of the C-P4H catalytic domain would help in the rational design of potent inhibitors for the treatment of fibrotic diseases. The catalytic domain of HIF-P4H-2 is not a suitable model for this purpose because of its low amino acid sequence identity (14%, starting from HIF-P4H-2 Thr-185, Fig. 2) and the remarkably different substrate specificity. We report here on the determination of several apo and liganded structures of an algal P4H of C. reinhardtii (Cr-P4H-1) (8), which shows 26% sequence identity to the catalytic domain of C-P4H-I (starting from Cr-P4H-1 Val-29, Fig. 2), and which, like the C-P4Hs, uses proline-rich peptide-substrates (8). Furthermore, Cr-P4H-1 and the C-P4Hs are inhibited by both pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate, whereas the latter compound does not inhibit the HIF-P4Hs (1, 2, 8, 25). Therefore, the structure of Cr-P4H-1 seems important to gain a better understanding of the properties of the catalytic domain of the C-P4Hs. The Cr-P4H-1 structure identifies the residues involved in cosubstrate binding and reveals a groove formed by an extension after the second jellyroll β-strand. The catalytic site is in a pocket in the middle of this shallow groove. Mutagenesis studies and crystallographic data on the crystal contact interactions of loops of neighboring molecules in this groove also suggest that it is important for peptide-substrate binding.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Analysis of Wild-type and Mutant Recombinant Cr-P4H-I Polypeptides—Because NMR analysis of full-length Cr-P4H-1 (lacking the signal peptide and, thus, starting from Ala-17) with an N-terminal histidine tag (8) indicated that the N terminus of the polypeptide was disordered (data not shown), a truncated construct starting from Val-29 and containing a Met-His6-Met sequence at the N terminus (referred to as His6-Cr-P4H-1-V29) was generated, expressed in Escherichia coli, and purified to homogeneity as

![FIGURE 1. The reaction catalyzed by P4H (A) and the structures of 2-oxoglutarate (B) and pyridine 2,4-dicarboxylate (C). For consistency in nomenclature, the atoms in pyridine 2,4-dicarboxylate are shown using the same numbering as in 2-oxoglutarate.](image)

![FIGURE 2. Amino acid sequence comparison of Cr-P4H-1 with A. thaliana P4H-1 (At-P4H-1) and -2 (At-P4H-2) and the catalytic domains of the α subunits of human C-P4Hs I–III (Hsα(I)–α(III)) and HIF-P4H-2. The secondary structure elements of Cr-P4H-1 and HIF-P4H-2 are indicated above and below the sequences, respectively. The β-strands forming the jellyroll core motif are shown with red arrows, whereas the other secondary structures are in blue. The residues that are predicted to bind the Fe2+ ion and the 1- and 5-carboxylate moieties of 2-oxoglutarate are highlighted with filled red arrows and black squares, respectively. The putative peptide-substrate binding residues subjected to mutagenesis are highlighted with cyan dots. The first amino acids of the crystallized His6-Cr-P4H-1-V29 and HIF-P4H-2 constructs are shown with black asterisks. The residues conserved throughout all sequences are in red, residues with 70–99% identity between the sequences are shaded in magenta, and those with 40–69% identity are in yellow.](image)
Prolyl 4-Hydroxylase Structure

described previously (8). SeMet-labeling of His$_{6}$-Cr-P4H-1-V29 was performed by expressing it in a methionine-requiring auxotrophic E. coli strain B384 (DE3) using 0.5 mM isopropyl 1-thio-β-δ-galactopyranoside induction for 20 h at 20 ºC. Mutations were introduced into the His$_{6}$-Cr-P4H-1-ing auxotrophic E. coli strain B384 (DE3) using a QuikChange™ site-directed mutagenesis kit (Stratagene). The mutagenesis oligos were the following: Y134F, 5’-CTGAGGTTCTTCTACCCATGACGTCAG-AAG-3’ + 5’-CTTCTGAGCCTAGTAGGAAATGCGAAACCTGCAG-3’; Y168F, 5’-GTCAGGTTCTTCTACCCATGACGTCAG-AAG-3’ + 5’-GTGGAGCTTCTGAGTGGCGATCTCGCTGTGC-3’; S95A, 5’-GAGATCCGCACCGCAGGCA-CCTGG-3’ + 5’-CCAGGTCCGCGGTTGCGGATCTC-3’; W243F, 5’-ACCCAGCGCCCGGCGGTTGCTG-GTGG-3’ + 5’-CTGACCTTTAGACCGCGGA-GGCGTGAC-3’; Q130A, 5’-CCATGAGGGGCTGGCCTCGCTGACTAC-3’ + 5’-GATGTCGAGTAGATGCTCGCTGACTAC-3’; E127A, 5’-GGGCCGCTGGTGCTGGTGCTGGTGGCGGCTGGATCTC-3’; H11032, 5’-ACCCAGCGCCCGGCGGTTGCTG-GTGG-3’ + 5’-CTGACCTTTAGACCGCGGA-GGCGTGAC-3’; H11001, 5’-CTGACCTTTAGACCGCGGA-GGCGTGAC-3’ + 5’-CTGACCTTTAGACCGCGGA-GGCGTGAC-3’.

