Active Site Directed Mutagenesis of 3β/17β-Hydroxysteroid Dehydrogenase Establishes Differential Effects on Short-Chain Dehydrogenase/Reductase Reactions†

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ABSTRACT: Mutagenetic replacements of conserved residues within the active site of the short-chain dehydrogenase/reductase (SDR) superfamily were studied using prokaryotic 3β/17β-hydroxysteroid dehydrogenase (3β/17β-HSD) from *Comamonas testosteroni* as a model system. The results provide novel data to establish Ser138 as a member of a catalytically important "triad" of residues also involving Tyr151 and Lys155. A Ser → Ala exchange at position 138 results in an almost complete (>99.9%) loss of enzymatic activity, which is not observed with a Ser → Thr replacement. This indicates that an essential factor for catalysis is the ability of side chain 138 to form hydrogen bond interactions. Mutations in the NAD(H) binding region, in strands βA, βD, and adjacent turns, reveal two additional residues, Thr12 and Asn87, which are important for correct binding of the coenzyme and with a differential effect on the reactions catalyzed. Thus, mutation of Thr12 to Ala results in a complete loss of the 3β-dehydrogenase activity, whereas the 3-oxoreductase activity remains unchanged. On the other hand, a T12S substitution yields a protein with unaltered catalytic constants for both reactions, revealing that a specific hydrogen bond is critical for the dehydrogenase activity. Our interpretation of the available crystal structure of 3α/20β-HSD from *Streptomyces hydrogenans* suggests a hydrogen bond in that enzyme between the Thr12 side chain and the backbone NH of Asn87 rather than the coenzyme, indicating that this hydrogen bond to the βD strand might determine a crucial difference between the reductive and the oxidative reaction types. Similarly, mutation of Asn87 to Ala results in an 80% reduction of k_{cat}/K_m in the dehydrogenase direction but also unchanged 3-oxoreductase properties. It appears that the binding of NAD^+ to the protein is influenced by local structural changes involving strand βD and turn βA to αB.

Short-chain dehydrogenases/reductases (SDRs) constitute a protein family with highly diverse functions in pro- and eukaryotes [for recent reviews, cf. Krozowski (1994) and Jörnvall et al. (1995)]. All known three-dimensional structures reveal a highly similar one-domain α/β pattern. Well-conserved primary structure elements are restricted to certain segments in the sequence, indicating a possibly common fold, active site, reaction mechanism, and coenzyme and substrate binding regions (Persson et al., 1991). Essential parts of the coenzyme binding site, as established by X-ray crystallography (Ghosh et al., 1991, 1994, 1995), and comparisons (Persson et al., 1991; Jörnvall et al., 1995), are located in the N-terminal part and consist of a "Rossmann fold" structure (Rossmann et al., 1974) with a conserved but variable (Jörnvall et al., 1984) Gly-X-X-X-Gly-X-Gly pattern (residues 13−19 in the 3β/17β-HSD sequence). Comparisons, chemical modifications, and site-directed mutagenesis of different SDR enzymes have revealed a conserved Tyr-X-X-X-Lys segment (residues 151−155 in the 3β/17β-HSD sequence) essential for catalytic activity of SDR proteins (Jörnvall et al., 1981; Krook et al., 1990, 1992; Persson et al., 1991; Obeid & White, 1992; Chen et al., 1993; Ensor & Tai, 1994). However, recent investigations (Ghosh et al., 1994; Jörnvall et al., 1995) also highlight several other amino acid residues as important in substrate binding and catalytic conversion. Structure alignments of short-chain dehydrogenases/reductases, based on the available data of hydroxysteroid and prostaglandin dehydrogenases and other SDR enzymes (Ghosh et al., 1994, 1995; Krook et al., 1993, Varughese et al., 1992; Tanaka et al., 1996), demonstrate that these residues are close to the catalytic center but also propose a more complicated architecture of the active site.

In order to establish the roles for these residues, which are strictly conserved in most SDR structures thus far known (Ghosh et al., 1994; Jörnvall et al., 1995), we performed mutagenetic replacement at these positions, i.e. replacement of Thr12, Ser16, Asn87, and Ser138, in the prokaryotic 3β/17β-hydroxysteroid dehydrogenase (3β/17β-HSD), which is a SDR protein (Yin et al., 1991) derived from the Gram-negative bacterium *Comamonas testosteroni* ATCC 11996.

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[1] *C. testosteroni* is used instead of *Pseudomonas testosteroni* throughout the text (Marcus & Talalay, 1956; Tamaoka et al., 1987).

