Published in final edited form as:

Ann N Y Acad Sci. 2005 December ; 1061: 135–148.

Determining Structure and Function of Steroid Dehydrogenase Enzymes by Sequence Analysis, Homology Modeling, and Rational Mutational Analysis

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Abstract

The short-chain oxidoreductase (SCOR) family of enzymes includes over 6,000 members identified in sequenced genomes. Of these enzymes, ~300 have been characterized functionally, and the three-dimensional crystal structures of ~40 have been reported. Since some SCOR enzymes are steroid dehydrogenases involved in hypertension, diabetes, breast cancer, and polycystic kidney disease, it is important to characterize the other members of the family for which the biological functions are currently unknown and to determine their three-dimensional structure and mechanism of action. Although the SCOR family appears to have only a single fully conserved residue, it was possible, using bioinformatics methods, to determine characteristic fingerprints composed of 30–40 residues that are conserved at the 70% or greater level in SCOR subgroups. These fingerprints permit reliable prediction of several important structure-function features including cofactor preference, catalytic residues, and substrate specificity. Human type 1 3 β -hydroxysteroid dehydrogenase isomerase (3 β -HSDI) has 30% sequence identity with a human UDP galactose 4-epimerase (UDPGE), a SCOR family enzyme for which an X-ray structure has been reported. Both UDPGE and 3-HSDI appear to trace their origins back to bacterial 3 α ,20 β -HSD. Combining three-dimensional structural information and sequence data on the 3 α ,20 β -HSD, UDPGE, and 3 β -HSDI subfamilies with mutational analysis, we were able to identify the residues critical to the dehydrogenase function of 3-HSDI. We also identified the residues most probably responsible for the isomerase activity of 3 β -HSDI. We test our predictions by specific mutations based on sequence analysis and our structure-based model.

Keywords

rational mutational analysis; steroid dehydrogenase; homology modeling; prostate cancer; breast cancer; prediction of ligand specificity; testosterone 3-HSDII

INTRODUCTION

The short-chain oxidoreductase (SCOR) family of enzymes is an ancient family that includes many dehydrogenases and reductases vital to growth and development.¹ The features that have come to define the SCOR family are: (1) an approximate length of 250–350 amino acids that form a single-domain Rossmann fold; (2) NAD or NADP cofactor dependence; (3) a glycine-rich signature sequence (GxxxxxG) located near the N-terminus and involved in cofactor binding; and (4) a catalytic triad usually composed of a Ser residue and Tyr and Lys residues in a YxxxK sequence.^{1–3} Over 6,000 probable SCOR genes have been identified by sequence similarity, but only 300 of these have been biochemically characterized.⁵ We have identified structural subgroups of the SCOR family, determined the sequence fingerprints of these subgroups, and demonstrated that the cofactor specificity of 90% of all hypothetical members of the family can be reliably predicted.^{2,4}

Although the SCOR enzymes have a common Rossmann fold, bind NAD(P) cofactors, and have common catalytic residues and a postulated mechanism of action, there is at most one fully conserved residue in the family, and the substrates of the SCOR family include a wide range of structure in terms of size and hydrophobicity. Because the known substrates for SCOR enzymes include steroids, prostaglandins, sugars, dyes, porphyrins, acids, and alcohols, the substrate-binding pocket is expected to be highly variable. We have used covariance analysis methods to identify the substrates of 25% of the SCOR subfamily that has a TGxxxGIG signature in β 1 α 2 turn (Fig. 5).¹

