Porcine Carbonyl Reductase

STRUCTURAL BASIS FOR A FUNCTIONAL MONOMER IN SHORT CHAIN DEHYDROGENASES/REDUCTASES*

Debashis Ghosh††§§, Mark Sawicki‡‡, Vladimir Pletnev+, Mary Erman§, Shuji Ohno¶, Shizuo Nakajin‡‡, and William L. Duax‡

From the  †Department of Structural Biology, Hauptman-Woodward Medical Research Institute, Buffalo, New York 14203, the ‡Department of Molecular and Cellular Biophysics, Roswell Park Cancer Institute, Buffalo, New York 14263, the §Department of Clinical Pharmaceutics, Faculty of Pharmaceutical Sciences, Nihon University, Narashinodai, 7-7-1 Funabashi-shi, Chiba 274-8555, and the ¶Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

The short chain dehydrogenases/reductases (SDR) superfamily and catalyzes the NADPH-dependent reduction of ketones on steroids and prostaglandins. The enzyme shares nearly 85% sequence identity with the NADPH-dependent human 15-hydroxyprostaglandin dehydrogenase/carbonyl reductase. The tertiary structure of the enzyme at 2.3 Å reveals a fold characteristic of the SDR superfamily that uses a Tyr-Lys-Ser triad as catalytic residues, but exhibits neither the functional homotetramer nor the homodimer that distinguish all SDRs. It is the first known monomeric structure in the SDR superfamily. In PTCR, which is also active as a monomer, a 41-residue insertion immediately before the catalytic Tyr describes an all-helix subdomain that packs against interfacial helices, eliminating the four-helix bundle interface conserved in the superfamily. An additional anti-parallel strand in the PTCR structure also blocks the other strand-mediated interface. These novel structural features provide the basis for the scaffolding of one catalytic site within a single molecule of the enzyme.

The short chain dehydrogenases/reductases (SDR) catalyze critical steps of activation and inactivation of steroids, vitamins, prostaglandins, and other bioactive molecules by oxidation and reduction of hydroxyl and carbonyl groups, respectively (1). A member of the SDR superfamily, the multifunctional enzyme porcine testicular carbonyl reductase (PTCR/3α/β, 20β-hydroxysteroid dehydrogenase (20β-HSD), catalyzes the NADPH-dependent reduction of ketones on androgens, progestins, and prostaglandins, as well as aldehydes and ketones on a large number of xenobiotics (2, 3). The high level of 20β-HSD activity in the enzyme is demonstrated by the reduction of 20-carbonyl groups of C21-steroids, such as conversion of 17α-hydroxyprogesterone to 17α,20β-dihydroxy-4-pregnen-3-one, which is present in pig testes during the neonatal stage (4, 5). Purified PTCR also shows vigorous 3α- and 3β-HSD activities with 5α-androstan-17β-ol-3-one (5α-dihydrotesterone) as a substrate (6).

PTCR is highly homologous to NADPH-dependent human and rat CRs, except for its 13 additional amino acid residues at the C terminus (2). The sequence identity of PTCR with the human CR, also known as 15-hydroxyprostaglandin dehydrogenase/9-ketoprostaglandin reductase, is about 85% (2). Alignment of amino acid sequences of PTCR and human CR with those of other well known members of SDR superfamily, such as bacterial 3α,20β-HSD (7, 8), human 17β-HSD type 1 (9), human 11β-HSD (10), and Drosophila alcohol dehydrogenase (11), suggests that these CRs have a 41-residue insertion at a strategic location, before the conserved Tyr-X-X-X-Lys motif. Nearly all SDRs are known to utilize a Tyr-Lys-Ser triad as catalytic residues (1). The Tyr hydroxyl has been proposed to be the proton donor in an electrophilic attack on the substrate carbonyl in a reduction reaction (8).

