Critical Residues for Structure and Catalysis in Short-chain Dehydrogenases/Reductases*

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Short-chain dehydrogenases/reductases form a large, evolutionarily old family of NAD(P)(H)-dependent enzymes with over 60 genes found in the human genome. Despite low levels of sequence identity (often 10–30%), the three-dimensional structures display a highly similar α/β folding pattern. We have analyzed the role of several conserved residues regarding folding, stability, steady-state kinetics, and coenzyme binding using bacterial 3β/17β-hydroxysteroid dehydrogenase and selected mutants. Structure determination of the wild-type enzyme at 1.2-Å resolution by x-ray crystallography and docking analysis was used to interpret the biochemical data. Enzyme kinetic data from mutagenetic replacements emphasize the critical role of residues Thr-12, Asp-60, Asn-86, Asn-87, and Ala-88 in coenzyme binding and catalysis. The data also demonstrate essential interactions of Asn-111 with active site residues. A general role of its side chain interactions for maintenance of the active site configuration to build up a proton relay system is proposed. This extends the previously recognized catalytic triad of Ser-Tyr-Lys residues to form a tetrad of Asn-Ser-Tyr-Lys in the majority of characterized short-chain dehydrogenases/reductase enzymes.

Since the discovery of fundamental differences between insect-type and liver-type alcohol dehydrogenases (1), corresponding to the protein families of “short-chain” dehydrogenases/reductases (SDR)1 and “medium-chain” dehydrogenases/reductases (MDR), respectively, SDR enzymes have received much attention. They constitute a large protein family with well over 2000 annotated enzyme and species variant sequences in databases and are represented in all life forms with minimally 60 genes found in the human genome (2–4). The SDR enzymes span several EC classes, from oxidoreductases and lyases to isomerases, with NAD(P)(H)-dependent oxidoreductases constituting the majority of forms. In this class, many enzymes with different specificities act on steroids, prostaglandins, aliphatic alcohols, and xenobiotics.

The pairwise sequence identity between different enzymes is low, typically 10–30%, but all available three-dimensional structures (~20) display a highly similar α/β folding pattern (5–11). Most SDR enzymes have a 250–350-residue core structure, frequently with additional N- or C-terminal transmembrane domains or signal peptides. Conserved sequence regions cover a variable N-terminal Gly-X-Gly-X-Gly motif as part of the nucleotide binding region and the active site with a triad of catalytically important Ser, Tyr, and Lys residues, of which Tyr is the most conserved residue within the whole family (2, 8, 12) (Fig. 1). The functions of the residues at these particular sites have been elucidated by a combination of chemical modifications, sequence comparisons, structure analyses, and site-directed replacements (2, 13). However, several other conserved but still variable residues have not been analyzed in detail. To understand their role, we now carried out a mutagenesis study using bacterial 3β/17β hydroxysteroid dehydrogenase (3β/17β-HSD) analyzing effects on enzymatic function and the stability of replacements, assisted by x-ray crystallography and docking analysis. Other residues have been studied before (13), but novel segments investigated include Asp-60 (between βC and αD), an NNAG motif (Asn-86–Gly-89 in βD), and Asn-111 in αE (Fig. 1).

MATERIALS AND METHODS

Molecular Cloning, Site-directed Mutagenetic Replacements, and Purification of Wild Type and Mutants of 3β/17β-HSD—Molecular cloning of 3β/17β-HSD (EC 1.1.1.51) and purification of recombinant proteins from Comamonas testosteroni ATCC 11996 (DSM, Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany) was carried out as described (13) using metal-chelate chromatography of Histagged enzymes. Mutagenetic replacements were performed using the megaprimer method (14) with Pfu polymerase (Stratagene). Wild-type sequences used for amplification were: 5'-GGCAAGCTATGACGAATGCTTGGACAGGG (sense) and 5'-CTAGGGATCCCTATAGCCCCACGCCATGGCAGAACAGT (antisense). Mutagenesis primers covering the desired replacement region were as follows (5'-3'): CCTCGCTGCTCACGGCA-3

3-Gly-Gly motif as part of... CAGAAT (antisense). Mutagenesis primers covering the desired replacement region were as follows (5'-3'): CCTCGCTGCTCACGGCA-3

The abbreviations used are: SDR, short-chain dehydrogenases/reductases; MDR, medium-chain dehydrogenases/reductases; 3β/17β-HSD, 3β/17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; isoUDCA, iso-ursodeoxycholic acid.