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Table 1: Designations, Positions, and Sequences of Oligonucleotides used for Mutagenesis Reactions

designation	mutation	nucleotide sequence (5′ → 3′)
T12A	Thr 12 Ala	GGC ACC ACC AGC GAC CAG CGC C
T12S	Thr 12 Ser	CTG GCA CCA CCA GAG ACC AGC GCC
S16A	Ser 16 Ala	GAC CCA CAC CGG CGG CAC CAC CAG
N87A	Asn 87 Ala	CAG GAT GCC GGC AGC GTT GAC CAG CAC
S138A	Ser 138 Ala	CAG CTC GAT ACC GCG GCC ATA TTG
S138T	Ser 138 Thr	CAG CTC GAT ACC GTG GCC ATA TTG ATG ATG GAG CC

We analyzed the influence of these mutations on enzymatic activity, protein folding, stability, and immunological reactivity.

MATERIALS AND METHODS

Molecular Cloning and Mutagenetic Replacements in the Prokaryotic 3β/17β-Hydroxysteroid Dehydrogenase. Molecular cloning of 3β/17β-HSD (EC 1.1.1.51) from *C. testosteroni* ATCC 11996 (DSM, Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany), was carried out by generating a PCR product from the genomic DNA using Vent polymerase (New England Biolabs) with sequence specific primers encompassing the complete gene (Abalain et al., 1993) and containing compatible restriction sites for further cloning. The resulting PCR product was cloned into the *Nde*I/*Bam*HI restriction sites of the pET 29a vector (Novagen), containing an F′ site, thus allowing single-stranded DNA rescue by superinfection with helper phage R 408 (Stratagene). These constructs were used for mutagenetic replacements. The complete DNA sequence was determined by use of the Taq dye deoxy terminator cycle sequencing kit (Applied Biosystems) or the Sequenase Kit (Amersham) and was found to be identical to the sequence reported (Yin et al., 1991). Site-directed mutagenesis of the (+) strand was carried out with the Sculptor mutagenesis kit (Amersham), based on the phosphorothioate technique (Taylor et al., 1985) using the oligonucleotides shown in Table 1. Sequence analysis of the respective regions usually displayed mutation efficiencies of greater than 75%. DNA of selected mutant plasmids was digested with *Nde*I and *Bam*HI restriction endonucleases, and inserts released were cloned into the *Nde*I/*Bam*HI restriction sites of the pET 15b vector (Novagen), resulting in an N-terminal His tag sequence with an integrated thrombin cleavage site in the overexpressed recombinant protein. The DNA sequence and the correct reading frame of each mutant were confirmed by use of the sequencing methods described above. For molecular cloning and sequence analysis, XL Blue MRF′ cells (Stratagene) were used, whereas for subsequent expression of recombinant proteins, the constructs were transformed into BL21 DE 3 cells (Novagen).

Overexpression and Purification of Wild-Type and Mutant 3β/17β-Hydroxysteroid Dehydrogenase Proteins. Recombinant proteins were overexpressed in BL21 DE3 cells grown in LB medium by addition of 1 mM IPTG as soon as the liquid culture had reached an optical density at 600 nm of 0.6. After 2.5 h, the cells were harvested and lysed using a French Press or sonication. The resulting supernatant from a 10 min centrifugation at 15000*g* was loaded to a His-Bind resin column (Novagen). After a washing step, the protein was eluted essentially in a pure form with 0.5 M NaCl, 1 M imidazole, and 20 mM Tris/HCl (pH 7.9). To avoid precipitation and inactivation of the enzyme, the preparation was immediately subjected to a buffer change [20 mM Tris/HCl (pH 7.9), 100 mM NaCl, and 5 mM DTT] on Sephadex G-50 or G-25 (Nick or NAP columns; Pharmacia). The cleavage of the N-terminal His tag was achieved by use of thrombin (Boehringer Mannheim) in the recommended digestion buffer [20 mM Tris/HCl (pH 8.4), 150 mM NaCl, and 2.5 mM $CaCl_2$] for 16 h at 4 °C. Cleavage of the His tag and efficient purification were confirmed by separation on 15% SDS/PAGE using a tricine-based buffer system and subsequent amino acid analysis on an LKB Alpha Plus amino acid analyzer after hydrolysis of the protein. Purifications and subsequent kinetic experiments were performed minimally four times.