Because co-crystallization experiments of the His$_{6}$-Cr-P4H-1-V29 with ligands failed, another construct having an N-terminal SUMO partner fused to the Gly-30 of Cr-P4H-1 (referred to as Cr-P4H-1-G30) was generated using the Champion™ pET SUMO protein expression system (Invitrogen). The fusion protein was expressed and purified as described (8), after which the SUMO partner was cleaved by digestion overnight with SUMO protease (Invitrogen or LifeSensors Inc.) at 4 ºC in 0.01 M Tris-HCl, 0.1 M NaCl, 0.1 M glycine, and 2 mM β-mercaptoethanol, pH 7.8. The sample was reapplied to a nickel nitrotriacetic acid column after which the cleaved Cr-P4H-1-G30 obtained in the flow-through was applied to a SuperDex HR75 column by which the buffer was changed into the storage buffer consisting of 0.01 M Tris-HCl, 0.15 M NaCl, 0.1 M glycine, and 2 mM ZnSO$_{4}$, a n d 2 mM pyridine 2,4-dicarboxylate. An ~20-s soak in the cryoprotectant before freezing the crystal in liquid nitrogen resulted in the optimal diffraction pattern and allowed data collection to 1.85 Å at the X12 beamline, EMBL, DESY, Hamburg. All the datasets were processed using XDS (26). The data collection statistics are summarized in Table 1. The initial phaseset (2.8 Å resolution, average figure-of-merit being 0.69) was obtained from the SeMet(apo) multiple-wavelength anomalous dispersion dataset using SOLVE (27), and the initial model was constructed using RESOLVE (28). The data were subsequently processed with programs of the CCP4 package (29). The model was further refined using the data to 1.93 Å resolution, with REFMAC5 (30) and the iterative model building programs O (31) and COOT (32). The needle(apo) and Zn(complex) crystal forms were solved by molecular replacement using Phaser (33) and molecule A of the SeMet(apo) as a model. Both structures were refined using REFMAC5 (30), and the models were completed using COOT (32). In most of the apo subunits there was a strong electron density in the 2-oxoglutarate pocket. This density could not be modeled as a water molecule, but a chloride ion placed at this position refined to normal B-values. The presence of a chloride ion also agrees with the geometry of this site, with distances to the surrounding atoms of Nz (Lys-237) and Og1 (Thr-178) being 3.1 and 3.0 Å, respectively. The final refinement statistics for all three crystal forms are shown in Table 1. Non-crystallographic symmetry restraints were used in the refinement of the needle(apo) crystal form.

The structures were validated using COOT (32), WHATIF (34), and Molprobity (35). The figures were prepared using ICM (MOLSOFT, LLC) (see Fig. 5, A and B) and SwissPDBviewer (36). For the structure superimpositions with the program O (31), the central residues Val-131—Trp-238 of the Cr-P4H-1 jelly roll β-strands were used. The superimpositions with HIF-P4H-2 (PDB code 1G19) and clavaminate synthase (CAS) (PDB codes 1GVG and 1DRY)
resulted in root mean square differences for the corresponding Cα atoms of 0.7, 0.8, and 0.8 Å, respectively.

RESULTS

Determination of the Cr-P4H-1 Structures—Four structures of N-terminal-truncated Cr-P4H-1 were obtained, two of the apoenzyme from two crystal forms referred to as the SeMet(apo) and needle(apo), one of a binary complex with the competitive inhibitor Zn2+, and one of a ternary complex with Zn2+ and pyridine 2,4-dicarboxylate (Tables 1 and 2). These two complex structures were obtained from the third crystal form referred to as the Zn(complex) form (Tables 1 and 2). The SeMet(apo) structure was