The other distinguishing feature of all SDRs is that the functional units are either homotetramers or homodimers (8, 9, 12–15). Surprisingly, the active units of both PTCR and human CR were reported to be monomeric (5, 16). Although more than half of the 15 or so crystal structures of SDRs to date exhibit the same tetrameric quaternary structure, only the four-helix bundle or the Q axis interface that involves the largest surface area of association (8) is conserved among SDRs. The other surface of close association of monomers in tetrameric SDRs is the so-called P axis interface involving an anti-parallel pair of strands and a pair of helices (8) that has been observed only once in a dimeric SDR (17). The interfacial four-helix bundle that borders two catalytic sites and buries hydrophobic surfaces of helices is predicted to be important for the integrity of the active site clefts (18, 19). Here we present the first crystal structure of a monomeric SDR and explain how the functional determinants of oligomeric SDRs could be retained within a monomer of PTCR having the basic SDR fold.

EXPERIMENTAL PROCEDURES

Purification, Crystallization, and Data Collection—The enzyme was purified as described (5). Crystalization and data collection details have also been published (20). Briefly, the enzyme was crystallized from a solution of 36–37% saturated ammonium sulfate in 10 mM MES buffer, pH 6.0. Crystals of diffraction size were grown by macroseeding. The space group is P41212 and the unit cell parameters are a = b =
Structure of Porcine Carboxyl Reductase

58.53 Å and c = 165.64 Å with one molecule (288 amino acid residues) in the asymmetric unit. Data collection was carried out on a Rigaku R axis II image plate detector at ambient temperature and processed with the software package DENZO (21). Data from three crystals between resolution range 55.2 and 2.30 Å were merged to yield a data set of 10792 unique reflections (F > 0) with an average of about three observations per reflection. The overall Rmerge was 0.066 on the intensities. The data were 80% complete overall and 52% complete in the resolution shell between 2.50 and 2.30 Å.

Structure Solution—The structure was solved by the single isomorphous replacement and anomalous scattering method, combined with molecular replacement solution. Two platinum compounds, potassium chloroplatinate (PtCl4) and di-μ-iodobis(ethylenediamine)diplatinum(II) nitrate (PIP), were used to prepare heavy atom derivative crystals, both yielding the same major platinum-binding site. A total of 4351 reflections to 3.00 Å was phased using the PIP derivative, and 3564 reflections to 3.20 Å were phased using the PtCl4 derivative. The latter data set also contained anomalous signal for 1680 reflections to 3.40 Å. The centric R value and the phasing power for the PIP and PtCl4 derivatives were 0.56 and 1.64 and 0.63 and 1.41, respectively. The phasing for the anomalous data was 1.92. The software package PHASES (22) was used for calculating and combining phases.

The molecular replacement solution was obtained using a search model which was a superposition of equally weighted structures of 3a,20β-HSD (8), 17β-HSD1 (9), mouse lung carboxyl reductase (13), and 7α-HSD (14). A large number of rotation search solutions were computed using compact (without loop regions) and extended (all loops through the catalytic triad present) search models. The optimal solution was obtained with the CNS program suite (23) and the extended search model with data between 15.00 and 3.50 Å, against an E2E2 synthesis. The translation function search was conducted in both P43212 and P41212 space groups. Phases from the most likely solutions were combined with single isomorphous replacement and anomalous scattering phases from a molecular replacement solution. Two platinum compounds, potassium chloroplatinate (PtCl4) and di-μ-iodobis(ethylenediamine)diplatinum(II) nitrate (PIP), were used to prepare heavy atom derivative crystals, both yielding the same major platinum-binding site. A total of 4351 reflections to 3.00 Å was phased using the PIP derivative, and 3564 reflections to 3.20 Å were phased using the PtCl4 derivative. The latter data set also contained anomalous signal for 1680 reflections to 3.40 Å. The centric R value and the phasing power for the PIP and PtCl4 derivatives were 0.56 and 1.64 and 0.63 and 1.41, respectively. The phasing for the anomalous data was 1.92. The software package PHASES (22) was used for calculating and combining phases.