SDS/PAGE and Western Blot Analysis. SDS/PAGE was performed according to Laemmli (1970), with 10% separating gels. Blotting after electrophoresis was carried out using the semidry technique (Kyhse-Andersen, 1984), and immunoreactive proteins were detected with alkaline phosphatase-coupled secondary antibodies (Bio Rad) with nitro blue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) as chromogenic substrates. Primary antibody serum against purified 3β/17β-HSD from *C. testosteroni* was kindly supplied by Dr. S. Genti-Raimondi (University of Cordoba, Argentina) and was purified further by adsorption to protein A-Sepharose (Pharmacia) according to the manufacturer's instructions.

Analysis of Enzyme Activity and Determination of Kinetic Constants. Enzyme activities were measured as NAD(H)-dependent 3β- and 17β-oxidoreductase activities, by the absorbance at 340 nm and using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH. Confirmation of steroid products, after extraction with diethyl ether, was achieved by thin-layer chromatography on silica plates. Reactions were performed in 1.0 mL at 25 °C under the following conditions: dehydrogenase reactions, 20 mM Tris/HCl (pH 8.5) and 250 μM NAD^+, varying the amount of steroid; and reductase reactions, 20 mM Tris/HCl (pH 7.0) and 100 μM NADH, varying the amount of steroid. Steroids used as substrates for the activity measurements were testosterone (17β-HSD), androsterone (17-oxoreductase), 3β,-12α-dihydroxy-5β-cholanoic acid (3β-HSD), and 5α-dihydrotestosterone (3-oxoreductase). Determination of Michaelis constants for the reduced and oxidized coenzyme was performed at a 100 μM steroid substrate concentration with varied coenzyme concentrations but with other reaction conditions as described above. Cleavage of the His tag did not alter the enzymatic properties compared to those with the uncleaved recombinant protein. Enzymatic constants (K_m, k_{cat}, and k_{cat}/K_m) were calculated by use of the *Fig.P* software (Biosoft, Cambridge, U.K.) for the 3-oxo and 3β reaction, which displayed Michaelis−Menten kinetics. Statistical analysis was performed by variance analysis followed by the Bonferroni or least significance difference (LSD) test.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectroscopy was carried out using an AVIV model 62DS circular dichroism spectropolarimeter (Aviv, Lakewood), equipped with a dual-syringe titration device. Purified recombinant enzyme preparations were analyzed in 10 mM Tris/HCl buffer (pH 8.5), 100 mM NaCl, and 2 mM DTT at a concentration of 0.5 mg/mL by measurement of the ellipticity as a function of wavelength at 0.5 nm

increments between 260 and 190 nm at 4 °C.

Equilibrium Unfolding Experiments by GuHCl Denaturation. Equilibrium unfolding experiments for 3β/17β-HSD wild-type and mutant proteins were performed using guanidine hydrochloride (GuHCl). The extent of protein unfolding was measured by the change in ellipticity at 222 nm at constant protein and variable GuHCl concentrations. The resulting denaturation curves (θ_{222} vs [GuHCl]) were transformed into the fraction of unfolded protein, f_u, as a function of denaturant concentration using eq 1.

$$f_u = (\theta_N^\circ + m_N[\mathrm{GuHCl}] - \theta_{exp})/[\theta_N^\circ + m_N[\mathrm{GuHCl}] - (\theta_U^\circ + m_U[\mathrm{GuHCl}])] \quad (1)$$

where θ_N° and θ_U° represent the circular dichroism values (222 nm) of the native and unfolded states of the protein, respectively, at the concentration used in the experiment. θ_{exp} is the experimentally obtained CD value (222 nm) at a given denaturant concentration. m_N and m_U are the calculated slopes of the pre- and post-transition regions, respectively. The transformed data were fit using nonlinear regression to eq 2, which assumes a monomolecular two-state equilibrium (Santoro & Bolen, 1988).

$$f_u = e^{-(m/RT)([\mathrm{GuHCl}]-C_{mid})}/[1 + e^{-(m/RT)([\mathrm{GuHCl}]-C_{mid})}] \quad (2)$$

where R is the gas constant, C_{mid} is the midpoint of the denaturation curve, and m is the denaturant dependence of ΔG (m value calculated using the linear extrapolation method; Pace, 1986), where $\Delta G = \Delta G^\circ_{n-u} + m[\mathrm{GuHCl}]$. Alternatively, the raw experimental data were fit directly to eq 3 (Santoro & Bolen, 1988).

$$\theta_{exp} = [\theta_N^\circ + m_N[\mathrm{GuHCl}] + (\theta_U^\circ + m_U[\mathrm{GuHCl}])e^{-(m/RT)([\mathrm{GuHCl}]-C_{mid})}]/[1 + e^{-(m/RT)([\mathrm{GuHCl}]-C_{mid})}] \quad (3)$$

The use of eq 3 is preferred as estimates of the error in the fitted parameters are more rigorous. Reversibility of the reaction was demonstrated by coincidence of unfolding and folding experiments. The concentration of the GuHCl stock solution was determined by refractometry (Nozaki, 1972).