3 α ,20 β -HYDROXYSTEROID DEHYDROGENASE

The first member of the SCOR family for which the crystal structure was determined was 3 α ,20 β -hydroxysteroid dehydrogenase (3 α ,20 β -HSD) from *Streptomyces hydrogenans*, an enzyme involved in the reversible oxidation of the 3 α -group of androstane derivatives and the 20 β -group of pregnane derivatives. At least two models had been proposed to explain the dual activity of the enzyme.⁷ One model invoked a single stereospecific steroid-binding pocket with cofactor binding sites at either end, accounting for the 3 α and 20 β activity. A second model proposed a single cofactor-binding site and a substrate-binding pocket that would permit steroids to bind in two different orientations. The X-ray structure of the complex of the tetrameric enzyme and cofactor⁸ revealed that each subunit of the tetramer contains a cofactor binding site and a putative steroid-binding site. The 245-amino acid monomer has essentially a single domain. The first 145 residues have the characteristic Rossmann fold,⁹ composed of a five-stranded parallel β -sheet with two helices on either side (Fig. 1). The rest of the single-domain structure consists of two additional β -strands added to the β -sheet and two more helices. The cofactor resides on one side of the β -sheet in an extended conformation. The adenine-ribose end of the cofactor lies in a cleft surrounded by five peptide segments from one monomer of the protein. Hydroxy groups of the adenine-ribose ring form hydrogen bonds with the Asp37 side chain, and the *bis*-phosphate moiety interacts with the Arg16 side chain.

On the basis of the observed structure, we proposed a mechanism of action involving highly conserved Ser(139) and Tyr(152) xxx Lys(156) residues (Fig. 2). Numerous mutation studies have demonstrated that these residues are critical to oxidoreductase activity. Subsequent structure determinations and mutation studies suggested that as many as four additional residues [Asp(111) and Pro(184)-GlyxxxThr(187)] that are closely linked to the cofactor or between the cofactor and the substrate-binding pocket may have important roles in the mechanism of action. Without altering the structure of the enzyme it was possible to model a cortisol molecule in the substrate-binding cleft with either the 3 β - or 20 β -carbonyl oriented near the cofactor and catalytic tyrosine, suggesting that the dual function of 3 α ,20 β -HSD is due to the fact that the binding site accepts steroids in two orientations.⁸

LICORICE EFFECTS ON STEROID METABOLISM

For over 50 years, physicians have warned people with hypertension not to eat licorice. Although a correlation between licorice consumption and elevation of blood pressure was well established, the physiological basis for the effect was not understood.¹⁰ When glycyrrhizic acid, the active ingredient responsible for the hypertensive response, was isolated, its ability to compete for the mineralocorticoid receptor was tested, but no evidence of binding could be detected. In the 1970s, some newborn infants were found to have clinical signs of aldosteronism, no detectable circulating aldosterone, and an inability to oxidize cortisol to cortisone. Urinary 11β -hydroxycortisol metabolites in these infants were greatly increased, and the corresponding 11β -oxocortisone metabolites were diminished. This condition was named apparent mineralocorticoid excess (AME).^{11,12} The altered steroid metabolism in AME was traced to a defective 11β -hydroxysteroid dehydrogenase (11β -HSD). The defect leads to a persistently active mineralocorticoid receptor saturated by excess accumulated cortisol. The discovery of the critical role of 11β -HSD in mineralocorticoid receptor binding and activity led investigators to question whether glycyrrhizic acid might inhibit 11β -HSD, producing an increased concentration of cortisol and associated hypertension. As predicted, inhibitor studies revealed that glycyrrhizic acid is a potent competitor for binding to the active site of 11β -HSD. The interactions between 11β -HSD, cortisol, cortisone, aldosterone, glycyrrhizic acid, and the mineralocorticoid receptor are illustrated in Figure 3.

Because 11β -HSD is a member of the SCOR family, we tested glycyrrhizic acid and its metabolite, carbenoxolone, and found them to be very potent inhibitors of $3\alpha,20\beta$ -HSD.¹³ Single crystals of the complex of carbenoxolone and $3\alpha,20\beta$ -HSD were prepared, and the structure determination revealed the presence of the inhibitor in the previously postulated binding site.¹⁴ The carbenoxolone molecule is anchored in the catalytic cleft by hydrogen bonding (Fig. 4) at both ends of the molecule. The hemisuccinate terminal oxygen forms a hydrogen bond (2.8Å) to Tyr152, one of the two strictly conserved residues of the catalytic triad. The cofactor is either prevented from binding or displaced by the inhibitor molecule. The fact that carbenoxolone occupies the catalytic cleft, displaces the cofactor, and hydrogen bonds directly to a member of the catalytic triad makes it a very potent high-affinity inhibitor and a good model upon which to base the design of selective inhibitors for other enzymes, particularly other members of the SCOR family.

