Article

Molecular Mechanism Study on Stereo-Selectivity of α or β Hydroxysteroid Dehydrogenases

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Abstract: Hydroxysteroid dehydrogenases (HSDHs) are from two superfamilies of short-chain dehydrogenase (SDR) and aldo-keto reductase (AKR). The HSDHs were summarized and classified according to their structural and functional differences. A typical pair of enzymes, 7 α -hydroxysteroid dehydrogenase (7 α -HSDH) and 7 β -hydroxysteroid dehydrogenase (7 β -HSDH), have been reported before. Molecular docking of 7-keto-lithocholic acid (7-KLA) to the binary of 7 β -HSDH and nicotinamide adenine dinucleotide phosphate (NADP⁺) was realized via YASARA, and a possible binding model of 7 β -HSDH and 7-KLA was obtained. The α side of 7-KLA towards NADP⁺ in 7 β -HSDH, while the β side of 7-KLA towards nicotinamide adenine dinucleotide (NAD⁺) in 7 α -HSDH, made the orientations of C7-OH different in products. The interaction between Ser193 and pyrophosphate of NAD(P)⁺ [Ser193-OG...3.11Å...O1N-PN] caused the upturning of PN-phosphate group, which formed a barrier with the side chain of His95 to make 7-KLA only able to bind to 7 β -HSDH with α side towards nicotinamide of NADP⁺. A possible interaction of Tyr253 and C24 of 7-KLA may contribute to the formation of substrate binding orientation in 7 β -HSDH. The results of sequence alignment showed the conservation of His95, Ser193, and Tyr253 in 7 β -HSDHs, exhibiting a significant difference to 7 α -HSDHs. The molecular docking of other two enzymes, 17 β -HSDH from the SDR superfamily and 3(17) α -HSDH from the AKR superfamily, has furtherly verified that the stereospecificity of HSDHs was related to the substrate binding orientation.

Keywords: hydroxysteroid dehydrogenases; short-chain dehydrogenase superfamily; aldo-keto reductases superfamily; stereospecificity

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1. Introduction

Hydroxysteroid dehydrogenases (HSDHs) are a type of nicotinamide adenine dinucleotide (phosphate) NAD(H)/NADP(H)-dependent oxidoreductase that can specifically catalyze the reduction of carbonyl to hydroxy of steroid-skeleton structures such as steroid hormones and bile acids, in addition to a reversible reaction. All HSDHs can be divided into two different protein superfamilies—the short-chain dehydrogenase/reductase (SDR) superfamily and aldo-keto reductase (AKR) superfamily [1,2]. A variety of HSDHs from different species have been reported. Most of them belong to the SDR superfamily, such as 7 α -HSDH (EC 1.1.1.159), 7 β -HSDH (EC 1.1.1.201), and 11 β -HSDH (EC 1.1.1.146), which have been identified in *Escherichia coli*, *Collinsella aerofaciens*, and *Homo sapiens*, respectively [3–5], usually with a length of 250–350 amino acids [6]. They all have a core composed of seven parallel β -sheets and several α -helices wraps around the core (Figure 1), which form two typical domains named Rossmann fold (each composed of two $\beta\alpha\beta$ units) for coenzyme binding [6,7]. Aldo-keto reductases (AKRs) catalyze the carbonyl reduction reaction with a wide range of substrate selectivity, including sugar aldehydes, keto-steroids, keto-prostaglandins, and retinals [8]. These enzymes are usually with a length of 320 residues on average, forming an (α/β)₈-barrel fold [8,9]. According to the differences of sources, HSDHs such as 3 α -HSDH (EC 1.1.1.145), 17 α -HSDH (EC

1.1.1.148) and 17 β -HSDH (EC 1.1.1.51, EC 1.1.1.357, EC 1.1.1.270, etc.) usually belong to two superfamilies. For example, 3 α -HSDHs from *Comamonas testosteron* [10] and *Pseudomonas* sp. [11] belong to the SDR superfamily, while from *Rattus norvegicus* [12] and *Homo sapiens* [13] belong to the AKR superfamily (Figure 2).

Figure 1. Comparison of structures (A,C) and folding topologies (B,D) of hydroxysteroid dehydrogenases (HSDHs) from short-chain dehydrogenase/reductase (SDR) superfamily (7 β -HSDH(PDB:5GT9)) and aldo-keto reductase (AKR) superfamily (3 α -HSDH(PDB:4L1X)). α -helices are represented as cylinders (in red) and β -sheets are represented as arrows (in blue). Several important loops related to coenzyme or substrate binding between α -helices and β -sheets are labeled, which are loop1–6 between β (A–F)– α (A–F) in 7 β -HSDH and loop1 (β A– α A), loop2 (β G– α G1) and loop3 (β H– α H1) in 3 α -HSDH. Figures A and C were created by Pymol [14].

This article reviews the HSDHs from SDR and AKR superfamilies that were identified in mammals and bacteria and then classifies the HSDHs according to their functions in steroid hormone metabolism or bile acid biosynthesis. The differences of NAD(P)(H)-binding models to enzymes and enzymatic reaction characteristics between SDR and AKR superfamilies were also summarized. A semi-flexible molecular docking was used for further exploration of differences of substrate binding model to enzyme–coenzyme complex between SDR and AKR superfamilies, in addition to the reasons of stereospecificity of substrate or product in HSDHs.

Figure 2. Classification of common HSDHs from SDR and AKR superfamilies according to their source differences. HSDHs related to secondary bile acids biosynthesis are mainly from bacteria, existing in the SDR superfamily, whereas HSDHs related to steroid hormone biosynthesis are mainly from mammals, existing in both SDR and AKR superfamilies. More detailed information is shown in Table S3.

2. Classification and Functions of HSDHs

HSDHs can be classified according to their structural or functional differences. From a structural perspective, HSDHs belong to two protein superfamilies—short-chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR) superfamily [1,2]. Most SDRs are active as dimers or tetramers. They usually have a core composed of seven parallel β -sheets, with several α -helices wrapped around, which form two typical domains named Rossmann fold (each composed of two $\beta\alpha\beta$ units) for coenzyme binding [7]. In contrast, most AKRs are soluble, monomeric proteins and have an architecture of $(\alpha/\beta)_8$ -barrel fold called TIM-barrel, which was named after a prototypical member of AKRs, triosephosphate isomerase (TIM) [15,16].

