The structures of the unique sulfotransferase retinol dehydratase with product and inhibitors provide insight into enzyme mechanism and inhibition

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The structures of the unique sulfotransferase retinol dehydratase with product and inhibitors provide insight into enzyme mechanism and inhibition

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Abstract

The structure of retinol dehydratase (DHR) from Spodoptera frugiperda, a member of the sulfotransferase superfamily, in complexes with the inactive form of the cofactor PAP 3′-phosphoadenosine 5′-phosphate (PAP) and (1) the product of the reaction with retinol anhydroretinol (AR), (2) the retinoid inhibitor all-trans-4-oxoretinol (OR), and (3) the potent steroid inhibitor androsterone (AND) have been determined and compared to the enzyme complex with PAP and retinol. The structures show that the geometry of the active-site amino acids is largely preserved in the various complexes. However, the β-ionone rings of the retinoids are oriented differently with respect to side chains that have been shown to be important for the enzymatic reaction. In addition, the DHR:PAP:AND complex reveals a novel mode for steroid binding that contrasts significantly with that for steroid binding in other sulfotransferases. The molecule is displaced and rotated ∼180° along its length so that there is no acceptor hydroxyl in close proximity to the site of sulfate transfer. This observation explains why steroids are potent inhibitors of retinol dehydratase activity, rather than substrates for sulfonation. Most of the steroid-protein contacts are provided by the α-helical cap that distinguishes this member of the superfamily. This observation suggests that in addition to providing a chemical environment that promotes the dehydration of a sulfonated intermediate, the cap may also serve to minimize a promiscuous sulfotransferases activity.

Keywords: X-ray crystallography; retinol dehydratase; sulfotransferase; anhydroretinol

Cytosolic sulfotransferases (STs) catalyze the transfer of a sulfate group from the universal sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) onto a hydroxyl or amino group of a large number of structurally diverse acceptor molecules, such as steroids, hormones, neurotransmitters, and other endogenous or xenobiotic compounds (Weinshilboum et al. 1997; Strott 2002; Chapman et al. 2004). Sulfation of metabolites commonly leads to their inactivation and elimination by increasing their water solubility and decreasing biological activity (Falany 1997). Recent data have also implicated STs in a number of disease states, such as chronic inflammation (Kansas 1996), entry of HIV (Farzan et al. 1999; Cormier et al. 2000), and various forms of cancer (Falany 1997; Weinshilboum et al. 1997; Armstrong and Bertozzi 2000). For example, STs have been shown to produce highly reactive intermediates (e.g., N-hydroxy heterocyclic and aromatic arylamines) that bind DNA and promote mutations (Yamazoe et al. 1999). For this reason, there is growing interest in the study of substrate binding and inhibition mechanisms of STs.

Crystal structures of several STs have been reported (Gamage et al. 2003, and references therein; Lee et al. 2003). Structural studies have shown that despite low to
moderate sequence identity between ST subfamilies, the overall molecular fold of all known STs is surprisingly similar. As one might infer by the diverse substrates recognized by individual members of the superfamily, the acceptor substrate binding pockets are the least conserved parts of the ST molecules.

Retinol dehydratase (DHR) from *Spodeptera frugiperda* is currently the only insect sulfotransferase for which a structure has been determined. The enzyme catalyzes the conversion of all-trans-retinol (RTL) to anhydroretinol (AR) (Fig. 1), a retro-retinoid that appears to play a role in morphogenesis. AR is also present in a number of mammalian cell types including liver, kidney, and lung (Buck et al. 1993). However, mammalian enzymes responsible for the production of AR have yet to be identified.

DHR has several unique features that distinguish it from other members of the ST family. The enzyme has low-sequence identity to the most homologous sulfotransferase rat aryl sulfotransferase (30%) and is significantly larger (41 kDa) than mammalian STs (30–36 kDa). However, sequence-alignment analyses and kinetic studies invariably classify this enzyme as a member of the sulfotransferase superfamily (Vakiani et al. 1998). As a typical cytosolic sulfotransferase, DHR sulfonates a wide variety of different hydroxycompounds, such as p-nitrophenol, phenol, vanillin, and serotonin. Some steroids bind tightly to DHR, in particular hydrocortisone and androsterone. However, these compounds do not appear to be substrates, as no sulfonated products are detected under standard assay conditions (Vakiani et al. 1998). On the other hand, other sulfotransferases do not seem to utilize retinol or other retinoids as substrates (Vakiani et al. 1998). The feature that most distinguishes DHR from other STs is that the end product of the enzymatic reaction, anhydroretinol, is not sulfonated. Retinyl sulfate appears to be a transient intermediate in the transformation of retinol to anhydroretinol.