Licorice has also been linked to decreases in serum testosterone levels in women. This effect may be due to inhibition of 17β -hydroxysteroid dehydrogenase and $17-20$ lyase in the adrenal glands of healthy young women.¹⁵

SHORT-CHAIN OXIDOREDUCTASE SUPERFAMILY

The three-dimensional crystal structures of over 40 unique SCOR enzymes had been reported and archived in the Protein Data Bank (PDB).¹⁶ Although at most one residue in the SCOR enzyme is conserved in all 6,000 members of the superfamily, we have identified a group of ~40 sequence positions exhibiting 70% or greater conservation that define the fold fingerprint of the largest subgroup of the SCOR family (Fig. 5). Of these 40 fingerprint residues, 12–15 make hydrogen-bond contacts with the cofactor (Fig. 6). In current databases, the majority of proteins with SCOR fingerprint sequence patterns are identified as putative, hypothetical, or probable oxidoreductases/dehydrogenases without a defined function or simply as open reading frame products of unknown function.

We have shown that NAD or NADP cofactor preferences can be reliably predicted for ~70% of the SCORs by the presence of Asp or Arg recognition residues, respectively, in specific adjacent sequence positions in the $\beta 2\alpha 3$ turn near the N-terminus (Fig. 5). The residues in the $\beta 1\alpha 2$ turn of the Rossmann fold distinguish different subfamilies. The 1,651 structures having

sequence homology with 3 α ,20 β -HSD are characterized by the TGxxxGIG sequence in the β 1 α 2 turn.² Because the known substrates for SCOR enzymes include steroids, prostaglandins, sugars, dyes, porphyrins, acids, and alcohols, the substrate-binding pocket is highly variable. In many crystal structures, competitive inhibitors have been cocrystallized with the enzyme steroid, permitting the determination of the substrate-binding site. Three flexible loops of hypervariable composition create a pocket on the surface of the enzyme that controls access to the catalytic residues and nicotinamide end of the cofactor. We have used covariance analysis⁶ to determine groups of amino acids that line this pocket and covary with the type of substrate (Fig. 7). In contrast to the fingerprint of highly conserved residues that determine the Rossmann fold, interacting with the cofactor and including the catalytic residues, the substrate-defining residues are not highly conserved.

A search for covariance among residues predicted to line the substrate-binding pocket led to identification of a conserved set of 10 residues that define this pocket for the β -ketoacyl-[acyl carrier protein] (β -k-ACPR) subfamily⁶ (Fig. 7). An identical pattern of 10 residues in three similarly spaced segments was found in 118 protein sequences in the SWISS-PROT/TrEMBL database. The majority of these structures had previously been identified as known or hypothetical β -k-ACPRs. These structures come from 95 different species ranging from bacteria to humans. Fourteen gene products previously assigned ambiguous or erroneous identifications can now be reliably predicted to be β -k-ACPRs based on this fingerprint. For example, one of these proteins (Ke 6) is a gene product previously identified as a type 8 17 β -hydroxysteroid dehydrogenase that is downregulated in a murine model for polycystic kidney disease.⁶ This enzyme is likely to be involved in fattyacid processing rather than sex steroid metabolism. We subsequently discovered that different combinations of amino acid residues in the same 9 sequence position on the 3 flexible loops define the substrate specificity of 11 different SCOR enzyme subfamilies.

RATIONAL MUTATIONAL ANALYSIS BASED ON PROTEOMIC FINGERPRINTS

In humans, there are two isoforms of 3 β -hydroxysteroid dehydrogenase encoded by separate genes and expressed in a tissue-specific manner. Human type 1 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSDI) catalyzes the two sequential enzyme reactions on a single protein that converts dehydroepiandrosterone or preg-nenolone to androstenedione or progesterone, respectively, in placenta, mammary gland, breast tumors, prostate, prostate tumors, and other peripheral tissues. Human type 2 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSDII) is expressed in testis, ovary, and the adrenal gland and has 90% homology with human 3 β -HSDI.^{17,18} Amino acid sequence analysis suggests that 3 β -HSDI is a member of the SCOR family. Similarities include the presence of a YxxxK sequence, a Serine doublet, and a Gly-rich sequence near the N-terminus. However, 3 β -HSDI is significantly larger than the typical SCOR enzyme (372 verses 250 amino acid residues). 3 β -HSDI is an isomerase as well as a dehydrogenase, and the Gly-rich sequence in the β 1 α 2 turn is different in detail. When the sequence of 3 β -HSDI was compared with the sequence of all SCOR enzymes for which crystal structures have been reported, the structure found to have the greatest percentage identity was the UDP galactose-4-epimerase (UDPGE) structure from *Escherichia coli* (PDB code 1NAH).¹⁹