All HSDHs can specifically catalyze the redox of steroid-skeleton substrates, such as bile acids and a series of steroid hormones [17,18]. According to the differences of reacting positions and hydroxyl orientations of substrates or products, HSDHs can be divided into 11 types at least, which are 3α -HSDH (EC 1.1.1.50, EC 1.1.1.51, EC 1.1.1.53, etc.) and 3β -HSDH (EC 1.1.1.145, EC 1.1.1.270, EC 1.1.1.391, etc.), 7α -HSDH (EC 1.1.1.159) and 7β -HSDH (EC 1.1.1.201), 11β -HSDH (EC 1.1.1.146), 12α -HSDH (EC 1.1.1.176) and 12β -HSDH (EC 1.1.1.238), 17α -HSDH (EC 1.1.1.148) and 17β -HSDH (EC 1.1.1.51, EC 1.1.1.357, EC 1.1.1.270, etc.) and 20α -HSDH (EC 1.1.1.149) and 20β -HSDH (EC 1.1.1.53), catalyzing the reduction of carbonyl on C3, C7, C11, C12, C17, and C20 of steroid-backbone substrates (producing hydroxyl with an α or β orientation) and their reverse reactions, respectively. A part of HSDHs only exists in SDR or AKR superfamilies (Table S1 and Table S2). All 7α -HSDHs, 7β -HSDHs, and 11β -HSDHs were reported to exist in the SDR superfamily, while most 17α -HSDHs and 20α -HSDHs belong to AKRs. However, enzymes such as $3\alpha/\beta$ -HSDHs and 17β -HSDHs could be found in both superfamilies. Numerous 3α -HSDHs of AKRs have functions of 17α -HSDH, 17β -HSDH or 20α -HSDH [12,19]. In particular, 3α -HSDHs (AKR1C31, AKR1C32, AKR1C33) from *Oryctolagus cuniculus* (rabbit) have other two functions of 17β -HSDH and 20α -HSDH [20].

2.1. Characteristics and Functions of HSDHs from SDRs

Consisted of more than 163,120 members [21], SDR enzymes are found in different classes of oxidoreductases, isomerases, and lyases [21,22], with multiple functions

such as carbonyl–alcohol oxidoreduction, steroid isomerase, enoyl–CoA reduction, decarboxylase, dehalogenase, dehydratase, C=N reduction and epimerase [22–24], covering broad substrates such as sugars, steroids, retinoids, lipids, polyols, prostaglandins and androstenedione [24,25]. SDRs could be further classified into six types at least according to their sequence differences, i.e., “Classical,” “Extended,” “Atypical,” “Intermediate,” “Divergent,” “Complex” and some members “Unassigned” (Table 1) [21,22,26]. Sequence alignment was used to analyze structural differences among SDR–HSDHs (HSDHs from SDR superfamily), demonstrating that most SDR–HSDHs are of the “Classical” type except 3 β –HSDH, which is more similar to the “Intermediate” type (Table 1, Figure S1). Even though SDR–HSDHs share a low sequence identity of 23–53% (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=3AGHXAGR114> (accessed on 29 January 2021)), all SDR–HSDHs are conservative in structures which composed of a core of Rossmann fold, with several α –helices wrapped around it (Figure 1). SDR–HSDHs are mainly able to catalyze the dehydrogenation/hydrogenation of substrates with steroid–skeleton, participating in the pathways such as steroid hormone biosynthesis, steroid degradation, and secondary bile acid biosynthesis in organisms (Table S3) [27].

Table 1. Features of six subfamilies of the SDR superfamily.

HSDHs	Monomer Size	Coenzymes	Catalytic Reaction	Coenzyme Binding	Active Site	Sequence Identity
Divergent		NAD(H)	Enoyl reductases	GxxxxSxA	YxxMxxxK	28–99%
Classical	~250 aa	NAD(P)(H)	Oxidation/reduction	TGxxxGxG	YxxxK	8–99%
Intermediate	~250 aa		Drosophila ADH	G/AxxGxxG/A		27–99%
Extended	~350 aa	NAD(P)(H)	Epimerases Dehydratases	TGxxGxxG		10–99%
Complex		NADP(H)	β –ketoacyl reduction		YxxxN	20–74%
Atypical			NmrA-like		Lacks catalytic Tyr	

“x” signalizes any amino acid residues.

2.1.1. HSDHs in Primary or Secondary Bile Acid Biosynthesis

Bile acids serve significant roles in cholesterol metabolism and the digestion of lipids for mammals [28]. The composition of bile acids pools is different in different species. For example, the human bile acids pool is usually composed of hydrophobic bile acids cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA), whereas the mice bile acids pool mainly consists of hydrophilic bile acids, muricholic acids(β –MCA) and ursodeoxycholic acid (UDCA) [17,29,30]. All these bile acids can be divided into two forms–primary bile acids (CA and CDCA) and secondary bile acids (DCA, LCA, UDCA, etc.), whose production is related to the activities of the liver and intestinal microbiota, respectively [31].

A number of SDR–HSDHs related to secondary bile acid synthesis were found in intestinal bacteria. The metabolic pathway of secondary bile acids was shown in Figure 3. 3 α –HSDHs found in species *Pseudomonas* sp. [11], *Comamonas testosterone* [32], *Corynebacterium glutamicum*, etc. [33] are able to catalyze the hydrogenation/dehydrogenation of carbonyl and hydroxyl on C3 position of bile acids, participating in the conversion of deoxycholate and lithocholate to isodeoxycholate and isolithocholate (<https://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-7755> (accessed on 29 January 2021)). A pair of epimerases, 7 α –HSDH and 7 β –HSDH, could specifically catalyze the reduction of C7=O or the oxidation of C7–OH on steroid–skeleton [34,35], with a substrate or product of CDCA(7 α –OH) and UDCA(7 β –OH). These two epimerases work together for the conversion of CDCA to UDCA in industry, with an intermediate 7–keto–lithocholic acid (7–KLA). UDCA has significant pharmaceutical applications in the treatment of gallstones, biliary cirrhosis, and even liver cancers [36].

Figure 3. HSDHs-related bile acids synthesis and metabolism. Primary bile acids (cholic acid (CA) and chenodeoxycholic acid (CDCA)) in the liver are usually conjugated with glycine (G), which can be deconjugated by bile salt hydrolases (BSH). Primary bile acids can be biotransformed to secondary bile acids (deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA), etc.) through the 7 α -dehydroxylation pathway and the oxidation and epimerization of 3 α -, 7 α - and 12 α -HSDHs.

2.1.2. HSDHs in Steroid Hormone Biosynthesis

3 β -HSDHs (EC 1.1.1.145) have been identified in *Homo sapiens* (type 1–2), *Mus musculus* (type 1–6), *Rattus norvegicus* (type 1–4), *Mycobacterium tuberculosis*, etc., usually with activities of dehydrogenase and isomerase (Δ^5 – Δ^4) [37]. Both Human type 1 3 β -HSD1/isomerase and type 2 3 β -HSD2/isomerase are NAD(H)-dependent enzymes, catalyzing the conversion of 3 β -hydroxy-5-ene-steroids to 3-oxo-4-ene-steroids with two sequential enzymatic reactions. Firstly, 3 β -HSDH catalyzes the dehydrogenation of 3 β -hydroxysteroid to a 3-oxo intermediate, and then the Δ^5 -form intermediate was isomerized to Δ^4 -form via their isomerase activity (Figure 4). This catalytic function of 3 β -HSDH is usually related to the conversion of dehydroepiandrosterone (DHEA) to androstenedione, 17 α -hydroxypregnenolone to 17 α -hydroxyprogesterone and pregnenolone to progesterone [38–40]. In humans, 3 β -HSD1 (type 1) and 3 β -HSD2 (type 2) are encoded by two distinct genes which are expressed in a tissue-specific pattern [41]. 3 β -HSD1 is expressed in peripheral tissues such as breast, placenta, prostate, and endometrium, with roles of production of some downstream steroids such as testosterone, dihydrotestosterone (DHT), and estrogens [42]. The testosterone and estradiol will promote the growth of breast tumors [43] and prostate tumors [44]; therefore, human 3 β -HSD1 is a potential target for the inhibition of estradiol production in breast tumors and testosterone production in prostate tumors. 3 β -HSD2 is expressed in adrenals, ovaries, and testis, which are related to the synthesis of steroid precursors required for the production of cortisol, aldosterone, and the female and male sex hormones [17,29,31].