| TABLE 1 | Crystallization, data collection and refinement statistics of the three crystal forms of Cr-P4H-1  |
|-----------------------------------------------|--------------------------------------------------|
| Dataset | SeMet(apo) | Needle(apo) | Zn(complex) |
| PDB code | 2V4A | 2JIJ | 2JIG |
| Crystallization | His6-CrP4H-1-V29 | His6-CrP4H-1-V29 | CrP4H-1-G30 |
| Protein concentration (mg/ml) | 15 | 5.5 | 3.5 |
| Method | Hanging drop | Hanging drop | Sitting drop |
| Well solution | 2.5 m (NH4)2SO4 | 2.5 m (NH4)2SO4 | 2.0 m (NH4)2SO4 |
| 9% Me2SO | 9% Me2SO | 0.1 M Tris, pH 8.5 |
| Additives | 0.24 mM N-octanoyl-sucrose | 2 mM ZnSO4 | 2 mM pyridine 2,4-dicarboxylate |
| Temperature | +22 °C | +22 °C | +4 °C |
| Crystal shape | Bipyramid | Needle | Bipyramid |
| Crystal size (µm) | 350 x 350 x 350 | 500 x 50 x 50 | 170 x 150 x 100 |
| Data collection | BW7A, EMBL DESY, ID14-3, ESRF X12, EMBL DESY | ID14-3, ESRF X12, EMBL DESY | X12, EMBL DESY |
| Temperature (K) | 100 | 100 | 100 |
| Wavelength (Å) | 0.942 | 0.931 | 0.900 |
| Space group | P4,2,2 | P2,2,2 | P2,2,2 |
| Unit cell parameters (Å) | a = 137.5, b = 137.5, c = 88.1 | a = 98.3, b = 117.5, c = 72.0 | a = 57.9, b = 60.3, c = 116.9 |
| Redundancy | 7.6 | 4.1 | 4.6 |
| Resolution range (Å) | 25–1.93 (2.05–1.93) | 35–2.90 (3.0–2.90) | 32–1.85 (1.95–1.85) |
| Completeness (%) | 99.1 (98.1) | 98.8 (99.0) | 98.1 (89.2) |
| I/σ(I) (σ(I)) | 11.8 (3.8) | 9.7 (1.8) | 6.3 (2.7) |
| Rmerge (%) | 6.4 (33.6) | 7.6 (44.6) | 9.9 (52.0) |
| Vm (Å³/D) | 1.8 | 2.4 | 1.8 |
| Molecules/asymmetric unit | 4 | 3 | 2 |
| Wilson B (Å²) | 33.5 | 48.3 | 26.0 |
| Refinement | | | |
| Resolution | 19.9-1.93 | 33.8-2.90 | 29.2-1.85 |
| R (%) | 18.8 | 21.2 | 18.3 |
| Rfree (%) | 22.3 | 26.1 | 23.8 |
| Number of reflections | 60,331 | 17,898 | 33,301 |
| Number of atoms | 6,127 | 4,673 | 3,687 |
| Protein | 5,771 | 4,640 | 3,225 |
| Waters | 315 | 31 | 435 |
| Zn²⁺ | 4 | | 4 |
| Chloride | 2 | | |
| Pyridine 2,4-dicarboxylate | | | 12 |
| (SO₄)₂⁻ | 5 | | 5 |
| Glycerol | 24 | | 6 |
| Me₂SO | 8 | | |
| Geometry statistics | | | |
| Root mean square deviations, bonds (Å) | 0.017 | 0.016 | 0.011 |
| Root mean square deviations, angles (°) | 1.5 | 1.7 | 1.2 |
| Average B-factor (Å²) | 26.7 | 39.4 | 23.2 |
| Main/side chain | 25.2/26.9 | 39.1/39.9 | 21.3/22.9 |
| Waters | 37.1 | 29.3 | 31.6 |
| Ligands | 60.2 | | 19.0 |
| Ramachandran plot (Molprobity) | | | |
| Favored (%) | 98.9 | 96.5 | 99.5 |
| Allowed (%) | 1.1 | 3.5 | 0.5 |

| TABLE 2 | The four conformational states of Cr-P4H-1 and their properties |
|---------------------------------------------------------------|
| Conformational state | Crystall form | Reference molecule | Residue range | Metal-binding site | 2-Oxoglutarate-binding site | Peptide binding groove | β3-β4 Hairpin loop | Tyr-140 of ββ1 | ββ1-ββIII loop |
| SeMet(apo) | needle | D | 28–249 | Empty | Chloride ion + waters | Empty | Disordered | Out | Open |
| Needle(apo) | Needle(apo) | A | 28–251 | Water | Chloride ion | Waters | Neighboring ββ1-ββIII loop | Disordered | In | Open |
| Binary complex | Zn(complex) | B | 36–251 | Zn²⁺ | Own ββ1-ββIII loop | | Disordered | Out | Open |
| Ternary complex | Zn(complex) | A | 36–251 | Zn²⁺ | Pyridine 2,4-dicarboxylate | Own ββ1-ββIII loop | Ordered | In | Closed |

* These modes of binding are caused by crystal contacts; the bound loops belong to a neighboring molecule.
determined by the multiple-wavelength anomalous dispersion phasing method and was used to solve the needle(apo) and the Zn(complex) crystal forms by molecular replacement.

The SeMet(apo) crystal form was refined at 1.93 Å resolution, whereas the needle(apo) and Zn(complex) forms were refined at 2.90 and 1.85 Å, respectively (Table 1). Each crystal form has multiple copies per asymmetric unit, representing altogether four conformational states differing with respect to the presence of ligands and the conformation of some of the active site loops (Table 2). The cores of each conformational state superimpose well, with the root mean square deviations of Ca atoms in a pairwise comparison being 0.3–0.5 Å. Analysis of each of the structures by means of Molprobity (35) shows that there are no Ramachandran outliers (Table 1). The SeMet(apo) form has four similar molecules per asymmetric unit, of which molecule D is best defined, whereas the needle(apo) form has three similar molecules per asymmetric unit, of which A is best defined (Table 2).

