Cofactor Hydrogen Bonding onto the Protein Main Chain Is Conserved in the Short Chain Dehydrogenase/Reductase Family and Contributes to Nicotinamide Orientation*

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Human estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1), a member of the short chain dehydrogenase/reductase (SDR) family, is responsible for the biosynthesis of all active estrogens. The crystal structures of two C19-steroid ternary complexes (17β-HSD1-androstanedione-NADP and 17β-HSD1-androstenedione-NADP) reveal the critical role of Leu149 in regulating the substrate specificity and provide novel insight into the different fates of a conserved glutamate residue in the estrogen-specific proteins upon the binding of the keto and hydroxyl groups of steroids. The whole NADP molecule can be unambiguously defined in the NADP binary complex, whereas both ternary complexes show that the nicotinamide moiety of NADP cannot be located in the density maps. In both ternary complexes, the expected position of carboxamide oxygen of NADP is occupied by a water molecule, which makes a bifurcated hydrogen bond with the O3 of C19-steroid and the main chain nitrogen of Val188. These results demonstrate that the hydrogen bonding interaction between the main chain amide group and the carboxamide group of NAD(P)(H) plays an important role in anchoring the nicotinamide ring to the enzyme. This finding is substantiated by structural analyses of all 33 NAD(P)(H) complexes of different SDR proteins, because 29 structures of 33 show this interaction. This common feature reveals a general mechanism among the SDR family, providing a rational basis for inhibitor design against biologically relevant SDR targets.

Short chain dehydrogenases/reductases (SDR) form a large, functionally heterogeneous protein family presently with about 3000 primary sequences deposited in databases and the corresponding genomes represent all forms of life (1). The enzymes in the SDR family span several EC classes, from oxidoreductases and lyases to isomerases, with oxidoreductases as the majority (1). As a member of the SDR family (2), human estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1, EC.1.1.1.62) catalyzes the conversion of an inactive estrogen, estrone (E1), to the biologically active estrogen, 17β-estradiol (E2) (3). Breast cancer is one of the most common malignancies in women worldwide, and estrogens play an important role in the development of hormone-dependent breast cancer (4). This enzyme has been demonstrated to be involved in maintaining high E2 levels in breast tumors of postmenopausal women (5, 6). Because E2 has a stimulatory effect on the proliferation of breast cancer cells (7), blocking its formation by specific inhibition of 17β-HSD1 should be of paramount importance for the control of breast tumor growth.

To develop new compounds capable to inhibit 17β-HSD1, a series of structure/function studies were conducted in several laboratories focusing on the understanding of steroid binding to the enzyme. The mechanism for estrogen (C18-steroid) recognition by 17β-HSD1 was previously studied using the crystallographic structure of the enzyme with estradiol (8, 9). Despite the similar hydrophobicity existing between estrogens (C18-steroid) and androgens (C19-steroid), however, 17β-HSD1 exhibits a high substrate specificity and catalytic efficiency toward estrogens such as estrone (E1) but insignificant ability to catalyze the conversion of androgens (10, 11). Recent investigation showed that androgens (C19-steroid) can bind in an alternative mode to the protein through a combined study of enzyme kinetics and x-ray crystallography (12). The original aim of our investigation is to further study the mechanism of steroid recognition and discrimination adopted by 17β-HSD1. Here we report the crystal structures of two ternary complexes: 17β-HSD1-androstanedione (A-dione)-NADP and 17β-HSD1-androstenedione (4-dione)-NADP, as well as the binary complex of 17β-HSD1-NADP. Surprisingly, these NADP complexes, combined with all available crystal structures of SDR family enzymes, reveal a critical interaction between the enzyme and the cofactor, which is highly conserved among SDR family enzymes.