Protein Determination. Protein concentrations were measured by analysis of the absorbance at 280 nm and by analysis of the total amino acid composition with an LKB Alpha Plus analyzer after hydrolysis in evacuated tubes with 6 M HCl and 0.5% phenol. Calculations from the spectrophotometric measurements were performed by using a molar extinction of the protein of 18.02×10^3 mol^{-1} cm^{-1}, corresponding to a protein concentration of 1.5 mg/mL at an OD of 1.0 in 6 M GuHCl.

RESULTS

Mutagenesis and Expression of Recombinant Enzymes. Cloning of the 3β/17β-HSD gene from *C. testosteroni* ATCC 11996 into the vector pET 15b and subsequent overexpression resulted in a 30 kDa fusion protein with an N-terminal His tag sequence. The recombinant protein could be purified in one step using metal chelate chromatography followed by removal of the His tag sequence by use of thrombin. The kinetic constants K_m, k_{cat}, and k_{cat}/K_m were determined and found to be of the same order of magnitude as for the wild-type protein. Six independent mutagenetic replacements at four positions (Table 1) were constructed in the pET 29a plasmid. The mutants were reinserted into the pET 15b system, expressed, and purified as described above. Typically, 15−20 mg of purified protein per liter of culture was obtained. All mutants could be expressed to a similar extent using the BL21 DE3 *Escherichia coli* strain. The purity of the protein samples was analyzed by SDS/PAGE (Figure 1A). No contaminating proteins were detected, confirming the efficiency of the purification scheme. The mutations did not change the overall recognition of the mutant proteins by anti-3β/17β-HSD antibodies, directed against the wild-type enzyme (Figure 1A).

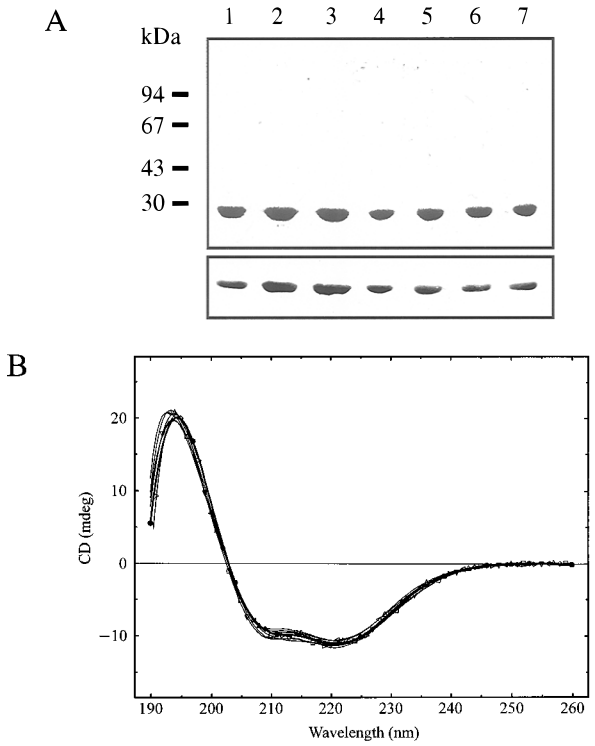

FIGURE 1: SDS/PAGE, immunoblot, and CD spectra of recombinant 3β/17β-HSD wild-type and mutant enzymes. (A, upper panel) SDS/PAGE: lane 1, wild type; 2, T12A; 3, T12S; 4, S16A; 5, N87A; 6, S138A; and 7, S138T. Protein (0.75 μg) was applied to each lane. Coomassie Blue staining. (A, lower panel) Western blot: The same protein-loading pattern as in SDS/PAGE was used. Samples were transferred to nitrocellulose membranes and incubated with anti-3β/17β-HSD antibodies. For visualization of the immunoreactive protein, an alkaline phosphatase-coupled detection system was utilized. (B) CD spectra of wild-type and mutant proteins measured from 260 to 190 nm. (●) wild type, (◇) T12S, (□) T12A, (△) S16A, (triangle pointing left) N87A, (▽) S138A, and (triangle pointing right) S138T.