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† The abbreviations used are: SDR, short-chain dehydrogenases/reductases; MDR, medium-chain dehydrogenases/reductases; 3β/17β-HSD, 3β/17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; isoUDCA, iso-ursodeoxycholic acid.

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determined by amino acid analysis of pure samples after hydrolysis in 6 M HCl followed by ninhydrin-based quantification on an LKB Alpha plus analyzer.

Determination of Kinetic and Binding Constants—Enzyme activities were measured as NAD(H)-dependent 3β- and 17β-oxidoreductase activities by determination of the change of absorbance at 340 nm and using a molar extinction coefficient for NADH of 6.22 mM·cm⁻¹. Recordings were carried out with a Cary 300Bio instrument. Reactions were performed in 1.0-ml volumes at 298 K. Conditions for dehydrogenase activities were: 20 mM Tris/HCl (pH 8.5) and 250 μM NAD, varying the amount of iso-ursodeoxycholic acid (3β,12α-dihydroxy-5β-cholan-24-oic acid, isoUDCA) or dehydroepiandrosterone (5α-androstene-3β-ol, 17-one, DHEA; 3β-HSD activity) and testosterone (4-androstene-17β-ol-3-one; 17β-HSD activity). Conditions for reductase activities were: 20 mM Tris/HCl (pH 7.0) and 250 μM NADH, varying the amount of 5α-dihydrotestosterone (5α-androstan-3α-ol-17-one; 3-ketoreductase activity) and androsterone (5α-androstan-3α-ol-17-one; 17-ketoreductase activity). Kinetic constants were calculated with the EnzPack software (Biosoft). Binding of NADH was determined by monitoring fluorescence energy transfer as a function of nucleotide concentration in 20 mM Tris-HCl, pH 7.0, at 298 K using a Shimadzu RF5000 spectrophotometer. Binding of NAD⁺ was assessed in a similar manner by displacement of bound NADH in 20 mM Tris/Cl, pH 8.5. Dissociation constants were obtained by non-linear regression using a 1:1 binding model as described previously (12).

Structure Analysis of 3β/17β-HSD and Comparison with SDR Members—The structure of the wild-type 3β/17β-HSD apoform was determined by x-ray crystallography to a final resolution of 1.2 Å.

FIG. 1. Sequence alignment of SDR enzymes based upon determined three-dimensional structures. The secondary structure elements of 1hxh are indicated as arrows for β-strands and as cylinders for α-helices. Mutated residues are indicated by black boxes and black downward arrows. Fabg protein members included in Table III (Protein Data Bank accession numbers 1bvr, 1qsg, and 1d70) were omitted from the alignment for clarity. Abbreviations and Protein Data Bank accession numbers are as follows: 1dhx: dihydropterdine reductase; 1en7: Homo sapiens 17β-hydroxysteroid-dehydrogenase; 1e5w: ratius norwegicus short-chain 3-hydroxyacyl-CoA dehydrogenase; 1d0b: Magnaporthe grisea trihydroxynaphthalene reductase; 1egq: Klebsiella pneumoniae acetoin reductase; 1ed0: Brassica napus β-keto acyl carrier protein reductase; 1egx: Bacillus megaterium glucose dehydrogenase; 1hdx: Streptomyces hydrogenans 3α,20β-hydroxysteroid dehydrogenase; 1hxh: C. testosteroni 3β/17β-hydroxysteroid dehydrogenase; 1cyd: Mus musculus carbonyl reductase; 1aee: Datura stramonium tropinone reductase-i; 2ae1: D. stramonium tropinone reductase-ii; 1bbd: Pseudomonas spec. cis-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase; 1a4u: Drosophila alcohol dehydrogenase; 1nas: m. musculus sepiapterin reductase; 1fjh: C. testosteroni 3α-hydroxysteroid dehydrogenase/carbonyl reductase.
RESULTS AND DISCUSSION