EXPERIMENTAL PROCEDURES

Purification and Crystallization Procedures—17β-HSD1 was purified from fresh human placenta according to a previously described rapid purification procedure (13, 14). Protein concentration for the homogeneous 17β-HSD1 was determined using the Bradford assay. The protein sample was concentrated to 15–17 mg/ml in a buffer containing 40 mM Tris, pH 7.5, 1 mM EDTA, 0.2 mM dithiothreitol, 20% glycerol, and 0.06% (w/v) β-OG. The optimized crystallization conditions were derived from previous results (15). For the A-dione ternary complex, the reservoir solution contained 30% PEG-4K, 0.16 M MgCl₂, 60 mM Hepes, pH 7.5 and 20% glycerol. For the 4-dione complex, the reservoir solution
Conserved Protein Main Chain/Cofactor Interaction in the SDR Family

Table I

Summary for data collection, structural, and refinement statistics

|                      | NADP binary complex | A-dione ternary complex | 4-dione ternary complex |
|----------------------|---------------------|-------------------------|-------------------------|
| Unit cell dimensions |                     |                         |                         |
| a, b, c (Å)          | 122.40, 43.92, 60.45| 122.65, 43.93, 60.79    | 122.70, 44.03, 60.79    |
| α, β, γ (°)          | 90, 99.86, 90       | 90, 99.76, 90           | 90, 99.52, 90           |
| Space group          | C2                  | C2                      | C2                      |
| Number of reflections| 101,931 (7521)      | 97,028 (9599)           | 65,964 (6385)           |
| Resolution (Å)       | 40 – 1.81 (1.67 – 1.81) | 20 – 1.63 (1.69 – 1.63) | 30 – 1.89 (1.96 – 1.89) |
| Completeness (%)     | 98.1 (92.2)         | 95.3 (98.9)             | 95.4 (93.6)             |
| I/σ(I)              | 20.1 (2.0)          | 20.4 (2.0)              | 15.4 (2.1)              |
| R_merge             | 0.049 (0.397)       | 0.044 (0.454)           | 0.046 (0.289)           |
| Multiplicity        | 3.55 (2.82)         | 2.53 (2.47)             | 2.70 (2.58)             |
| B value from Wilson plot (Å²) | 21.5 | 24.1 | 21.4 |
| R-factor            | 0.191 (0.260)       | 0.209 (0.268)           | 0.188 (0.240)           |
| R-free              | 0.235 (0.326)       | 0.249 (0.335)           | 0.227 (0.329)           |
| RMSD from target geometry |                |                         |                         |
| Bond lengths (Å)     | 0.012               | 0.011                   | 0.012                   |
| Bond angles (°)      | 1.5                 | 1.5                     | 1.7                     |
| Number of non-H2O atoms |             |                         |                         |
| All atoms            | 2328                | 2392                    | 2300                    |
| Protein              | 2123                | 2120                    | 2131                    |
| NADP                 | 48                  | 27                      | 27                      |
| C19-steroid          | /                   | 21                      | 21                      |
| Glycerol             | 6                   | 6                       | 6                       |
| Water                | 151                 | 218                     | 115                     |
| Average B factors (Å²) |                  |                         |                         |
| All atoms            | 35.3                | 32.9                    | 37.7                    |
| Protein main chain   | 31.7                | 29.2                    | 34.3                    |
| Protein side chain   | 37.2                | 34.5                    | 39.8                    |
| C19-steroid          | /                   | 49.2                    | 62.0                    |
| NADP                 | 43.7                | 35.1                    | 43.7                    |
| Glycerol             | 48.4                | 41.1                    | 47.0                    |
| Water                | 44.8                | 47.3                    | 45.1                    |

Data statistics for the last shell are given in parentheses.

R_merge = \sum F_o(h) - \sum F_c(h) / \sum F_o(h), where \sum F_c(h) is the mean intensity after rejections.

R-factor = \sum F_o(h) - \sum F_c(h) / \sum F_o(h), where F_o(h) and F_c(h) are the observed and calculated structure-factor amplitudes for the reflection with Miller indices h = (h,k,l).

The free R-factor is calculated for a test set of reflections which were not included in atomic refinement.

RESULTS

Overall Structure and Model Quality—Crystals employed in this investigation belonged to space group C2 and contained one subunit per asymmetric unit. Two C19-steroid ternary complexes, 17β-HSD1-A-dione-NADP, and 17β-HSD1-4-dione-NADP, were refined at 1.63 and 1.89 Å, respectively. The 17β-HSD1-NADP binary complex was refined at 1.81 Å. All three models show good stereochemistry (22) and the quality of the refined models can be assessed from the statistics in Table I. As observed in previous models (8, 9, 12, 23, 24), no clear electron density was present for the C-terminal end of the protein (residues 286–327) and the flexible loop between residues 191 and 198 in these three complexes. The protein parts of these three complexes are very similar to the previously published structures, with root mean-square deviations (RMSD) between these complexes and the E2 complex (8) at 0.55, 0.52, and 0.53 Å, respectively, for the α-carbon of 276 amino acids excluding the above-mentioned flexible parts.