Although the percent conservation of identities between 1NAH and 3 β -HSDI is only 20% and the alignment incorporates 11 insertions and 6 deletions (Fig. 8), there is no doubt about the fit because of the conservation of:

1. The catalytic YxxxK and Ser.
2. The presence in the 3 β -HSDI sequence of 35 of the 95 fingerprint residues of UDPGE.

3. The presence in the 3 β -HSDI of the **TGxxGxxG** signature sequence in the β 1 α 2 turn of the UDPGE family.²
4. The conservation of many of the conserved residues in the UDPGE structural family that contact the NAD/NADP cofactor.
5. The presence of an aspartate (D) residue in the β 2 α 3 turn of 3 β -HSDI isomerase that predicts NAD preference in cofactor binding.²

The validity of using the three-dimensional structure of UDPGE as a model for 3 β -HSDI was tested biochemically by mutation studies. The superposition of the active site residues and cofactor positions in 3 α ,20 β -HSDI and UDPGE (Fig. 9) together with the sequence alignment between UDPGE and 3 β -HSDI allowed us to identify the probable catalytic residues in the 3 β -HSDI. There are two YxxxK sequences in 3 β -HSD [(Y(154)xxxK(158) and Y(269)xxxK(273)]. Mutation studies proved that the Y(154) and K(158) were the catalytic residues, and the superposition of numerous SCOR enzymes including UDPGE is consistent with this result and further validates UDPGE as a suitable model for 3 β -HSDI.²⁰ We were able to change the cofactor dependence of 3 β -HSDI from NAD to NADP by the double mutation D36A K37R.²¹ This demonstrated that the model correctly identified the residues that distinguish between NAD and NADP binding. There are over a dozen serine residues in 3 β -HSDI (Fig. 10), but the model indicated that the second serine in the doublet S123 S124 was the most probable candidate to be the catalytic serine. Mutation studies revealed that the S124 was the catalytic serine (Fig. 10).²²

Sequence similarity between 3 β -HSDI and 3 α ,20 β -HSD is greatly reduced beyond the catalytic YxxxK. This is probably due to the fact that 3 β -HSDI is a dehydrogenase and an isomerase. Our data indicated that the NAD binding associated with the D36 residue is critically important to both reactions. We postulate that the dual action is associated with the 120 additional residues on the carbonyl end of the enzyme compared to the other members of the SCOR family.

There is a strict tissue-specific distribution of the two human 3 β -HSD isoforms. 3 β -HSDI is expressed in mammary gland, breast tumors, prostate, prostate tumors, and placenta, and 3 β -HSDII is expressed in the adrenals, testes, and ovaries. Comparison of the sequences of type 1 and type 2 3 β -HSD (Fig. 11) reveals only 23 differences. Some of these variations in sequence are responsible for the profound differences measured for the kinetics of substrate utilization and inhibitor steroid binding by 3 β -HSDI relative to 3 β -HSDII. Tissue specificity is probably due to factors controlling expression of isomorphs in different tissues. Our initial efforts to probe the impact of amino acid differences between the isoforms have focused on the Tyr versus the His residue in the x_2 position in the helical turn containing the catalytic Y x_1 x x_2 x x_3 K sequences. We find that His156 contributes to the 14- to 17-fold higher affinity that 3 β -HSDI exhibits for substrate and inhibitor steroids compared to the 3 β -HSDII containing Tyr156 in the catalytic domain. Our structural model of 3 β -HSDI correctly predicted that Glu105 on one enzyme subunit interacts differently with the Tyr156 and H156 residue and that the difference in dehydrogenase substrate kinetics has to do with dimerization of the enzyme.²³ Additional studies are in progress to further characterize the structural reasons for the substantially higher affinity for steroids of 3 β -HSDI compared with 3 β -HSDII. Because human 3 β -HSDI is selectively expressed in breast and prostate tumors, the development of highly specific inhibitors will block the production of estradiol or testosterone to slow tumor growth without compromising the production of cortisol and aldosterone by 3 β -HSDII in the adrenals.²³