Overexpression, Purification, Folding, and Stability Analysis of Wild Type and Mutants of 3β/17β-HSD—Wild-type and mutant 3β/17β-HSD forms were overexpressed in Escherichia coli strain BL21 and purified by metal-chelate chromatography (Fig. 2). Proteins were analyzed regarding folding, stability, and conformational stability (data not shown). Stability measurements were performed by titration with guanidine-HCl using a titration spectropolarimeter. Conformational stability was determined by titration of the individual proteins with guanidine-HCl using a titration spectropolarimeter. The transition temperature for dehydrogenase reactions are an order of magnitude higher (1.7 × 10^6 s^-1 M^-1 for 17β-HSD activity, 1.0 × 10^6 s^-1 M^-1 for 3β-HSD activity with isoUDCA) as com-

| Wild-type | 3β-HSD | 17β-HSD | IsoUDCA | Testosterone | 5α-Androstan-3α,17β-diol |
|-----------|--------|---------|---------|-------------|--------------------------|
| K_m (M)   | k_cat (min^-1) | k_cat (min^-1) | k_cat (min^-1) | k_cat (min^-1) | k_cat (min^-1) |
| 2.25 ± 0.23 | 0.14 ± 0.01 | 1.0 ± 0.3 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 28.4 ± 1.8 | 1.8 ± 0.1 | 1.1 ± 0.2 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 47.3 ± 0.17 | 4.1 ± 0.16 | 1.0 ± 0.2 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 125 ± 0.47 | 0.14 ± 0.01 | 0.49 ± 0.02 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 118.8 ± 0.26 | 0.24 ± 0.03 | 0.24 ± 0.03 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 21.5 ± 0.39 | 0.08 ± 0.02 | 0.08 ± 0.02 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 38.5 ± 0.18 | 0.078 ± 0.008 | 0.078 ± 0.008 | 100 ± 5 | 100 ± 5 | 100 ± 5 |
| 26.9 ± 0.12 | 0.064 ± 0.007 | 0.064 ± 0.007 | 100 ± 5 | 100 ± 5 | 100 ± 5 |

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pared with the reductive reactions (0.13 \times 10^6 s^{-1} M^{-1}, 3-oxo-reductase, 0.11 \times 10^6 s^{-1} M^{-1}, 17-oxoreductase).

The mutants compared for dehydrogenase and reductase activities have residue exchanges at positions Thr-12, Asp-60, Asn-86, Asn-87, Ala-88, Asn-111, Ser-138, and Tyr-151, surrounding the coenzyme binding region or located at the active site. Depending on the amino acid substitution, differential effects on enzymatic constants were observed. Completely inactive enzymes were obtained with the N111L, S138A, and Y151F mutants, whereas partially active enzymes with significant changes were observed for the other substitutions (Table I).

Replacement of Thr-12 by Ala results in an enzyme that is only able to catalyze the reductive reaction at position C3 and the corresponding dehydrogenase activity with DHEA as substrate. All other activities are not detectable. Exchange to Ser results in an enzyme with enzymatic properties largely similar to those of the wild-type form (Table I). A profound change in cofactor binding (Table II) is observed with the T12A mutant; the $K_d$ for NADH is altered from 1.0 to 41 \mu M, whereas the $K_d$ for NAD$^+$ changes from 1.5 \mu M for the wild type to 0.1 \mu M for T12A. This altered binding is reflected in a lowered $K_m$ value for NAD$^+$ (8.96 versus 29.6 \mu M for wild type) and an increased $K_m$ value for NADH (36.7 versus 21.6 \mu M for wild type).

Mutants D60A, N86A, N87A, and A88S display reduced catalytic efficiencies over the wild type with the exception of the A88S mutant, which shows a 20% higher relative $k_{cat}/K_m$ value.