17β-HSD1-NADP Binary Complex—The NADP molecule is well defined in the electron density maps (Fig. 1), and the location and overall conformation of NADP are very similar to that of previously determined 17β-HSD1(A221L)-E2-NADP complex (25). It binds to the enzyme in an extended conformation with the adenine ring in an anti conformation and the nicotinamide ring in a syn conformation. Most interactions between NADP and the nicotinamide dinucleotide binding pocket of 17β-HSD1 have been described earlier (24, 25). Stabilization of the nicotinamide ring is achieved through hydrogen interactions and hydrophobic contacts. The nicotinamide ring packs against the side chains of Val138 and Cys135 and its members were retrieved from the RCSB Protein Data Bank (21). These structures were analyzed using the program O (18).

Summary for data collection, structural, and refinement statistics

|                          | NADP binary complex | A-dione ternary complex | 4-dione ternary complex |
|--------------------------|---------------------|-------------------------|-------------------------|
| Unit cell dimensions     |                     |                         |                         |
| a, b, c (Å)             | 122.40, 43.92, 60.45| 122.65, 43.93, 60.79    | 122.70, 44.03, 60.79    |
| α, β, γ (°)             | 90, 99.86, 90       | 90, 99.76, 90           | 90, 99.52, 90           |
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| Number of reflections   | 101,931 (7521)      | 97,028 (9599)           | 65,964 (6385)           |
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| RMSD from target geometry |                  |                         |                         |
| Bond lengths (Å)        | 0.012               | 0.011                   | 0.012                   |
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| All atoms               | 2328                | 2392                    | 2300                    |
| Protein                | 2123                | 2120                    | 2131                    |
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| C19-steroid            | /                   | 21                      | 21                      |
| Glycerol               | 6                   | 6                       | 6                       |
| Water                  | 151                 | 218                     | 115                     |
| Average B factors (Å²) |                     |                         |                         |
| All atoms               | 35.3                | 32.9                    | 37.7                    |
| Protein main chain     | 31.7                | 29.2                    | 34.3                    |
| Protein side chain     | 37.2                | 34.5                    | 39.8                    |
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| Glycerol               | 48.4                | 41.1                    | 47.0                    |
| Water                  | 44.8                | 47.3                    | 45.1                    |
carboxamide group forms hydrogen bonds with the Val<sup>188</sup> peptide amide group and the side chain of Thr<sup>190</sup>. In addition, the intramolecular hydrogen bond between the carboxamide group and the pyrophosphate moiety also contributes to the stabilization of the glycosidic bond in the syn conformation, burying the A face of the ring against the protein and exposing the B face to the active site.

**17β-HSD1–A-dione–NADP Complex**—The electron density for the steroid was clearly visible from the initial Fourier difference map. The model of dihydrotestosterone molecule retrieved from the Protein Data Bank (Ref. 21, accession code: 1D2S) was fitted to the (m<sub>F</sub> — d<sub>F</sub>) electron density map. As observed in the testosterone complex, this C19-steroid also adopts the reverse orientation, which differs from the binding mode of estradiol. However, the atoms in the D-ring of dihydrotestosterone cannot fit well into the density although all other atoms fit very well. The density map strongly suggests that the atoms O17, C17, C13, and C16 are located on the same plane, which is characteristic of a ketone group. This phenomenon demonstrates that DHT has been oxidized into A-dione in the presence of NADP during the soaking procedure. The previous kinetic studies have pointed out that the 17β-hydroxyl group of DHT can be oxidized to a 17-keto group by 17α-oxidoreductase. However, the 17-keto group cannot make a direct hydrogen bond with the carboxylate group of Glu<sup>282</sup> because of the lack of a hydrogen donor. As a consequence of the potential repulsion between them, the side chain of Glu<sup>282</sup> rotates away to provide space for a bridging water molecule (Fig. 2, W149), which is within the hydrogen bonding distance of the following atoms: O17 of A-dione (3.30 Å), OE1 of Glu<sup>282</sup> (2.74 Å), NE2 of His<sup>221</sup> (2.87 Å) and thus eliminates the potential polar repulsion between O17 and the carboxylate group of Glu<sup>282</sup>. Superposition of the residues in the binding pockets of A-dione, testosterone, and estradiol complexes reveals little change on the conformations for these residues. Except for the two residues (His<sup>221</sup> and Glu<sup>282</sup>), the steroid binding pocket of A-dione and testosterone complexes are essentially the same. A-dione and testosterone are also located in very similar positions.