RESULTS AND DISCUSSION

The Tertiary Structure of PTCR, an Overview of SDRs—All 288 amino acid residues of one polypeptide chain of PTCR (excluding the N-terminal methionine) were located from electron density maps. Amino acid residues are numbered 1–288 starting with the N-terminus at position 2 in the DNA sequence (2). A ribbon diagram of the tertiary structure of PTCR is shown in Fig. 1a. A schematic description of the structure is provided in Fig. 1b. The basic SDR fold includes a seven-stranded parallel β-sheet (βA to βG) flanked by three parallel helices on each side (αB, αC, αG and αD, αE, αF). The segment βA to βF is a doubly wound α/β motif, with alternating β-strands and α-helices. Whereas the βA to βF segment constitutes the classic “Rossman fold” associated with the binding of the coenzyme NADPH, the βD to βG segment, in addition to being partially within the Rossman fold, governs quaternary association and substrate binding. The two longest helices αE and αF form the interfacial four-helix bundle, typical of dimeric and tetrameric forms.

The view in Fig. 1a is along the central β-sheet of the PTCR molecule, overlooking the active site delineated by the coenzyme NADPH and catalytic residues Tyr-193, Lys-197, and Ser-139. As shown in Fig. 1, a and b, the basic SDR fold described above is well preserved in PTCR, except for two important differences. First, the 41-residue insertion before Tyr-193 describes four helices αF-1 (residues 140–148), αF-2 (residues 151–158), αF-3 (residues 164–179), and αF-4 (residues 182–186). The first turn of αF-1 coincides with the helical turn immediately following the catalytic serine (Ser-139 in PTCR) present in nearly all SDRs. This all-helix subdomain folds away from the active site cleft, towards the dimer interface of other SDRs. Second, after a tight turn the polypeptide following βG describes an additional strand βH (residues 272–275) that is anti-parallel to βG. The last 13 C-terminal residues form an extended tail-like structure that approaches the active site of a 2-fold symmetry-related molecule. The terminal carboxyl group forms a salt bridge with the Arg-37 side chain of the second molecular. A similar eighth anti-parallel strand is present only in one other member of the SDR family, dihydropteridine reductase (12).

Fig. 1c is a superposition of monomers of crystal structures of all four steroid dehydrogenases belonging to the SDR superfamily (bacterial 3a,20β-HSD, human 17β-HSD1, Escherichia coli 7α-HSD (14), and PTCR). The viewing direction in this figure is roughly the same as in Fig. 1a. 3a,20β-HSD and 7α-HSD have homotetrameric structures, whereas 17β-HSD1 is homodimeric (Fig. 2). The structural diversity among monomers of all members of the SDR family is well represented by this group. The overlap of the Rossman fold and the catalytic triad among these four enzymes, excluding the 41-residue insertion in PTCR, is striking. Furthermore, two long helices αE and αF from 3a,20β-HSD, 7α-HSD, and 17β-HSD1 that make up the four-helix bundle dimer interface superimpose remarkably well with the ones from PTCR, which are not involved in dimer formation.

The other important observation from this superposition is the diversity of the three-dimensional structures of the regions primarily contributed by the C-terminal halves of polypeptide chains. This region, which constitutes the outer walls of the
substrate-binding cleft of the active site, provides specific interactions that are critical to the selectivity of substrates and to the mechanism of molecular recognition by the enzyme (19, 29). Although this region is well developed for 17β-HSD1 having additional structural elements from an insertion between βF and αG, and from an extended C terminus that surrounds the catalytic cleft, fewer residues from similar regions of 3α,20β-HSD and 7α-HSD are present in the area. In 3α,20β-HSD, a segment of the polypeptide chain from the loop between βF and αG (Met-184 to Pro-206) and C-terminal residues Trp-243 to Gln-255 border the active site cleft (8). Similarly, in 7α-HSD, residues Ala-191 to Pro-214 between βF and αG and C-terminal residues Gly-250 to Asn-255 line the active site (14). Thus, the outer wall structures in 3α,20β-HSD and 7α-HSD differ significantly from that of 17β-HSD1. These differences in the architecture of active sites probably explain why 17β-HSD1 has high specificity for estrone as a substrate, whereas the other two enzymes are not known to be substrate-specific.