### Table II

| Substrate     | $K_m$ for NADH | $k_d$ for NADH | $K_m$ for NAD$^+$ | $k_d$ for NAD$^+$ |
|---------------|----------------|----------------|-------------------|-------------------|
|              | \(5\alpha\)-DHT \(\mu \text{mol/liter}\) | \(\mu \text{mol/liter}\) | \(\mu \text{mol/liter}\) | \(\mu \text{mol/liter}\) |
| Protein       |                |                |                  |                  |
| WT$^b$        | 21.6           | 1.0            | 83.2             | 29.6             |
| T12A          | 36.7           | 41             | NA$^c$           | 8.96             |
| T12S          | 21.7           | 1.9            | 90.4             | 13.4             |
| D60A          | 52.3           | 19             | 651              | 87.9             |
| N86A          | 7.44           | 3.1            | 843              | 122              |
| N87A          | 25.5           | 3.8            | 81.2             | 259              |
| A88S          | 30.4           | 9.5            | 1550             | 685              |
| N111L         | NA$^c$         | 5.2            | NA$^c$           | NA$^c$           |
| S138A         | NA$^c$         | 12             | NA$^c$           | NA$^c$           |
| S138T         | 23.1           | ND$^d$         | 59.0             | ND$^d$           |
| Y151F         | NA$^c$         | 4.3            | NA$^c$           | NA$^c$           |

$^a$ \(5\alpha\)-DHT, \(5\alpha\)-dihydrotestosterone.

$^b$ WT, wild type.

$^c$ NA, no activity detectable.

$^d$ ND, not determined.

**FIG. 3.** Details of the central \(\beta\)-sheet (displaying strands \(\beta\)A, \(\beta\)D, and \(\beta\)E) of \(3\beta/17\beta\)-HSD in relation to the NAD$^+$ molecule. Residues analyzed in this study include Thr-12, Asn-86, Asn-87, and Ala-88. Hydrogen bond distances (in Å) between side chain and backbone atoms are given as dotted lines. The figure was created with RIBBONS (26).

**FIG. 4.** Close-up view of active-site residues in \(3\beta/17\beta\)-HSD determined by x-ray crystallography in relation to modeled NAD$^+$ (red) and steroid substrate (\(3\beta\)-hydroxy-\(5\alpha\)-androsten-17-one, blue) molecules. Tyr-151 is in catalytic distance to the substrate hydroxyl (2.7 Å), further stabilized by Ser-138 interaction to the substrate hydroxyl (2.6 Å). A network of residues and interactions comprising Lys-155, Ser-154, Asn-111, and Ile-90 via a conserved water molecule is shown. For clarity, NAD$^+$ contacts are omitted (cf. “Results and Discussion”). The figure was created with RIBBONS (26).
for 3-oxoreductase activity. The drop in $k_{cat}/K_m$ values is more pronounced for dehydrogenase activities (ranging from 2.3 to 50% versus wild type) than for reductase values (ranging from 25 to 120% versus wild type). The relative change in the ratio of dehydrogenase/reductase reaction for the different comparable substrate pairs (i.e. similar ring configurations and substritutents) at positions C3 (DHEA/5α-dihydrotestosterone) and C17 (testosterone/androstenedione) ranges from about 3-fold (N87A, position C3) to over 25-fold for the A88S form. Here the relative $k_{cat}/K_m$ for reactions at C17 is shifted from 72% for the reductase reaction to 2.6% for dehydrogenase activity as compared with the corresponding wild-type values. Significant changes in $k_{cat}/K_m$ values are also observed with the 5β-reduced steroid isoUDCA as substrate for 3β-HSD activity, supporting the conclusion that Asn-86, Asn-87, and Ala-88 mutants significantly attenuate oxidative kinetic constants and show a less pronounced effect in the reductive reactions catalyzed.