We have previously shown (Pakhomova et al. 2001) that the insertion of the 32-residue helical “lid” domain into the canonical sulfotransferase fold is responsible for the functional transformation of a sulfotransferase to a dehydratase. We also showed that a DHR deletion mutant that lacks the lid domain retains sulfotransferase activity, but no longer functions as a dehydratase. On the basis of biochemical and structural data, a mechanism for the enzymatic reaction was proposed. However, structural information was limited to the enzyme:PAP:substrate complex. In this article, we present the results of three crystal structure determinations of ternary complexes of the enzyme with (1) the potent steroid inhibitor androsterone, (2) the product of the reaction anhydroretinol, and (3) the retinoid inhibitor (all-trans-4-oxoretinol) (Fig. 2), all in the presence of PAP. These structures provide a more detailed picture of the enzymatic reaction and help to explain differences between DHR and other members of the ST super-family.

### Results and Discussion

As previously described (Pakhomova et al. 2001), the overall molecular fold of DHR is an α/β motif with a central
five-stranded parallel β-sheet surrounded by α-helices (Fig. 3). The lid domain, the addition to the canonical sulfotransferase fold, closes the entrance to the enzyme’s active site. The fully encapsulated active site is predominately hydrophobic with a high proportion of aromatic residues (two phe and four tyr) in addition to the highly conserved amino acids that participate in sulfate transfer (R73, K162, H164, and H197), which may serve to promote desulfonation of the sulfonated intermediate. A comparison of the three crystal structures determined here reveals that active-site geometry is preserved in the various complexes, i.e., the binding of ligands that differ in chemical structure (the steroid androstosterone vs. retinol) leads to only limited structural differences in the active site of the enzyme. The helical lid appears to allow the enzyme to trap substrates and inhibitors in distinct positions in the active site.

The DHR:androstosterone complex

Steroids that serve as substrates for steroid sulfotransferases are potent inhibitors of retinol dehydratase activity. Despite the fact that steroids bind to the active site of DHR, the enzyme does not sulfonate these compounds. To elucidate the mechanism of inhibition of DHR by androstosterone ($K_i = 0.11 \mu M$), we solved the structure of DHR in the ternary complex with PAP and AND. The structure reveals a novel mode for steroid binding that contrast significantly with that for steroid binding in other sulfotransferases (Fig. 4). The steroid is displaced and rotated ~180° along the length of the molecule, so that there is no acceptor hydroxyl in close proximity to the site of sulfate transfer, consistent with the fact that steroids are not converted into sulfonated products. Most of the steroid–protein contacts are provided by the α-helical cap that distinguishes this member of the superfamily. This observation suggests that in addition to providing a chemical environment that promotes the dehydration of a sulfonated intermediate, the cap may also serve to minimize a promiscuous sulfotransferase activity. STs, in general, accommodate chemically diverse substrates in active sites with unobstructed access. The lid may help to restrict access to the catalytic machinery, and in this case, holds a potential substrate just out of reach (Fig. 5A). Only F130 is positioned differently (with respect to its orientation in the DHR:PAP:RTL structure previously described) in the inhibited complex—in the DHR:PAP:AND structure the rotamer of F310 is that most commonly observed. This difference is likely a consequence of the fact that this end of the binding site is not occupied by the steroid inhibitor, but is occupied in the DHR:substrate structure by the isoprene tail of the retinol.

Specific contacts that restrain AND include water-mediated hydrogen bonds between the C3 hydroxyl and the carbonyl oxygen of Y120 and the hydroxyl of Y135 of the lid domain. Additional stabilization of the complex is provided by van der Waals contacts contributed by both residues from the helical insert (I111, L139, L138) and the canonical ST fold (F31, L201, Y298, M295). The lack of any direct H-bond interactions between the protein and inhibitor, or more extensive hydrophobic interactions, is somewhat surprising, given the affinity of the inhibitor for the enzyme, but not without precedent. The family of intracellular fatty acid-binding proteins that includes proteins specific for fatty acids and retinoids encapsulate their ligands in oversized cavities with limited chemical complementarity to their respective substrates (Newcomer et al. 1998). Similarly, AND is fully encapsulated in the DHR cavity.