Effects of Mutations on Protein Folding and Stability. To exclude the possibility that incorrectly folded proteins were produced, CD spectroscopy with 3β/17β-HSD wild type and mutants was performed. These proteins had essentially indistinguishable CD spectra between 260 and 190 nm (Figure 1B), indicating that the loss of enzyme activity in the S138A and T12A mutants (Table 2) is not due to major structural changes in the protein. The integrity of the mutant 3β/17β-HSD compared to that of the wild-type protein could also be judged by comparison of GuHCl denaturation curves (Figure 2). The midpoints (C_{mid}) obtained by nonlinear regression (eq 3) are presented in Table 3. Except for T12S and N87A, all mutant proteins exhibited a stability toward GuHCl similar to that of the wild-type protein. The

Table 2: Kinetic Constants of Wild-Type and Mutant Forms of 3β/17β-HSD from *C. testosteroni*[a]

		3β-HSD			3-oxoreductase		
mutation	location	K_m ($\times 10^{-6}$ M)	K_{cat} ($\times 10^3$ min^{-1})	k_{cat}/K_m ($\times 10^{-6}$ s^{-1} M^{-1})	K_m ($\times 10^{-6}$ M)	K_{cat} ($\times 10^3$ min^{-1})	k_{cat}/K_m ($\times 10^{-6}$ s^{-1} M^{-1})
wild type	–	22.3	1.4	1.1	16.1	0.1	0.09
Thr12 → Ala	cofactor binding	na[c]	–	–	15.8	0.1	0.08
Thr12 → Ser	cofactor binding	28.3	1.7	1.0	13.9	0.06	0.07
Ser16 → Ala	cofactor binding	27.6	1.4	0.9	23.8[b]	0.1	0.08
Asn87 → Ala	cofactor binding	28.8	0.3[b]	0.2[b]	18.6	0.09	0.11
Ser138 → Ala	active site	na	–	–	na	–	–
Ser138 → Thr	active site	18.8	1.5	1.3	14.2	0.06	0.07

[a] Enzymatic constants for the 3β/3-oxo steroid substrates were determined with 3β,12α-dihydroxy-5β-cholanoic acid (3β-HSD) and 5α-dihydrotestosterone (3-oxoreductase) as described in Materials and Methods. Statistical significance was calculated by analysis of the variances followed by Bonferroni or least significance difference (LSD) tests. [b] $p < 0.05$; $n = 5$. [c] na, no activity.

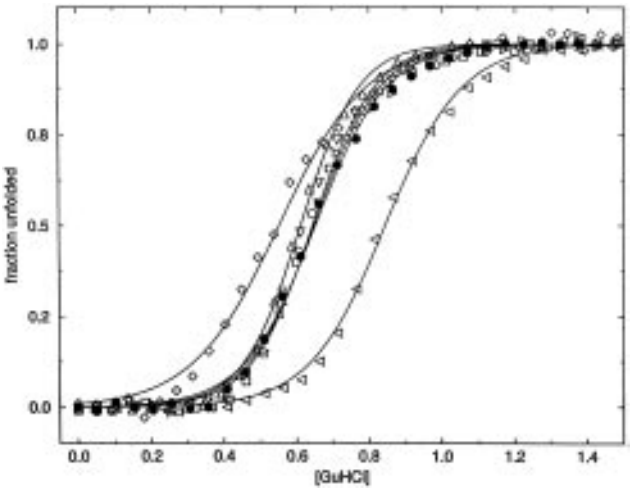

FIGURE 2: GuHCl denaturation of 3β/17β-HSD and mutant forms followed by CD (222 nm). Symbols are as in Figure 1B. For details, see Materials and Methods.

Table 3: Values for C_{mid} and m as Obtained from Nonlinear Regression of the GuHCl Denaturation Curves for 3β/17β-HSD Wild-Type and Mutant Proteins Using eq 3[a]

protein	C_{mid} (mol/L)	$-m \times 10^{-3}$ (kcal/mol)/(mol/L)
wild type	0.65 ± 0.01	5.58 ± 0.02
T12A	0.65 ± 0.01	6.43 ± 0.02
T12S	0.55 ± 0.01	4.57 ± 0.02
S16A	0.61 ± 0.01	6.84 ± 0.02
N87A	0.85 ± 0.01	5.21 ± 0.02
S138A	0.63 ± 0.01	6.23 ± 0.02
S138T	0.65 ± 0.01	6.00 ± 0.02

[a] Data were transformed using the equations defined in Materials and Methods.

midpoints obtained for T12S and N87A (0.55 and 0.85, respectively), taken together with the CD data (Figure 1B), indicate that the proteins are indeed folded.