NAD$^+$ binding constants for the N86A and N87A mutants are in the same range as the wild-type value (1.5 and 1.1 μM, respectively, versus 1.5 μM). Slightly lowered values are observed for the D60A and A88S forms (0.5 μM). Depending on the substrate for determination of 3β-HSD activity (isoUDCA or DHEA), significant increases (up to 1550 μM, A88S, isoUDCA substrate) in $K_m$ for NAD$^+$ are observed except for N87A (81.2 versus 83.2, isoUDCA substrate). Weaker NADH binding is observed for Asp-60, Asn-86, Asn-87, and Ala-88 mutants, ranging from 3.1 to 19 μM, which is accompanied by a moderate increase in $K_m$ for NADH (range from 25.5 to 52.3 μM) except for the N86A mutant displaying a $K_m$ of 7.5.

Taken together, the drastic reduction in $k_{cat}/K_m$ values for the oxidative reaction of the Asp-60, Asn-86, Asn-87, and Ala-88 forms is mainly due to increased $K_m$ for NAD$^+$ without significant decrease in binding affinities, indicating inhibition of substrate formation without affecting association of the enzyme-substrate complex. The $k_{cat}/K_m$ values for the reductive reactions are slightly lowered, accompanied by a decrease in NADH binding. Steroid structure is a further contributing factor to the differential decrease in oxidation since differences between isoUDCA and DHEA are observed.

**Determination of Kinetic and Binding Constants of Active Site Mutants**—Mutations performed within or close to the active site were residues Ser-138 and Tyr-151, recognized previously to be part of a catalytic triad with Lys-155, and the conserved Asn-111, located within helix αE, which forms the main subunit interaction surface. Mutants N111L, S138A, and Y151F are enzymatically inactive. Coenzyme binding constants of these mutants are changed but within the range observed with the mutants described above (from 0.2 μM for NAD$^+$ for Y151F to 12 μM for NADH for S138A). As described earlier (13), substitution of Ser for Thr at position 138 yields an active enzyme. Loss of activity observed for S138A and Y151F mutants thus underscores the critical role of these residues in catalysis.

**Crystallographic Analysis of 3β/17β-HSD and Comparison with High Resolution SDR Structures**—The apo structure of wild-type 3β/17β-HSD was determined by x-ray crystallography to a resolution of 1.2 Å. The enzyme forms a tetramer with subunit interactions similar to those of tetrameric SDR structures (9, 10, 17). The geometry of coenzyme binding and active sites was sufficiently similar to allow docking analysis of a 3β/17β-HSD ternary complex with NAD$^+$ and a steroid substrate (3β-OH-5-androsten-17-one) (Figs. 3 and 4). Details of side chain and backbone interactions of residues Thr-12, Asp-60, Asn-86, Asn-87, and Ala-88 within the central β-sheet formed by strands βA, βD, and βE are given in Fig. 3 and illustrate the essential nature of these residues for correct coenzyme positioning and binding (Tables I and II). No direct interactions of Thr-12, Asn-86, and Asn-87 with the coenzyme are observed in our model and in most other coenzyme complexes (9, 10, 17–19). An exception is the recently determined porcine carbonyl reductase, where backbone carbonyl interactions of Asn-89 to the 3’-OH of the nicotinamide ribose are found (20). Based on structural and kinetic data, we conclude that these residues (Thr-12, Asn-86, Asn-87) supply a framework essential for keeping the strands oriented within the central β-sheet, important for coenzyme positioning (Fig. 3).

Multiple and critical side chain and backbone interactions are formed through Thr-12 to strand βD (ND2Asn-87-OThr-12: 3.05 Å; OG1Thr-12-NAsn-87: 3.03 Å; OG1Thr-12-NAsn-87: 2.98 Å) and between strands βD and βE through Asn-86 and Asn-87 (OAsn-86-NMet-136: 2.93 Å; NAsn-86-OLle-134: 2.83 Å; OD1Asn-135-ND2Asn-86: 2.87 Å).