11 β -HSDHs (EC 1.1.1.146) in humans can be generally divided into two types, 11 β -HSD1 and 11 β -HSD2, working together on glucocorticoid metabolism to regulate the availability of active glucocorticoids [45]. 11 β -HSD1 is NADP(H)-dependent enzyme, facilitating the conversion of inactive glucocorticoids, cortisone in human and 11-deoxycorticosterone in rodents, to their active 11-keto metabolites, cortisol, and corticosterone, respectively [46] (Figure 5). 11 β -HSD1 is mainly expressed in liver, adipose tissues, brain and skeletal muscles [47], whose function associated with insulin resistance, dyslipidemia, obesity, hypertension, and other features of mineralocorticoid excess; hence, the

inhibition of 11 β -HSD1 is a potential method for the treatment of glucocorticoid (active)-related disorders [48,49]. 11 β -HSD2 was found primarily in mineralocorticoid target tissues, such as kidneys, sweat glands, salivary glands, and colonic mucosa [50], catalyzing the conversion of glucocorticoids to inactive 11-ketosteroids in a NAD(H)-dependent activity, in contrast to 11 β -HSD1 [51]. Another type of 11 β -HSDH, 11 β -HSD3, has been identified in zebrafish, fathead minnow, and some paralogous amphioxus [52].

Figure 4. 3 β -HSDHs (3 β -HSD1 and 3 β -HSD2) from the SDR superfamily have both activities of dehydrogenase and isomerase, catalyzing the oxidation of 3 β -hydroxy and the isomerization of Δ 5 to Δ 4-form.

Figure 5. Human 11 β -HSD1 from the SDR superfamily is able to catalyze the activation of glucocorticoids, while 11 β -HSD2 catalyzes a reverse reaction.

17 β -HSDHs are encoded by non-homologous genes with different subcellular localizations, coenzymes, and substrate preferences [53]. Up to now, over 14 different subtypes of 17 β -HSDHs (17 β -HSD1–14) have been identified, most of them belong to the SDR superfamily except 17 β -HSD5, which is a member of the AKR superfamily [54,55]. All 17 β -HSDHs are able to catalyze the reduced or oxidized reactions at position C17 of C18- or C19-steroids, involved in the activation and inactivation of steroid hormones (Figure 6) [54]. 17 β -HSD1 could catalyze the activation of estrone to estradiol in humans. However, the production of estradiol is linked to the development of diseases such as breast cancer, ovarian tumor, endometriosis, endometrial hyperplasia, and uterine leiomyoma [56]. It was demonstrated that estradiol can be converted to genotoxic metabolites in breast tissue. Consequently, the inhibition of 17 β -HSD1 is one of the treatments for breast cancer in clinical [57]. Meanwhile, the decreasing of estradiol will lead to osteoporosis. 17 β -HSD2 have opposing actions to 17 β -HSD1, catalyzing the inactivation of estradiol to estrone, which means the inhibition of 17 β -HSD2 is one of the therapies for osteoporosis [58]. Otherwise, 17 β -HSD1 could catalyze the conversion of dehydroepiandrosterone (DHEA) to Δ 5-androstane-3 β , 17 β -diol while 17 β -HSD2 catalyze a reverse reaction. Δ 5-androstane-3 β , 17 β -diol can be converted to estradiol with functions of 3 β -HSDH and aromatase [59]. 17 β -HSD3 exists predominantly in testis and catalyzes the conversion of Δ 4-androstenedione to testosterone. It was confirmed that its mRNA was overexpressed in prostate cancer tissue. Testosterone is responsible for cell proliferation in androgen-dependent diseases, and the inhibition of 17 β -HSD3 is effective for the treatment of diseases such as prostate cancer [60]. The functions of other subtypes of 17 β -HSDH were described in Table S3.

Figure 6. HSDHs-related steroid hormones synthesis and metabolism. “3βDH/iso” signals the dehydrogenase and isomerase activities of 3β-HSDH.

2.2. Characteristics and Functions of HSDHs from AKRs

Most aldo–keto reductases (AKRs) are soluble monomeric NAD(P)(H) oxidoreductases that catalyze a NAD(P)H-dependent reduction of aldehydes and ketones, yielding primary and secondary alcohols [61]. Over 190 members belonging to AKRs have been reported, with a high sequence identity of 40–60% [2,61,62]. The (α/β)₈-TIM barrel provides a common architecture for a NAD(P)(H)-dependent catalytic activity [63]. AKRs have broad substrate specificity, which was determined by the variation of loops on the C-terminal. Their functions were related to the metabolism of sugar, lipid aldehydes, steroids, and prostaglandins, etc. [61]. All AKRs can be further classified into 16 families (AKR1–AKR16) [61,62] and HSDHs reported from AKRs (AKR–HSDHs) belong to AKR1 [63]. Up to now, four isoforms of human 3α–HSDHs and one isoform of rat 3α–HSDH belonging to AKRs have been reported, which are AKR1C1 (human 3α(20α)–HSDH), AKR1C2 (human type 3), AKR1C3 (human type 2), AKR1C4 (human type 1) and AKR1C9 (rat liver), respectively [64–67]. The human type 2 3α–HSDH (AKR1C3) is also known as type 5 17β–HSDH, while type 3 (AKR1C2) is also known as bile acid-binding protein [64–66].

Human aldo–keto reductase AKR1C3 (type 2 3α–HSDH/type 5 17β–HSDH) is also known as prostaglandin F synthase [68] and possesses activities in the reduction of Δ 4–androstene-3,17-dione to testosterone, 5α–dihydrotestosterone(5α–DHT) to 3α– and 3β–androstenediol, and estrone to 17β–estradiol, respectively (Figure 6) [69], which is expressed in sex hormone-dependent tissues (testis, breast, endometrium, and prostate) and sex hormone-independent tissues (kidney and urothelium) [70], whose functions are associated with breast cancer, endometrial cancer and the development of endometriosis and dysmenorrhea [71]. AKR1C2 is another AKR1C isozyme that is highly expressed in the prostate and catalyzes the oxidation of 3α–androstenediol to the active hormone 5α–DHT, opposite to AKR1C3 [66,72].

AKR1C4 can work in concert with steroid 5β–reductase to produce the 5β,3α–tetrahydrocholestanes in the liver, play a pivotal role in the bile acid biosynthesis, and steroid hormone metabolism [66]. AKR1C1 is usually expressed in lung, liver, testis, mammary gland, endometrium, brain, kidney, adipose cells, skin, osteoblasts, and optic nerve head astrocytes [73,74], exhibiting both 3α/β– and 20α–HSD activities and mainly reducing progesterone and 5α–pregnan–3α–ol–20–one to their corresponding inactive metabolite 20a–hydroxyprogesterone with a function of 20-ketosteroid reductase [73,74]. Progesterone plays a crucial role in preventing the onset of premature birth, blunting the estrogenic

effects in endometriosis and endometrial cancer [75,76]. Thus, the inhibition of AKR1C1 is a potential agent for the treatment of these disorders [77,78].