Effects of Mutagenetic Replacements on Enzymatic Activities. Enzyme kinetic constants k_{cat}, K_m, and k_{cat}/K_m for steroid carbonyl reduction and hydroxysteroid dehydrogenase reactions involving position C3 of the steroid molecule were determined (Table 2) with the wild-type and mutant proteins. The 3β/17β-HSD wild-type enzyme efficiently catalyzes the β-hydroxy dehydrogenation of steroids of the androstane and bile acid series with k_{cat}/K_m values of 1.1×10^6 s^{-1} M^{-1} for the 3β oxidation, whereas the reverse oxo reduction is 10 times less efficient (0.1×10^6 s^{-1} M^{-1}) under the conditions employed.

Depending on the amino acid substitution, several effects on enzymatic constants were observed. Replacement of Ser16 with Ala does not significantly change the catalytic efficiency for any of the reactions tested, indicating the nonessential nature of the Ser16 hydroxyl moiety for catalysis in 3β/17β-HSD. Only the K_m value was significantly elevated for the 3-oxo-reductase reaction (23.8 vs 16.1) but without effecting the overall k_{cat}/K_m value. On the other hand, substitution of Thr with Ala at position 12, located between strand βA and helix αB (Benach et al., 1996), abolishes all enzymatic activity except the 3-oxoreductase reaction, which is unaffected. However, substitution of Thr12 with Ser does not alter the activities and gives values not significantly different from those of the wild-type enzyme, indicating that a hydroxyl group at position 12 is essential for catalysis.

Exchange of Asn87 for Ala also results in a mutant protein with altered dehydrogenase properties, whereas the reductive reactions are not influenced. The k_{cat}/K_m values for the oxidative reactions are reduced by >80% (1.1 vs 0.2 for the 3β-HSD reaction). The change in 3β-HSD activity can be attributed to a lowered k_{cat} value (1.4 vs 0.3 for the wild type and N87A, respectively).

A replacement of Ser138 with Ala results in a close to complete loss of all enzyme activities (<0.04% residual activity compared to wild-type activities). However, substitution of Ser138 with Thr yields a mutant enzyme, which is fully active. Enzyme constants for this mutant protein are not significantly different from those for the wild-type enzyme, indicating that a side chain hydroxyl at position 138 is essential for catalytic activity.

We also determined the enzyme constants for the C17 position using testosterone and androsterone as substrates. The results from these measurements are similar to those obtained for the 3β/3-oxo reactions, with the additional effect that the T12A mutant enzyme did not exhibit 17-oxoreductase activity with androsterone as substrate. However, the compounds used in the C17 reactions display substrate inhibition (Talalay & Marcus, 1956), and Michaelis constants for this steroid position were not determined.

To find out if the 3β/3-oxo activity differences of the mutants vs those of the wild-type enzyme may be derived from pH effects on the reactions catalyzed, we tested all reactions over a pH range from 7.0 to 9.5. The relative activities of the mutants compared to that of the wild type remained unchanged, showing that the functional effects observed are due to side chain differences rather than to pH influences.

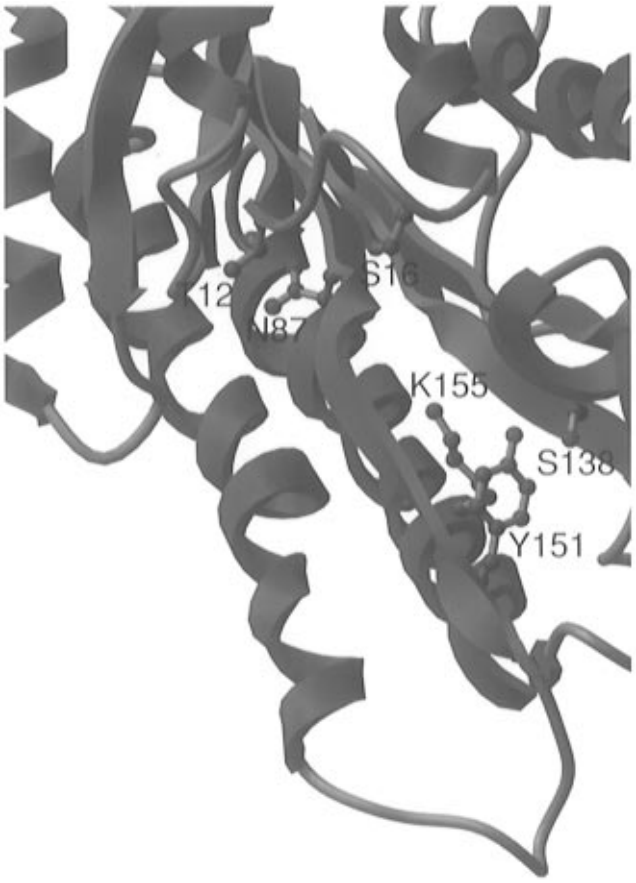

FIGURE 3: View of the 3β/17β-HSD active site as determined by crystallographic analysis (Benach et al., 1996). Residues now mutated, as well as the conserved Tyr151 and Lys155, are highlighted by inclusion of their side chains. The figure was generated by use of the program ICM (version 2.5, Molsoft LLC, Metuchen, NJ, 1996). The numbering of residues is based upon the protein sequence determined (Yin et al., 1991).

