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SHORT-CHAIN DEHYDROGENASES/REDUCTASES

Structure, Function and Motifs of Hydroxysteroid Dehydrogenases

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ABSTRACT

Short-chain dehydrogenases/reductases (SDR) constitute a superfamily of proteins catalysing reactions of a wide range of substrates in all life forms. During the past years, a large number of new proteins belonging to this superfamily has been detected. They are important in e.g. the metabolism of steroids and bile acids, β-oxidation, and retinol metabolism, and are critical in health and disease. In this thesis, I have focused on hydroxysteroid dehydrogenases in general and three proteins in particular: 3β/17β-hydroxysteroid dehydrogenase (3β/17β-HSD) from *Comamonas testosteroni,* and human and *Drosophila* 17β-hydroxysteroid dehydrogenases type 10/short chain hydroxyacyl coenzyme A dehydrogenases (17β-HSD10/SCHAD II). 3β/17β-HSD was studied to obtain information about structure and function relationships and reaction mechanisms within the SDR superfamily. 17β-HSD10/SCHAD II was studied to obtain knowledge about targeting motifs, subcellular organisation of proteins, the functional role in health and disease, and species relationships.

3β/17β-HSD was studied by site-directed mutagenesis of conserved amino acid residues in the SDR superfamily. Data obtained by steady-state kinetics, stability measurements, X-ray crystallography and docking analysis define roles for several residues regarding different conserved motifs in the cofactor binding site, the catalytic site and the substrate binding site.

The critical role of residues Thr 12, Asp 60, Asn 86, Asn 87, and Ala 88 in coenzyme binding and catalysis is highlighted. The importance of residues regulating the reaction direction, Thr 12 and Asn 87, and the role of Ser 138 involved in the catalytic mechanism, were demonstrated. The data also reveal essential interactions of Asn 111 with the active site residues. A general role of its side-chain interactions in the 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 and Lys in the majority of characterised SDR enzymes. The largely conserved residue Asn 179 was concluded to connect the active site and the substrate binding loop, through a conserved water molecule, indicated by loss of function in an N179A mutant in 3β/17β-HSD. Importantly, this structural motif was detected in 16 out of 21 determined SDR crystal structures. The tertiary and quaternary structures of the apoform of 3β/17β-HSD have been determined at 1.2 Å resolution. The structure, kinetic analysis of wild-type and mutants (Y148F and H182L) with residue exchanges in the binding loop, and docking experiments make it possible to explain the steroid-protein recognition site, with novel structural motifs.

The mitochondrial multifunctional enzyme 17β-HSD type 10, with activities on 17β- and 3α-OH positions on steroids, displays short-chain L-3-hydroxyacyl CoA dehydrogenase activity. This constitutes an essential step in the β-oxidation of fatty acids, and correspondingly the enzyme is also classified as type 2 short chain hydroxyacyl CoA dehydrogenase (SCHAD II). 17β-HSD10 is structurally related to bacterial 3β/17β-HSD, with about 35% sequence identity. The

mitochondrial targeting of the human enzyme was studied by using hybrid constructs with green fluorescent protein. The N-terminal part, residue 1-34, was shown to consist of a non-cleavable mitochondrial target sequence. The first 15 residues are specific for the enzyme but not sufficient to import the functional protein. Residues 16-34 can be replaced by similar structures such as the corresponding sequence in 3β/17β-HSD. The orthologous, essential protein in *Drosophila* has been studied in a similar manner and its activities with different steroid substrates, acetoacetyl CoA and DL-β-hydroxybutyryl CoA, were determined.

Material from six patients with defective L-3-hydroxyacyl CoA dehydrogenase activity (the third step in the β-oxidation) for short-chain fatty acids has been studied. Sequences for exons and exon-intron boundaries for SCHAD I and SCHAD II were determined. No mutations in SCHAD II were found, whereas a known SCHAD I polymorphism in two of the six patients was detected. Kinetic comparison of SCHAD I and II reveals that type I is the main enzyme form responsible for β-oxidation of short-chain hydroxyacyl derivatives of fatty acids in humans. These data suggest that the observed biochemical deficiency leading to the lethal metabolic complications is not a defective SCHAD enzyme, but most likely resides in defective interactions with other proteins, regulating the SCHAD activity.

Keywords: short-chain dehydrogenases/reductases, site-directed mutagenesis, steady-state kinetics, X-ray structure, enzyme function, steroid, motif, reaction mechanism, 3β/17β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, short chain hydroxyacyl coenzyme A dehydrogenase

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CONTENTS

LIST OF ORIGINAL PAPERS

The thesis is based on the following articles, which will be referred to by their Roman numerals:

I. Oppermann, U. C. T., Filling, C., Berndt, K. D., Persson, B., Benach, J., Ladenstein, R. & Jörnvall, H. (1997). Active site directed mutagenesis of 3β/17β-hydroxysteroid dehydrogenase establishes differential effects on short-chain dehydrogenase/reductase reactions. *Biochemistry*, 36, 34-40.

II. Filling, C., Nordling, E., Benach, J., Berndt, K. D., Ladenstein, R., Jörnvall, H. & Oppermann, U. (2001) Structural role of conserved Asn179 in the short-chain dehydrogenase/reductase scaffold. *Biochem. Biophys. Res. Comm.* 289, 712–717.

III. Filling, C., Berndt, K. D., Prozorovski, T., Knapp, S., Benach, J., Nordling, E., Ladenstein, R., Jörnvall, H. & Oppermann, U. (2002) Critical residues for folding and catalysis in short-chain dehydrogenases/reductases (SDR). *J. Biol. Chem.* 277, 28, 25677-25684.

IV. Benach, J., Filling, C., Oppermann, U., Roversi, P., Bricogne, G., Berndt, K. D., Jörnvall, H. & Ladenstein, R. Structure of bacterial 3β/17β-hydroxysteroid dehydrogenase at 1.2 Å resolution: explanation of multiple steroid recognition. *Biochemistry*, in press.

V. Filling, C., Shafqat, N., Wu, X., Björk, L., Thyberg, J., Salim, S., Mårtensson, E., Jörnvall, H. & Oppermann, U. Comparison of type 10 17β-hydroxysteroid dehydrogenases from human and *Drosophila*. Manuscript.

VI. Filling, C., Keller, B., Kalaitzakis, E., Marschall, H.-U., Jörnvall, H., Bennett, M. & Oppermann, U. Kinetic analysis of human hydroxyacyl-CoA dehydrogenase genes for short-chain hydroxyacyl CoA dehydrogenase (SCHAD) deficiency suggests the type 1 enzyme to be the main form involved in β-oxidation. Manuscript.

Related publications not included in the thesis

- Oppermann, U. C. T., Persson, B., Filling, C., Jörnvall, H. (1997) Structure-function relationships of SDR hydroxysteroid dehydrogenases. *Adv. Exp. Med. Biol.*, 414, 403–415.

- Filling, C., Marschall, H.-U., Prozorovski, T., Nordling, E., Persson, B., Jörnvall, H. & Oppermann, U. C. T. (1999) Structure-function relationships of 3β-hydroxysteroid dehydrogenases involved in bile acid metabolism. *Adv. Exp. Med. Biol.*, 463, 389–394.

- Salim, S., Filling, C., Mårtensson, E. & Oppermann, U. C. T. (2000) Lack of quinone reductase activity suggests that amyloid-β peptide/ERAB induced lipid peroxidation is not directly related to production of reactive oxygen species by redoxcycling. *Toxicology*, 144, 163–168.

- Oppermann, U. C. T., Filling, C. & Jörnvall, H. (2001) Forms and functions of human SDR enzymes, *Chemico-Biological Interact.* 130-132, 699–705.

- Filling, C., Wu, X., Shafqat, N, Hult, M, Mårtensson, E., Shafqat, J., Oppermann, U. C. T. (2001) Subcellular targeting analysis of SDR-type hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* 171, 99–101.

- Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kallberg, Y., Persson, B. & Jörnvall, H. Short-chain dehydrogenases/reductases (SDR): the 2002 update. *Chemico-Biological Interact.*, in press.

ABBREVIATIONS

One and three letter codes for amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ABAD	amyloid-β binding alcohol dehydrogenase
androsterone	5α-androstan-3α-ol-17-one
ATP	adenosine triphosphate
C. testosteroni	*Comamonas testosteroni*
CD	circular dichroism
CoA	coenzyme A
CPT-I	carnitine palmitoyl transferase I
CPT-II	carnitine palmitoyl transferase II
D. lebanonensis	*Drosophila lebanonensis*
DH	dehydrogenase

DHEA	dehydroepiandrosterone (5-androsten-3β-ol-17-one)
5α-DHT	5α-dihydrotestosterone (5α-androstane-17β-ol-3-one)
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E2	estradiol (1,3,5(10)-estratriene-3,17β-diol)
E. coli	*Escherichia coli*
ER	endoplasmic reticulum
ERAB	endoplasmic reticulum amyloid-β associated binding protein
estriol	16α-hydroxyestradiol
estrone	Δ1,3,5(10)-estratrien-3-ol-17-one
FAD/$FADH_2$	flavin adenine dinucleotide (oxidised/reduced)
FAO	fatty acid oxidation
FPLC	fast performance liquid chromatography
GALE	UDP-galactose 4' epimerase
GFP	green fluorescent protein
GuHCl	guanidinium hydrochloride
HSD	hydroxysteroid dehydrogenase
IPTG	isopropyl-β-D-thiogalactopyranoside
isoUDCA	iso-ursodeoxycholic acid (3β,17β-dihydroxy-5β-cholan-24-oic acid)
M. grisea	*Magnaporthe grisea*
MDR	medium-chain dehydrogenase/reductase
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidised/reduced)
20αP	20α-progesterone (Δ^4-pregnen-20α-ol-3-one)
Prog	Progesterone (Δ^4-pregnen-3,20-dione)
PCR	polymerase chain reaction
Red	reductase
S. hydrogenans	*Streptomyces hydrogenans*
SDR	short-chain dehydrogenase/reductase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T	testosterone (4-androsten-17β-ol-3-one)
Tris	tris(hydroxymethyl)-aminomethane
UDCA	ursodeoxycholic acid (3α,17β-dihydroxy-5β-cholan-24-oic acid)
3D	Three-dimensional

EC number	Enzyme name	Abbreviation (PDB number)
1.1.1.1	Alcohol dehydrogenase	ADH (1a4u)
1.1.1.4	Acetoin reductase	(1geg)
1.1.1.14	Sorbitol dehydrogenase	
1.1.1.35	Hydroxyacyl-CoA dehydrogenase	HAD
	Short-chain hydroxyacyl-CoA dehydrogenase type I	SCHAD I
	Short-chain hydroxyacyl-CoA dehydrogenase type II	SCHAD II (1e3w)
1.1.1.47	Glucose dehydrogenase	(1gco)
1.1.1.50	3α-hydroxysteroid dehydrogenase	(1fjh)
1.1.1.51	3β/17β-hydroxysteroid dehydrogenase	3β/17β-HSD (1hxh)
1.1.1.53	3α/20β-hydroxysteroid dehydrogenase	20β-HSD (1hdc)
1.1.1.62	17β-hydroxysteroid dehydrogenase	17β-HSD (1a27)
1.1.1.103	Threonine dehydrogenase	
1.1.1.146	11β-hydroxysteroid dehydrogenase type 1	11β-HSD1
	11β-hydroxysteroid dehydrogenase type 2	11β-HSD2
1.1.1.153	Sepiapterin reductase	Sepiapterin Red (1nas)
1.1.1.159	7α-hydroxysteroid dehydrogenase	7α-HSD
1.1.1.184	Carbonyl reductase	Carbonyl Red (1cyd)
1.1.1.206	Tropinone reductase I	Troponine Red1 (1ae1)
1.1.1.211	Long-chain hydroxyacyl-CoA dehydrogenase	LCHAD
1.1.1.236	Tropinone reductase II	Troponine Red2 (2ae1)
1.1.1.252	1, 3, 6, 8-Tetrahydroxynaphthalene reductase	THNR (1doh)
1.3.1.9	β-Keto acyl carrier protein reductase	(1edo)
1.3.1.53	*cis*-Biphenyl-2,3-dihydrodiol-2,3-dehydrogenase	
	Electron transfer flavoprotein	ETF
1.5.5.1	Electron transfer flavoprotein dehydrogenase	ETFD
1.6.99.10	Dihydropteridine reductase	DHPR (1dhr)

1 INTRODUCTION

Proteins and peptides are central to life and proteins often work as enzymes. An enzyme is a biological catalyst. They can be divided into six classes; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. In this thesis, I have focused on enzymes from the first class, oxidoreductases. They are enzymes catalysing oxidation-reduction reactions:

$A^- + B \Leftrightarrow A + B^-$

In this class, alcohol dehydrogenases are found, which can be further divided into superfamilies, medium-chain dehydrogenases/reductases (MDR) and short-chain dehydrogenases/reductases (SDR). In particular, protein members covered in this thesis include the superfamily of SDR with emphasis on hydroxysteroid dehydrogenases. SDR forms a superfamily of more than 3 000 proteins annotated in databases. In the past few years, a number of three dimensional structures have been determined, giving a large amount of data. The SDR superfamily is involved in many metabolic steps and is critical in human health and disease.