Nonetheless, all three enzymes possess the so-called substrate entry-loop between βF and αG, which tightens on the substrate upon its entry into the active site providing additional substrate-specific interactions (8, 14, 19, 29).

The entrance to the active site in PTCR is more open than any other HSD. This is a direct consequence of a shortened three-residue linkage between βF and αG, connecting Gly-236 and Pro-240 (corresponding to residues Thr-190 and Val 210, respectively, in 3α,20β-HSD). Consequently, the substrate-entry loop is almost non-existent in PTCR. Moreover, only residues Glu-281 to Ala-288 from the C-terminal tail are in the vicinity of the active site. The number of residues that border the substrate-binding cleft is thus only 15 in PTCR, as opposed to 37 in 3α,20β-HSD, 30 in 7α-HSD, and 60 in 17β-HSD1. The openness of the substrate-binding cleft is consistent with the observed promiscuity in substrate specificity of PTCR.

The Lys-238 side chain in human placental CR is autocatalytically modified to carboxyethyllysine (30). This side chain in
PTCR is in a highly exposed region, and its electron density is not discernible beyond the Cδ atom owing probably to dynamic disorders.

The Quaternary Structure of SDRs and How a PTCR Monomer Mimics It—The Q axis dimer interface involving the four-helix bundle (a pair of αE and αF helices) (a), and the P axis interface (a pair of strands βG and helices αG) (b). The same four-helix bundle interface is also shown in the ribbon diagram of homodimeric 17β-HSD1 in c. The views are down the molecular 2-fold rotation axes in all three figures. a and c, active sites across the four-helix bundle are highlighted by bound inhibitors and/or coenzyme. A close-up view of the stacking of new subdomain helices (αF'-2 and αF'-3) of monomeric PTCR against αE and αF is shown in d.