The Zn(complex) form has two molecules of different conformation per asymmetric unit; molecule A (the ternary complex) has the competitive inhibitor pyridine 2,4-dicarboxylate in the 2-oxoglutarate-binding pocket and a Zn$^{2+}$ ion at the iron-binding site, whereas molecule B (the binary complex) has only the bound Zn$^{2+}$ (Table 2). The ternary complex is the best defined structure in terms of its corresponding electron density map and will mainly be used for the subsequent description of the overall fold. The Overall Structure—Cr-P4H-1 consists of 13 β-strands and 3 α-helices, which form a compact one-domain molecule (Figs. 2 and 3A). The C-terminal part (about 120 residues) is formed by 8 β-strands (β6–β13) folded into the jellyroll fold typical of the 2-oxoglutarate dioxygenases (22). These eight β-strands are labeled as βI–βⅢ for consistency in nomenclature (Figs. 2 and

FIGURE 3. The crystal structure of Cr-P4H-1. A, stereoview of the ribbon diagram of the ternary structure. The active site residues His-143, Asp-145, and His-227 that bind the Zn$^{2+}$ (gray ball), the Lys-237 that binds the 5-carboxylate moiety of the inhibitor pyridine 2,4-dicarboxylate (black stick), and the disulfide bond between Cys-195 and Cys-230 are shown in more detail. B, schematic structure of Cr-P4H-1. Color-coding and numbering of the secondary structures in A and B are identical to Fig. 2. C, stereoview of the superimposed structures of the four conformational states trapped in the three crystal forms. The ternary structure is shown in gray, and red; the rigid part of the Cr-P4H-1 fold is in gray, and the β3–β4 hairpin loop, β1–βII loop, βIII, and βII–βIII loop are in red. The latter regions have high conformational flexibility; the corresponding regions of the binary structure and the two apo structures are shown (if ordered) in magenta (binary), blue (SeMet(apo)) and green (needle(apo)), respectively. The Zn$^{2+}$ and pyridine 2,4-dicarboxylate in the ternary structure are shown as a gray ball and black stick, respectively. Some important side chains, discussed under “Results” and “Discussion,” of each structure are also shown.
formed between the open βIII-βIII loop and the strands β5, βI, and βII (Table 2).

The Catalytic Site—The catalytic site is best defined in the binary and ternary complexes with bound Zn$^{2+}$ and with Zn$^{2+}$ and pyridine 2,4-dicarboxylate, respectively (Figs. 3A and 4). The positions of the Zn$^{2+}$ ion and the pyridine 2,4-dicarboxylate molecule are clearly defined by the electron density maps. Zn$^{2+}$ and pyridine 2,4-dicarboxylate are competitive P4H inhibitors with respect to Fe$^{3+}$ and 2-oxoglutarate, respectively (2, 25, 37), with $K_i$ values for Cr-P4H-1 of 10 and 70 μM (8). Zn$^{2+}$ takes the position of Fe$^{3+}$ and is bound to His-143 (the proximal histidine) and Asp-145 of the His-X-Asp motif of βII and His-227 (the distal histidine) of βVII, with bond distances of 2.1, 2.1, and 2.2 Å, respectively (Fig. 3A). The inhibitor is positioned so that its 5-carboxylate moiety is bound in a deeply buried pocket inside the jellyroll core. This moiety forms four direct hydrogen bonds with the side chains of Tyr-134 (2.9 Å) of βI, Thr-178 (2.7 Å) of βV, and Lys-237 (2.8 Å) and Ser-239 (3.3 Å) of βVIII (Fig. 4). Lys-237 is a conserved basic residue corresponding to Lys-493 in the human C-P4H-I, which has been shown by site-directed mutagenesis to bind the 5-carboxylate of 2-oxoglutarate (21). Hydrogen bonds are also formed between the 1-carboxylate of the inhibitor and the side chains of Thr-241 (2.7 Å) and Trp-243 (3.3 Å) of βVIII and a water molecule (2.9 Å) (Fig. 4). The only apolar residues close to the bound inhibitor are Tyr-140 and Leu-166. Tyr-140 covers the binding pocket by a stacking interaction with the aromatic ring of the inhibitor. Interestingly, in the binary complex with no bound inhibitor Tyr-140 has flipped outwards and points to the bulk solvent, similar to the situation in molecule D of the SeMet(apo) structure (Fig. 3C and Table 2). At the back of the molecule, the pocket for binding of the 5-carboxylate moiety is sealed off from the bulk solvent by the hydrophobic side chains of Tyr-168 of βIII, Leu-200, Leu-202, and Pro-204 of βV, and Leu-210 of βVI. Residues from the N-terminal part of Cr-P4H-1, such as Val-43 and Leu-45 of βI and Leu-53 of β2, further extend this cluster of hydrophobic residues at the back of the molecule. The outer rim of the 2-oxoglutarate binding pocket, near the catalytic site, is shaped by Thr-241 and Trp-243 of βVIII, Gln-130 and Leu-132 of βI.
Arg-93 of β5, Tyr-140 of βII, and the metal ion binding residues (Fig. 5, A and B).

In the needle(apo) structure the side chains of the three iron binding residues superimpose well on those in the binary and ternary complexes (Fig. 3C). It appears that in this crystal form a loosely bound water molecule has replaced the Zn$^{2+}$, having distances of 3.0, 3.1, and 2.7Å to His-143, Asp-145, and His-227, respectively. In contrast, in the SeMet(apo) form the iron binding residues His-143 and Asp-145 of βII are only visible in subunit D but have high B factors. The two iron binding histidines point inwards, whereas Asp-145 points away from the catalytic site leading to a highly irregular structure (Fig. 3C) which is different from those seen in the binary and ternary complex.