USING RATIONAL MUTATIONAL ANALYSIS TO PROBE FUNCTION AND SPECIFICITY

1. Identify a homologous sequence having a PDB structure.

2. Determine fingerprints for fold and cofactor.
3. Use LIGPOT²⁴ to determine the amino acids that directly contact the cofactor substrate and inhibitor in any crystal complexes.
4. Use the three-dimensional structure to predict the role of highly conserved fingerprint residues.
5. Test the predictions by mutations.
6. Use results to refine models for mechanism of action and to design inhibitors.

The technique described in this paper and summarized in the list above identifies the amino acid residues in an enzyme-binding protein or receptor that are critical to various aspects of function including cofactor and substrate specificity and can be applied to any protein for which a homologous sequence exists in the protein database.¹⁶ A rational mutational analysis mechanism of protein function and the design of potent agonist, antagonist, or inhibitors involves the preceding sequence of steps.

Acknowledgements

This work was supported by National Institutes of Health Grants DK26546 (W.L.D.) and CA114717 (J.L.T.).

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FIGURE 1.
Stereo illustration of the crystallographically observed complex of 3 α ,20 β -HSD and the NAD cofactor.

FIGURE 2.
Proposed mechanism of action of 3 α ,20 β -HSD.

FIGURE 3.

Schematic representation of interactions between aldosterone, the mineralocorticoid receptor, cortisol, cortisone, NADH-dependent 11 β -hydroxysteroid dehydrogenase, and glycyrrhizic acid related to proper modulation of steroid balance in the corticoid-collecting duct of the kidney.

FIGURE 4.
Stereo illustration of the monomer of 3 α ,20 β -HSD in complex with carbenoxolone.

FIGURE 5.

Fingerprint of a 1,651 member 3α,20β-HSD subfamily with 40 conserved identities. Capital letters identify residues conserved to 70%. The cofactor fingerprint residues (GxxxGxG) and catalytic residues Serine (S) and Tyrosine (Y) xxx Lysine (K) are underlined.

FIGURE 6.

(a) A schematic diagram and (b) an atomic project of residues contacting the NAD cofactor either directly or through water molecules included 13 of the 34 fingerprint residues conserved at 65% identity in 1,651 structures that align with 3 α ,20 β -HSD (1HDC). The numbers over the capital letters are the % conserved identity of that residue in the 1,651 gene products found to have 25% identity with 3 α ,20 β -HSD.

FIGURE 7.

Illustrations of the residue on three flexible hypervariable loops of members of the SCOR family of enzymes that make up the substrate-binding pocket. **(A)** Schematic illustration of the sequences of residues in the three flexible loops including the identities of residues in the sequence of the 3 α ,20 β -HSD (*dark upper fingerprint*) and the 118 member β -keto acyl carrier reductase subfamily (*light lower fingerprint*) and **(B)** a projection of the residues in the flexible loop that are in contact with the carbonoxolone in the crystalline complex of 3 α ,20 β -HSD and the inhibitor. With the exception of a hydrogen bond involving the catalytic Tyr152, all interactions are van der Waals contacts.


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          G x x G x x G
3βHSD1  MTGWSCLVTGAGGFLGQRIIRLLVKEKELKEIRVLDKAFGPELREEFSKLQNKTKLTVLE
1NAH    ---MRVLVTGGSGYIGSHTCVOLLQNGHDVILDNLCNSKRSLPVIERLGGKHP-TFVE

3βHSD1  GDILDEPFLKRACQD--VSVIIHTACIIDVFG-VTHRESIMNVNVKGTQLLLEACVOASV
1NAH    GDIRNEALMTEILHDHAIDTVIHFAGLKAVGESVQKPLEYYDNNVNGTLRLISAMRAANV

          S S                      Y x x S K
3βHSD1  PVFIYTSSIEVAGPNSYKEIIQNGHEEEPLENTWPAFYPHSKKLAEKAVLAANGWNKNG
1NAH    KNFIFSSATVYVDN-PK--I-PYVESFP-TGTPQSPYGVSK-L---MV-EQILTDLQKA