The coenzyme is bound through few specific contacts, performed through other residues than Thr-12, Asn-86, and with indicated exceptions, Asn-87. Among the residues investigated, Asp-60 and Ala-88 contribute directly to coenzyme binding, a view supported by the significantly altered coenzyme constants. In the modeled structure, the carboxyl side chain of Asp-60 is in weak H-bonding distance to the adenine ring (OD1 Asp-60-N6A 3.99 Å) (Fig. 2), and similar interactions between Asp-60 and coenzyme are observed in, for example, MLCR, 7α-HSD, or CR (9, 10, 20). Side chain to backbone interactions are also observed (OD2Asp-60-OGSer-62: 2.7 Å), and the main role of Asp-60 appears to be in the stabilization of the turn between βC and αD as part of the adenine ring binding pocket (10). Ala-88 can make hydrophobic contacts to the ade-
nine ring and thus contributes to binding of the coenzyme. These hydrophilic interactions of Ala-88 appear to be an important characteristic since mutation to Ser significantly changes activities.

**Reaction Mechanism of 3β/17β-HSD**—The previously determined triad of Ser-Tyr-Lys residues (positions 138, 151, and 155, respectively in 3β/17β-HSD) constitutes the active site (2) (Fig. 4). Our data extend this concept by addition of an essential Asn-111 to this triad to form an active site tetrad. Previous studies support the concept that Tyr-151 functions as the catalytic base (2), whereas Ser-138 stabilizes the substrate, and Lys-155 forms hydrogen bonds with the nicotinamide ribose moiety and lowers the $pK_a$ of the Tyr-OH to promote proton transfer (Fig. 5). In the 3β/17β-HSD apo structure, water molecules are bound to the Tyr-OH and Lys side chain, thus mimicking substrate and ribose hydroxyl group positions. Determination of the *Drosophila* alcohol dehydrogenase structure (21) revealed interaction of the conserved Asn-111 via a water molecule, binding to the active-site Lys-155, and this interaction is also observed in the present structure (OAas111-H2O: 2.76 Å). In the *Drosophila* alcohol dehydrogenase structure, a large hydrogen-bonded solvent network including the water molecule bound by Asn-111 and Lys-155 was found (21), thereby substantiating our assumption of a proton relay with access to bulk solvent molecules (cf. below). Inspection of available three-dimensional SDR structures reveals interactions of Asn-111 (Table III) similar to those in 3β/17β-HSD and *Drosophila* alcohol dehydrogenase, indicating a homologous role of Asn-111 in all these cases. Out of 20 SDR structures retrieved from the Protein Data Bank, 16 contain Asn at the position homologous to the one in 3β/17β-HSD, show similar side chain/backbone interactions, and display the feature of having a connecting water molecule to the active site lysine. Moreover, the four structures without a homologous Asn-111 contain a Ser residue, which is connected through a water molecule to the active site Lys. An extended network is created in mouse sepiapterin reductase built (Protein Data Bank accession number 1nas) with an additional Arg residue involved. Based on this general configuration, we conclude that Asn-111 is important to stabilize the position of Lys-155, and furthermore, that a proton relay is formed in most if not all SDR structures at the active site, including coenzyme, substrate, Tyr-151, ribose 2’OH, Lys-155, water, and Asn-111 or a corresponding Ser (Figs. 4–6). A proton relay system involving water and essential ribose contacts to the catalytic base similar to that found in horse liver alcohol dehydrogenase (22) has been postulated earlier also for SDR (7); however, here we provide direct evidence for a critical involvement of Asn-111 in this process. The stabilization of the active site geometry is thus achieved through maintaining the Lys-155 position and furthermore through Asn side chain interactions with main chain atoms.