Unlike the metal ion binding residues, all the 2-oxoglutarate binding residues superimpose well when the apo structures are compared with the binary and ternary structures. The 2-oxoglutarate binding pocket is shaped by residues of β (for example, Leu-132) and βII (for example Tyr-140). Farther above the Trp-99/Trp-243 center are the side chains of Arg-93 and Ser-95, which are located immediately after the β3-β4 hairpin loop. Arg-93 is stacked to the Tyr-140 side chain, the hydroxyl group of which points into the peptide binding groove. The Tyr-140 side chain is also stacked to the catalytic His-143 side chain. On the bulk solvent side, below the Trp-99/Trp-243 center, Glu-127 is also hydrogen-bonded to His-245, which is subsequently hydrogen-bonded to the main chain oxygen of Gly-157 and His-159 of the βII-βIII loop. These hydrogen bonds do not exist in the ternary complex with the closed βII-βIII loop, but instead there are hydrogen bonds between Arg-93 and Gln-130 and the closed βII-βIII loop.

Due to the crystallographic contacts, the putative peptide-substrate binding groove in the needle(apo) and the binary complex is occupied by a loop region of a neighboring molecule. The modes of binding of the polypeptide chain in this groove in the needle(apo) and binary complex are different from each other and also different from the mode of binding of the closed βII-βIII loop of the ternary complex. In all these complexes, however, hydrogen bonds are formed between the polar side chains adjacent to the Trp-99/Trp-243 center and the peptide bound in the groove.

**Mutagenesis Studies**—Based on the structural analyses, we selected for mutagenesis studies nine solvent exposed and two buried residues, predicted to be involved in peptide-substrate and a chloride ion (Table 2). The chloride ion is hydrogen-bonded to Lys-237 and Thr-178, thus replacing an oxygen of the 5-carboxylate moiety of pyridine 2,4-dicarboxylate. The other 5-carboxylate oxygen atom is replaced by a water molecule, which is hydrogen-bonded to Tyr-134 and Ser-239 in the apo structures.

**The Putative Peptide Binding Groove**—The βI-II/III loop and the β3-β4 hairpin loop shape a long shallow groove that stretches from Trp-99 (β5) to βII. The shape of this possible peptide binding groove is illustrated in Fig. 5, A and B. Its center, near the catalytic site, is dominated by Trp-243, which is covered by the Arg-161–Glu-127 salt bridge on the bulk solvent side and by the side chains of Gln-130 and Thr-241 on the bulk protein side. Adjacent to Gln-130 and Thr-241 (above in the standard view of Fig. 5B) this peptide binding pocket is shaped by residues of β (for example, Leu-132) and βII (for example Tyr-140). Farther above the Trp-99/Trp-243 center are the side chains of Arg-93 and Ser-95, which are located immediately after the β3-β4 hairpin loop.

The groove in the needle(apo) and the binary complex is occupied by a loop region of a neighboring molecule. The modes of binding of the polypeptide chain in this groove in the needle(apo) and binary complex are different from each other and also different from the mode of binding of the closed βII-βIII loop of the ternary complex. In all these complexes, however, hydrogen bonds are formed between the polar side chains adjacent to the Trp-99/Trp-243 center and the peptide bound in the groove.

**FIGURE 5. The putative peptide-substrate binding groove of Cr-P4H-1.** Stereoview of this groove (marked by two arrows) in the needle(apo) structure shown as an electrostatic surface potential representation (A) and a zoomed-in ribbon representation (B). The red and blue colors in A refer to negative and positive potential, respectively, whereas the gray color refers to a neutral potential. The geometry of the Zn$^{2+}$ (brown sphere) and pyridine 2,4-dicarboxylate (green stick) are from the ternary complex after superimposition on the needle(apo) structure. In B the three metal ion binding residues and the residues shaping the putative peptide binding groove are shown in stick representation. Note that the β3-β4 hairpin loop is disordered in this structure; the C and N termini, respectively, of the ordered β3 and β5 strands are labeled in both panels.
2-oxoglutarate binding, respectively (Table 3). Analysis of the purified mutant enzymes by circular dichroism (CD) spectropolarimetry confirmed that these mutations had no substantial effects on the secondary structures and thermal stabilities of the variants as compared with the wild-type enzyme (data not shown). The structures suggested that tyrosines 134 and 168 might be involved in 2-oxoglutarate binding, and their mutations to phenylalanines indeed increased the $K_m$ values for 2-oxoglutarate 6- and 3.5-fold, respectively, whereas they did not affect the $K_m$ for poly(L-proline) (Table 3). Mutations of Arg-93, Ser-95, Tyr-140, Arg-161, and His-245 to alanines totally inactivated the enzyme (Table 3), in line with the proposal that these residues may have a crucial role in peptide binding. Likewise, the E127A mutation reduced the enzyme activity so dramatically that it was impossible to determine reliable kinetic constants. Mutations of the proposed peptide binding residues Trp-99 and Gln-130 to alanine and Trp-243 to phenylalanine led to about 2.5- and 1.8-fold increases and no increase in the $K_m$ for poly(L-proline), respectively, and about 3.5-, 2-, and 12-fold decreases in $k_{cat}$ (Table 3). These surface mutations did not affect the $K_m$ for 2-oxoglutarate.