3. Coenzyme Binding Modes of HSDHs

Nearly 150,000 different sequences were annotated as or predicted to be NAD(H)/NADP(H)-dependent oxidoreductases/reductases [79]. The binding model of NAD(H)/NADP(H) in the enzyme is significant for the proton transfer. Several structures were reported for coenzyme binding, which are Rossmann fold, $(\alpha/\beta)_8$ -TIM-barrel, dihydroquinoate synthase-like, and FAD/NAD-binding folds [79–81].

3.1. Coenzyme Binding Modes of the HSDHs from SDRs

The sequence alignment of HSDHs in the SDR superfamily shows a low sequence identity of 23–53% (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=3AGHXAGR114> (accessed on 29 January 2021)). However, all SDR-HSDHs share similar and conservative motifs for NAD(P)(H) binding. The basic structure of SDRs is composed of two Rossmann folds, which is stable and no loss of function occurs even with mutations at some sites of the folding and that is why SDRs retain similar catalytic functions even with such low sequence identities [82–84].

The binding sites of the adenosine ring of coenzyme to enzyme are on the loop1, loop2, and loop3 (Figure 1), close to the C-terminal part of SDRs, while the nicotinamide-binding region is on loop 4 and loop 6, close to the N-terminal part. A conserved glycine-rich pattern GxxxGxG between the first β -strand and α -helix is usually interacted with pyrophosphate [84–86]. The individual subfamilies of the SDR superfamily differ regarding the spacing of the three glycine residues [87]. For example, it was demonstrated that 3 β -HSDHs have a motif of X1-Gly-X2-X3-Gly-X4-X5-Gly (Figure S1), which is similar to the “Intermediate” type [88], while most SDR-HSDHs have a motif of X1-Gly-X2-X3-X4-Gly-X5-Gly and belong to the “Classical” type. In the meantime, the sequence alignment of SDR-HSDHs from different sources showed that the residue X1 in 7 β -HSDHs is L/C, which is obviously different from the residue T/S in other SDR-HSDHs (Figure S1). The features among different subfamilies of SDRs were described in Table 1. The motif YxxxK on α E-helix has two functions of coenzyme binding and catalysis [89], showing differences among different types of SDRs as well, which will be described in detail below.

There is no doubt that NAD(H)/NADP(H) can act as a proton donor or acceptor during redox reactions [90] and can participate in the remodeling of the substrate-binding pocket. The common belief is that the broad substrate specificity of HSDHs is related to the substrate-binding loop in previous studies, but are the interactions between NAD(P)(H) and the enzyme also responsible for the substrate specificity, especially the stereospecificity? The distance between C6 of adenine and C2 of nicotinamide in a syn conformation is frequently used to express the degree of extension of coenzyme molecule [91], which is 13.4 Å, 13.7 Å, 14.1 Å, and 14.3 Å in 7 α -HSDH (PDB: 1FMC) [92], 7 β -HSDH (PDB: 5GT9) [93], 11 β -HSDH (PDB: 3G49) and 17 β -HSDH (PDB: 3QWH), respectively, showing little difference (Figure 7). The dihedral angles of PA–O3–PN–O5D (pyrophosphate group), C4B–C2B–C1B–N9A (adenosine ring), and C4D–C2D–C2N–C5N (nicotinamide ring) were used for a further study on conformation change of NAD(P)(H) (Table 2). In the pyrophosphate binding region, Ile23 and Thr194 of 7 α -HSDH, Val20 and Thr191 of 7 β -HSDH, Ile21 and Thr195 of 11 β -HSDH, in addition to Ile30 and Thr202 of 3 α (17 β)-HSDH, are equivalent in function. However, the dihedral angle PA–O3–PN–O5D (−80.6°) in the 7 β -HSDH binary is apparently different compared with others, which may lead to the difference of substrate binding pocket. Another crystal structure of 7 α -HSDH from *Clostridium absonum* was obtained as a tetramer in the study by Lou et al., which exhibited a coenzyme-dependent of NADP(H) [94]. The dihedral angle PA–O3–PN–O5D in the pyrophosphate region is −143.4° (Figure 8), which is similar to 7 α -HSDH–NAD⁺ complex (−147.8°) from *E. coli*, although the coenzymes of these two crystals are completely different. Both of these two dihedral angles PA–O3–PN–O5D are different from 7 β -HSDH–NADP⁺ complex (−80.6°),

and this is probably able to prove that the stereospecificity of substrate/product is related to the coenzyme conformation.

Figure 7. Residues that interacted with NAD(P)(H) in the SDR superfamily, in which the residues in yellow, green, and red color interact with the adenosine region, pyrophosphate region, and nicotinamide region of coenzyme, respectively. The distance between C6 of adenine and C2 of nicotinamide and the dihedral angles of PA–O3–PN–O5D (pyrophosphate group), C4B–C2B–C1B–N9A (adenosine ring), and C4D–C2D–C2N–C5N (nicotinamide ring) in blue color are also shown. This figure was created by Pymol [14].

Table 2. Residues that interacted with NAD(P)(H) in the SDR superfamily and the NAD(P)(H)-conformation differences among them.

HSDHs	Interaction between NAD(P)(H) and HSDHs			Distance/Å	Dihedral Angle/°		
	Adenosine Ring	Pyrophosphate	Nicotinamide Ring		A	B	C
7α-HSDH	Adenine: Asp68, Ile69	Ile23, Thr194	Nicotinamide: Ile192, Thr194; Ribose: Tyr159, Lys163	13.4	138.7	47.8	116.5
7β-HSDH	Adenine: Phe67, Asp66; Ribose: Thr17, Glu18, Arg40, Arg41	Val20, Thr191, Ser193	Nicotinamide: Thr189, Thr191; Ribose: Tyr156, Lys160	13.7	143.4	80.6	146.2
11β-HSDH	Adenine: Met68; Ribose: Ser18, Arg41, Ser42	Ile21, Thr197	Nicotinamide: Ile193, Thr195; Ribose: Tyr158, Lys162	14.1	134.6	156.5	142.6
3α(17β)-HSDH	Adenine: Arg78, Asp76, Ile77, Ser104; Ribose: Gly25, Gly27, Arg28, Ala50, Asn51, Ser52	Arg28, Ile30, Thr202, Asp203	Nicotinamide: Thr200, Thr202; Ribose: Asn103, Tyr167, Lys171	14.3	143.7	130.6	125.6

Dihedral angles A, B and C represents PA–O3–PN–O5D (pyrophosphate group), C4B–C2B–C1B–N9A (adenosine ring), and C4D–C2D–C2N–C5N (nicotinamide ring), respectively.

Figure 8. The structural alignment (A) of 7 α -HSDH (PDB: 5EPO) and 7 β -HSDH (PDB: 5GT9) and the NADP(H) conformation differences between them (dihedral angles of PA–O3–PN–O5D of pyrophosphate group) (B). Figure (C) exhibits the NADP(H)-interacted residues in the 7 α -HSDH complex. This figure was created by Pymol [14].