The residues in the cofactor binding site do not show any significant conformational change compared with the above NADP binary complex. In the density map, there is clear electron density for the adenine and the adenine ribose of the NADP molecule. However, the electron density corresponding to the nicotinamide mononucleotide (NMN) moiety of NADP molecule is poorly defined in the density maps (Fig. 3). The presence of only a few disconnected density peaks in this region led to the conclusion that the nicotinamide and its attached ribose are disordered in the crystal. Thus, these atoms were omitted from further refinement cycles. One water molecule (W51), occupying the position of the carboxamide oxygen of the NADP molecule in the above cofactor binary complex, is well defined because of its strong spherical density that persisted during cycles of refinement. Unlike the previously described NADP binary complex, the electron density for the 2′-phosphate group attached to the adenine ribose is remarkably poor. This is not difficult to understand considering the fact that the side chain of Arg<sup>27</sup> has moved to the protein surface and is out of the hydrogen bonding distance of the 2′-phosphate group. The loss of the stabilization interaction by Arg<sup>27</sup> releases the phosphate group, which is thus distributed more randomly in the cavity. To obtain comparable temperature factors for the adenine nucleotide moiety, the occupancy of these atoms is set to 0.75 and that of the phosphate group to 0.01 because of its invisibility in the electron density maps. Refined B factors for the well defined portion of NADP with occupancy fixed at 0.75 range from 29 to 49 Å<sup>2</sup>. The average B factor for these atoms is 37 Å<sup>2</sup>, compatible with that of all protein atoms (32 Å<sup>2</sup>).

**17β-HSD1–4-dione–NADP Complex**—The structure of the 17β-HSD1–4-dione–NADP ternary complex displays high similarity to that of the A-dione complex and the C19-steroid molecule adopts the same orientation as A-dione. The clearly

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**Fig. 1.** Stereoview of the 2m<sub>F</sub> — d<sub>F</sub> electron density map around the NADP molecule in the 17β-HSD1–NADP binary complex. The map computed with 1.81 Å resolution data is contoured at a 1.0σ level. Some residues surrounding the NADP molecule are also shown in standard colors (yellow for carbon, red for oxygen, and blue for nitrogen). Hydrogen bonding interaction between the carboxamide oxygen of NADP and the main chain amide group of Val<sup>188</sup> is represented by cyan dotted lines. This figure and the figures that follow were produced using Molray (37).
interpretable electron density for the steroid indicates that 17β-hydroxyl has been oxidized to 17-ketone, that is, testosterone has been converted into 4-dione under crystallization conditions. Similar to the oxidation of DHT, a very small percentage of testosterone molecules can bind to the enzyme with its 17β-hydroxyl group approaching the catalytic residues and subsequently be oxidized into 4-dione, which is also supported by the previous kinetic studies (10). After 4-dione leaves the

Fig. 2. A, stereoview of the steroid binding site in the 17β-HSD1-A-dione-NADP ternary complex. The 2mF_o−dF_c electron density map around the A-dione molecule and two bridging water molecules (W51, W149), computed with 1.63 Å data, is contoured at a 1.5σ level. Hydrogen bonding interactions involving the two hydrophilic ends (O3 and O17) of A-dione are represented by cyan dotted lines. B, side view of the 2mF_o−dF_c electron density map (1.5σ level) around the A-dione molecule.