The DHR:anhdroretinol and DHR:all-trans-4-oxoretinol complexes

The substrate-binding pocket accommodates the product anhydroretinol in basically the same orientation as retinol in the DHR:PAP:RTL crystal structure (Fig. 5B). However, the β-ionone ring of anhydroretinol is rotated with respect to that of the substrate. This conformational difference, which requires no repositioning of the amino acids in the lid, is in all probability a consequence of the fact that the ring–isoprene chain dihedral angle is fixed by the C6–C7 double bond in the product, but not the substrate. Amino acids Y120 and Y130 from the lid domain were identified to be crucial for AR production. The β-ionone ring in DHR: PAP:AR is positioned closer to Y135 (4.8 Å versus 5.6 Å in RTL complex), but farther away from Y112 (5.2 Å versus 3.6 Å), while its distance to Y120 (4.2 Å versus 3.9 Å) is only slightly changed. The hydroxyl group of Y120 is further removed from the putative proton abstraction site (6.9 Å versus 6.5 Å). These observations might suggest that Y135 permits proton abstraction, while Y120 provides carbocation stabilization, although at this point, this is only

Figure 3. A secondary structure rendering of a monomer of the DHR-PAP:RTL complex. Retinol and PAP molecules are shown as space-filled renderings. The helical lid, which is the addition to the canonical sulfotransferase fold, is represented by the top three helices. (Pictures 3–5 were created using SETOR [Evans 1993].)
speculation, since our structures do not provide details of the transition state, and small distance differences may not be significant. In any case, it appears that the binding site can permit the transformation of retinol to anhydroretinol with only modest protein conformational changes; only the side chain of Y298 differs in its position in the active site.

In the DHR:PAP:OR complex, the orientation of the β-ionone ring with respect to amino acids Y112, Y120, and Y135 of the lid approximates that in the enzyme:product complex; however, the ring is flipped such that the 4-oxo group is positioned where the dimethyl-substituted C1 of the product is positioned in AR. The three dehydratase:retinoid structures (retinol, anhydroretinol, and 4-oxoretinol) reveal three orientations for the retinoid ring-tail dihedral.

**Figure 4.** (A) Superposition of the androsterone binding site in DHR:PAP:AND complex with estrogen molecule (shown in magenta) from mouse estrogen sulfotransferase (EST). Electron density $F_o-F_c$ simulated annealed omit 2.35 Å resolution map for the omitted androsterone molecule contoured at 3.3σ. (B) Electron density $F_o-F_c$ simulated annealed omit 2.75 Å resolution map for the omitted anhydroretinol molecule in DHR:PAP:AR complex contoured at 3.0σ. (C) Electron density $F_o-F_c$ simulated annealed omit 2.10 Å resolution map for the omitted all-trans-4-oxoretinol molecule in DHR:PAP:OR complex contoured at 3.0σ.

**Figure 5.** The stereodiagram of the substrate binding site of DHR with retinol molecule superimposed with corresponding parts of the (A) DHR:PAP:AND, (B) DHR:PAP:AR, and (C) DHR:PAP:OR complexes. The lid domain is shown as a ribbon diagram, superimposed substrate molecules as well as corresponding protein side-chain residues which line the substrate binding site are shown in magenta.
As mentioned above, this angle is fixed by the C6–C7 double bond in AR, while in the 4-oxo derivative and retinol, a range of dihedral angles is accessible. In the earlier DHR:substrate structure, the ring-tail dihedral is 98°, close to that observed in the high-resolution structure of RBP-retinol (Calderone et al. 2003). However, the β-ionone ring of the substrate is flipped with respect to its orientation in RBP. It appears that in the dehydratase binding site, the ionone ring of the substrate is prepositioned for transformation to anhydroretinol. In contrast, the β-ionone ring for the inhibitor oxo-derivative is in accordance with the most commonly observed conformation. Transformation to product from this position would require a 180° rotation about the C6–C7 bond.

The different structures reveal alternate placement of the isoprene tail of the retinoid as well; the position of the C9 methyl group is displaced 2.8 Å in both AR and OR complexes in comparison to the RTL structure. Accompanying changes in the protein structure are localized to the side chain of Tyr 298, which rotates to permit the repositioning of the C9 methyl group of the isoprene chain. Thus, one might propose that during the course of catalysis, the conformational change in the retinoid necessitated by the conversions of the ring-tail single bond to a double bond is compensated by movement of the isoprene tail and repositioning of Tyr 298. The ensemble of dehydratase structures now available might suggest that it is the substrate that provides the conformational flexibility to proceed through the transition state.

Is retinol dehydratase the odd member in the sulfotransferase family?