The pyrophosphate-interacted residues, Ser193 in 7 β -HSDH, Thr197 in 11 β -HSDH and Ser11 in 3 α (17 β)-HSDH, are quite special compared with 7 α -HSDH ternary. The interaction [Thr194–OG1 $\cdots$ 2.79 Å $\cdots$ O1N–PN] in 7 α -HSDH ternary was replaced by [Ser193–OG $\cdots$ 3.11 Å $\cdots$ O1N–PN] in 7 β -HSDH, leading to a rotation of the phosphate group PN to the outside, which made the coenzyme conformation different (Figure 7).

In the adenosine ring binding region, the existence of O2' ribose phosphate group in NADP(H) makes the interactions between the adenosine ribose region and β -HSDHs complicated. A hydrogen-bond network between coenzyme, residues, and soluble molecules maintains the adenosine ribose conformation of NADP⁺ in binary or ternary complexes.

These residues in 7 β -HSDH are Arg40–Arg41, in 11 β -HSDH are Arg41–Ser42, and in 3 α (17 β)-HSDH are Ala50–Asn51–Ser52 (Table 2). In the nicotinamide ring binding region, the interactions between nicotinamide ribose and residues seem to be conserved. Motifs Tyr–X–X–Lys [89–94] and Thr/Ile–X–Thr have conserved interactions with NAD(P)(H) (Figure 7 and Table 2). The hydrogen bonds existed among PN–phosphate group of pyrophosphate region, nicotinamide, and Thr contribute to maintaining the orientation of pro-S side of nicotinamide towards catalytic sites, for example, the interactions [Thr189–N $\cdots$ 2.9 Å $\cdots$ O7N–Nicotinamide], [Thr189–O $\cdots$ 3.2 Å $\cdots$ N7N–Nicotinamide], [Thr189–O $\cdots$ 2.9 Å $\cdots$ N–Thr191], [Thr191–OG1 $\cdots$ 3.0 Å $\cdots$ N7N–Nicotinamide], [Thr191–OG1 $\cdots$ 2.7 Å $\cdots$ O2N–PN], and [Nicotinamide–O7N $\cdots$ 2.9 Å $\cdots$ O2N–PN] in the holo-form of 7 β -HSDH, which making the happen of reaction possible.

3.2. NAD(H)/NADP(H) Binding Modes of the HSDHs from AKRs

HSDHs from AKRs (AKR–HSDHs) are not only conserved in structure, but also share a high sequence identity of 64–93% (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=3AH34HGD114> (accessed on 29 January 2021)). The basic structure of AKRs is composed of eight β -sheets. At least one α -helix exists between every two β -sheets, which surround the barrel around outside [95,96]. This structure was named after TIM-barrel fold, consisted of an eightfold repeat of (α/β) units ((α/β)₈), was first seen in triosephosphate isomerase (TIM) [97,98].

Loops between β -sheets and α -helices are used for the binding of the coenzyme and substrate. In contrast with SDR–HSDHs, the residues that interacted with NADP(H) adenosine are closer to the N-terminal, whereas residues that interacted with NADP(H) nicotinamide are closer to the C-terminal [99]. The NAD(P)H binds AKRs in an extended anti-conformation [61], stretching through the channel formed by Van der Waals con-

tacts [100], which composed of loop 1(β A– α A, residues 22–31), loop 2(β G– α G, residues 216–235), and loop 3(β H– α H, residues 269–274) (Figure 1).

The interactions between AKR–HSDHs and coenzymes in the binary or ternary structures of 3 α –HSDH (PDB: 1J96), 3(17) α –HSDH (PDB: 2P5N), 17 β –HSDH (PDB: 1ZQ5), and 20 α –HSDH (PDB: 1Q5M) were analyzed using Pymol (distributed by Schrödinger, New York, NY, US) (Figure 9) [14], to explore the conformation changes of NADP(H) after binding to the enzyme and whether these changes are conservative in all AKR–HSDHs. The conformation of NADP(H) is highly conserved in AKRs, showing little difference. The distance between C6 of adenine and C2 of nicotinamide is 17.0 Å, which means the occurrence of structural extension of NADPH (15.6 Å) due to the interactions between residues and coenzyme, such as hydrogen bonds, π – π interactions, and hydrophobic interactions [101], and it is definitely different from SDR–HSDHs, which is roughly 14 Å. Other structural changes and interacted residues are described in Table S4, which demonstrated high similarity and conservation in 3 α –HSDH, 3(17) α –HSDH, 3 α (17 β)–HSDH, 17 β –HSDH, and 20 α –HSDH.

Figure 9. Residues that interacted with NAD(P)(H) in AKR superfamily, in which the residues in green, red, and yellow color interact with the adenosine region, pyrophosphate region, and nicotinamide region, respectively. The distance between C6 of adenine and C2 of nicotinamide and the dihedral angles of PA–O3–PN–O5D (pyrophosphate group), C4B–C2B–C1B–N9A (adenosine ring), and C4D–C2D–C2N–C5N (nicotinamide ring) in blue color are also shown. This figure was created by Pymol [14].

Taking the 3 α –HSDH holo-form (PDB: 1J96), for example, the adenine ribose group in AKR–HSDHs is embedded between α G1-helix and α H-helix, interacted with residues of Ala253 from α G1-helix, Arg276, and Gln279 from α H-helix, while the O2' ribose phosphate group was contained in the cavity formed by Lys70–Ser271–Tyr272 on the loop 1 and Arg276 on α H-helix. These residues and the water molecules formed a hydrogen bond network with the adenosine ribose group, which helps to fix the adenosine ribose group at the N-terminal. All these interactions are highly conserved in AKR–HSDHs, especially the interactions between Ala253–N...3.1Å...H2O...3.1Å...N1A–adenosine] and [Arg276–NH2...3.0Å...O2X–Phosphate group] [102–104].

The pyrophosphate group crosses through the tunnel composed of loop 1, loop 2, and loop 3, connecting the adenosine ribose at the outside of the barrel and nicotinamide ribose

in the center. On the inner wall of the tunnel, the residues Ser217–Ala218–Leu219–Ser221 on loop 2, Leu268, Lys270 on loop 3, and Tyr24 on loop 1 formed a hydrogen bond network with water molecules and pyrophosphate groups and maintained the conformation of this region (Figure 9). Among them, Ser/Thr217–Ala218–Leu219 and Leu268 are highly conserved. The residue Tyr24 has hydrogen-bond linkage to nicotinamide ribose while Leu219 has hydrogen-bond linkage to adenosine ribose, respectively.