In order to obtain more information about structure–function relationships and to interpret the general architecture and reaction mechanism, we have studied conserved motifs in this enzyme family. As a model we used the bacterial 3β/17β-hydroxysteroid dehydrogenase.

To go more into details of the targeting and localisation of enzymes in this superfamily, we chose to investigate a human 17β-hydroxysteroid dehydrogenase localised to the mitochondria and the orthologous protein from fruit fly. The role of these enzymes in the metabolism has not clearly been understood. A correlation of the human enzyme to the neurodegenerative Alzheimer's disease has been observed, and the enzyme from fruit fly has been correlated to development, and lipid accumulation in the cells. Therefore, the functional relationship between these enzymes needed to be determined. Lipid accumulation in different forms is a clinical observation that has been detected in the liver of children with sudden infant death syndrome. This prompted us to investigate the role of this enzyme in one disorder contributing to early infant death.

1.1 Alcohol Dehydrogenases

Alcohol dehydrogenases (E.C.1.1.1.1) of different types are common in nature and present in all life forms. They encompass at present five dehydrogenase/reductase superfamilies; medium-chain dehydrogenases/reductases (MDR), short-chain dehydrogenases/reductases (SDR), the superfamily of iron-activated alcohol dehydrogenases, long-chain dehydrogenases/reductases, and aldo-keto reductases (AKR).
They catalyse the reversible oxidation of alcohols to aldehydes/ketones using $NAD(P)^+$/ NAD(P)H as electron acceptor and donor:

$$\text{Alcohol} + NAD(P)^+ \Leftrightarrow \text{Carbonyl} + NAD(P)H$$

Alcohol dehydrogenases are involved in degradative pathways as a detoxification system and in synthetic pathways. Many of the reactions can be carried out also with other enzymes like the cytochrom P450-enzyme system. However, the dehydrogenase reaction proceeds without radical formation and is essential to life.

1.1.1 Medium-Chain Dehydrogenases/Reductases

The medium chain dehydrogenase/reductase superfamily consists of dimeric zinc metalloenzymes of 350-odd amino acid residues. The first enzyme pair recognised was mammalian liver alcohol dehydrogenase and sorbitol dehydrogenase (Jörnvall *et al.*, 1981). In addition to alcohol and sorbitol dehydrogenases, the MDR family includes threonine and xylitol dehydrogenases and reductases like ζ-crystallin with quinone reductase activity. These enzymes are distantly related, reflecting ancient duplicatory origins.
So far six, possibly eight, classes of alcohol dehydrogenase in the MDR family, have been found, with sequence identities of 50-70% between classes. Some classes are divided into isozymes, which are further subdivided into allelic variants. These have sequence identities of 80-95%, and more than 95%, respectively (Hjelmqvist, 1995).
The subunits consist of two domains, one catalytic and one nucleotide-binding, with a catalytic zinc atom that usually is liganded to three residues, often two Cys and one His (Eklund et al., 1985; Aronson et al., 1989), and a structural zinc atom. However, all family members do not contain zinc.

1.1.2 Aldo-keto reductases

Aldo-keto reductases constitute a superfamily that contains proteins expressed in all life forms. So far, fourteen families have been detected within this superfamily, designated AKR1-AKR14. Seven of these include families defined by a >60% identity in amino acid sequence. The largest family, AKR1, comprises aldehyde dehydrogenases, aldose reductases, hydroxysteroid dehydrogenases (HSDs) and Δ^4-3-ketosteroid-5β-reductases. AKR6 contains the potassium channel β-subunits, and AKR7 contains aflatoxin aldehyde reductases. Allelic variation occurs as in the MDR mammalian alcohol dehydrogenases. Alleles have an amino acid sequence identity of >97%.

Aldo-keto reductases adopt a $(\beta/\alpha)_8$ barrel structural fold with the active site located in a hydrophobic cavity at the C-terminal end of the β-barrel. Residues involved in catalysis are Asp 50, Tyr 55, Lys 84 and His 117 (numbering refers to rat liver 3α-HSD: AKR1C9). Although the majority of the AKRs are monomeric proteins of about 320 amino acid residues, multimeric forms are found in AKR6 and AKR7.

AKRs are NAD(P)(H)-dependent enzymes metabolising a wide range of substrates. The nicotinamide cofactor binds the protein without a Rossmann-fold motif (Wilson et al., 1992; 1995; Hoog et al., 1994; El-Kabbani et al., 1995). Many contacts observed are nearly identical between the enzymes as well as between the nucleotide cofactor and inhibitors of the AKRs (Jez et al., 1997).

There is little or no information about the potential catalytic activities of several of the AKR members. There are at least 125 potential AKR genes identified by genome sequencing projects, many of which have no assigned function. Toxic aldehydes are produced during lipid peroxidation and oxidative stress and as by-products of food and drug metabolism. Their toxicity depends on their reactivity. The AKRs appear to function as reductases in the detoxification of these toxic aldehydes. Other roles are in the metabolism of sugars, steroids, prostaglandins, polycyclic aromatic hydrocarbons and isoflavinoids (Petrash et al., 1995; Penning et al., 1996; Bruce et al., 1994; Welle et al., 1991).

1.1.3 Short-Chain Dehydrogenases/Reductases

The first enzyme recognised in this superfamily was the *Drosophila* alcohol dehydrogenase. Consequently, this superfamily was originally called “insect-type alcohol dehydrogenases”. Later, other enzymes where included and with the incorporation of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase from human placenta (Krook et al., 1990), a

mammalian enzyme was added to the short-chain dehydrogenases/reductases. In 1991, the properties of these enzymes were summarised (Persson et al., 1991). Since then there has been an explosion of new SDR sequences.

The SDR superfamily comprises proteins from all life forms and is one of the largest protein superfamilies known today with more than 3 000 sequences known from databanks. Over 60 genes encoding SDR forms have been identified within the humane genome (Oppermann et al., 2001; Kallberg et al., 2001). Some of these have defined physiological roles comprising several "housekeeping" functions, involved in amino acid, fatty acid, steroid, and sugar metabolism.

SDR enzymes have a broad set of substrates (figure 1.1). They are involved in bile acid metabolism, and steroid hormone metabolism constituting an important pre-receptor control mechanism by catalysing the switch between hormone receptor ligands and non-binding

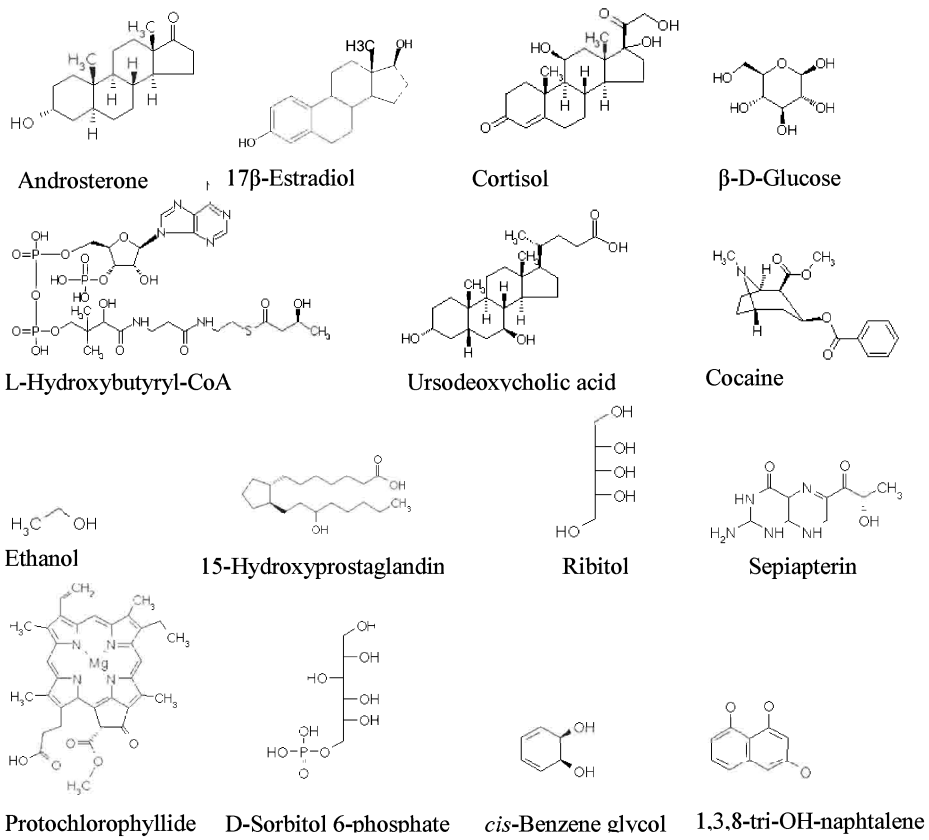

Figure 1.1. SDR enzymes have very broad substrate specificity.

metabolites. They also have metabolic functions such as β-oxidation, retinol metabolism, metabolism of neurotransmitters, synthesis of serotonin and synthesis of tropane alkaloids such as cocaine.

Several genetic defects in SDR enzymes have been identified and linked to human diseases (table 1.1). Some of these are the apparent mineralocorticoid excess syndrome, male pseudohermaphroditism, galactosemia type III and tetrahydrobiopterin deficiency.

Table 1.1. SDR enzymes linked to human diseases.

Disease	Gene	Reference
Apparent mineralocorticoid excess syndrome	HSD11B2	New and White, 1995
Male pseudohermaphroditism	17β-HSD3	Geissler et al., 1994
Galactosemia type III	UDP-galactose 4' epimerase	Maceratesi et al., 1998
Tetrahydrobiopterin deficiency	Sepiapterin reductase	Dianzani et al., 1998
Tetrahydrobiopterin deficiency	Dihydropteridine reductase	Dianzani et al., 1998

The degree of sequence identity between different forms is low, usually between 15 and 30%, but the conformations for the available structures show a highly similar architecture with a one domain α/β folding pattern in the thus far analysed 3D structures (Ghosh et al., 1991; Varughese et al., 1992; Tanaka et al., 1996a, 1996b; Benach et al., 1998; Hulsmeyer et al., 1998; Mazza et al., 1998; Nakajima et al., 1998; Rozwarski et al., 1999; Roujeinikova et al., 1999; Stewart et al., 1999; Grimm et al., 2000; Powell et al., 2000; Fisher et al., 2000; Ghosh et al., 2001; Liao et al., 2001; Otagiri et al., 2001; Price et al., 2001; Yamamoto et al., 2001; paper IV). They also show a similar quaternary structure with either a dimer or a tetramer. An exception is carbonyl reductase that is a monomer (Ghosh et al., 2001). Each subunit is formed of about 250 amino acid residues and has a Ser, Tyr, Lys triad of active site residues: Ser^{138}-X_{12}-Tyr^{151}-X-X-X-Lys^{155}. Other conserved segments and residues are the cofactor binding regions: Gly^{14}-X-X-X-Gly^{18}-X-Gly^{20}, the Rossmann fold, a βαβ motif common to nucleotide binding enzymes (Rossmann et al., 1974), and positions in addition to the triad that surround the catalytic pocket (Jörnvall et al., 1995) (table 1.2; figure 1.2).

Table 1.2. Conserved sequence elements of SDR proteins.

Sequence	Position	Secondary structure
GxxxGxG	13–19	turn between βA and βB
NNAG	86–89	end of βD
N	111	αE
GxxxxxxS	131–138	βE
YxxxK	151–155	αF
PG	183–184	turn after αF

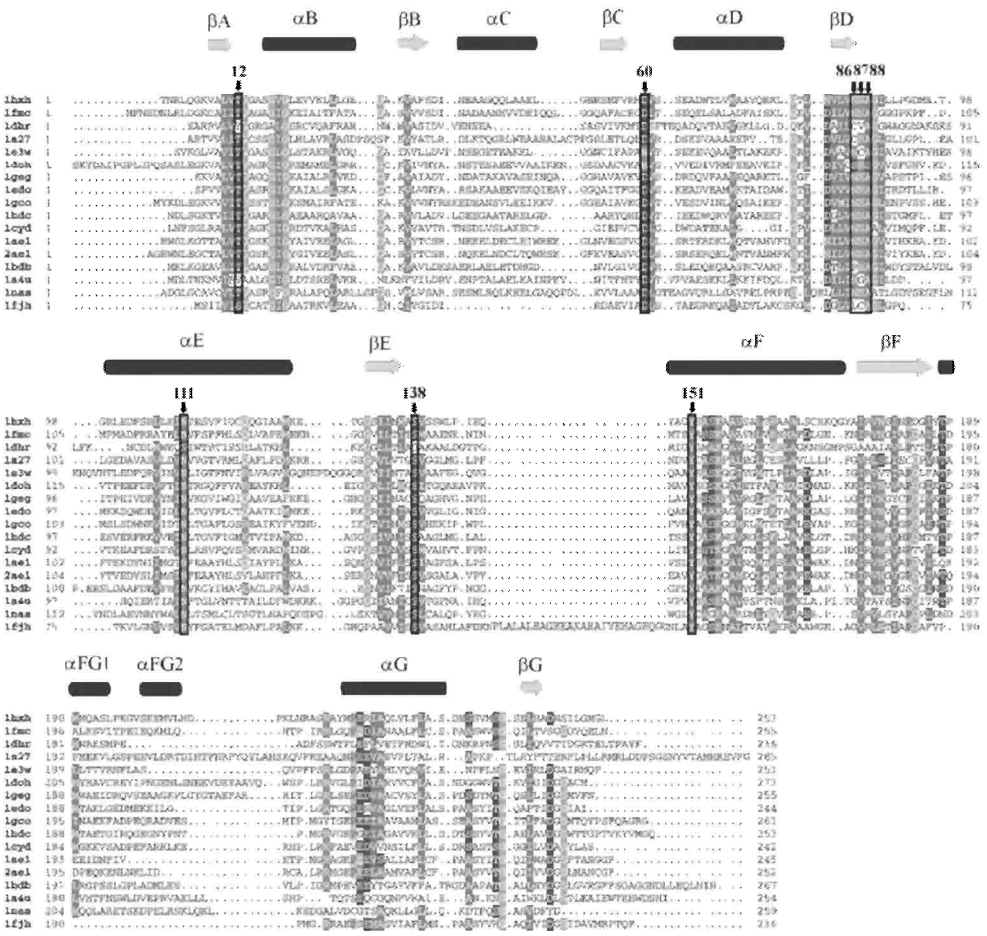

Figure 1.2. Structural alignment of SDR sequences. Secondary structure elements are indicated as arrows for β-strands and cylinders for α-helices. Some of the more conserved residues mutated in this thesis are indicated by black boxes and black downward arrows. Abbreviations: page 10.