The superfamily of sulfotransferases currently comprises 47 mammalian isoforms, one insect isoform, and eight plant isoforms (Blanchard et al. 2004). Although DHR is clearly a member of the ST superfamily (as it utilizes the same universal sulfate donor PAPS, is able to sulfonate many hydroxycompounds, and has the typical sulfotransferase fold), the biochemical activity of DHR appears to indicate that it is a unique enzyme. A search against the GenBank database with the DHR sequence reveals two proteins which...
appear to be like DHR, unconventional siblings in the superfam-
ily: a Drosophila melanogaster gene product (GenBank code AAF58309, 37% sequence identity with DHR), and one from Anopheles gambiae (mosquito, GenBank code EAA01764, 33% sequence identity). Both proteins have yet unknown structure and function, but possess the DHR-like lid domain insertion in a ST sequence (Fig. 6). The moderate sequence identity and quite different amino acid composition of the lid domains in these proteins in comparison to that of DHR may indicate that they are not necessarily retinol dehydratases. But the presence of the similar lid domain does suggest that these two proteins with yet undiscovered catalytic activities could exploit a sulfonation step as an intermediate, as in the case of DHR. An alternative interpretation may simply be that the insertion of the helical cap in the insect enzymes further restricts the substrate specificity of the sulfotransferases, or allows for a mechanism of regulation by inhibitory compounds such as steroids.

Materials and methods

Crystallization
The double mutant C258S:C279S of DHR was used for crystallization. It has the same activity as wild-type enzyme, but produces crystals of better quality. The crystals of the ternary enzyme complexes were obtained using the hanging-drop vapor-diffusion method by mixing equal volumes of protein at 10 μg/μL (incubated overnight with PAP, ethylmercurithiosalicylate, and androsterone or all-trans-4-oxoretinol solution in dimethylsulfoxide) and the reservoir solution (8%–12% PEG3350, 100 mM sodium Hepes at pH 6.2–6.8, 8% glycerol) at 12°C. Crystals usually appear in 2 wk.
Data collection, structure solution, and refinement

Prior to data collections, crystals were coated with mineral oil and flash frozen in a stream of liquid nitrogen at $-170^\circ$C. X-ray data were collected on a Raxis IV mounted on a Rigaku RU-200 rotating anode. The images were processed and scaled using DENZO and SCALEPACK (Otwinowski and Minor 1997). Data collection and data processing statistics are summarized in Table 1.

Since crystals of all studied DHR complexes were isostructural to the previously determined structure of DHR:PAP:RTL complex (Pakhomova et al. 2001), we used rigid body refinement to position the initial model (PDB accession code 1FLM; retinol and water molecules were omitted from the model). The same $R_{free}$ set from the DHR:PAP:RTL structure was used in every case because of isomorphous character of all data sets. The positioned models were refined using the maximum likelihood refinement in REFMAC (CCP4 1994) (DHR:PAP:AR, DHR:PAP:OR complexes) or CNS (Brünger et al. 1998) (DHR:PAP:AND complex). No $\sigma$ cutoff was applied to the data. Twofold noncrystallographic symmetry restraints, as well as bulk solvent corrections, were applied in every case. The program O (Jones et al. 1991) was used to build the models throughout the refinement. Refinement statistics are listed in Table 1. Details of refinement of each of the structures follow.

DHR:PAP:AND

The final model consists of residues 7–349 for both protein monomers, two cofactor PAP, two androsterone molecules, two Ca$^{2+}$ ions, and 134 water molecules. H239 (monomer A) was modeled in two alternate conformations. Two cis peptide bonds (residue P226 in both molecules) were detected.

DHR:PAP:AR

The final structure includes residues 6–349 for both protein molecules, two cofactor PAP, two anhydroretinol molecules, two Ca$^{2+}$ ions, and 74 water molecules. Additional electron density on cysteines 318 (both molecules) was interpreted as from covalently bound ethylmercury residues. Two cis peptide bonds (residue P226 in both molecules) were detected.

DHR:PAP:OR

The final model consists of residues 8–349 for both protein monomers, two cofactor PAP, two all-trans-4-oxoretinol molecules, two Ca$^{2+}$ ions, two Hg$^{2+}$ ions (bound to C318), and 301 water molecules. Two cis peptide bonds (residue P226 in both molecules) were detected, similarly as in previous complexes.

Data deposition

The atomic coordinates have been deposited to the Protein Data Bank with the accession codes: DHR:PAP:AND, 1X8J; DHR:PAP:AR, 1X8K; DHR:PAP:OR, 1X8L.

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References