### Table III

| Structure | Interacting residues | Distance | Distance | Distance |
|-----------|----------------------|----------|----------|----------|
| 1hxh      | Asn-111 ND2–Ile-90 O | 2.98 Ang | Asn-111 OD1–Ile-90 N | 2.80 Ang |
| 1fmc      | Asn-118 ND2–Gly-98 O | 2.98 Ang | Asn-118 OD1–Gly-98 N | 2.95 Ang |
| 1c2t      | Asn-114 ND2–Leu-93 O | 2.65 Ang | Asn-114 OD1–Leu-93 N | 2.84 Ang |
| 1e3w      | Asn-121 ND2–Ile-94 O | 2.96 Ang | Asn-121 OD1–Ile-94 N | 2.87 Ang |
| 1doh      | Asn-130 ND2–Val-17 O | 2.82 Ang | Asn-130 OD1–Val-17 N | 2.84 Ang |
| 1gge      | Asn-110 ND2–Val-89 O | 3.00 Ang | Asn-110 OD1–Val-89 N | 2.92 Ang |
| 1edc      | Asn-126 ND2–Ile-105 O | 2.83 Ang | Asn-126 OD1–Ile-105 N | 3.18 Ang |
| 1hdo      | Asn-116 ND2–Leu-95 O | 3.07 Ang | Asn-116 OD1–Leu-95 N | 2.95 Ang |
| 1hc       | Asn-111 ND2–Ile-90 O | 3.14 Ang | Asn-111 OD1–Ile-90 N | 2.89 Ang |
| 1cyd      | Asn-107 ND2–Leu-96 O | 3.15 Ang | Asn-107 OD1–Leu-96 N | 2.97 Ang |
| 1a1e      | Asn-130 ND2–Val-109 O | 3.35 Ang | Asn-130 OD1–Val-109 N | 3.10 Ang |
| 2a1e      | Asn-118 ND2–Ile-97 O | 3.10 Ang | Asn-118 OD1–Ile-97 N | 2.72 Ang |
| 1bdc      | Asn-115 ND2–Ile-89 O | 2.88 Ang | Asn-115 OD1–Ile-89 N | 2.93 Ang |
| 1a4u      | Asn-107 ND2–Ile-94 O | 2.93 Ang | Asn-107 OD1–Ile-94 N | 2.97 Ang |
| 1g        | Asn-86 ND2–Leu-72 O | 3.07 Ang | Asn-86 OD1–Leu-72 N | 2.85 Ang |
| 1nas      | Asn-128 ND2–Thr-104 N | 3.17 Ang | Asn-128 OD1–Thr-104 OG1 | 3.14 Ang |
| 1drh      | Ser-107 OG–Water | 2.65 Ang | Water–Gly-85 O | 2.67 Ang |
| 1bwr      | Ser-123 OG–Phe-97 N | 2.97 Ang | | |
| 1qge      | Ser-120 OG–Phe-94 N | 3.21 Ang | | |
| 1d7o      | Ser-162 OG–Asn-139 N | 3.07 Ang | | |
| 1hxh      | Asn-111 O-Water | 2.76 Ang | Lys-155 NZ–Water | 2.74 Ang |
| 1fmc      | Asn-118 O-Water | 3.02 Ang | Lys-163 NZ–Water | 2.38 Ang |
| 1c2t      | Asn-114 O-Water | 3.60 Ang | Lys-159 NZ–Water | 2.85 Ang |
| 1e3w      | Asn-121 O-Water | 2.90 Ang | Lys-172 NZ–Water | 2.84 Ang |
| 1doh      | Asn-130 O-Water | 3.56 Ang | Lys-182 NZ–Water | 2.84 Ang |
| 1gge      | Asn-110 O-Water | 3.25 Ang | Lys-156 NZ–Water | 2.94 Ang |
| 1edc      | Asn-126 O-Water | 2.63 Ang | Lys-171 NZ–Water | 2.85 Ang |
| 1h        | Asn-116 O-Water | 2.78 Ang | Lys-162 NZ–Water | 2.69 Ang |
| 1a4u      | Asn-107 O-Water | 2.76 Ang | Lys-153 NZ–Water | 2.85 Ang |
| 1a1e      | Asn-130 O-Water | 2.99 Ang | Lys-175 NZ–Water | 2.98 Ang |
| 2a1e      | Asn-118 O-Water | 3.14 Ang | Lys-163 NZ–Water | 2.56 Ang |
| 1bdc      | Asn-115 O-Water | 2.86 Ang | Lys-159 NZ–Water | 2.76 Ang |
| 1a4u      | Asn-107 O-Water | 2.83 Ang | Lys-155 NZ–Water | 2.68 Ang |
| 1g        | Asn-86 O-Water | 2.83 Ang | Lys-159 NZ–Water | 2.66 Ang |
| 1nas      | Asn-128 O-Arg-175 NH1 | 3.05 Ang | Lys-175 NZ | 2.75 Ang |
| 1drh      | Ser-107 O-Water | 3.44 Ang | Lys-150 NZ–Ser-107 OG | 2.85 Ang |
| 1bwr      | Ser-123 O-Water | 2.78 Ang | Lys-175 NZ–Water | 2.79 Ang |
| 1qge      | Ser-120 O-Water | 2.79 Ang | Lys-173 NZ–Water | 2.81 Ang |
| 1d7o      | Ser-162 O-Water | 2.75 Ang | Lys-206 NZ–Water | 2.89 Ang |