A similar reaction mechanism for each individual protein has been proposed based on the findings with the conserved Tyr acting as a general base in the deprotonation process. This is facilitated by the adjacent protonated Lys residue and the oxidized form of the coenzyme: $NAD(P)^+$, which lowers the pK_a of the phenolic Tyr-OH group. The Lys residue binds to the ribose hydroxyl groups of the coenzyme and fixes the coenzyme position during catalysis. The function of the serine in the catalytic triad is less apparent, but it could be assigned to stabilize the transition state of the reaction or the initial orientation of the substrate by hydrogen bonding (paper I).

1.1.3.1 Hydroxysteroid Dehydrogenases

The majority of the hydroxysteroid dehydrogenases (HSDs) so far characterised belong either to the SDR superfamily or the AKR superfamily, with the majority belonging to the former (table 1.3).
As indicated above, a comparison between these two enzyme superfamilies shows differences in respect to protein folding and nucleotide cofactor stereospecificity. Despite these differences, SDR and AKR enzymes share a similar reaction mechanism and represent an example of convergent evolution (Jörnvall et al., 1995; Oppermann and Maser, 2000). Both have an acid-base mechanism with Tyr as catalytic acid/base, and a Lys residue, presumably lowering the pKa of the Tyr side chain hydroxyl group (Jörnvall et al., 1995; Penning, 1997) (figure 1.3).

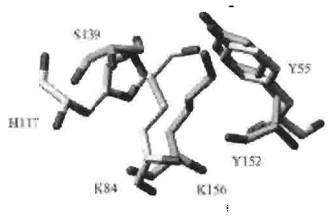

Figure 1.3. Overlapping active site of SDR and AKR enzymes. Structures displayed are from the Protein Data Bank. Residues important in catalysis are for the SDR enzyme 3α/2β-HSD (2hsd): Ser 139, Tyr 152 and Lys 156 (dark grey) and for the AKR enzyme rat liver 3α-HSD/DHD (1afs): Lys 84, His 117 and Tyr 55 (light grey).

HSDs have many functions in organisms. In vertebrates they participate in the synthesis of all classes of vertebrate steroid hormones, where crucial steps are performed by the enzymes: 3β-HSD/Δ4-5 isomerase, 17β-HSD and 11β-HSD. Therefore, deficiencies of these enzymes correspond to inborn errors of steroid synthesis and metabolism (figure 1.4). HSDs regulate the bioactivation and inactivation processes of steroid hormones. They are responsible for the

Table 1.3. HSD classification and main function.

Enzyme Form	Protein family	*In vivo* reaction	Main function	Other functions
3α-HSD				
1[a]	AKR	reductase	androgen inactivation	DDH, PGDH
2[a]	AKR	reductase	5α-DHT inactivation	identical to 17β-HSD5
3[a]	AKR	dehydrogenase	5α-DHT inactivation	
3β-HSD				
1[b]	SDR	dehydrogenase/isomerase	steroid synthesis	
2[b]	SDR	dehydrogenase/isomerase	steroid synthesis	
3[c]	SDR	dehydrogenase/isomerase	steroid synthesis	
4[c]	SDR	dehydrogenase/isomerase	steroid synthesis	
5[c]	SDR	dehydrogenase/isomerase	steroid synthesis	
6[c]	SDR	dehydrogenase/isomerase	steroid synthesis	
11β-HSD				
1[d]	SDR	reductase	GC activation	microsomal CR
2[d]	SDR	dehydrogenase	GC inactivation	
17β-HSD				
1[e]	SDR	reductase	E2 production	20α-HSD
2[e, f]	SDR	dehydrogenase	sex steroid inactivation	20α-HSD, 3β-HSD
3[e]	SDR	reductase	T production	
4[e]	SDR	dehydrogenase	E2 inactivation	β-oxidation
5[e, a]	AKR	reductase	T production	3α-HSD, DDH
6[e]	SDR	dehydrogenase	5α-DHT inactivation	3α-HSD
7[e]	SDR	reductase	E2 production	
8[e]	SDR	dehydrogenase	E2 inactivation	
9[g]	SDR	dehydrogenase	sex steroid inactivation	3α-HSD, retinol DH
10[h]	SDR	dehydrogenase?	sex steroid inactivation?	β-oxidation, 3α-HSD
11[i, j]	SDR	dehydrogenase	sex steroid inactivation	
20α-HSD[a]				
	AKR	reductase	P inactivation	DDH, aldose reductase

[a]Penning *et al*., 2000; [b]Schwarz et al., 2000; [c]Konig *et al*., 2000; [d]Stewart *et al*., 1999; [e]Peltoketo *et al.,* 1999; [f]Suzuki et al., 2000; [g]Su *et al*., 1999; [h]He *et al*., 2000; [i]Li et al., 1998; [j]Brereton *et al*., 2001. Abbreviations: page 8-10.

local activation of inactive steroid hormone precursors like the conversion of the sex steroids dehydroepiandrosterone (DHEA) and androstenedione into the potent androgens testosterone and dihydrotestosterone (DHT), thereby regulating the intracellular levels of "active" steroid hormones (Labrie, 1991, Nobel et al., 2001).

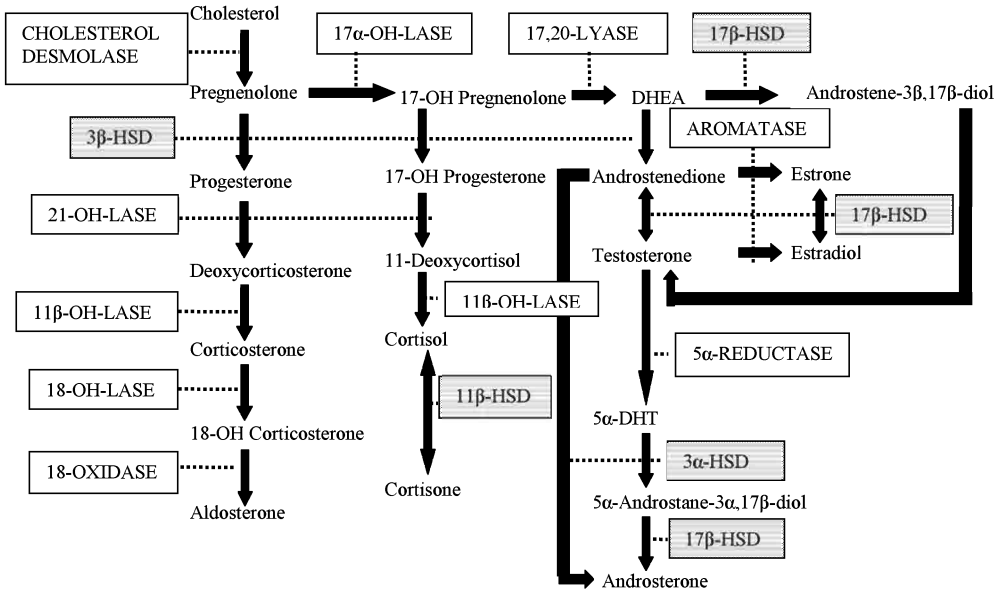

Figure 1.4. Steroid metabolism

HSDs are important in synthesis, binding and hepatocellular transport of bile acids. In the AKR superfamily a cytosolic bile acid binding protein with 3α-HSD activity has been characterized (AKR1C2) (Stolz et al., 1995). In intestine, the conversion of bile acids to corresponding isobile acids is dependent on microbial HSDs.
Another function of HSDs is the participation in xenobiotic phase I metabolism of exogenous substances including drugs, pesticides and carcinogens (Felsted and Bachur, 1980; Maser, 1995). Xenobiotic metabolism is divided in phase I and phase II reactions, where phase I reactions include the activation that prepare a molecule for conjugation, e.g. oxidation – reduction or hydrolysis, and phase II reactions are the conjugation to other molecules, e.g. by methylation or adding a thiol- or a sulphategroup.

1.1.3.2 3β/17β-Hydroxysteroid dehydrogenase from *Comamonas testosteroni*

The Gram-negative bacterium, *Comamonas testosteroni*, can grow on steroids as the only carbon source and expresses different steroid-inducible steroid dehydrogenases (Marcus and

Talalay, 1956; Watanabe et al., 1980). Some of these enzymes have been purified from *C. testosteroni*. 3β/17β-hydroxysteroid dehydrogenase (E.C.1.1.1.51) catalyses the reversible NAD(H)-dependent oxidoreduction of β-hydroxy/oxo-groups at position 3 and 17 of the steroid skeleton (figure 1.5) of a variety of steroid compounds, including hormones and isobile acids. This is achieved at a common catalytic site for 3β- and 17β-activities (Minard et al., 1985). Other functions of this enzyme are to act as a xenobiotic carbonyl reductase and a morphine dehydrogenase.

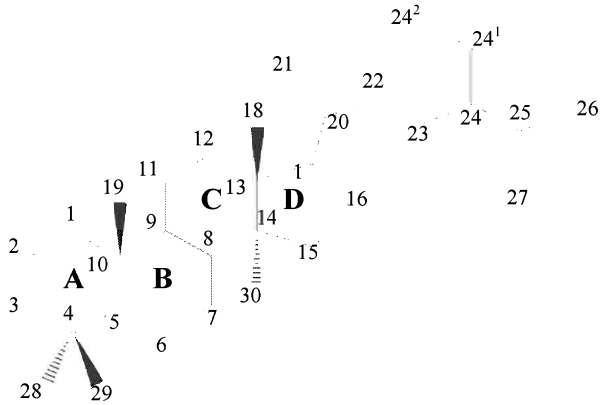

Figure 1.5. Carbon numbering system in steroids.

3β/17β-HSD is a tetrameric enzyme with identical subunits of a molecular mass of 27 kDa each, and belongs to the short-chain dehydrogenases/reductases (Yin et al., 1991; Jörnvall et al., 1995). The reaction follows an ordered bi-bi mechanism with the coenzyme NAD^+ binding prior to the steroid substrate or inhibitor in the dehydrogenase reaction (Schultz et al., 1977; Levy et al., 1987). Dissociation of the reduced coenzyme is the rate-limiting step in the overall dehydrogenase reaction.

1.1.3.3 17β-Hydroxysteroid dehydrogenase type 10 / SCHAD II from human

In 1997, a study about an enzyme mediating neurotoxicity in Alzheimer's disease was reported (Yan et al., 1997). The enzyme was called endoplasmic-reticulum-associated binding protein (ERAB), is a tetrameric enzyme with identical subunits of 261 residues each, and belongs to the short-chain dehydrogenases/reductases (Yan et al., 1997). Since then, the protein has received several other names depending on its different activities towards

estrogens, androgens, L-3-hydroxyacyl-CoAs and alcohols: 17β-hydroxysteroid dehydrogenase (17β-HSD10), short-chain hydroxyacyl-CoA dehydrogenase type II (SCHAD II), 3-hydroxyacyl-CoA dehydrogenase type II (HADH) and amyloid β-peptide-binding alcohol dehydrogenase (ABAD). Furthermore, it has 3α-HSD activity (He et al., 1999).
At present, at least 11 distinct forms of 17β-HSDs have been described in mammals, differing in substrate specificities, tissue, developmental and subcellular distribution patterns and the preferred *in vivo* reaction direction. All, except for 17β-HSD5, belong to the SDR superfamily (table 1.4). So far 17β-HSD6 is the only form that has not been cloned from human.