Fig. 3. 2mF_o−dF_c electron density map contoured at a 1.0σ level for the ligands (A-dione and NADP) in the 17β-HSD1-A-dione-NADP ternary complex. The well-defined portion of the NADP molecule is represented in standard color. For comparison, the position of the NADP molecule in the 17β-HSD1-NADP binary complex is shown in cyan. Hydrogen-bonding interaction between the O3 of the A-dione molecule and the main chain amide group of Val188 (via a bridging water molecule W51) is represented by magenta dotted lines.
around the 4-dione molecule and one bridging water molecule (W57), computed with 1.89 Å data, is contoured at a 1.0σ level. Hydrogen-bonding interactions involving the two hydrophilic ends (O3 and O17) of 4-dione are represented by cyan dotted lines.

active site, it will rebind to the enzyme in a reverse orientation as shown in the present ternary complex. Although the previous high-resolution 17β-HSD1-testosterone binary complex structure unambiguously showed that testosterone binds in a reverse orientation within the cavity, this is not contradictory to the kinetic results, because a very small percentage of substrate bound in a different orientation may be undetectable in the X-ray structure while it could contribute significantly to the enzymatic activity observed under catalytic conditions.

The 4-dione molecule basically stays at the same position as testosterone in the testosterone complex with only a very small rotation around the long axis (O3-O17). For example, the shifted distances of the relative oxygen atoms are less than 0.4 Å. The 3-keto group (O3) of the 4-dione molecule, identical to those of testosterone and A-dione, is anchored to the main chain of Val188 by a bridging solvent molecule (W57) (Fig. 4). In the other hydrophilic end, unlike the A-dione complex, the present 4-dione complex does not show the existence of a bridging water molecule between the O17 and the Glu282 side chain. To avoid the polar repulsion from the O17 of 4-dione, the OE1 of Glu282 moved about 1.2 Å compared with that of the testosterone complex. Thus, the hydrogen bonding interaction made by the O17 of 4-dione only involves His221 (2.65 Å). Without the hydrogen bonding interaction, the Glu282 side chain might be moved more flexible, as indicated by its increased B factors (−61 Å²) compared with that of the A-dione complex (−47 Å²) although the overall B factors are quite similar (37 Å² for the 4-dione complex versus 33 Å² for the A-dione complex). Similarly, the relatively high B factors of the 4-dione molecule (62 Å²), compared with that of A-dione and testosterone, could also be partly explained by the absence of hydrogen bonding interaction with Glu282 because both testosterone and A-dione make hydrogen bonds to Glu282 either directly or via a bridging solvent molecule.

No apparent difference can be observed for the cofactor binding site between the 4-dione complex and the A-dione complex. Similarly, we are unable to see the NMN moiety of the NADP molecule in the electron density maps. Thus, no attempt was made to refine the positions of the NMN group. The apparent existence of two ordered solvent molecules (W57, W112) in this cavity suggests that, in the crystal, the NMN moiety spends little or no time in that site.

Nicotinamide Ring Binding Site of the SDR Family—As of September 2003, crystal structures have been determined and coordinates deposited in the Protein Data Bank for 35 members of SDR family (two of them are structural neighbors of the SDR family and the same enzyme in different species is just counted once), among which 33 are of complexes with the cofactor NAD(P)(H) (21). The corresponding PDB accession codes for these 33 NAD(P)(H) complexes are listed as follows: 1AHH, 1AE1, 1B14, 1BDB, 1G1A, 1BVR, 1CYD, 1DHR, 1DOH, 1E3W, 1ED0, 1EQ2, 1E7W, 1GCO, 1GEG, 1GZ6, 1H5Q, 1JA9, 1N5D, 1N7H, 1N2S, 1NFF, 1OAA, 1QR, 1QYV, 1K6X, 1HDO, 2HSD, 2AE2, 1BSV, 1EK5, 1FK8, and 1Y8. Investigation of these 33 structures reveals that at least as many as 29 proteins (all of the above list except the last four) share the common binding interaction with the carboxamide group of the nicotinamide ring. All these 29 cofactors are bound in the classical “Rossman fold” of SDR family proteins and demonstrate high similarity in their orientation and conformation regardless of their redox states. The carboxamide group of NAD(P)H plays an important role in the specific orientation of the nicotinamide ring relative to enzyme catalytic groups. As observed for the 17β-HSD1-NADP binary complex in which the peptidic amide group of Val188 forms a hydrogen bond with the carboxamide group of NADP, a main chain amide group, found in all of these 29 proteins and which is located at the binding site of the nicotinamide ring, functions as a hydrogen donor to anchor the carboxamide group, thus stabilizing the binding of the cofactor. Nevertheless, this main chain amide does not belong to one specific residue type. In fact, the corresponding residues are quite variable, ranging from as small as serine to as bulky as phenylalanine. Among them, 9 of 29 are valine and another 9 are isoleucine. The residues in the other 11 enzymes consist of asparagine, leucine, threonine, tyrosine, phenylalanine, histidine, serine, and alanine.