4. Catalytic Mechanism of HSDHs

4.1. Catalytic Mechanism of HSDHs from SDRs

It is recognized that the motif Tyr–X–X–X–Lys is associated with the catalytic function of SDRs. This conserved motif attracted much attention as early as 1981 [26,105], and its function was reported in the studies of 3 α (20 β)–HSDH from *Streptomyces hydrogenans* [106] and 7 α –HSDH from *E. coli* [92]. However, the conservative motif shows differences in different types of SDRs. The most common motif Tyr–X–X–X–Lys exists in “Classical” SDRs, while the motif in the “Divergent” type is Tyr–x–x–Met–x–x–Lys [26], the features of these six types of SDRs were shown in Table 1. Tyr is strictly conserved even in the whole SDR superfamily, while Lys and Ser are only conserved in most SDRs. With the assistance of Lys–NH₂ to decrease the pK_a of Tyr–OH, the side chain phenolic group of Tyr acts as a general acid/base catalyst. The hydride of the substrate is released to the pro-S side of the nicotinamide group of NAD(P)(H) in an oxidated reaction [107]. Apart from being a proton donor or acceptor, NAD(P)(H) plays a vital role in the formation and stability of the substrate-binding pocket. A strict binding order of substrate and coenzyme to drosophilid alcohol dehydrogenase (DADH) was reported [108]. DADH could convert alcohol to aldehyde/ketone. In an oxidated reaction, the coenzyme is bound to a free enzyme to form a binary complex firstly and then the substrate binds to the binary complex and the rate-limiting step is the release of the coenzyme product (NADH) [109–111]. This mechanism is similar to the “bi–bi” mechanism of AKRs in which coenzyme binds first and leaves last [16] and probably applies to all SDRs.

The catalytic mechanism (Figure 10) of SDRs was investigated through X-ray crystallographic structure determination of 3 α (20 β)–HSDH in a study by Ghosh et al. [106]. It was proposed that the catalytic function of 3 α (20 β)–HSDH was realized by a triad Ser–Tyr–Lys. Tyr–OH acts as a proton donor applying for an electrophilic attack on the 20-keto oxygen in the reduction of 20-keto [106]. Lys–NH₃⁺ is close to Tyr, facilitating the transformation of the proton. Ser participates in catalysis through direct interaction with Tyr–OH or just as part of a proton-relay network [106]. Tanaka et al. proposed a three-step catalytic mechanism for 7 α –HSDH and confirmed it through the mutation of Tyr159, Lys163, and Ser146 [92]. Firstly, the Lys163 binds to NAD(H) and lowers the pK_a value of Tyr159 and then Tyr159 was deprotonated and interacted with the C7 position of 7-oxoglycochenodeoxycholic acid (7-oxo–GCDCA), the intermediate was stabilized by Ser146 with a hydrogen bond to C7–O. Secondly, the deprotonated Tyr acts as a catalytic base and extracts the hydrogen from C7–OH. NAD⁺ accepts another hydrogen released from position C7 in the same time [92,112]. In the studies of DADH, the theoretical calculations suggested that the proton was released from a coupled irregular ionization of the active site groups Tyr151 and Lys155 instead of a single residue Tyr151 [91,108].

Other amino acid residues participating in the catalytic reactions were proposed. Lou et al. elaborated the mechanism in the study of 7 α –HSDH from *Clostridium absonum* [94], it was found that the serine in Ser–Tyr–Lys triad was replaced by threonine, without a complete activity loss of 7 α –HSDH. In the study of 3(20) β –HSDH, it was found that the Asp participated in the catalysis, which formed an active site tetrad Ser–Tyr–Lys–Asp.

Figure 10. The catalytic mechanism of HSDHs from the SDR superfamily.

4.2. Catalytic Mechanism of HSDHs from AKRs

All HSDHs catalyze the oxidations of hydroxyl to ketone/aldehyde on steroid-skeleton substrates or their reversible reaction, with the assistance of NAD(P)(H) and sharing a similar catalytic mechanism no matter in SDR or in AKR. However, unlike in SDRs, it was a tetrad Asp–Tyr–Lys–His instead of a triad Ser–Tyr–Lys play a catalytic role in AKRs. AKR–HSDHs accept 4-pro-R hydride from A-face of NADPH, while SDR–HSDHs accept 4-pro-S hydride from B-face NAD(P)H [61,113,114]. In the verification experiment of these sites, no activity of the enzyme was detected with a mutation of Y48F, while 1460-fold less active in K77M mutation and 8000-folds increasing of K_m in H110N mutation was observed in aldose reductase (EC 1.1.1.21) [115–117].

An ordered “bi–bi” kinetic mechanism of AKRs was proposed, in which coenzyme binds first and leaves last [16,118]. Cooper et al. described the mechanism detailed in the structure of rat 3α -HSDH (AKR1C9), which contains nine discrete steps in the kinetic mechanism and is associated with 16 rate constants. Firstly, the coenzyme binds to the enzyme and forms a loose structure of the coenzyme factor complex, and then the complex undergoes two conformational changes to make the binding tighter. It was found that an 86-fold and 110-fold increase of binding affinity for NADPH and NADP⁺ was realized after successive conformational changes, via the verification of steady-state, transient state kinetics, and kinetic isotope effects [103,117].

A “push–pull” catalytic mechanism was proposed to explain the reduced and oxidated reactions of AKRs (Figure 11) [119]. In a reduction direction at pH 6.0, the side chain amino group (–NH₂) of Lys was protonated, which was then interacted with the phenolic group (–OH) of Tyr. In the meantime, the imidazole group of His attracted hydrogen at 3-N position, which was also interacted with the phenolic group (–OH) of Tyr. These two interactions ultimately reduce the pK_a value of Tyr and promote the transfer of protons. Meanwhile, the Asp also participates in the reaction through a salt bridge to Lys. In an oxidation direction, the interaction between –COO[−] of Asp and –NH₂ of Lys promotes the abstraction of Tyr phenolate anion to hydrogen from the steroid alcohol [113–120].

Figure 11. The catalytic mechanism of HSDHs from the AKR superfamily.

5. Stereospecificity Study of HSDHs through Molecular Docking

In the reduction direction of HSDHs, the carbonyl is reduced to α -OH or β -OH. The stereoisomeric differences at different positions will lead to functional divergences of steroid molecules. For example, both UDCA and CDCA are reduction products of 7-keto-LCA (7-KLA), with an α -OH in CDCA and β -OH in UDCA. However, UDCA exhibits much better performance than CDCA in dissolving cholesterol, with less toxicity. In the process of UDCA synthesis with a substrate of CDCA, 7-KLA (C7=O) usually occurs as an intermediate. The two-step process including the oxidization of 7 α -OH firstly and then the reduction of 7-keto group [121–123]. Both reactions require NAD(P)(H) as a proton acceptor or donor to participate. Other crystal structures of HSDHs were reported, such as 3 α -HSDH, 3 β -HSDH, 17 α -HSDH, 17 β -HSDH, etc. In the stereospecificity study of 7 β -HSDH, Savino et al. proposed a possibility that the substrates were bound to 7 α -HSDH and 7 β -HSDH in opposite orientations, which caused opposite stereoselectivity of products, based on a structural alignment of 7 β -HSDH (apo-form) [124] and 7 α -HSDH (holo-form) [92].