* Predicted to be 2.7 Å by comparisons to water-containing crystal structures of other SDR members.

*Lys-175 NZ–Water 2.93 Å, Water–Arg-178 ND2 2.90 Å.*
of residues located within the segment preceding helix αE (Table III).

Asn-111 is located within αE, the main dimerization interface in oligomeric SDRs. Notably, at this position, the helix forms a sharp kink. This motif created by the side chain of Asn-111 presumably forces its backbone carbonyl group to bind a water molecule instead of the amide group (Val-115) that would have been expected in an α-helical structure. Moreover, we found other water molecules in the same cavity, forming a small water-rich enclosure inside the protein fold. In 3β/17β-HSD, we observed four water molecules, and in other SDR structures, the number ranges from two to five. This water-filled hydrophilic cavity is lined by other well conserved amino acids: the side chains of Thr-12 (for Protein Data Bank accession number 1a4u, Thr-114; for 1dhr, Thr-110; and for 1a27, Thr-118), Asn-86, and Ser-114 and the main chain carbonyl group of Ala-88. This hydrophilic pocket serves as a proton relay system by apparently acting as a proton bridge between Lys-155 and the bulk solvent, similar to a temporary proton relay system by apparently acting as a proton bridge between Lys-155 and the bulk solvent, similar to a temporary proton relay and stabilizes the geometry of active site residues. This view is supported by mutational analysis of a homologous Ser (Ser-154 in 3β/17β-HSD) in type 1 11β-hydroxysteroid dehydrogenase, showing a critical involvement of this residue in catalysis (23).

Role of Conserved Residues in SDR Enzymes—With over 2000 sequences annotated in databases and about 20 crystal structures determined, a picture of the general SDR architecture and mechanism emerges. Considering all sequences, no strict positional conservation is noted. However, multiple sequence alignments revealed several conserved motifs, the most conserved being the N-terminal TGXGXXG around Thr-12, as part of the nucleotide binding fold, and the active site SYK triad, now shown to form a tetrad with the conserved Asn-111. The NNAG motif around residue 86, the conserved Asn-111, defined in this study, and further motifs (comprising a conserved Asn-179 in strand βF, the PG motif, and the conserved Thr-188; Fig. 1), identified through structural alignments and functional analyses (6, 12, 24) reveal the critical involvement of conserved elements for coenzyme binding, maintenance of the SDR scaffold, and catalysis. Notably, the recent structure determination of sequence-unrelated proteins displaying the SDR fold considerably extend structure-activity relationships (25). Thus the SDR domain structure appears to be a generic scaffold not only including dehydrogenases/reductase, lyase, epimerase, and hydratase activities but also comprising RNA binding proteins, kinases, and transcription factors (25). Greater understanding of the mechanistic and structural principles governing the SDR architecture will reveal novel substrate and protein-protein interactions and will facilitate the development of inhibitors directed against biologically relevant SDR targets. These efforts constitute avenues currently pursued at several pharmaceutical sites.

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