Other than this interaction, the second next residue (Thr190) in the case of 17β-HSD1 downstream from the previous residue is also conserved in 21 of 35 proteins. These 21 proteins share a common residue threonine at this position, which is found, in 12 of 19 cofactor containing crystal structures, to form a bifurcated hydrogen bond with the carboxamide group and one phosphoryl oxygen of the cofactor.

DISCUSSION

To understand the recognition and discrimination of sex steroids by 17β-HSD1, great efforts have been made via different approaches including enzyme kinetics, mutagenesis, and X-ray crystallography. Consistent with all the other available 17β-HSD1-steroid complexes, our present models demonstrate that hydrophobic interactions between the steroid and the substrate-binding pocket are required to maintain the efficient binding of steroids. It was reported that testosterone was forced to bind in the alternative binding mode to avoid the potential steric hindrance with the side chain of Leu149 (12). Now we have shown that two other C19-steroids, A-dione and
hydrogen bond (2.77 Å complex, the phenolic hydroxyl of estradiol makes a strong interaction with the residue at the recognition end of the cleft. In the E2/17β-HSD1 ternary complex, the 3-hydroxyl group of estrogens (28) interacts with the main chain amide group of Val188 via bridging water molecules (labeled by WAT). For clarity, the hydrogen-bonding interactions between the steroid and the residues His221 and Glu282 are not shown.

4-dione, can also be bound in the alternative mode in the hydrophobic tunnel. Inspection of the active site architecture for all available crystal structures of 17β-HSD1 reveals that the steroid binding site is very rigid and that no big conformational changes have been observed for the residues in the binding site in the presence of different steroids. Thus, the lack of flexibility of the steroid-binding cavity has forced the C19-steroid to shift its position so as to accommodate the increased steric bulk of the C19-methyl group. Our results provide further evidence that 17β-HSD1 utilizes steric hindrance by Leu149 with the C19-methyl group of androgens to confer substrate specificity. In fact, steric hindrance by a critical residue could be a more general principle regulating substrate specificity of enzymes using hydrophobic substrates, as indicated in the studies of P450 enzymes and estrogen sulfotransferase (26, 27).

The crystal structures reported here, together with the previously determined E2 and testosterone complexes of 17β-HSD1, constitute an impressive example of the power of molecular recognition and discrimination by proteins. Moreover, the two C19-steroid complexes, in combination with the 17β-HSD1-testosterone and 17β-HSD1-E2 complexes, provide novel pictures for the estrogen-specific proteins on how the critical estrogen-recognition residue Glu282 responds to the hydroxyl and keto groups (Fig. 5), which are characteristic of the 3-end of estrogens and androgens, respectively. Located in the substrate recognition site, His221 is found, in all steroid complexes of 17β-HSD1, to be involved in hydrogen bonding interaction with the hydrophilic end of the steroid. However, different fates have been exhibited for Glu282, another important residue at the recognition end of the cleft. In the E2 complex, the phenolic hydroxyl of estradiol makes a strong hydrogen bond (2.77 Å) with the carboxylate group of Glu282 (8). Similarly, a strong interaction has also been observed between Glu282 and the 17β-hydroxyl group of testosterone (2.66 Å) although it adopts an alternative binding mode in the 17β-HSD1-testosterone complex (12). In contrast to what is observed for the above two complexes, the distance between the carboxylate group of Glu282 and the 17-keto group of the steroid in the structures presented in this report is longer because of the potential polar repulsion.