DISCUSSION

To understand the role of amino acid residues lining the catalytic cavity in SDR proteins (Ghosh et al., 1994; Jörnvall et al., 1995), we exchanged these residues by site-directed mutagenesis and investigated the corresponding effects on the enzymatic activity, protein conformation, and stability. For orientation, Figure 3 provides a view of the 3β/17β-HSD active site, based on the partially refined three-dimensional structure of 3β/17β-HSD (Benach et al., 1996) showing the positions of the mutated residues in relation to the conserved Tyr151 and Lys155. Our results reveal several aspects of importance in relation to the SDR superfamily.

First, the concept of Ser138 adding to previously recognized Tyr151 and Lys155 (Jörnvall et al., 1995; Ensor & Tai, 1996), to form a catalytic triad (Ghosh et al., 1995) in the SDR family, is strongly supported. The present results demonstrate the importance of hydrogen bonding at position 138 involving the side chain hydroxyl. In most SDR proteins, the Ser hydroxyl group appears to be able to form hydrogen bonds to the reaction product (as in mouse lung carbonyl reductase; Tanaka et al., 1996) and/or to the hydroxyl group of the conserved Tyr (Ghosh et al., 1994). The role of this Ser residue might most likely be derive from a stabilization by hydrogen bonding to the substrate, reaction intermediate, and product. It is not clear why in all SDR sequences thus far characterized Thr is conspicuously absent

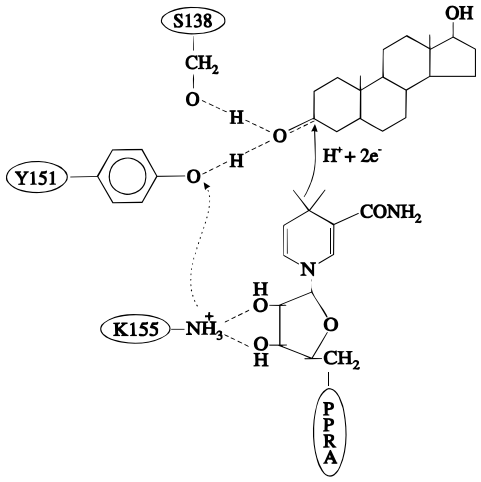

FIGURE 4: Proposed reaction mechanism of SDR HSDs with possible interactions according to atomic distances as determined from the crystal structures of 3α/20β-HSD (Ghosh et al., 1994) and mouse lung carbonyl reductase (Tanaka et al., 1996). The dotted arrow indicates the influence of Lys155 in lowering the pK_a of the hydroxyl group of Tyr151. PPRA depicts the pyrophosphate−ribosyl−adenine moiety of NADH.

at this position, since our *in vitro* replacement of Ser138 with Thr yielded an active protein with identical catalytic constants. However, some SDR proteins, like rat dihydropteridine reductase or *Klebsiella aerogenes* ribitol dehydrogenase, contain neither Ser nor Thr at this position. Our results support the conclusion that a hydroxyl is important for the reaction to take place, but in some proteins, a modified reaction mechanism might be involved in catalysis, since Ser is not completely conserved throughout all SDR structures.

Figure 4 gives a possible mechanism for the SDR dehydrogenase reaction with respect to the catalytic triad residues, coenzyme, and substrate. Notably, a similar reaction mechanism is found in the related monomeric aldo-keto reductase superfamily (Bohren et al., 1989) with conserved Tyr and Lys residues. The role of Ser138 in SDR proteins in stabilizing and/or polarizing the carbonyl substrate, however, seems to be achieved by a conserved His residue in the aldo-keto reductases (Barski et al., 1995).