Table 1.4. 17β-Hydroxysteroid dehydrogenases/ketosteroid reductases

Type	Protein family	Species cloned	Dehydrogenase/ reductase	Putative function
1	SDR	Human, rat, mouse	Reductase	E2 production E2, T production
2	SDR	Human, rat, mouse	Dehydrogenase	E2, T inactivation, (20αP activation)
3	SDR	Human, mouse	Reductase	T production
4	SDR/ MFE*	Human, rat, mouse, porcine, chicken, guinea pig	Dehydrogenase	β-oxidation of fatty acids, E2 inactivation
5	AKR	Human	Reductase	T production, bile acid production., 20αP activation, detoxification
		Mouse		T production, detoxification
6	SDR	Rat	Dehydrogenase	5α-DHT inactivation
7	SDR	Human, rat, mouse	Reductase	E2 production
8	SDR	Human, mouse	Dehydrogenase	E2 inactivation (E2 production), androgen inactivation
9	SDR	Mouse	Dehydrogenase	E2 inactivation, androgen inactivation
10	SDR	Human, rat, mouse, *Drosophila*	Dehydrogenase?	β-oxidation of fatty acids, E2 inactivation
11	SDR	Human, mouse	Dehydrogenase	Androgen inactivation, (E2 inactivation)

*MFE = multi-functioning enzyme. Abbreviations: pages 8-10.

17β-HSD10/SCHAD II (EC 1.1.1.35) shares the common NAD(H)-binding motif and catalytic triad residues of other SDR enzymes: GxxxGxG and serine, tyrosine and lysine residues. It is expressed in mitochondria of normal tissues and over-expressed in neurons affected in Alzheimer's disease. Its location in mitochondria has been confirmed and differs from the other 17β-HSD isozymes (He et al., 1999).

1.1.3.4 17β-Hydroxysteroid dehydrogenase type 10/SCHAD II/*scully* from *Drosophila melanogaster*

17β-HSD10/SCHAD II/*scully* from *Drosophila melanogaster* is homologous to human 17β-HSD10/SCHAD II. It was detected in 1998 by phenotypic analysis of *D. melanogaster* mutants. The wild type expression pattern was studied and was found to be present in many tissues, including the central nervous system (Torroja et al., 1998). Highest expression was noted in gonadal primordial, mature ovaries and testes, suggesting a role in germ line formation (Torroja et al., 1998). 17β-HSD10/SCHAD II/*scully* consists of 253 amino acid residues, and homology searches show 60% identity to mammalian 17β-HSD10/SCHAD II, suggesting it to be 17β-HSD/L-3-hydroxyacyl-CoA dehydrogenase. However, the N-terminal signal sequence is shorter than the mammalian orthologous proteins (figure 1.6). The location therefore, has to be determined experimentally to be established (paper V). A lethal *Drosophila* mutant has been described with mutations in the *scully* gene (Torroja et al., 1998).

```
              1         11        21        31        41        51
Human         MAAACRSVKGLVAVITGGASGLGLATAERLVGQGASAVLLDLPNSGGEAQAKKLGNN
Bovine        MAAACRSVKGLVALITGGASGLGLATAERLVGQGATAVLLDLPNSDGETQAKKLGKS
Rat           MAAACRSVKGLVAVITGGASGLGLSTAKRLVGQGATAVLLDVPNSEGETEAKKLGGN
Mouse      MCKMAAAVRSVKGLVAVVTGGASGPWLATAKRLVGQGATAVLLDVPDSEGESQAKKLGES
C elegans       MSALRSTKGLVALVTGGASGLGKGAAEVLAKAGAQVAILDLPQSKGADVAKEIGGI
D melanogaster        MIKNAVSLVTGGASGLGRATAERLAKQGASVILADLPSSKGNEVAKELGDK
3β/17βHSD       MTNRLQGKVALVTGGASGVGLEVVKLLLGEGAKVAFSDINEAAGQQLAAELGER

positive amino acids: R, K
hydroxylated amino acids: S, T, Y
negative amino acids: d, e
```

Figure 1.6. N-terminal sequences of 17β-HSD10/SCHAD II from different species and 3β/17β-HSD from *C. testosteroni*.

1.1.3.5 SCHAD I from human

Short chain L-3-hydroxyacyl-Coenzyme A (CoA) dehydrogenase type I (EC 1.1.1.35) (SCHAD I) plays an essential role in the mitochondrial β-oxidation of short chain fatty acids where it catalyses the third reaction (Clayton et al., 2001) (figure 1.7). It exerts its highest activity toward 3-hydroxybutyryl-CoA (Kobayashi et al., 1996), and belongs to the 3-hydroxyacyl-CoA dehydrogenase (HAD) family (Birktoft et al., 1987). SCHAD I possess a signature sequence that defines this family: HxxxPxxxMxLxE (He and Yang, 1998), where a catalytic His – Glu pair is included. The histidine serves as a general acid/base in the catalytic mechanism of HAD and the glutamate is required for proper orientation of the same histidine (Barycki et al., 2001). All the HADs utilize L-3-hydroxyacyl-CoAs of varying chain lengths from C4 to C16 (Kobayashi et al., 1996), and have a two-domain structure (Holden and Banaszak, 1983) with a consensus sequence for NAD^+/NADH binding of GxGxxG in the N-terminal domain (Wierenga et al., 1985). Other members of this family are the mitochondrial trifunctional protein, where the homology resides within the α-subunit and the peroxisomal bifunctional enzyme. The three-dimensional structure is similar to other HAD but also to 6-phosphogluconate dehydrogenase despite low sequence identity (Barycki et al., 1999). HADs invariably bind L-3-hydroxyacyl-CoAs at a cleft between two domains (Birktoft et al., 1987). SCHAD I has a mitochondrial import signal peptide of 12 amino acids and 302 amino acid residues of mature protein located in the mitochondrial matrix (Vredendaal et al., 1996). It is expressed at different levels in skeletal and cardiac muscle, liver, kidney and pancreas and is mapped to chromosome 4q22-26 (Vredendaal et al., 1996). In comparison to the SCHAD II there are a number of differences in structure, function and motifs (table 1.5).

Table 1.5. Comparison of SCHAD I and SCHAD II/17β-HSD10

SCHAD I	SCHAD II/17β-HSD10
314 amino acids (34 kDa)	261 amino acids (xx kDa)
Homodimer	Homotetramer
3-OH-acyl-CoA DH family	SDR family
Two-domain structure	One-domain
NAD binding domain: GXGXXG	NAD binding domain: GXXXGXG
Catalytic residues: H, E	Catalytic residues: S, Y, K
Expressed in liver, kidney, pancreas, heart and skeletal muscle	Expressed in normal tissues, over-expressed in neurons affected in Alzheimer's Disease

1.2 Subcellular localisation of SDR enzymes

About 30 soluble SDR proteins have been crystallised, the three dimensional structures determined and deposited in the Protein Data Bank. No membrane bound SDR proteins have been crystallised, and studied yet regarding the transmembrane motifs. Neither have proteins that are further transported to other organelles regarding the targeting motifs. Some of these proteins are microsomal 11β-hydroxysteroid dehydrogenase (11β-HSD), which has an N-terminal transmembrane region, mammalian 3β-hydroxysteroid dehydrogenase/Δ4-5 isomerase (3β-HSD) with a C-terminal transmembrane region and mammalian 17β-HSD10/SCHAD II. These enzymes consequently have signal peptides for transport into these membranes and/or organelles.

Table 1.6. Cofactor preference and location of some SDR enzymes.

Enzyme form	Dehydrogenase/ reductase	NADPH	NAD^+	Location
3β-HSD				
1	Dehydrogenase		+	Membranebound
2	Dehydrogenase		+	Membranebound
11β-HSD				
1	Reductase	+		Microsomal toward lumen
2	Dehydrogenase		+	Microsomal toward cytosol
Rat RDH1	Reductase	+		Microsomal
Human all-*trans*-retinal Red	Reductase	+		Membranebound/associated
Human all-*trans*-RDH	Dehydrogenase		+	Microsomal
17β-HSD				
1	Reductase	+		Cytosolic
2	Dehydrogenase		+	Microsomal toward lumen
3	Reductase	+		Microsomal
4	Dehydrogenase		+	Peroxisomal
6	Dehydrogenase		+	Membranebound
7	Reductase	+		Membranebound/associated
8	Dehydrogenase		+	
9	Dehydrogenase		+	ER
10	Dehydrogenase?		+	Mitochondrial matrix
11	Dehydrogenase		+	

Another feature that can be observed is the preference of cofactor in biological systems (table 1.6). Reaction direction is largely dependent on cofactor availability and ratio. Reductases

mostly use NADPH and dehydrogenases mostly use NAD^+ (Luu-The et al., 1995; Luu-The, 2001). It is likely that this phenomenon is linked to the intracellular ratio of $NADP^+$/NADPH and NAD^+/NADH. These ratios are in the cytosol around 0.01 and 1 000, respectively and in the mitochondria around 12 and 8 (both in the cristae and in the matrix), respectively (Krebs and Veech, 1969). In the lumen of the endoplasmic reticulum (ER) the redox state is more oxidised than in the cytosol (Hwang et al., 1992). This could explain the dehydrogenase activity of the luminal ER 17β-HSD2 over the reductase activity of the cytosolic 17β-HSD1. Another explanation of this is the evolution of the different proteins (Baker et al., 1995). Human 17β-HSD1 is more related to human all-*trans*-retinal reductase, human 11β-HSD1 and 17β-HSD7 and all of these are reductases that prefer the $NADP^+$/NADPH couple. The human 17β-HSD2 is more related to the human 11β-HSD2, human all-*trans*-retinol DH, mouse 17β-HSD9 and human 17β-HSD6 and these enzymes prefer the NAD^+/NADH couple and work as dehydrogenases (Baker et al., 2001).

1.3 Protein import into mitochondria

Proteins imported into mitochondrial matrix are usually taken up within a minute or two of their release from free ribosomes by cytosolic factors that help to maintain import and prevent aggregation. The proteins have a signal peptide (20-60 residues) at the N-terminus that directs the protein to a receptor on the mitochondria. These signal peptides have abundant positive charges, very few if any negative charges, and frequent hydroxylated residues. Sometimes the signal peptide is cleaved off by a signal peptidase in the mitochondrial matrix and is then degraded in the matrix. Physical studies show that the signal peptide can form amphiphatic α-helical structures in membranes in which positively charged residues line up on one side of the helix while uncharged hydrophobic residues line up toward the opposite side. In aqueous solution they show little structural organization.

Energy for transport and vectorial movement require energy from ATP (adenosine triphosphate) or electrochemical gradient maintained by electron transport chain. There is no electrochemical gradient across the outer mitochondrial membrane as it is freely permeable to ions. The proteins pass through both membranes at certain contact sites, i.e. translocases of the outer and the inner membrane, more known as TOM and TIM complexes. The membrane potential is only necessary for initial penetration, whereas ATP hydrolysis and physiological temperatures are necessary for movement of the remainder of the chain into the matrix.

The proteins presumably unfold before crossing the two mitochondrial membranes. Unfolding requires energy, probably *via* ATP. Certain members of the hsp70 family of stress-

response proteins are essential for the unfolding reaction of the mitochondrial membranes. (Neupert, 1997 and references therein)

1.4 Fatty acid transport and mitochondrial oxidation

Fatty acid oxidation provides 80% of all energy for heart and liver function at all times (Eaton *et al.,* 1996) and at muscular activity, fasting and febrile illness it represents a major source of energy. Degradation of fatty acids is an oxidative process that takes place in both the peroxisome and the mitochondrion (figure 1.7). It is primarily performed through a cycle of

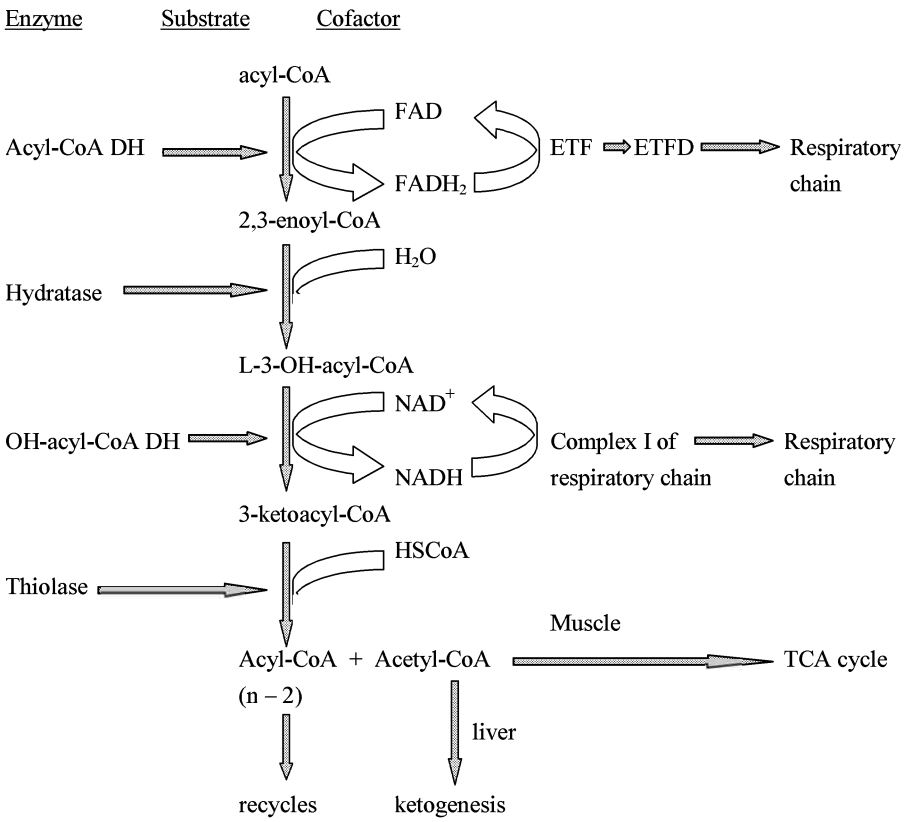

Figure 1.7. β-oxidation of fatty acids in mitochondria.

reactions known as β-oxidation. Carbons are released, two at a time, from the carboxyl end of the fatty acid (Knoop, 1905; Dakin, 1909). The major end-products are acetyl coenzyme A

(CoA) and the reduced forms of the nucleotides, flavin adenine dinucleotide and nicotinamide adenine dinucleotide ($FADH_2$ and NADH) (Drysdale and Lardy, 1953).
Free fatty acids only occur at low concentrations in the human body. In blood, fatty acids are bound to albumin and in the cytosol to a series of fatty-acid-binding proteins and enzymes.
As the first step of catabolism, fatty acids have to be activated to their CoA derivative, using adenosine triphosphate (ATP) as their energy source, catalysed by fatty acyl CoA synthetase. The length of the fatty acid dictates in which organelle it is activated, and how it is transported through the mitochondrial and peroxisomal membrane (table 1.7).