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Why Is PTCR NADPH-specific?—Although no coenzyme was added in the crystallization medium, a molecule of NADPH was bound to the protein molecule. The coenzyme is in the symmetry-related helices αE and αF in 3α,20β-HSD, 17β-HSD1, and 7α-HSD, blocking the interface and preventing dimer formation. However, as shown in a close-up view of the area in Fig. 2d, this arrangement does not completely shield the interface leaving αE and αF partially exposed. Selective substitutions of residues on these helices in PTCR render the exposed surface more hydrophilic than in oligomeric SDRs, as shown in Fig. 2d (substitutions from 3α,20β-HSD to PTCR are as follows: Phe-116 to Arg-118, Thr-121 to Glu-123, Val-168 to Arg-209, Gly-171 to Arg-212, and Thr-172 to Glu-213). All four helices of the insertion domain are amphipathic; their hydrophobic surfaces face the outer hydrophobic surfaces of αE and αF, and their hydrophilic surfaces are exposed to the solvent, as shown for αF'-3 in Fig. 2d. Interestingly, the inner hydrophobic surface of αF'-3 contains Met-171 which packs against Met-110, in close proximity to the Met-115 side chain, both from αE. The Sδ atom of Met-171 is at a distance of 3.76 Å from Sδ of Met-110, which is 6.04 Å from Sδ of Met-115. This partially exposed “methionine trap” is responsible for the high affinity binding of platinum ions (mono- and di-platinate) to the crystalline protein that resulted in the only useful isomorphous derivatives for solving the phase problem (see under “Experimental Procedures”).
extended conformation as observed for all other NAD(P)(H) bound to SDRs (8, 12–15, 29). The distance between C-2 of the nicotinamide and C-6 of the adenine ring, a parameter used to compare the degree of extension of coenzymes, is 14.65 Å, well within the range for SDRs. The nicotinamide ring is in syn conformation, with the 4-pro-S hydride facing the catalytic site, typical of all SDRs. The coenzyme molecule and its electron density are shown in Fig. 3a, along with side chains in the vicinity. Puckering of the nicotinamide ring was not discernible at the working resolution, and we used parameters of an oxidized coenzyme to restrain its geometry during refinement. Hydrogen bonding and charge interactions between NADPH and neighboring amino acid residues are drawn schematically in Fig. 3b. The high specificity of the enzyme for reduced β-NADPH has been established. At pH 7, PTCR has the highest affinity for β-NADPH as evidenced by the lowest $K_m$ of 7.2 μM and the highest $V_{max}/K_m$ ratio of 16.2 nmol min$^{-1}$ mg$^{-1}$ μM$^{-1}$ of all coenzymes (6). In the crystal structure, we find that the negatively charged 2'-phosphate moiety is surrounded by three positively charged side chains (Arg-37, Arg-41, and Lys-14), one hydrogen bond forming polar side chain (Asn-13), and a water molecule. All of these make specific contacts with the 2'-phosphate group, either through hydrogen bond formation or electrostatic charge neutralization, as shown in Fig. 3. The Asn-13 side chain carbonyl also accepts a proton from the 3'-hydroxyl of the adenine ribose. In addition to the presence of charged Arg and Lys residues near the 2'-phosphate of NADPH noted before (13, 29), specific interactions of the Asn-13 side chain with phosphate and ribose groups may play an important role in coenzyme selectivity. The location of Asp-62 near the amide group of the adenine ring is analogous to the Asp-60 in 3α,20β-HSD (8) and the Asp-68 in 7α-HSD (14). The carboxamide group of the nicotinamide ring interacts with the main chain atoms of Val-230, whereas the Thr-232 side chain makes a hydrogen bond with the bridging pyrophosphate. Interestingly, a sulfhydryl group from Met-234 is also present in the general vicinity, as has been observed in other SDR structures (Met-189 in 3α,20β-HSD and Met-193 in 17β-HSD1). Two water molecules are hydrogen-bonded to the phosphates. Catalytic side chains Tyr-193 and Lys-197 and the main chain carbonyl group of Asn-89 have contacts with the 2'- and 3'-hydroxyls of the nicotinamide ribose moiety, as shown in Fig. 3. Not shown in the figure is a short contact (3.1 Å) between the main chain carbonyl oxygen of Gly-228 of PTCR.
conserved in all steroid dehydrogenases and many other SDRs, and C-4 of the nicotinamide ring. This interaction and the orientation of carbonyl with respect to the nicotinamide ring have been proposed to have an important role in driving the hydride transfer from the coenzyme to the substrate (19).

**Crystal Packing**—The most interesting intermolecular interaction among crystallographically related monomers of PTCR is the formation of a crystallographic dimer by the packing of two loops between βD and αE (Ile-92 to Pro-101) about a 2-fold rotation axis. Because of the openness of the active site cleft, the loops penetrate each other’s active site and the Asp-97 side chain from one loop makes two strong hydrogen bonds to the catalytic residues Tyr-193 and Ser-139 (2.57 and 2.58 Å, respectively) of the other molecule, as shown in Fig. 4a. Interaction of the free carboxyl end of one monomer with the Arg-37 side chain of the other. The Arg-37 side chain also have specific contacts with 2′-phosphate of the bound NADP molecule.

Fig. 4. a, interactions between two PTCR monomers related by a crystallographic 2-fold rotation axis. The Asp-97 side chain of one monomer forms hydrogen bonds with catalytic Tyr-193 and Ser-139 side chains of the other (distances are 2.57 and 2.58 Å, respectively). NADP and other side chains have been removed for clarity. The backbones of two loops between βD and αE are shown. The view is down the 2-fold axis. b, a close-up of the superposition of a section of this loop for four SDRs, illustrating the difference in its conformation between PTCR and other SDRs. The backbone atoms of PTCR are color-coded, whereas those of 3α,20β-HSD, 17β-HSD1, and 7α-HSD are shown in green, blue, and magenta, respectively. c, interaction of the free carboxyl end of one monomer with the Arg-37 side chain of the other. The Arg-37 side chain also have specific contacts with 2′-phosphate of the bound NADP molecule.
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