Analyses of all available crystal structures of estrogen-specific proteins have revealed that these proteins share a common estrogen-binding architecture, among which is a conserved glutamate residue acting as a proton acceptor that interacts with the 3-hydroxyl group of estrogens (28). A mutagenesis study on the estrogen receptor-α (ERα) showed that Glu282 plays a significant role in binding the A-ring phenolic group of estradiol and in receptor discrimination between estrogens and androgens (29). This interaction was further proved by the crystal structure of the ER-estradiol complex (30). These authors also proposed that the matching of hydrogen bonding character between ligand and receptor is an underlying factor for the receptor proteins to recognize their cognate ligands (29). That is to say, in the case of estrogen-ER recognition, the phenolic hydroxyl group of the estrogen is a potent hydrogen donor and the carboxylate group of the corresponding glutamate residue is a potent hydrogen acceptor. On the contrary, in the androgen-androgen receptor recognition, the 3-keto group of the androgen is a potent hydrogen acceptor and the carboxamide group of glutamine is a potent hydrogen donor. This notion was buttressed by clinical evidence. Failure of such matching, as shown by the naturally occurring mutation Q711E in the androgen receptor, would cause androgen insensitivity syndrome (AIS) (31). The different response of the Glu282 carboxylate group to the corresponding hydroxyl or keto group of steroids, illustrated by our crystal structures, strongly supports the above concept.

Other than the above observations, the most striking result from these structures resides in the binding of the NADP molecule in the presence of C19-steroid. For comparison, we determined the crystal structure of the 17β-HSD1-NADP binary complex which displays well defined electron density for the whole NADP molecule and similar cofactor-protein interactions as observed in the previous 17β-HSD1(H221L)-E2-NADP ternary complex (25). In both the A-dione and the 4-dione complex, however, it is surprising to see that only the moieties corresponding to adenosine 2′,5′-diphosphate could be reliably located in the density maps. It seems impossible that the nicotinamide moiety has been cleaved during the soaking procedure because the crystallization conditions for the binary and ternary complexes are nearly identical except for the additional C19-steroid in the latter. Inspection of the electron density maps for the two C19-steroid ternary complexes reveals that one water molecule, stabilized by the main chain amide group of Val188 and the 3-keto group of the C19-steroid, occupies the position of the carboxamide oxygen of NADP, thus preventing stable binding of the cofactor. This implies that the conserved water molecule is an integrated part of C19-steroid reverse binding because we also observed the existence of such water molecule in the testosterone binary complex (12) (Fig. 5). This water molecule may also be involved in the procedure of cofactor release, if we consider its close proximity to the flexible loop and the fact that it excludes the stable binding of the cofactor. At the end of the reaction cycle, this water molecule might enter the cavity, via the flexible loop, to approach the main chain nitrogen of Val188 and consequently make the NMN...
moiety of the cofactor more mobile, finally leading to the release of cofactor. The above suggestion and the precise picture of the cofactor release remains to be further studied.

What if the NMN moiety of NADP in the ternary complexes occupied the same position as observed in the NADP binary complex? That is to say, the carboxamide oxygen is within the hydrogen bond distance of the main chain amide of Val188. In this proposed case, the distance between the carboxamide oxygen of NADP and the 3-keto group of the C19-steroid would be less than 3.0 Å, leading to an unacceptable polar repulsion because of the lack of hydrogen atom for both groups. And what if the carboxamide group of NADP rotated around 180 degrees to avoid the potential repulsion with the 3-keto of C19-steroid? This is also unlikely, considering that although the carboxamide nitrogen can make a hydrogen bond with the 3-keto group of steroid, the carboxamide oxygen would be in a position too close to the nearby phosphoryl oxygen atom. As observed in the NADP binary complex, the intermolecular hydrogen bonding interaction between NADP and the main chain amide group of the enzyme, as well as the intramolecular hydrogen bond between the carboxamide nitrogen and the nearby phosphoryl oxygen, are both important to maintain the nicotinamide ring in the syn-conformation, as might be expected for a B-side-specific enzyme. Thus, the stable binding of the C19-steroid and of the whole NADP is almost mutually exclusive. On this regard, as a consequence of the potential steric interference with the O3 of C19-steroids, the carboxamide group of NADP in the ternary complexes has to be in positions that fall out of the hydrogen-bonding distance from the main chain amide group. Besides, this steric hindrance may also make it impossible for the cofactor to form the intramolecular hydrogen bond between its carboxamide and pyrophosphate groups. However, as shown in the NADP binary complex, these specific hydrogen-bonding interactions are required for the enzyme to bind the NADP nicotinamide moiety order within the cavity. As a conclusion then, the potential steric hindrance with the O3 of C19-steroid results in the loss of the hydrogen-bonding interactions, which in turn causes the NADP disorder in the ternary complexes. Note that because the binding region of the NMN moiety in the NADP binary complex is not far from the flexible loop composed of residues 190–200, we may speculate that the NMN part of NADP in the ternary complex swung away from the expected binding region as shown in the NADP binary complex and became more accessible to the solution. Thus, it is very likely that the NMN moiety of NADP would adopt multiple conformations because of the variability of the glycosidic bond.