Table 1.7. Metabolism of fatty acids.

Size class	Number of carbons	Site of degradation	Membrane transport
Short chain	2-4	Mitochondria	Passive diffusion
Medium chain	4-12	Mitochondria	Passive diffusion
Long chain	12-20	Mitochondria	Carnitine shuttle
Very long chain	> 20	Peroxisome	Unknown

Peroxisomes are able to conduct β-oxidation of medium to very long chain fatty acids by a pathway similar to mitochondrial oxidation, but with significant differences. They produce H_2O_2 instead of $FADH_2$ in the step where acyl CoA dehydrogenase acts. They are relatively inefficient in the catabolism of short chain fatty acids that have to be transported to the mitochondria.
Long chain fatty acids enter the mitochondria by the carnitine shuttle including three components; the extra- and intramitochondrial carnitine palmitoyl transferases (CPT-I and CPT-II) located in the outer and the inner mitochondrial membranes, respectively, and the carnitine-acyl carnitine translocase located in the inner mitochondrial membrane. The carnitine shuttle operates by an antiport mechanism in which free carnitine and the acyl carnitine derivative move in opposite directions across the inner mitochondrial membrane.
The first reaction in the β-oxidation involves a member of the acyl-CoA dehydrogenase family of FAD-requiring oxidoreductases responsible for the reduction across the 2,3 position of the acyl-CoA moiety to produce a 2,3-enoyl-CoA (Lynen and Ochoa, 1953). The electrons are transferred via electron transfer flavoprotein (ETF) to ETFD and ultimately to the oxidative phosphorylation where ATP is produced. The second reaction involves hydration of the double bond in the 2,3 position to produce L-3-hydroxyacyl-CoA. The third step of fatty acid oxidation involves the reduction at the L-3-hydroxy position to yield a 3-ketoacyl-CoA (Wakil et al., 1954). In this reaction, NAD^+ is reduced to NADH. The final step involves cleavage of the 3-ketoacyl-CoA between the α- and β-carbons by a thiolase reaction to yield acetyl-CoA and a two-carbon shortened acyl-CoA. (Eaton et al., 1996 and references therein)

The end-product, acetyl CoA, of each round of β-oxidation enters the TCA cycle, where it is further oxidised to CO_2 with the concomitant generation of reduced nucleotides as well as ATP. In the liver acetyl CoA enters the ketogenesis pathway.

1.4.1 Mitochondrial fatty acid oxidation disorders

Disorders in mitochondrial β-oxidation of fatty acids are becoming increasingly recognized as important causes of infant mortality and morbidity (Bennett *et al.*, 1999). Signs and symptoms may vary greatly in severity and typically include hypoketotic hypoglycemia, liver disease, skeletal myopathy and sudden and unexpected death in early life (Rinaldo et al., 2002). The symptoms appear at different ages, from birth to adult life, and may occur in variable combinations that often lead to life-threatening complications. All mitochondrial fatty acid oxidation (FAO) disorders known today are autosomal recessive. The complexity of FAO disorders can be described by enzymes that have been postulated to be involved in diseases (table 1.8).

Table 1.8. Some known enzymes involved in disorders of FAO.

Enzyme	Function	Disorder
Hepatic carnitine transporter	Carnitine shuttle	Carnitine transporter deficiency
Carnitine-acylcarnitine translocase	Carnitine shuttle	Carnitine translocase deficiency
Liver CPT I	Carnitine shuttle	CPT I deficiency
Muscle CPT I	Carnitine shuttle	CPT I deficiency
CPT II	Carnitine shuttle	CPT II deficiency
SCAD	1st step in β-oxidation	SCAD deficiency
MCAD	1st step in β-oxidation	MCAD deficiency
VLCAD	1st step in β-oxidation	VLCAD deficiency
LCAD	1st step in β-oxidation	LCAD deficiency
Trifunctional protein	2nd step in β-oxidation	LHYD deficiency
Trifunctional protein	3rd step in β-oxidation	LCHAD deficiency
SCHAD I	3rd step in β-oxidation	SCHAD deficiency
MCKAT	4th step in β-oxidation	MCKAT deficiency
ETFD	Electron transport chain	MAD: Glutaric aciduria type II
ETF	Electron transport chain	MAD: Ethylmalonic-adipic aciduria
HMG CoA lyase	Ketone body formation	HMG CoA lyase deficiency

1.4.2 Short chain hydroxyacyl Coenzyme A dehydrogenase deficiency

A deficiency of short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase (SCHAD) is a congenital metabolic error that occurs as fatty acids derived from fat stores in the body are metabolised. As a result, fatty acids can enter the liver but cannot generate energy. SCHAD deficiency may be responsible for a small portion of otherwise unexplained deaths in children younger than 1 year old. A study managed by Dr. Bennett (Texas University) among 150 infant deaths determined the frequency of SCHAD deficiency to 2% of the cases (Voelker, 1999).

This disorder of mitochondrial β-oxidation has been identified to different tissues: the muscle, the fibroblasts and the liver (Tein et al, 1991; Bennett *et al.*, 1996; Bennett et al., 1999). Elevated urinary levels of short chain L-3 hydroxybutyryl CoA indicate a defect in the third step of mitochondrial fatty acid β-oxidation:

$$\text{L-3-hydroxyacyl-CoA} + NAD^+ \quad \Leftrightarrow \quad \text{3-oxoacyl-CoA} + NADH$$

As described earlier (pages 20–22) two genes coding for mitochondrial short-chain L-3-hydroxyacyl-CoA dehydrogenases (SCHAD) with overlapping tissue-expression have been identified so far in human (Vredendaal *et al.*, 1996, He *et al.*, 1998). In an initial study of three patients no mutations or abnormalities in the type 1 gene were found (Bennett *et al.*, 1999). A separate enzyme, long chain L-3-hydroxyacyl-CoA dehydrogenase appears to be responsible for the oxidation of L-3-hydroxyacyl-CoA of chain lengths with six carbons and longer.

A recent report describes the case of a homozygous carrier with heterozygous parents for a P258L mutation in SCHAD I, resulting in a severely reduced enzymatic activity (Clayton et al., 2001).

These data suggest an isozyme to be linked to the patient phenotype. Therefore, we hypothesised a genetic defect in type II SCHAD/17β-HSD10 of SCHAD patients. The biochemical and histological profiles of 3 patients are given in table 1.9. The hypothesis was further strengthened by data of the *Drosophila scully* mutants, displaying lipid accumulation in the cells (Torroja et al., 1998). Therefore, we performed a study to identify genetic defects in type I and II SCHAD, and to establish candidate genes for SCHAD deficiency (paper VI).

Table 1.9. Profile for SCHAD deficient patients.

Patient data	**Healthy**	**Patient 1**	**Patient 2**	**Patient 3**
Activity in skeletal muscle	normal	normal	normal	normal
Activity in liver	100%	6.7%	11%	3.4%
Hepatic C4:C16 activity ratio	2.95	0.28	0.37	0.17
Muscle C4:C16 activity ratio	normal	normal	normal	normal
C16 activities in liver & muscle	normal	normal	normal	normal
Size of type 1 enzyme	31	31	31	30
Lipid accumulation in liver	normal	marked	moderate	no fat
Lipid storage		steatosis		

2 SPECIFIC AIMS OF THIS THESIS

The aims of this thesis were three-fold and define:

1. to study structural motifs of the SDR family, using site-directed mutagenesis, steady-state kinetics, stability measurements and structure determination (paper I-IV).

2. to analyse targeting motifs in SDR enzymes (paper V).

3. to study genetic defects in fatty acid oxidation, with a possible involvment of a multifunctional hydroxysteroid dehydrogenase (paper VI).

3 RESULTS AND DISCUSSION

Detailed information about the methods used to obtain the results described in this section is provided in the Materials and Methods sections of the original articles and manuscripts.

3.1 General architecture of SDR hydroxysteroid dehydrogenases

The enzyme 3β/17β-hydroxysteroid dehydrogenase from *C. testosteroni* served as a model for hydroxysteroid dehydrogenases in the SDR family. This was chosen as a model for the SDR family since it contains all the characteristic motifs, and signatures, and was well characterised in terms of its mechanism as described in the introduction. The sequence identity is low, but the tertiary structure shows a highly similar architecture with the thus far analysed 3D structures. Mutagenetic replacements of conserved amino acid residues within the active site (Asn 111, Ser 138, Tyr 148 and Tyr 155), the cofactor binding region (Thr 12, Ser 16, Asp 60, Asn 86, Asn 87 and Ala 88) and the substrate binding region (Asn 179 and His 182) give new insight into structure – function relationships in the SDR family and in hydroxysteroid dehydrogenases in particular. In paper I six different mutations are discussed (T12A, T12S, S16A, N87A, S138A and S138T), in paper II one mutation (N179A), in paper III ten mutations (T12A, T12S, D60A, N86A, N87A, A88S, N111L, S138A, S138T and Y151F), and in paper IV two mutations (Y148F and H182L). The common secondary structure elements in the SDR subunits, and the mutated positions in 3β/17β-HSD are shown in figure 3.1.

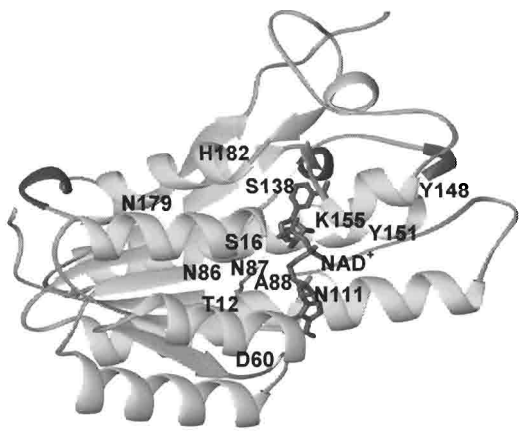

Figure 3.1. The common secondary structure elements in the SDR subunit. The mutated positions are indicated as well as NAD^+.

The wild-type and the mutant 3β/17β-HSD forms were overexpressed in *E. coli* BL21 at a level of 1-10 mg/l culture. Metal chelate chromatography yielded correctly folded and pure enzymes according to CD spectroscopy and SDS/PAGE (data not shown). The activities were measured by spectrophotometer at 340 nm in 1.0 ml at 25°C and the catalytic constants, K_m and k_{cat}, were calculated for the 3β- and 17β-dehydrogenase activities and the 3-oxo- and 17-oxo-reductase activities (table 3.1). As substrates an isobile acid, isoursodeoxycholic acid (iso-UDCA) and the steroid hormone, dehydroepiandrosterone were used for 3β-HSD activities, 5α-dihydrotestosterone (5α-DHT) was used for 3-oxo-reductase activities, testosterone was used for 17β-HSD activities and androsterone was used for 17-oxo-reductase activities.

Table 3.1. Kinetics of 3β/17β-HSD with constants for steroid substrates.

Activity	**3β-HSD**			**3β-HSD**			**3-oxo-reductase**			**17β-HSD**			**17-oxo-reductase**		
	iso-UDCA			DHEA			5α-DHT			testosterone			androsterone		
			relative			relative			relative			relative			relative
Mutation	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
Wild type	22.3	1.4	100	8.17	0.62	100	16.1	0.10	100	11.8	1.2	100	31.7	0.10	100
Thr12Ala	na			321	0.039	0.15	15.8	0.10	110	na			na		
Thr12Ser	28.4	1.7	100	17.7	0.74	54	13.9	0.060	72	13.7	0.96	75	26.9	0.10	80
Ser16Ala	27.7	1.4	84				23.8	0.10	70	24.7	1.4	59	26.0	0.060	100
Asp60Ala	47.3	0.47	17	12.6	0.49	50	35.5	0.12	56	23.9	0.25	11	38.5	0.18	78
Asn86Ala	125	0.37	4.9	10.9	0.064	7.5	24.9	0.11	74	19.5	0.45	24	37.8	0.072	32
Asn87Ala	28.8	0.30	17	24.4	0.42	22	18.7	0.090	80	25.7	0.46	19	24.6	0.10	80
Ala88Ser	116	0.17	2.4	19.7	0.30	19	90.5	0.26	48	68.1	0.18	2.8	54.0	0.15	46
Asn111Leu	na			na			na			na			na		
Ser138Ala	na			na			na			na			na		
Ser138Thr	18.9	1.5	130				14.2	0.060	70	15.1	0.92	63	25.1	0.090	60
Tyr148Phe	28.3	0.73	41	11.8	0.65	98	10.4	0.13	150	9.5	2.5	260	22.3	0.28	190
Tyr151Phe	na			na			na			na			na		
Asn179Ala	na			na			na			na			na		
His182Leu	130	0.12	1.5	15.9	0.038	4.3	53.5	0.027	6.3	10.8	0.06	5.6	27.1	0.026	14

K_m values in μM, k_{cat} values in 10^{-3}min^{-1} and relative k_{cat}/K_m values in %. na, not active.