3βHSD1  GTLYTCALRPMYIYEGSRFLSASINEALNNNGILSSVGKFSTVNPVYVGNVAUAHILAL
1NAH    QPDWSIAL--LRYFNPVGAHPSGDMGEDPQ--GIPN-----NLMP-YIAQVAVGRRDSL

          Y x x S K
3βHSD1  RALQDPKKAPSIRGQFYIISDDTPHQSYDNLNVTLSKEFGLRLDSRWSFPLSLMYWIGFL
1NAH    AIFGNDYPTEDGTGVVDYI-----HVMDLADGHVAMEK---LANKPGVHI-YNLGAGVG

3βHSD1  LEIVSFLLRPIYTYRPPFNRHIVTLNSVFTFSYK---KAQRDLAYKPLYSWEEAKQKTV
1NAH    NSVLDVVNAFSKACGKPVNYHFAPRREGDLPAYWADASKADRELNURVTRTLDEMAQDTU

3βHSD1  EWVGS�VDRHKETLKSQTQ
1NAH    HWQS----RHPQGYPD---

                                CLUSTAL W(1.60)

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FIGURE 8.

Sequence alignment of UDP galactase epimerase (1NAH) and 3β-HSDI. The positions of the Rossmann fold signature (TGxxGxxG) and the catalytic residues (SS and YxxxK) are highlighted. A second YxxxK sequence found in 3β-HSDI is also identified.

FIGURE 9.

Overlap of the three-dimensional structure of 3 α ,20 β -HSD (1HDC) and our model of 3 β -HSD based on the UDP galactose epimerase (1NAH), illustrating (a) similarity in Rossmann fold cofactor-binding domain and catalytic S,YxxK,PGxxxT residues and (b) the significant difference in the C-terminal of the two structures associated with an additional 125 residues common to 1NAH and 3 β -HSDI that are presumed to be associated with the isomerase activity of the latter.

FIGURE 10.

Our model suggests that the second serine in the S(123) S(124) doublet is the catalytic serine. In the model, S123 and S124 are on opposite sides of a β -strand with S124 proximal to the catalytic Tyr154, the nicotinamide ring, and the probable site of the substrate carbonyl. Mutational analysis confirmed the prediction that S124 is catalytic, but it also revealed that S123 influences the kinetics.²⁵ (Reprinted with permission.)

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      1      13      19      24      33      39
Human Type 1 T G W S C L V T G A G G F L G Q R I I R L L V K E K E L K E I R V L D K A F G P E L R E E
Human Type 2 . . . . . L . . . . . V . . . . . E . . . . . A . . . . . R . . . . .
      52      78
      F S K L Q N K T K L T V L E G D I L D E P F L K R A C Q D V S V I I H T A C I I D V F G V
      . . . . . R . . . . . V . . . . .
      124
      T H R E S I M N V N V K G T Q L L L E A C V Q A S V P V F I Y T S S I E V A G P N S Y K E I
      . . . . .
      152 156      177
      I Q N G H E E E P L E N T W P A P Y P H S K K L A E K A V L A A N G W N L K N G G T L Y T C
      . . . . . T . . . . . Y . . . . . . . . . . D . . . . .
      187 194 195
      A L R P M Y I Y G E G S R F L S A S I N E A L N N N G I L S S V G K F S T V N P V Y V G N V
      . . . . . T . . . . . G P . . . . .
      240 248      270
      A W A H I L A L R A L Q D P K K A P S I R G N F Y Y I S D D T P H Q S Y D N L N Y T L S K
      . . . . . R . . . . . V . . . . . I . . . . .
      285 288      299      305      309 311      318
      E F G L R L D S R W S F P L S L M Y W I G F L L E I V S F L L R P I Y T Y R P P F N R H I V
      . . . . . L . . . . . T . . . . . V . . . . . S . . . . . S . . . . . Q . . . . . T .
      . . . . .
      T L S N S V F T F S Y K K A Q R D L A Y K P L Y S W E E A K Q K T V E W V G S L V D R H K
      . . . . .
      372
      E T L K S K T Q

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FIGURE 11.
Sequence alignment of 3 β -HSDI Types 1 and 2 with the 23 differences that influence substrate specificity.