**TABLE 3**

| Mutation and its proposed function | $K_m$ Poly(L-Pro)$^a$ | $K_m$ 2-Oxoglutarate$^a$ | $k_{cat}$ 2-Oxoglutarate$^a$ |
|-----------------------------------|----------------------|--------------------------|-------------------------------|
| Wild type                         | 250 ± 35             | 160 ± 45                 | 70 ± 10                       |
| 2-oxoglutarate binding            |                      |                          |                               |
| Y134F                             | 280 ± 90             | 1000 ± 230               | 3 ± 1                         |
| Y168F                             | 270 ± 15             | 570 ± 160                | 30 ± 2                        |
| Peptide binding                   |                      |                          |                               |
| RV93A                             | Inactive             |                          |                               |
| S95A                              | Inactive             |                          |                               |
| W99A                              | 650 ± 80             | 130 ± 50                 | 20 ± 3                        |
| E127A                             | ND$^b$               | ND                       | ND                            |
| Q130A                             | 460 ± 60             | 150 ± 40                 | 35 ± 4                        |
| Y140A                             | Inactive             |                          |                               |
| R161A                             | Inactive             |                          |                               |
| W243F                             | 280 ± 30             | 140 ± 60                 | 6 ± 1                         |

$^a$Values are the means ± S.D. from 4–8 independent experiments. 
$^b$ND, not determined because of too low enzyme activity for reliable determination of kinetic constants.

**FIGURE 6.** Structure-based sequence alignment of the jellyroll core fold of 2-oxoglutarate dioxygenases. The locations of the eight jellyroll $\beta$-strands (I–VIII) are shown above the sequence alignment. The approximate location of $\beta$II is shown with a dashed arrow because in some superfamily members it is very short (as in Cr-P4H-1) or totally irregular (as in HIF-P4H-2). The Fe$^{2+}$ and 2-oxoglutarate binding residues of Cr-P4H-1 are marked using red arrows and black squares, respectively. The boxed residues highlight the conserved 2-oxoglutarate binding residues. The sequence identifiers and PDB codes are: HIF-P4H-2 (38) to 7 (CAS) (41). The substrates of most of these enzymes are small molecules, whereas those of Cr-P4H-1, HIF-P4H-2, and the HIF asparaginyl hydroxylase (factor-inhibiting HIF (FIH)) are polypeptides and that of the (AlkB) is a polynucleotide (15, 38, 42–44).

All superfamily members have the conserved eight-stranded jellyroll core fold (Fig. 6), but in addition they can have exten...
sions or domains at the N terminus, C terminus, or within the core between the βIV and βV (22). The longest N-terminal and C-terminal extensions are observed in HIF-P4H-2 and proline 3-hydroxylase, respectively (Fig. 6) (15, 45). The highest sequence similarity within the superfamily, apart from the four catalytically critical residues, is found in the βV-βVI region (Fig. 6). In all these structures there is a tight turn between βV and βVI, and the hydrophobic residues in this region are well conserved. βV and βVI are the edge strands of the jellyroll core fold, and this region defines the bottom of the 2-oxoglutarate binding pocket (as shown in Fig. 3, A and B). The high sequence conservation suggests that this region may be important for proper folding and stability of the jellyroll framework. A unique feature of the Cr-P4H-1-fold is the disulfide bridge (Cys-195—Cys-230) between the extended βIV-βV loop and βVII of the jellyroll core (Fig. 3A). In HIF-P4H-2, as in four other members, this loop region is very short (only 5 residues), whereas in Cr-P4H-1 it has 18 residues and forms a small helix (α3) that contains the unique cysteine.

The jellyroll core fold, the β2- and β3-strands, and the two N-terminal helices of Cr-P4H-1 and HIF-P4H-2 superimpose well (Fig. 7), but large deviations exist at the N terminus and C terminus and in some of the loop regions. The crystallized HIF-P4H-2 starts with an α-helix, whereas the corresponding Cr-P4H-1 region is a β-strand (β1). Also the C-terminal tail of HIF-P4H-2 is longer and adopts a helical conformation. These structural differences as well as the longer α3-βI loop in HIF-P4H-2 (α2-βI loop in Cr-P4H-1) and the longer βVI-βVII and βIV-βV loops in Cr-P4H-1 are not close to the catalytic site (Fig. 7). However, two important differences in loop structures are present near the putative peptide binding region. Closest to the catalytic site is the longer extension after βII (βII-βIII loop) in Cr-P4H-1, which is a very short turn in HIF-P4H-2 (Figs. 2 and 7). The second difference near the catalytic site concerns the β3-β4 hairpin loop, which has a different conformation in the ternary structure of Cr-P4H-1 than in the corresponding HIF-P4H-2 structure (Fig. 7). It is noteworthy that this region is fully disordered in the apo structures and the binary structure of Cr-P4H-1, indicating high structural plasticity.

The Catalytic Site—Cr-P4H-1 is one of the few 2-oxoglutarate dioxygenases that has been crystallized in two apo forms without any bound ligand as well as in a binary complex with a bound metal and a ternary complex with a bound metal and a 2-oxoglutarate analogue (Table 2). The disorder in the Fe²⁺ binding region in the absence of bound metal ion, in the SeMet(apo) form, highlights the importance of the metal ion for obtaining the correct geometry of a competent catalytic site.