Our analyses for all available crystal structures of the SDR enzymes reveal that the interaction between a main chain amide group and the carboxamide group of cofactor is highly conserved in this super family. Despite their low sequence identity (about 15–30%), all available crystal structures for this family display highly similar α/β folding patterns with a central β-sheet, typical of the Rossmann fold. A relatively conserved threonine residue, located in the NMN moiety binding region and proposed to be involved in the interaction with the carboxamide group and/or phosphoryl oxygens, is found in 19 structures (32, 33). However, only 12 of 19 structures display the interaction between this threonine residue and the cofactor. In contrast, inspection of the above 33 structures reveal an identical pattern of hydrogen bonding interaction between a main chain nitrogen, located at the end of βF, and the carboxamide oxygen of cofactor, regardless of the type of coenzyme, in 29 of 33 structures. This finding cannot be achieved through sequence alignment due in part to the low sequence identity for these enzymes. The more significant reason is the high variability of the residue involved in this interaction, as evidenced by the fact that this residue type is composed of as many as 10 different residues (Fig. 6). This interaction can tolerate a high degree of sequence variation and can still be maintained because the functional group is the peptide main chain, not the side chain. Such an arrangement, thus, emphasizes the important role of this interaction for the positioning and function of cofactors in the SDR family. Nevertheless, this position may have a preference for hydrophobic residues over hydrophilic ones. This may result from the fact that the nicotinamide ring can increase its stability by stacking against the hydrophobic side chains.

The interaction involving the main chain amide group cannot presently be found in the other four structures (PDB accession codes: 1BSV, 1EK5, 1FK8, and 1IY8). Except for UDP-glucose-4-epimerase (1EK5), in which the binding environment for the NMN moiety is quite different, most probably, the interaction will show up upon the binding of substrate in the enzymes involved in the other three complex structures.

The recently determined structure of a negative transcriptional regulator (Nmra, included in our above structural anal-
yses and which also maintains the conserved interaction we found) revealed that this sequence-unrelated protein displays the SDR fold, thus extending the structure-function relationship of SDR (34). The physiological functions of SDR enzymes establish some particular SDR members as pharmacological drug targets (1). For example, among the hydroxysteroid dehydrogenases belonging to the SDR family, 17β-HSDs are attractive targets for structure-based rational drug design for the prevention and control of sex steroid-dependent cancers and 11β-HSD1 is another good target for drug design for the therapy of several metabolic syndromes (insulin resistance, hyperlipidemia, arterial hypertension, and obesity). A recent study showed that two highly selective inhibitors can occupy the dihydronicotinamide binding site and thus displace the dihydronicotinamide moiety in dihydrofolate reductase (35). Also, in 3α,20β-hydroxysteroid dehydrogenase, a member of the SDR family, the hemisuccinate side chain of the inhibitor, carbeneoxolone, makes a hydrogen bond with the hydroxyl group of the conserved residue Tyr152 and occupies the position of the nicotinamide ring of the cofactor (36). Considering the fact that the backbone amide group discussed here is important for the positioning and function of cofactors in the SDR family and is in the vicinity of substrate binding site, specific inhibitors could be designed to compete with the substrate, as well as to displace the stable binding of cofactors simultaneously in some target enzymes. Thus, our finding on the conserved interaction between the cofactor and the main chain amide group in SDR broadens our understanding on the structure-function relationship for this super family facilitating the development of inhibitors directed against biologically relevant SDR targets.

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Cofactor Hydrogen Bonding onto the Protein Main Chain Is Conserved in the Short Chain Dehydrogenase/Reductase Family and Contributes to Nicotinamide Orientation

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