The kinetic constants K_m and k_D have been determined for the cofactor couple NAD^+/NADH with the 3β/17β-HSD wild-type and mutant forms (table 3.2). The dissociation constant for NADH were determined by spectrofluorometer by monitoring fluorescence energy transfer as a function of nucleotide concentration (excitation at 286 nm, emission at 450 nm). For NAD^+ this was performed similarly by displacement of bound NADH (excitation at 345 nm, emission at 450 nm).

Table 3.2. Kinetics of 3β/17β-HSD with constants for NAD^+ and NADH.

	K_m for NADH	k_D for NADH	K_m for NAD^+		k_D for NAD^+
Substrate	5α-DHT		Iso-UDCA	DHEA	
	μM	μM	μM	μM	μM
Protein					
Wild-type	21.6	1.01	83.2	29.6	1.47
Thr13Ala	36.7	40.9	na	8.96	0.135
Thr13Ser	21.7	1.94	90.4	13.4	1.94
Asp60Ala	52.3	19.4	651	87.9	0.304
Asn86Ala	7.44	3.14	843	122	1.51
Asn87Ala	25.5	3.81	81.2	259	1.11
Ala88Ser	30.4	9.52	1550	685	0.291
Asn111Leu	na	5.15	na	na	2.34
Ser138Ala	na	12.0	na	na	2.13
Ser138Thr	23.1		59.0		
Tyr148Phe	12.6	3.0	168	23.2	2.6
Tyr151Phe	na	4.34	na	na	0.235
Asn179Ala	na		na	na	
His182Leu	25.5		484	180	

na, not active.

Conformational stability of the wild-type and the mutant 3β/17β-HSDs was analysed by equilibration unfolding experiments using guanidinium chloride (GdmCl) denaturation. The extent of protein unfolding was measured by the change in ellipticity at 222 nm at constant protein and variable GdmCl concentrations at 4°C. No significant differences between the wild-type and the mutant proteins were detected except for the N179A mutant showing a two-fold increase in unfolding midpoint.

3.1.1 Active site and reaction mechanism: N111, S138, Y151, K155

Residues located at the active site that were subjected to site-directed mutagenesis were Asn 111, Ser 138, Tyr 148 and Tyr 151. The exchange of Tyr 148 is more of specific interest for 3β/17β-HSD and will be dealt with in a later context. Here I will discuss more about the general features for the active site and the reaction mechanism of the SDR family and the HSDs in particular.

Ser 138 is highly conserved in all SDR members together with Lys155. It was suggested to be part of a catalytic triad by alignment comparisons of the known SDR structures, at that time around 50 (Jörnvall et al., 1995), and have since then been mutated in several studies (Cols et al., 1993; Ensor and Tai, 1996; paper I and III). In this thesis Ser 138 was exchanged to an alanine or a threonine. The alanine exchange resulted in loss of activity. However, the exchange to threonine resulted in activities similar compared to the wild-type (paper I and III). Threonine and serine both have a hydroxyl group. This functional group may be responsible for stabilisation by hydrogen bonding of the substrate, the reaction intermediate, and the product in catalysis.

Tyr 151 is the only residue completely conserved in SDR enzymes. It is probably the residue mutated most frequently in this family (Ensor and Tai, 1991; 1994; Obeid and White, 1992). An exchange to a Phe yielded a completely inactive enzyme.

Asn 111, located within helix αE, the main subunit interaction area, is also highly conserved in SDR enzymes. We exchanged this asparagine to a leucine with the result of a protein enzymatically inactive. To exclude the fact that this mutation effect was due to an oligomerisation different than that in the wild-type, we performed sedimentation analysis of the wild-type and the mutant form and found no changes. When Asn 111 was analysed in the available 3D structures we noticed a hydrogen bond network surrounding this residue. In a few cases a serine could be observed instead of this asparagine, but with a homologous binding pattern. Especially important was the well-ordered water molecule between this residue and the active site residue Lys 155. This large hydrogen-bonded solvent network was first observed in the *Drosophila* alcohol dehydrogenase crystal structure (Benach et al., 1998).

Ser 138 has been proposed to be part of a catalytic triad of SDR enzymes together with Lys 155 and Tyr 151. These three residues were until the N111L mutant the only residues that by site-directed mutagenesis and chemical modification had proven a role in catalysis.

From the results of the mutated Asn 111, Ser 138 and Tyr 151, together with chemical modifications of the Tyr and crystallographic results from the 3β/17β-HSD together with analysis of other determined 3D structures, we propose a reaction mechanism for the SDR hydroxysteroid dehydrogenases. It is a general acid-base mechanism, where Tyr 151 functions as a catalytic base. It is facilitated by the protonated Lys 155, which lowers the pK_a of the hydroxyl group on the tyrosine. Lys 155 interacts with the nicotinamide ribose on the coenzyme. Ser 138 forms hydrogen bonds to the substrate to stabilise or polarise the substrate group. A proton is transferred from the tyrosine and a hydrid transfer takes place from the coenzyme (figure 3.2).

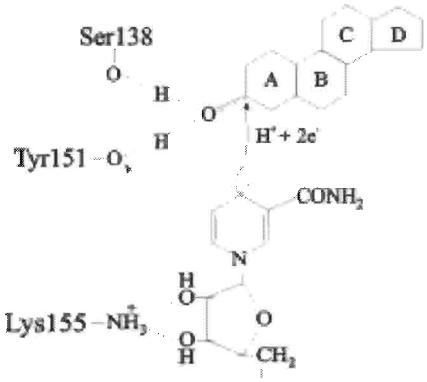

Figure 3.2. Suggested reaction mechanism for SDR-type HSDs.

We extend this concept by Asn 111 that stabilises the position of Lys 155 and in such a manner a proton relay is formed in most SDR structures, including as substantial elements: substrate, Tyr 151, ribose2'-hydroxyl, Lys 155, H_2O and Asn 111 (figure 3.3).

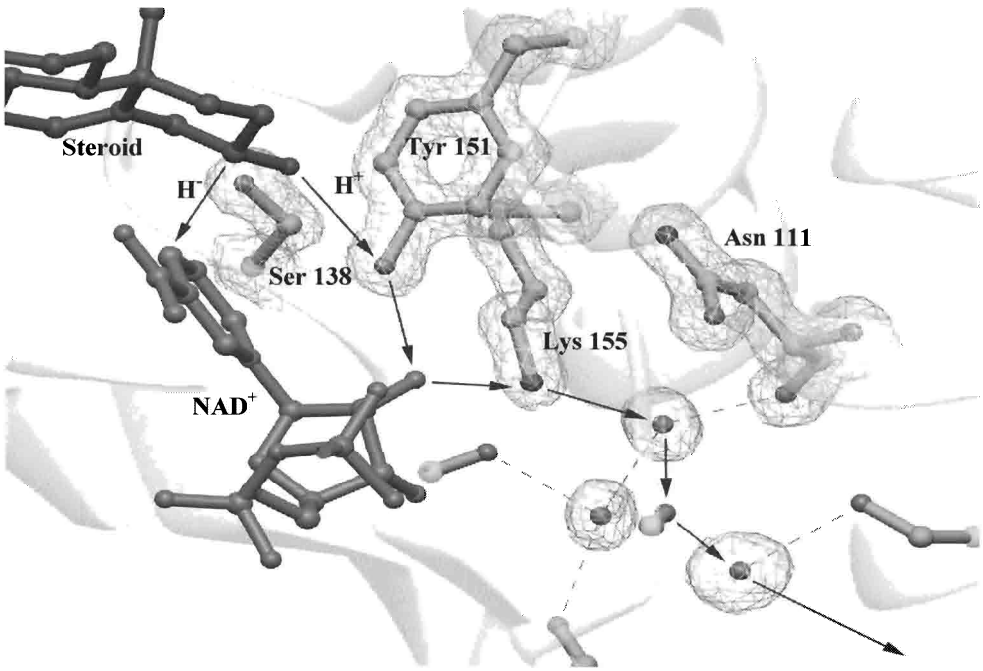

Figure 3.3. Reaction mechanism including the catalytic tetrad and a proton relay mechanism.

3.1.2 β-Sheet orientation: T12, D60, N86, N87, A88

In the coenzyme binding region several positions were mutated, Thr 12, Asp 60, Asn 86, Asn 87 and Ala 88 (paper I and paper III). These are all highly conserved in the SDR family. Residues Thr 12, Asn 86, Asn 87 and Ala 88 are located within the central β-sheet in SDR enzymes and Asp 60 is located between β-strand C and α-helix D. Analysis of the crystal structure reveal a critical network of hydrogen bonds and interactions between these residues (figure 3.4).

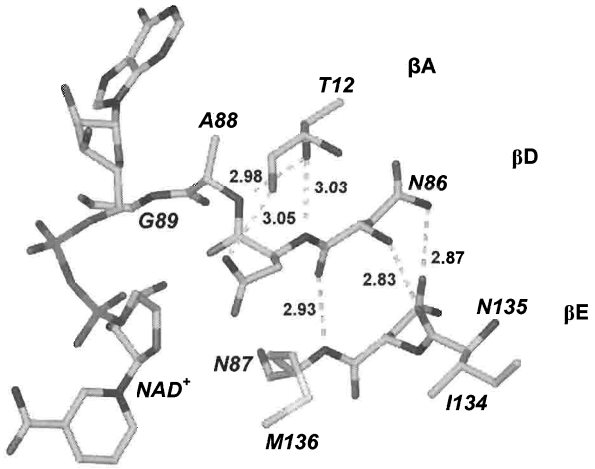

Figure 3.4. Central β-sheet in 3β/17β-HSD important for coenzyme binding and positioning in SDR-type HSDs.

The activities for T12A were similar to the wild-type for the 3-ketoreductase activity, highly reduced for the 3β-dehydrogenase activity with DHEA as substrate, but inactive for the other activities. The cofactor binding properties are changed for this mutant, K_M and k_D for NADH are increased and K_M (DHEA as substrate) and k_D for NAD^+ are decreased. A serine replacement of this residue results in activities more similar to the wild-type in both directions. This indicates that a hydrogen bond is important in this position for the direction of the reaction.

The activities for D60A, N86A, N87A and A88S are reduced compared to wild-type, except for A88S were it is increased for the 3-ketoreductase activity. The reduction is more pronounced in the dehydrogenase directions than in the reductase directions. In general the

K_M and the k_D values for the cofactors are increased for these mutant proteins compared to wild-type values with some exceptions, N86A has a decreased K_M for NADH, N87A has similar K_M for NAD^+ as the wild-type and D60A and A88S have decreased k_D for NAD^+ compared to wild-type.

Based on structural and kinetic data, the conclusions regarding these residues are the contribution of a framework that is essential to keep the strands oriented within the central β-sheet to position the coenzyme correctly. Asp 60 and Ala 88 contribute directly to the coenzyme binding, the aspartate by stabilising the turn between βC and αD and the alanine by hydrophobic interactions to the adenine ring of the coenzyme.

3.1.3 Central scaffold: N179

Asn 179 is located in the substrate binding region in β-strand F. It is a less conserved amino acid residue previously unknown as a functionally important motif (paper II). It is part of a motif, described as Ile-Arg-Val-Asn in SDR enzymes. Crystallographic studies show that this motif is well conserved in the 3D structures. The N179A mutant in 3β/17β-HSD is folded but enzymatically inactive. Binding experiment by spectrofluorometer shows that it does bind the cofactor in a similar manner as the wild-type. GdmCl titration to unfold the enzyme is significantly changed for the mutant compared to the wild-type. The unfolding midpoint is considerably higher for N179A than for the wild-type, 1.6 M *versus* 0.65 M, indicating a much more stable mutant protein. According to analysis of the wild-type at 1.2 Å resolution and other available 3D structures of SDR enzymes in databases this residue is hydrogen bonded to a buried conserved well-ordered water molecule, connecting β-strands E and F (figure 3.5).

This region is in contact with the active site and the substrate binding site. When the asparagine is replaced to an alanine this creates a hydrophobic environment that probably disturbs the network surrounding the water molecule in such a way that the H_2O is lost.

The corresponding asparagine in 11β-HSD1 has previously been mutated to a Glu or an Asp with completely abolished dehydrogenase and reductase activity (Agarwal et al., 1995). They suggested that N-linked glycosylation at this site would be the reason for the inactive mutants. However, sugar attachment was later determined not to be the reason for the lost activity. Based on these results we predict that the glycosylation site (Asn-Val-Ser) in 11β-HSD1 in the similar way as for 3β/17β-HSD and other analysed structures in the SDR family is necessary to maintain the central scaffold surrounding this asparagine residue in the SDR family.