In the ternary structure the N-moiety of the pyridine 2,4-dicarboxylate (corresponding to the 2-oxo moiety of 2-oxoglutarate, Fig. 1) is bound to the metal ion opposite to the carboxylate group of Asp-143 in the His-X-Asp motif (Figs. 4 and 8, A and B). This geometry is seen in most of the other structures of corresponding complexes of the superfamily members. A geometry where the 1-carboxylate is bound opposite to the distal histidine is seen in a subset of superfamily members, for example in CAS, cephalosporin synthase and phytanoyl-CoA-2 hydroxylase (22). In this subset of enzymes the putative oxygen-binding site is proposed to be opposite to the histidine of the His-X-Asp motif. This is best established for CAS. In the complex of CAS with 2-oxoglutarate, Fe²⁺, and nitric oxide (NO), the NO is bound to this site mimicking the binding of O₂ (46). Interestingly, in CAS the binding of NO induces a small rearrangement in the mode of 2-oxoglutarate binding (46). Without NO the 1-carboxylate moiety is bound to the iron opposite the proximal histidine, whereas in the presence of NO it moves up, more opposite to the distal histidine (Fig. 8B). These observations in CAS suggest that 2-oxoglutarate may also be bound somewhat further up, more opposite to distal His-227 in the actual active complex of Cr-P4H-1 with bound O₂. In CAS, the shifted position of the 1-carboxylate moiety replaces a water molecule, whereas in Cr-P4H-1 such an adjustment in the mode of 2-oxoglutarate binding would require a structural rearrangement of the Tyr-140 side chain. Interestingly, this tyrosine adopts a completely different conformation in the Cr-P4H-1 binary complex (Fig. 3C), pointing away from the catalytic site.

The structural homology between Cr-P4H-1 and the other superfamily members suggests that the oxygen-binding site in Cr-P4H-1 is opposite to the proximal His-143, near the Asp-145 and Trp-243 side chains in an otherwise hydrophobic pocket that is also shaped by Thr-164, Leu-166, Phe-212, and Thr-241 (Figs. 4 and 8, A and B). Each of the latter residues protrudes out of the major sheet, whereas each of the three Fe²⁺ binding residues protrudes out of the minor sheet. In the
ternary complex this pocket is empty, but in the HIF-P4H-2 complex structure the sixth coordination site of iron (trans to the proximal histidine) is occupied by a water molecule (Fig. 8A). The situation is different in the binary structure of Cr-P4H-1, which has no bound pyridine 2,4-dicarboxylate. In this complex the architecture of the catalytic site residues coordinating Zn$^{2+}$ is the same, but the coordination sites occupied by the pyridine 2,4-dicarboxylate atoms are now occupied by two loosely bound water molecules, 2.0 and 3.0 Å away from the Zn$^{2+}$ ion. No water is bound in the putative oxygen binding pocket opposite the proximal His-143 in this structure.

The 2-Oxoglutarate-binding Site—Although pyridine 2,4-dicarboxylate is a known P4H inhibitor, no 2-oxoglutarate dioxygenase structures complexed with it have been reported so far. Its 5-carboxylate moiety (Fig. 1) is bound deep inside the 2-oxoglutarate binding pocket and is salt-bridged to Lys-237 and hydrogen-bonded to Tyr-134, Thr-178, and Ser-239 (Fig. 4). Each of the hydrogen-bonding residues is a common 2-oxoglutarate binding residue in the superfamily (Fig. 6). Lys-237 (βVII) corresponds to an arginine in all other superfamily members except for FIH, in which case Lys-214 of another β-strand (βIv) is the corresponding residue (42). In some superfAMILY members (such as HIF-P4H-2 and CAS but not Cr-P4H-1), a buried water molecule also provides a hydrogen-bonding partner for the 5-carboxylate moiety (Fig. 8A and B). The positioning of this moiety with respect to the jellyroll core is indeed similar in each superfAMILY member, as shown for Cr-P4H-1, HIF-P4H-2, and CAS in Fig. 8, A and B. This highlights the conserved, anchoring mode of binding of the 5-carboxylate moiety. The Y134F mutation increased the $K_m$ of Cr-P4H-1 for 2-oxoglutarate 6-fold and reduced the $k_{cat}$ about 20-fold, whereas Y168F increased the $K_m$ for 2-oxoglutarate 3.5-fold and reduced the $k_{cat}$ about 2-fold (Table 3). These data show that Tyr-134, which is directly hydrogen-bonded to the 5-carboxylate moiety, is more critical for catalytic efficiency than Tyr-168, which is linked to the inhibitor only by indirect hydrogen bonding (Fig. 4).