Other SDRs, such as tropinone reductase (EC 1.1.1.236) TR-I and TR-II, are able to stereospecifically reduce the 3-keto group of tropinone to stereoisomeric forms the enantiomers tropine (α -hydroxy) and pseudotropine (β -hydroxy). It is believed that the reaction stereospecificities of TR-I and TR-II are related to the electrostatic environment of the substrate-binding pocket, which is caused by differently charged residues (positively charged His112 in TR-I while negatively charged Glu156 in TR-II). The substrate-binding directions are determined by charged residues in the pocket, which lead to the stereospecificity of reduction products [125,126]. (R)-hydroxypropyl-coenzyme M dehydrogenase (R-HPCDH) and (S)-hydroxypropyl-coenzyme M dehydrogenase (S-HPCDH) existing in epoxide metabolism of *Xanthobacter autotrophicus* Py2, usually catalyze the oxidization of (R)- and (S)-enantiomers of 2-hydroxypropyl coenzyme to the achiral product 2-ketopropyl-coenzyme M, respectively. A negatively charged sulfonate group, participating in the reaction as a convenient “handle” for substrate binding, was considered related to the substrate binding orientation [127,128]. Other enzymes, such as naphthalene dioxygenase (NDO) from *Pseudomonas* sp. are able to catalyze cis-dihydroxylation of multiple substrates, whose stereospecificity is related to Phe-352 [129–131].

5.1. The Methods of Molecular Docking

In order to explore the stereoisomeric characteristics of HSDHs, the holo-form of 7 β -HSDH (PDB: 5GT9), 17 β -HSDH (PDB: 3QWH) [132] from the SDR superfamily and 3(17) α -HSDH (PDB: 2P5N) [133] from AKR superfamily were used as receptors for molecular docking. The 7 β -HSDH (PDB: 5GT9) structure contains two monomers (A and B),

with each one binding one NADP⁺ molecule. The 17 β -HSDH (PDB: 3QWH) structure contains four monomers (A, B, C, and D), and each one is complexed with a NADP⁺, 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one and di(hydroxyethyl)ether. The 3(17) α -HSDH (PDB: 2P5N) contains two crystallographically independent monomers, with each one binding an NADPH and (4S)-2-methyl-2,4-pentanediol (Protein Data Bank: <https://www.rcsb.org/structure/3QWH> (accessed on 29 January 2021) and <https://www.rcsb.org/structure/2P5N> (accessed on 29 January 2021)).

For simplifying the docking, we used a single subunit as the receptor. In 5GT9, the conformations of NADP⁺ in subunits A and B are quite different, and subunit B was finally used as receptor after structure alignment or comparison with 7 α -HSDH (PDB:1FMC) and 11 β -HSDH (PDB:3G49), for they share similar NAD(P)(H) conformation. In 3QWH and 2P5N, the NADP(H) conformations are similar between subunits, and other molecules except NADP(H) were removed. Two ligands, 7-KLA and androstenedione, were downloaded from ZINC (<http://zinc.docking.org/substances/ZINC000004428526/> (accessed on 29 January 2021) and <http://zinc.docking.org/substances/ZINC000013547326/> (accessed on 29 January 2021)). Both ligands and receptors were processed for energy minimization before docking. A suitable cell around the substrate-binding pocket of receptors was set. The files should be named X_receptor and X_ligand (X represents the same letters or numbers) and the files were placed in the same folder.

The docking was realized via YASARA (distributed by YASARA Biosciences GmbH, Vienna, VIE, AUT. <http://www.yasara.org/> (accessed on 29 January 2021)) under pH 7.4 with a forcefield of AMBER03. In order to make the substrate bind the binding pocket better, several flexible residues were set, which in 5GT9 are Val 20, His 95, Tyr156, Lys160, Thr189, Thr191, Ser193, and Tyr253; in 3QWH are Val107, Phe109, Ser153, Asn154, Phe159, Tyr167, Pro197, Gly198, Gly199, Thr200, Phe205, His206, Glu207, Val208, Ser209, Tyr212, Ile213, Pro214, Met227, Ala228, Ala231, Asp266, and Ala269; in 2P5N are Lys31, Tyr55, His117, Tyr118, Phe219, Tyr224, Gly225 Gly226, and Trp227. Setting files X_receptor and X_ligand as the targets through YASARA, the docking was realized by dock_run.mcr, and then dock_play.mcr was used for further analysis.

5.2. Results and Discussion of Molecular Docking

A crystal structure of the ternary complex of 7 α -HSDH, NAD⁺ and 7-oxo-GCDCA was obtained in a study by Tanaka N et al. [92]. In the crystal structure, 7-oxo-GCDCA is bound to 7 α -HSDH–NADH complex in the orientation of α side toward catalytic residue Tyr159 and β side towards NADH. A hydrogen-bond network was constructed among the C24=O of 7-oxo-GCDCA, Gly97, the adenosine ribose region, and the pyrophosphate region of NADH as well as water molecules for substrate binding, with a binding direction of substrate C24=O towards C-terminal and C3–OH toward N-terminal of 7 α -HSDH. Hydrogen bonds directed or mediated by water molecules exist among C3–OH, Asn151, and Glu253. At the active sites, hydrogen-bond connections of [Ser146–OH $\cdots$ 2.6 Å $\cdots$ O–C7] and [Tyr163–OH $\cdots$ 2.8 Å $\cdots$ O–C7] were generated among C7=O of substrate and side chains of Ser146 and Tyr159. The structure binding model is analogous when in a tetramer crystal of 7 α -HSDH from *Clostridium absonum*, even though with a coenzyme of NADP⁺ and a substrate of taurochenodeoxycholic acid (TCDCA); even the Ser of Tyr–Lys–Ser was changed by Thr [94].

The semi-flexible molecular docking was realized using the holo-form structure of 7 β -HSDH–NADP⁺ as a receptor, and the 7-KLA as a ligand. The residues Val 20, His 95, Tyr156, Lys160, Thr189, Thr191, Ser193, and Tyr253 were set as flexible residues, and the results are shown in Figure 12. The substrate 7-KLA binds to the binary 7 β -HSDH–NADP⁺ in the orientation of β side toward catalytic residue Tyr156 and α side toward NADP⁺, with a distance of 4.0 Å and 3.8 Å between C7–O and Tyr156–OH and C4–nicotinamide group ([Tyr163–OH $\cdots$ 3.1 Å $\cdots$ O–C7] and [C7–O $\cdots$ 3.4 Å $\cdots$ C4–nicotinamide]), respectively, which is opposite to 7 α -HSDH. The binding energy is 7.79 kcal/mol. Three key residues His95, Ser193, and Tyr253 are probably related to the orientation of substrate bind-

ing, which conserved in 7β -HSDH and definitely different from 7α -HSDHs (Figure 13). An obstruction formed by the side chain of His95, Ser193 and the pyrophosphate group of NADP^+ makes the substrate only able to bind to the pocket of the nicotinamide region for the happening of reaction, which make the β side of the substrate toward the NADP^+ impossible. The orientation, with C3–OH toward C-terminal (or the adenosine region of NADP^+) and C24–O towards N-terminal (or nicotinamide region of NADP^+), helped to the formation of this binding model.

Figure 12. The substrate-binding model in 7α -HSDH (PDB: 1FMC) and the molecular docking results of 7-KLA to 7β -HSDH (PDB: 5GT9), androstenedione to 17β -HSDH (PDB: 3QWH) and 3(17) α -HSDH (PDB: 2P5N), respectively. The distances between the reaction position C7=O (in 7α -HSDH and 7β -HSDH) and Tyr–OH, C4 of nicotinamide and the reaction position C17=O (in 17β -HSDH and 3(17) α -HSDH) and Tyr–OH, C4 of nicotinamide were measured using Pymol and shown in blue color.