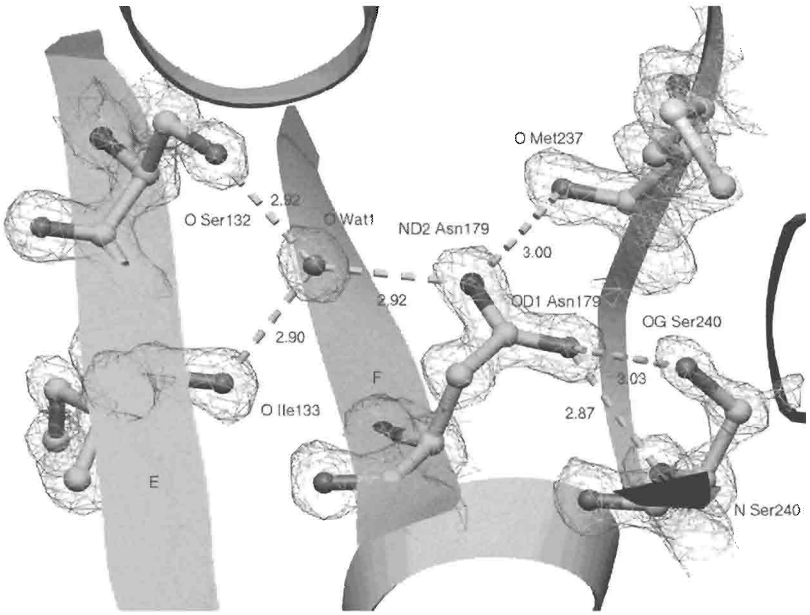

Figure 3.5. Asn 179 is part of a motif combining the active site with the substrate binding site.

3.1.4 Coenzyme specificity: S16, D37, K/R38

Different amino acid residues determine whether the enzymes binds NAD(H) or NADP(H) in the SDR family (table 3.3). This specificity towards the coenzymes is in part regulated by electrostatic interactions (Tanaka et al., 1996a; Tsigelny & Baker, 1996; Nakanishi et al., 1996, 1997; Mazza et al., 1998). Residue 16 is mostly basic or polar. In NAD(H) dependent enzymes like 3β/17β-HSD it can be replaced by a nonpolar residue like alanine without a major change in activity (paper I). This indicates that it is non-essential for catalysis to occur. Residue 37 is an aspartic acid in NAD(H) dependent enzymes. The aspartate contributes to repulsion of the phosphate group if NADP(H) would be used as a coenzyme instead of NAD(H) (Chen et al., 1991). For NADP(H) dependent enzymes residues 16 or 38 are always basic (Kallberg et al., 2002). The positive charge of the basic residues attracts the extra phosphate group in NADP(H).

Table 3.3. Positions important for contribution of specificity between NAD(H) and NADP(H) in different SDR enzymes are shown in bold.

Enzyme	Positions				References
	16	**37**	**38**	**39**	
NAD(H) dependent					
C. testosteroni 3β/17β-HSD	Ser	**Asp**	Ile	Asn	this thesis (paper I)
Rat SCHAD II/17β-HSD10	Ser	**Asp**	Leu	Pro	Powell et al., 2000
S. hydrogenans 3α/20β-HSD	Arg	**Asp**	Val	Leu	Ghosh et al., 1991
D. lebanonensis ADH	Gly	**Asp**	Arg	Val	Chen et al., 1991; Benach et al., 1998
E. coli 7α-HSD	Ala	**Asp**	Ile	Asn	Tanaka et al., 1996b
B. sp. LB400 2,3-di-OH-biphenyl DH	Ser	**Asp**	Lys	Ser	Hulsmeyer et al., 1998
K. pneumoniae 2,3-butanediol DH	Gln	**Asp**	Tyr	Asn	Otagiri et al., 2001
Rat DHPR	Gly	**Asp**	Val	Val	Varughese et al., 1992
NADP(H) dependent					
Mouse lung CR	**Lys**	Thr	**Arg**	Thr	Nakanishi et al., 1996,1997; Tanaka et al., 1996a
Rat retinol DH1	Ser	Glu	**Lys**	Gly	Tsigelny & Baker, 1996
Human 11β-HSD1	**Lys**	Ala	**Arg**	Ser	Tsigelny & Baker, 1995
Human 17β-HSD1	Ser	Leu	**Arg**	Asp	Mazza et al., 1998
M. grisea THNR	**Arg**	Tyr	Ala	Asn	Andersson et al., 1996
B. napus 3-oxoacyl Red	**Arg**	Tyr	Ala	**Arg**	Fisher et al., 2000
D. stramonium troponine Red II	**Arg**	Ser	**Arg**	Asn	Nakajima et al., 1998
Mouse Sepiapterin Red	Thr	Ala	**Arg**	Ser	Auerbach et al., 1997

C. testosteroni = Comamonas testosteroni, S. hydrogenans = Streptomyces hydrogenans, D. lebanonensis = Drosophila lebanonensis, E. coli = Escherichia coli, B. sp. LB400 = Burkholderia sp. LB400, K. pneumoniae = Klebsiella pneumoniae, M. grisea = Magnaporte grisea, B. napus = Brassica napus, D. Stramonium = Datura stramonium.

3.1.5 Crystal structure of the apostructure and residues of specific importance for 3β/17β-HSD; Y148, H182

The apostructure of 3β/17β-HSD from *C. testosteroni* was determined at 1.2 Å resolution (paper IV). Crystallographic analysis reveals the enzyme to have nearly identical subunits forming a tetramer. This is one of the largest oligomeric structures refined at this resolution. Each subunit is built up of a single α/β domain structure comprising a seven-stranded β-sheet flanked by three α-helices on either side of the sheet. The typical Rossmann fold can be located to the region between the βA and βF segment. The substrate binding region is well-

defined in the structure in contrast to other available SDR apostructures. This together with the substrate variability of 3β/17β-HSD and its activity toward both bile-acids and steroid hormones, i.e. steroid substrates with different steroid configuration provide a good tool to interpret protein-steroid interactions.

Residues identified to be specific to 3β/17β-HSD, with possible implications in substrate binding or catalysis are Tyr 148 and His 182. These two sites were studied by site-directed mutagenesis (paper IV).

His 182 is located at the end of β-strand F. When this histidine is exchanged to a leucine the activity is clearly reduced in both dehydrogenase and reductase direction compared to the wild-type enzyme. Binding constants of the cofactors could not be determined since no saturation of binding up to 100 μM NAD^+ or NADH was observed, in contrast to the wild-type. An explanation for this could be that His 182 connects the active site and the substrate binding region by hydrogen bonds to the main-chain of Ser 138 and side-chains of Ser 141 and Ser 180 and seems to form a salt bridge to the Glu 241 side-chain.

The substrate binding site is according to the crystal structure designed to account for the specificity toward 3β- and 17β-hydroxysteroids. Furthermore the cavity fits steroids in *cis*- or *trans*-configuration. This is even more apparent when compared with other steroid-binding enzymes in the SDR family. Substrates and inhibitors are modelled into the active site to demonstrate the interactions from these to the enzyme (figure 3.5). The reactive hydroxyl group at position 3 and 17 are within hydrogen bond distance to Tyr 151 and Ser 138 in the substrates but not in the inhibitors.

The tyrosine at position 148 located in the active site region was mutated to a phenylalanine. The 3β-dehydrogenase activity with the bile acid iso-ursodeoxycholic acid as substrate is reduced to half of the wild-type but with the hormone dehydroepiandrosterone (DHEA) as substrate it is like the wild-type activity. For the 3- and 17-ketoreductase and 17β-dehydrogenase activity the activity is increased in the Tyr 148 Phe mutant compared to wild-type activity. This could be explained by increased K_m-values for NAD^+ with iso-UDCA as substrate, similar K_m for NAD^+ with DHEA as substrate and decreased K_m for NADH with 5α-DHT as substrate.

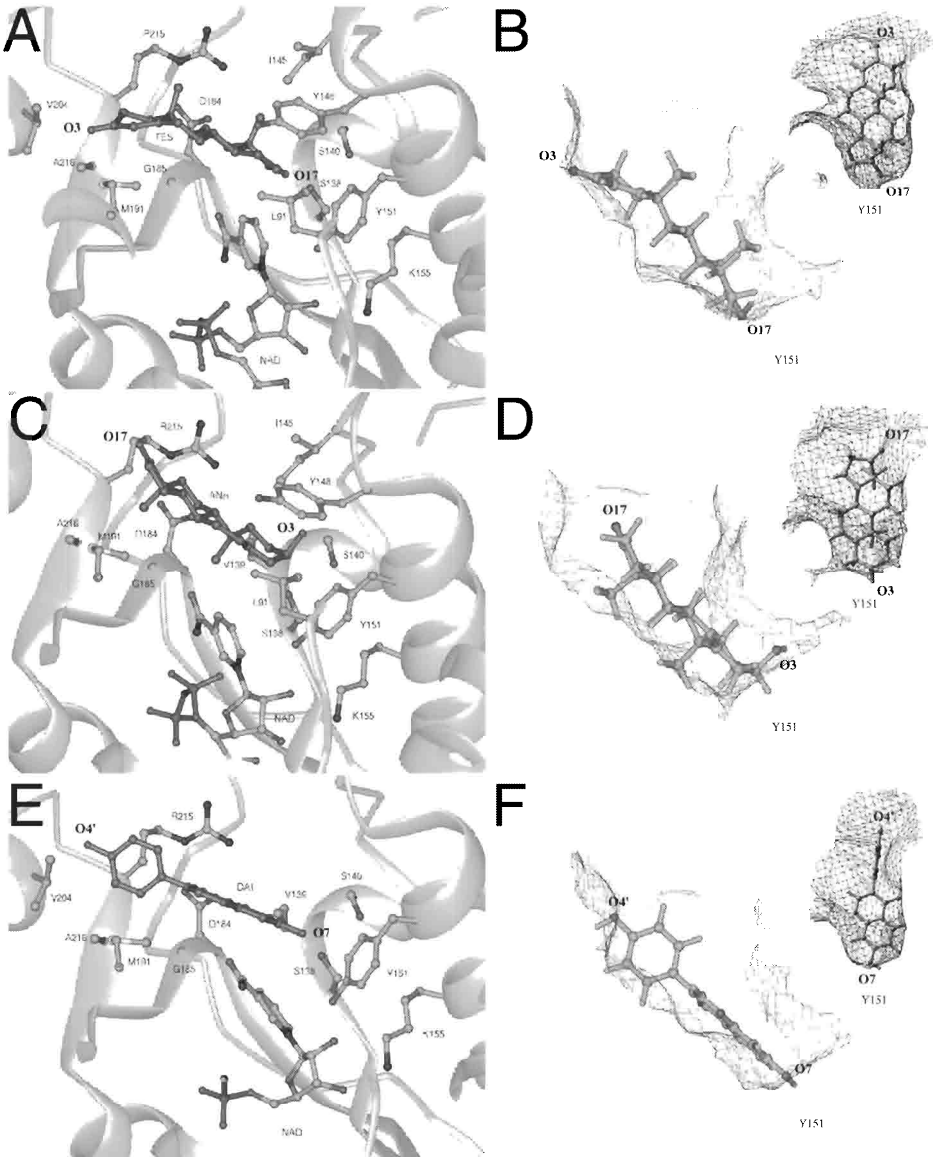

Figure 3.5. Binding of substrates and inhibitors in the active site of 3β/17β-HSD in relation to the catalytic triad and NAD^+. In the left panels, A, C, and E, the catalytic triad and residues forming van der Waals contact with the substrate or inhibitor are shown. In the right panels, B, D and F, the complementarity between the substrate or inhibitor and the accessible surface of the protein are shown in two orientations. A and B have testosterone (TES) as substrate, C and D have 3α-hydroxy-5-androsten-17-one (ANA) as substrate, and E and F have daidzein (DAI) as inhibitor.

3.2 Localisation and targeting of 17β-HSD10/SCHAD II to mitochondria

To study the subcellular localisation and targeting of 17β-HSD10/SCHAD II to mitochondria N-terminal domain shifts between that protein and the bacterial 3β/17β-HSD have been performed. 3β/17β-HSD is a cytosolic protein and thus lacks this sequence. The human enzyme show high sequence identity with 3β/17β-HSD and has in general the same length with exception of a short N-terminal region. The domain exchanges were studied by *in vitro* translation and fluorescence microscopy, using a fusion protein, GFP (= Green Fluorescent Protein). The GFP protein was fused to the C-terminal of the protein not to disrupt the N-terminal targeting signal sequence. To localise the two wild-types and the two recombinants (17β-HSD10/SCHAD II + 3β/17β-HSD and 3β/17β-HSD + 17β-HSD10/SCHAD II) in the cell fluorescence microscopy was used. The N-terminal part 1-34 of 17β-HSD10/SCHAD II has been determined to contain a noncleavable mitochondrial signal sequence. This contains part 1-15 that is specific for the human 17β-HSD10/SCHAD II but not enough for import and part 16-34 that can be replaced by similar structures as the corresponding region in 3β/17β-HSD. The results suggest that the N-terminal region work as a signal sequence directing the protein to the mitochondria but not to the endoplasmic reticulum. Electron microscopy confirmed these results. Electron microscopy pictures with 17β-HSD10/SCHAD II also show that the typical cristae in mitochondria are disrupted when this protein is colocalised to mitochondria. This can not be seen when 17β-HSD10/SCHAD II + 3β/17β-HSD is colocalised to mitochondria (data not shown).
Enzymology performed as described above for 3β/17β-HSD of the human 17β-HSD10/SCHAD II and the *Drosophila* ortholog show the similar multifunctionality. Both are active against estrogens, androgens and short chain hydroxy-fattyacyl-CoAs. The kinetic constants do not differ significantly, but the *Drosophila* enzyme seems to be cytosolic in contrast to the mitochondrial human enzyme. The shorter N-terminal sequence of the *Drosophila* enzyme appears not to be enough directing it to the mitochondria.