The two polar residues involved in hydrogen bonds with the 1-carboxylate moiety of pyridine 2,4-dicarboxylate, Thr-241 and Trp-243 (Fig. 4), are located near the entrance of the 2-oxoglutarate binding pocket, which is in the center of the extended peptide binding groove (see below). These two residues, protruding out of βVIII, are also found in HIF-P4H-2, but no hydrogen bonds are formed between them and the bound inhibitor in the HIF-P4H-2 crystal structure because the structure of the inhibitor is different, missing the 1-carboxylate moiety (Fig. 8A). However, as these residues are conserved in HIF-P4H-2 and are well aligned with Thr-241 and Trp-243 of Cr-P4H-1, they are likely to be hydrogen-bonded to the 1-carboxylate moiety of 2-oxoglutarate in HIF-P4H-2 as well. The structural superimposition shows that the inhibitor bound to HIF-P4H-2 cannot become bound in the same mode...
to Cr-P4H-1, because the entrance of its 2-oxoglutarate binding pocket is narrower and more polar due to Leu-132 and Gln-130 (Ala-301 and Met-298 in HIF-P4H-2, respectively) (Fig. 8A). Gln-130, Thr-178, and Lys-237 of the 2-oxoglutarate binding pocket are conserved in C-P4Hs but not in HIF-P4H-2 (Figs. 2 and 8A), indicating that the 2-oxoglutarate binding pocket of algal and C-P4Hs is uniquely different from the binding pocket of the HIF-P4H-2.

The Putative Peptide Binding Groove—The extended peptide binding groove in Cr-P4H-1 stretches from Trp-99 (β5) to βII (Fig. 5, A and B) and is further shaped by the long βII-βIII loop and the β3-β4 hairpin loop together with the Arg-93 side chain located immediately after the latter loop. The corresponding regions of HIF-P4H-2 are different. In HIF-P4H-2 the βII-βIII loop is much shorter and the β2-β3 hairpin loop points in a different direction from the corresponding β3-β4 hairpin loop in Cr-P4H-1 (Figs. 2 and 7). It has been suggested that the β2-β3 hairpin loop may have a role in the substrate specificity of HIF-P4Hs (15). Arg-93 is conserved in HIF-P4H-2 (Arg-252), but points away from the catalytic site, whereas in Cr-P4H-1 it points toward the catalytic site in the center region of the putative peptide binding groove, interacting with the side chains of Ser-77 and Ser-95. Ser-77, which is exposed on the surface, is at the beginning of β3 and the somewhat buried Ser-95 is part of β5 (Fig. 5B). Ser-95 is conserved in the C-P4H α subunits but not in HIF-P4H-2 (Fig. 2). Mutation of Arg-93 or Ser-95 to alanine inactivated Cr-P4H-1 (Table 3), indicating an important role in catalysis and/or substrate binding. The mode of peptide-substrate binding to HIF-P4H-2 has not been determined, but FIH has been co-crystallized with a fragment of the C-terminal transactivation domain of HIF-1α (43). This fragment is bound to a groove that has a distinctly different shape from the putative peptide binding groove of Cr-P4H-1 due to differences in the β3-β4 hairpin loop and to the very short βII-βIII loop, the elongated βIV-βV loop, and the longer C-terminal tail in FIH (43). These structural differences essentially fill up the open space present near the solvent-exposed βII of Cr-P4H-1.

Mutation of Trp-99 in the peptide binding groove to alanine increased the $K_m$ for poly(l-proline) about 2.5-fold and reduced the $k_{cat}$ about 3.5-fold (Table 3). The W243F mutation had no influence on the $K_m$ for poly(l-proline) but reduced the $k_{cat}$ about 3.5-fold (Table 3). The polar residues Glu-127, Gln-130, Tyr-140, and Arg-161 surrounding these aromatic residues were also found to be important for catalytic function. Mutation of Glu-127, Tyr-140, or Arg-161 to alanine led to complete or essentially complete inactivation, whereas the Q130A mutation led to similar but less severe changes in the catalytic properties to those brought about by the W99A mutation (Table 3). Each of the mutated polar side chains is probably important for substrate binding and are indeed hydrogen-bonded to the bound peptide from the neighboring molecule in the needle(apo) structure. The role of His-245 may also concern the stabilization of the open conformation of the βII-βIII loop via two hydrogen bonds to the main chain oxygens of this loop. This suggestion is supported by the inactivation seen by the H245A mutation (Table 3). The importance of this histidine is also highlighted by the fact that it is crucial for the catalytic activity of the human C-P4H-1 (21).

Concluding Remarks—The structural analysis of Cr-P4H-1 combined with its mutagenesis data provide strong evidence that the protein surface near Trp-243 is the central region of a peptide binding groove. It is seen that Cr-P4H-1 and the catalytic domain of C-P4Hs form a separate structural subgroup distinct from HIF-P4H-2. Each of the nine residues of the predicted peptide binding surface that were selected for mutagenesis is also conserved in each of the C-P4H α subunit isoforms (Fig. 2), and the lengths of the loops near the catalytic site are very similar (Fig. 2). The lengths of the βII-βIII loop and the β3-β4 hairpin loop seem to be particularly important for defining the shape of the peptide binding groove. These loops have a large conformational flexibility in the Cr-P4H-1 structure, and it appears that they can fold over the bound peptide-substrate, thus forming a closed active site. The importance of an active site isolated from the bulk solvent for efficient catalysis has also been proposed as being a key feature in other 2-oxoglutarate dioxygenases, for example carbapenem synthase (47) and cephalosporin synthase (48). Further mutagenesis and crystallographic studies on Cr-P4H-1 have been initiated to characterize the precise mode of peptide-substrate binding.

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