The differences of substrate binding between SDRs and AKRs were analyzed through a semi-flexible molecular docking of ligand androstenedione to 17β -HSDH-NADP⁺ (PDB: 3QWH) from SDR superfamily and $3\alpha(17\alpha)$ -HSDH-NADP⁺ (PDB: 2P5N) from AKR superfamily, with flexible residues Val107, Phe109, Ser153, Asn154, Phe159, Tyr167, Pro197, Gly198, Gly199, Thr200, Phe205, His206, Glu207, Val208, Ser209, Tyr212, Ile213, Pro214, Met227, Ala228, Ala231, Asp266, and Ala269 in 17β -HSDH, as well as Lys31, Tyr55, His117, Tyr118, Phe219, Tyr224, Gly225 Gly226, and Trp227 in $3\alpha(17\alpha)$ -HSDH. The results were shown in Figure 12. In AKRs, the substrate-binding pocket is composed of loops between α A- β A (residues 21–30), α B- β B (residues 52–60), and α G- β G (residues 220–228). The binding directions of substrate and coenzyme are vertical in space, while it is parallel in SDRs, which may be the reason why HSDHs with a catalytic function at positions 7, 11, and 12 are mainly present in SDRs. The relative position of substrate binding pocket and coenzyme binding pocket makes it difficult for the positions 7, 11, and 12 of the steroid-skeleton substrates to approach the catalytic center, which impacts the proton transfer among substrate, catalytic residues, and nicotinamide group of NAD(P)(H).

The catalytic residues in 17β -HSDH and $3\alpha(17\alpha)$ -HSDH are Ser-Tyr-Lys and Asp-Tyr-Lys-His, respectively. The hydrogen of hydroxyl (–OH) at substrate reaction position was from the catalytic residue Tyr when in a reduction direction. Androstenedione is bound to 17β -HSDH-NADP⁺ binary with an orientation of β side towards catalytic residue Tyr and α side towards B-face of nicotinamide group, with a distance of 3.1 Å and 3.4 Å between C17=O and Tyr–OH and C4–nicotinamide group in 17β -HSDH. The ligand androstenedione binds to $3\alpha(17\alpha)$ -HSDH-NADP⁺ binary in an opposite orientation to 17β -HSDH, with a distance of 3.8 Å and 3.6 Å between C17=O and Tyr–OH and C4–nicotinamide group in $3\alpha(17\alpha)$ -HSDH. A docking result of C3=O of the ligand toward catalytic residue Tyr and α side toward A-face of nicotinamide group was obtained as well, with a distance of 6.2 Å and 3.4 Å between C3=O and Tyr–OH and C4–nicotinamide group. Further determination of the binding method may require molecular dynamics simulation.

The catalytic mechanism of $3\alpha(17\alpha)$ -HSDH (AKR1C21) for different reactions on positions C3 and C17 has been studied through molecular docking. 3-keto/ 3α -hydroxysteroids and the 17-keto steroids were used for docking to $3\alpha(17\alpha)$ -HSDH. C3 and C17 steroid substrates exhibit different binding orientations due to hydrogen-bonding interactions affected by residues Lys31, Gly225, and Gly226 in the substrate-binding pocket. Human HSDHs of AKR1C1 and AKR1C2 usually act as $3\alpha/3\beta$ -HSDHs in the reduction of 5α -dihydrotestosterone (5α -DHT), catalyzing the reduction of 5α -DHT to produce 3α - and 3β -androstenediol. The molecular docking was realized using 5α -DHT as a ligand and AKR1C1 and AKR1C2 as receptors. It was found that the A-ring of steroid 5α -DHT presented its β -face to the 4-pro-R hydrogen when docked into AKR1C2(3β -HSDHs),

Whereas A-ring of 5α -DHT presented its α -face to the 4-pro-R hydrogen of coenzyme when docked into AKR1C1(3α -HSDHs). The different orientations may be related to different residues of the steroid-binding pockets, which is Leu of AKR1C1 while Val in AKR1C2 [100,133,135].

6. Conclusions

In this article, the HSDHs from the SDR and AKR superfamilies were summarized and classified according to their structural and functional characteristics. The different NAD(P)(H)-binding models of these two types of HSDHs were analyzed, with the pro-S side of nicotinamide group towards catalytic residues in SDR superfamily while pro-R side of nicotinamide group towards catalytic residues in AKR superfamily. It impacts the catalytic mechanisms of SDR-HSDHs and AKR-HSDHs. AKR-HSDHs accept 4-pro-R hydride from NADPH, while SDR-HSDHs accept 4-pro-S hydride from NAD(P)H. The majority of AKR-HSDHs are NADPH-dependent and conserved in coenzyme conformations. However, the conformations of the NAD(P)(H) show large differences in the SDR superfamily, especially between α -HSDHs and β -HSDHs. The different conformation in the pyrophosphate region of NAD(P)(H) between 7α -HSDH and 7β -HSDH attracted

much attention. The dihedral angle (PA–O3–PN–O5D) of the pyrophosphate region is roughly -140° in two structures of 7α -HSDH with different coenzymes, while it is -80.6° in 7β -HSDH. A typical pair of stereoisomeric enzymes, 7α -HSDH and 7β -HSDH, were used to analyze the stereospecificity of HSDHs. A semi-flexible molecular docking was realized using the holo-form of 7β -HSDH–NADP⁺ as a receptor and 7-KLA as a ligand via YASARA. A possible binding mode of 7β -HSDH and 7-KLA was obtained. The result was confirmed by comparison to a ternary complex of 7α -HSDH. A hydrophilic face (α side) of 7-KLA toward NADP⁺ in 7β -HSDH made the orientation of C7–OH different in the product, while a hydrophobic face (β side) towards NAD⁺ in 7α -HSDH. One reason leading to the different substrate orientations is the different conformation of the pyrophosphate region of coenzymes caused by Ser193 in 7β -HSDH. An interaction toward pyrophosphate [Ser193–OG $\cdots$ 3.11 Å $\cdots$ O1N–PN] caused the upturning of PN–phosphate, which formed a barrier with the side chain of His95 to prevent 7-KLA from passing through this region and could only be combined with NADP⁺ as α side towards nicotinamide group. A possible interaction existing in the Tyr253 of 7β -HSDH and C24–O of 7-KLA may contribute to the formation of substrate binding orientation. The results of sequence alignment showed the conservative of these three residues (His95, Ser193, Tyr253) in 7β -HSDH, which had a significant difference to 7α -HSDH. The molecular docking of the other two enzymes, 17β -HSDH from the SDR superfamily and $3(17)\alpha$ -HSDH from the AKR superfamily, has further verified the relation between stereospecificity of HSDHs and substrate binding orientation. However, the results still need a further mutation to confirm in future studies.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4352/11/3/224/s1>. Figure S1: Sequence alignment of HSDHs from SDR superfamily, Table S1: HSDHs identified belonging to SDR superfamily, Table S2: HSDHs identified belonging to AKR superfamily, Table S3: Functions of HSDHs and their related metabolic pathways, Table S4: Residues that interact with NAD(P)(H) in AKR superfamily and their conformation differences of coenzyme.

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