3.3 Short chain hydroxyacyl coenzyme A dehydrogenase deficiency

Six patients were diagnosed SCHAD deficiency according to symptoms, enzymatic measurements of tissue samples and histological examinations. The cDNA sequences for the two SCHAD genes have been determined for these six patients. The regions analysed are the exons and the flanking regions in the introns. SCHAD I is on chromosome 4 why both alleles have to be analysed. However, the SCHAD II gene is located on the X chromosome and the

patients were all male (genotype XY), why only one existing allele is to determine for this gene. The results were no difference in the sequence for the SCHAD II gene, but in two out of the six patients DNA for the SCHAD I gene, C257T in the precursor cDNA corresponding to a Pro 86 exchanged to a Leu in the mature protein. This mutation is a polymorphism existing in 5-7 % of the normal population (Bennett and O'Brien, unpublished results).

Western Blots of samples from the patients show SCHAD I expression (Bennett et al., 1999 for patient 1-3; Bennett, Strauss, O'Brien, unpublished results for patient 4-6) in all six patients and SCHAD II expression for patient 1-4. In patient 5 and 6 the proteins were degraded (paper VI).

The data indicate that these two genes are not responsible for the SCHAD deficiency in the patients. This suggests that other factors contributing to this disease must exist. It could be a third SCHAD enzyme or a so far unknown defect binding protein to any of the known SCHAD enzymes. There are two reports about mitochondrial proteins binding to SCHAD I in rat and in pig (Furuta and Hashimoto, 1995; Kispal et al., 1986). The rat binding protein was a homodimer of subunits of 60 kDa inhibiting SCHAD I, and the pig binding protein was a heterodimer with subunits of 69 and 70 kDa, activating SCHAD I. In human there is a report about interactions between glucose transporter 4 (GLUT4) C-terminus and SCHAD I at the plasma membrane, as well as the binding of amyloid β-peptide binding to SCHAD II (Yan et al., 1997). However, the functional consequence *in vivo* is unknown.

In paper VI we report about five human mitochondrial proteins binding to SCHAD I. The three smallest proteins were identified as degradation products of SCHAD I and the 60 kDa protein was identified as glutamate dehydrogenase type I, whereas the 70 kDa protein has not been identified yet.

From bovine liver two mitochondrial SCHAD have been purified and kinetic data obtained (Kobayashi et al., 1996). One protein is SCHAD I and the other is SCHAD II (Furuta et al., 1997). Kinetic data from these two enzymes in bovine indicate a more efficient SCHAD II than SCHAD I in the dehydrogenase reaction but the opposite for the reductase reaction. In human our results point to a different way (table 3.4) (paper VI).

Comparative kinetics of human SCHAD I and II with the substrates acetoacetyl CoA, DL-β-hydroxybutyryl CoA, NADH and NAD^+ were performed at two different pH values, at pH optimum and at mitochondrial pH to be able to compare the resulting activities as the enzymes operate at different pH optimum (table 3.4). These were pH 7.0 for reduction for both enzymes and for dehydrogenase pH 8.5 for SCHAD I and pH above 9.3 for SCHAD II (however measurements were performed at pH 9.3 in this case). One observation that should be done is that both the D- and the L-form of the dehydrogenase substrate, β-hydroxybutyryl CoA is used. The D-form might be an inhibitor for these two enzymes why the most realistic

substrate used in interpretations for these enzymes should be the reductase substrate, i.e. acetoacetyl CoA.

Table 3.4. Kinetics of SCHAD I and SCHAD II.

Substrate	**Acetoacetyl-CoA**			**NADH**	**β-hydroxybutyryl-CoA**			**NAD^+**
	K_M	k_{cat}	k_{cat}/K_M	K_M	K_M	k_{cat}	k_{cat}/K_M	K_M
SCHAD I								
pH optimum	16.6	77.2	77.5	10.6	22.5	7.14	5.29	109
pH 8.0	22.2	40.9	32.8	nd	32.0	5.02	2.61	nd
SCHAD II								
pH optimum	25.7	1.43	0.927	30.6	85.2	0.29	0.058	42.3
pH 8.0	31.5	1.28	0.677	nd	38.6	0.13	0.058	nd

K_M values in µM, k_{cat} values in $10^3 min^{-1}$ and k_{cat}/K_M in $10^6 s^{-1}M^{-1}$. Values shown are the average of 3-5 experiments. Abbreviation: nd = not determined.

Judged from these kinetic values the SCHAD I enzyme is the more efficient of these two enzymes performing both the dehydrogenase and the reductase reaction. SCHAD I is around 50-fold more efficient in both reactions at pH 8.0, the pH in mitochondrial matrix (Llopis et al., 1998). This is in agreement with a report where SCHAD I is mutated in a patient with SCHAD deficiency where the mutation C773T corresponding to Pro 246 exchanged to a Leu in mature protein. The resulting mutant protein when it is cloned shows no activity with acetoacetyl CoA as substrate. The short chain 3-hydroxyacyl-CoA activity in the patient was less than 5% in fibroblast mitochondria (Clayton et al., 2001). This residual activity in the patient could very well correspond to SCHAD II activity in the patient.

Overall, this suggests that SCHAD I is the main enzyme in the third step of the β-oxidation in human and that there are proteins binding to SCHAD I that are responsible for SCHAD deficiency.

4 CONCLUSIONS

4.1 General architecture of SDR enzymes

- Residues directly involved in the reaction mechanism are Tyr 151, Lys 155 and Ser 138. Moreover, data obtained with Asn 111 point to essential interactions with the active site residues, thus extending the known catalytic triad to a tetrad of Asn 111, Ser 138, Tyr 151 and Lys 155.
- Mutations in the coenzyme binding region demonstrate the importance of the central β-sheet and Asp 60 for stabilising the position and orientation of the coenzyme in SDR proteins as well as for the reaction direction.
- Mutation data from less conserved regions (substrate binding, Asn 179) highlight the important structural interactions between the active site and the substrate binding region *via* a connecting well-ordered water molecule.

Table 4.1 summarise the conserved amino acid residues in SDR enzymes and the role of these residues. It demonstrates further motifs within this enzyme family that are not covered in this thesis.

4.2 Comparison of human and *Drosophila* 17β-HSD10/SCHAD II

The enzymatic profiles of these two enzymes are similar. They both catalyse estrogens, androgens and short chain hydroxy-fattyacyl-CoAs. However, their subcellular localisation differs in such a way that the human enzyme is mitochondrial and the *Drosophila* enzyme is cytosolic. It is the shorter N-terminal signal sequence of the human enzyme that directs it to the mitochondrial compartment.

Table 4.1. Suggested mechanism of conserved amino acid residues in SDR enzymes.

	Position	Residue	Region	Suggested mechanism
*	12	T, S	cofactor binding	H-bond to backbone amide Asn 87
	13[a]	G	cofactor binding	βA/αB positioning: Rossmann fold
*	16[b-c]	S, R, K	cofactor specificity	interaction with 2'phosphate of coenzyme
	17[d]	G	cofactor binding	βA/αB positioning: Rossmann fold
	19[d]	G	cofactor binding	βA/αB positioning: Rossmann fold
	37[b-c, e]	D	cofactor specificity	interaction with 2'phosphate of NADP(H)
	38[b-c, e]	R, K	cofactor specificity	interaction with 2'phosphate of NADP(H)
*	60	D	between βC and αD	βC orientation?
*	86	N	cofactor binding	βD/αE positioning: Rossmann-fold
*	87	N	cofactor binding	βD/αE positioning: Rossmann-fold
*	88	A	cofactor binding	βD/αE positioning: Rossmann-fold
	89	G	cofactor binding	βD/αE positioning: Rossmann-fold
*	111[f]	N	catalytic site	H-bonds to active site H_2O; proximity to conserved Ser 154
*	138[g-h]	S	catalytic triad	H-bonds to substrate
*	*148*	*Y*	*catalytic region*	*active site cavity location*
*	151[i-k]	Y	catalytic triad	catalytic base
	154[l]	S	active site	proximity to conserved Asn 111
	155[b-c, e-f, m-p]	K	catalytic triad	lowering of Tyr pK_a; H-bond to NAD ribose
*	179	N	substrate binding	maintenance of SDR scaffold
*	182	H	end of βF	*connecting substrate interaction area and active site*
	183-184[c, e]	P-G	end of βF	substrate binding region; close to the nicotinamide moiety of the coenzyme
	188[b-c, q]	T	loop after βF	H-bond to NAD carboxamide, hinge region

Position numbering refers to 3β/17β-HSD. Residues given indicate the most frequent residues determined at that position. Asterics indicate residues studied in this thesis. Italics highlight residues which are not conserved but important in respect to catalysis for 3β/17β-HSD. [a]Chen et al., 1990; [b]Andersson et al., 1996; [c]Tanaka et al., 1996a; [d]Lesk, 1995; [e]Tanaka et al., 1996b; [f]Benach et al., 1998; 1999; [g]Cols et al., 1993; [h]Ensor and Tai,1996; [i]Persson et al., 1991; [j]Jörnvall et al., 1999; [k]Obeid and White,1992; [l]Obeyesekere et al., 1995; [m]Auerbach el al., 1997; [n]Ghosh et al., 1991; [o]Ghosh et al., 1994; [p]Varughese et al., 1992; [q]Zhou & Tai, 1999.

4.3 Comparison of SCHAD I and SCHAD II, and their role in β-oxidation and SCHAD deficiency

All the patients investigated showed a clearly defined biochemical defect in the third reaction of mitochondrial β-oxidation of fatty acids. Surprisingly, no genetic defects in the coding regions of the type 2 SCHAD gene could be detected. Sequence analysis of the type 1 gene in the same patients revealed no mutational defects either that could be responsible for SCHAD deficiency. At least four patients express SCHAD II as detected by Western blot, whereas the remaining two allow no conclusion due to proteolytic degradation.
Both SCHAD I and II are expressed in liver in healthy and SCHAD deficient patients. Thus the following conclusions can be drawn:

- the underlying defect in fatal SCHAD deficiency is probably not due to either lack of expression or presumably, lack of activity of SCHAD I or II.
- the block in β-oxidation at the level of hydroxyacyl-CoA dehydrogenase could be the consequence of defects in regulatory factors/protein interactions of SCHAD binding proteins.
- a thus far undescribed and mutated type 3 SCHAD in liver could be the cause of this disease.

Based on the kinetic comparison of human SCHAD I and II together with the report from Clayton et al, 2001 where the SCHAD I mutation decreased the mitochondrial fibroblast SCHAD activity to less than 5 % the following conclusion can be drawn:

- SCHAD I is the main protein involved in the third step in β-oxidation for short chain fattyacyl-CoA in human.

5 FUTURE PERSPECTIVES

General information about the structure – function relationships of proteins in the SDR family will provide new facts regarding mechanisms, protein – protein interactions, design of novel inhibitors, and determination of substrate specificities. This is of great importance because of the many and variable functions of enzymes in the SDR family and their involvement in metabolism and diseases.

Regarding the human enzyme 17β-HSD10/SCHAD II, its involvement in steroid metabolism and β-oxidation need to be studied further to be able to explain its role in these pathways, in diseases like SCHAD deficiency and Alzheimer's disease. Questions that should be answered are which proteins interact with each other? How relevant is the interaction for the pathway, disease etc.? Where does the interaction take place? Moreover, species variants and knockout models of the enzyme would be of great interest to study. Why are the *Drosophila* enzyme and the human enzyme located in different organelles? How is this regulated? Which sequence region is important for import? Do the enzymes have further substrates/inhibitors?

Concerning SCHAD deficiency a knockout model of SCHAD I would make interpretations regarding this disease easier. Furthermore, the indications of other factors than mutations in the two known SCHAD isozymes responsible for the observed phenotypes in the patient studied in this thesis need to be clarified. The interaction between SCHAD I and glutamate dehydrogenase type I needs to be determined. Other binding proteins need to be identified and their role clarified. Are there activating factors or inhibiting factors binding to SCHAD I?

Concurrently with the growth of the SDR family and increased knowledge, new tools for interpretations are developed. This together with knowledge about other pathways will help to define the role of proteins in pathological diseases and cancer forms and will have impact on treatment of these, since SDR enzymes play central roles in steroid hormone synthesis, steroid hormone-dependent cancer forms, regulation of blood pressure and intermediary metabolism.

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