Second, the mutations of residues found in strands βA and βD reveal factors important for the reaction direction of the SDR hydroxysteroid dehydrogenases. The residues chosen for mutation were Thr12, Ser16, and Asn87, all being parts of the coenzyme binding site. Position 16, which in most SDR sequences is a polar or charged residue like Ser, Lys, or Arg, can be replaced in the 3β/17β-HSD by a nonpolar residue like Ala without gross change in activity, indicating that it is not participating in important hydrogen-bonding interactions with NAD(H). However, in NADP(H)-dependent SDR enzymes, basic residues at position 16 and 38 (numbering in 3β/17β-HSD) confer specificity to NADPH by forming electrostatic interactions with the ribose 2′-phosphate (Tanaka et al., 1996). In NAD(H)-dependent enzymes such as 3β/17β-HSD, these basic residues are not strictly conserved and are preceded at position 37 by an aspartic acid residue. This carboxylate might result in electrostatic repulsion of the 2′-phosphate moiety, thereby conferring specificity to NAD(H) (Chen et al., 1991; Baker, 1994).

Located in the turn between βA and αB, Thr12 is highly conserved in all SDR structures known. Mutation to Ala

Table 4: Apparent Michaelis Constant K_m for NADH and NAD^+ with 3β/17β-HSD Wild-Type and Mutant Proteins[a]

protein	K_m for NADH (μmol/L)	K_m for NAD^+ (μmol/L)
wild type	21.6	83.2
T12A	36.7	–
T12S	21.7	90.4
S16A	22.1	85.3
N87A	25.5	81.2
S138A	–	–
S138T	23.1	59.0

[a] Number of experiments was three to five.

yields a protein that is only able to carry out the 3-oxoreductase reaction and loses all other steroid-metabolizing activities. Nevertheless, a replacement with Ser results in a fully active enzyme and points to the importance of hydrogen bonding also to this side chain. Since the crystal structure of 3β/17β-HSD is still under refinement (Benach et al., 1996), we chose the highly similar three-dimensional structure of 3α/20β-HSD (Ghosh et al., 1991) as the basis for the explanation of our results. Our interpretation of the structure of this enzyme complexed with the coenzyme suggests that the Thr/Ser12 OH group binds to the backbone NH of Asn87 (distance of 2.0 Å), rather than directly to the coenzyme. We propose that the role of Thr/Ser12 is to give the correct positioning of strand βD and adjacent residues toward the coenzyme. This is further demonstrated by mutation of Asn87 to Ala. This exchange yields a protein with unaltered reductase properties but with a 80% decrease in dehydrogenase activity, compatible with an important role of this part of the sequence in correct binding of the coenzyme. In the 3α/20β-HSD structure (Ghosh et al., 1994), we could not detect hydrogen bonding between the coenzyme and Asn87. However, in combination with a hydrated coenzyme, the carbonyl group of the Asn side chain could interact with the adenine ribose *via* a water molecule. Therefore, Asn87 probably contributes to the stabilization of the catalytic cleft and process. Positions 12 and 87 are also critical in terms of protein stability, since the T12S exchange results in a somewhat less stable mutant, whereas the N87A mutation yields a more stable protein (Figure 2, Table 3).

The interpretation of our data is compatible with former results, which show that *C. testosteroni* 3β/17β-HSD is a bifunctional, NAD(H)-dependent enzyme (Talalay & Marcus, 1956) with a single catalytic site (Minard et al., 1985) and an ordered reaction mechanism (Schultz et al., 1977). Experiments concerning the dehydrogenase type of reaction reveal that NAD^+ binds first, followed by the steroid substrate (Schultz et al., 1977). Dissociation of the reduced coenzyme is the rate-limiting step in the overall dehydrogenase reaction. Fluorescence titration experiments also revealed dissociation constants of 16 and 0.25 μM for NAD^+ and NADH, respectively, whereas the apparent Michaelis constant for NAD^+ was 83 μM (Schultz et al., 1977), which is in perfect agreement with our values (Table 4). Therefore, the Thr12 and Asn87 mutations most likely disturb the binding of NAD^+. The reductive reaction is not influenced at all, probably because binding of the reduced coenzyme is still sufficient to promote catalysis.

Taken together, these results highlight the importance of conserved residues in SDR structures, describe factors important for discrimination between the reductive and oxidative reaction direction in these proteins, and improve our knowledge of the catalytic requirements of the protein family. In this respect, three-dimensional structures of the Ser138 and Thr12 mutants in complex with cofactor and steroid substrate will allow a more detailed examination of the active site and give the opportunity to promote a general understanding of the catalytic mechanism and architecture of short-chain dehydrogenases/reductases. Such a knowledge is important for our insight into endocrinological and vascular diseases, since SDR enzymes, especially hydroxysteroid dehydrogenases like 11β-HSDs, 3β-HSDs, and 17β-HSDs, play central roles in steroid hormone synthesis, development of steroid hormone-dependent cancer forms, regulation of blood pressure, and phase I metabolism of hormones and xenobiotics, including carcinogens.

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