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Current physico-biochemistry in steroid research and status of structural biology for steroid-converting enzymes

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1. Rapid kinetics and single-molecule enzymology

In this special issue, pre-steady-state kinetics were used to assign rate constants to discrete steps for steroid converting enzymes [30]. This issue describes the principles of rapid kinetics and their application to elucidate ligand binding mechanisms that may involve more than one intermediate state. The k_{chem} can be measured in a single-turnover experiment and the interpretation of burst-phase kinetics is also described. The application of this technology is shown in the dissection of the P133 R mutant of steroid 5 β -reductase (AKR1D1), which is responsible for bile-acid deficiency. Ligand chase experiments on this mutant showed the rapid displacement of NADP⁺ from the AKR1D1 · NADP⁺ binary complex by NADPH. This showed that despite the proximity of Pro133 to the steroid binding site the enzyme had undergone a dramatic change in cofactor affinity (Fig. 1). Pre-steady-state kinetics were also used to dissect a steroidogenic cytochrome P450 reaction [41] where the authors went one step further to show how fast kinetics can be employed to measure discrete steps in the P450 reaction cycle: this is even more complex and challenging than measuring reactions performed by hydroxysteroid dehydrogenases (HSDs).

2. Enzymology, protein–protein interactions and inhibition of steroid converting enzymes

In the study of steroid-converting enzyme function and inhibition, Maltais et al. [26] reported a detailed structure-activity study for the inhibitor development of human 17 β -

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hydroxysteroid dehydrogenase type 1 (h17 β -HSD1), the enzyme with the highest catalytic efficiency for converting estrone to 17 β -estradiol. Fouad-Mansour et al. [13] report the characterization of 5 α -reductase activity and isozymes in human abdominal adipose tissues responsible for the formation for DHT (5 α -DHT) which inhibits pre-adipocyte differentiation. They found that androst-4-en-3,17-dione was a superior precursor of DHT than testosterone suggesting that 5 α -androstane-3,17-dione was the immediate precursor of DHT in this peripheral tissue.

An interesting review is provided by Biron and Bédard [6], which describes the development of small molecule inhibitors that will block the protein-protein interaction of the androgen receptor (AR) with its co-activators in castration-resistant prostate cancer. Manenda et al. [27] reviewed structural information on androgen-metabolizing enzymes, from both a mechanistic and inhibitor design perspective. The article reviews a series of P450 enzymes and oxidoreductases involved in androgen metabolism.

3. Estrogen receptors and intracrinology in medical applications

The structure-function of estrogen receptors (ERs) and ARs is described with respect to their roles in hormone dependent breast cancer (BC) and neuroprotection. Chan et al. [7] characterized the role of aromatase and ERs in endocrine-responsive and -resistant BC cells. Estrogens and ERs also play important and unexpected roles in brain neuroprotection in animal models of Parkinson's disease. In this disease glutamate overactivity is well-documented. Striatal metabotropic glutamate receptor 5 and brain-derived neurotrophic factor were affected by estrogen in wild-type, but not in ER-alpha or ER-beta knockout male mice [35]. These results demonstrate that in male mice ERs are important for neuroprotection affecting their response to neurotoxicants including MPTP.

In this issue, a new experimental approach is described to elucidate the role of steroid converting enzymes in the intracrine formation of steroid hormones [40,45]. The approach was used to study the source of hormone in estrogen-dependent BC, which accounts for the majority of BC cases occurring in post-menopausal women. The provision of different steroids to reductive steroid enzymes identified DHEA as an important hormone precursor. These studies mimic postmenopausal conditions where adrenal DHEA becomes the main source of steroid hormones during the post-menopausal period. It is likely that DHEA-SO₄ is an even more important source of hormone since this is 1000 times more abundant than DHEA in the serum.

In addition, early attempts to demonstrate enzyme mechanisms in living cells are attracting the interest of biologists. The progress of such attempts is described by Han et al. [20].

4. Structural biology of steroid-converting enzymes

Structural biology is an important aspect of physico-biochemistry and has witnessed significant advancement for steroid-converting enzymes and sex-hormone-receptors during the course of the last few decades. A large number of structures have been determined for these enzymes and receptor ligand-binding domains and some representative examples are reviewed in this preface.

During the previous two decades numerous efforts have concentrated on structural studies of steroid-converting enzymes, including P450 enzymes, short-chain dehydrogenase/reductase (SDR), aldo/keto reductase (AKR) enzymes, sulfotransferases (SULTs), and sulfatases to name a few.

These studies have significantly improved our understanding of their catalytic functions and active site architecture. Due to the pivotal role of steroid-converting enzymes in physiology, many of them are targets for drug design, for example in the fight against hormone dependent cancers. In this regard, structures of enzyme-inhibitor complexes have revealed the molecular basis of inhibition and guided subsequent efforts for further optimization of these inhibitors [24]. These structural studies have revealed that the steroid binding pockets are lined by a series of hydrophobic residues to accommodate the apolar steroid nucleus, whereas the two hydrophilic ends are often anchored by polar residues. Structural comparison of estrogen-specific proteins/enzymes has also shown the presence of a common narrow binding pocket to discriminate estrogens from androgens [29].

Among these, the sulfotransferases (SULTs) and sulfatases are two distinct enzyme families involved in the respective sulfonation and desulfonation of steroids—two vital biological processes that regulate circulating hormone reservoirs (*i.e.* deconjugation of DHEA sulfate and estrone sulfate) for the peripheral formation of active steroid hormones. The structures of human DHEA sulfo-transferase (SULT2A1) [8] and human estrogen sulfotransferase (SULT1E1) [17] have been determined in the presence of steroid ligand and/or phosphoadenosine phosphate (the product of PAPS, the sulfonate-donating cofactor). The cofactor binding site and the catalytic residues are highly conserved in this family, whereas their main difference lies in the region/loops governing substrate specificity. The steroid sulfatase crystal structure is available, which contains long hydrophobic transmembrane helices as well as a globular head harbouring the catalytic site and the Ca^{2+} cofactor [21]. Also revealed in this structure is the presence of sulfate group covalently linked to a hydroxyformylglycine residue resulting from post-translational modification, which provided insights into the catalytic mechanism.

P450 enzymes are heavily involved in steroid biosynthesis and metabolism and are responsible for several critical steps in the formation of both estrogens and androgens. Structural studies have been carried out for several major P450 enzymes including: P450_{sc} (CYP11A1), which catalyzes a multiple-step reaction (sequential hydroxylation followed by C—C bond cleavage) leading to the formation of pregnenolone, the first step that dedicates cholesterol to steroid hormone biosynthesis [37]; P450_{c17} (CYP17A1) responsible for the production of DHEA [10], the precursor molecule for all active sex steroid hormones; and aromatase (CYP19A1), which plays a central role in transforming androgens to estrogens [18]. These three P450 enzymes are membrane-bound and display the canonical fold of other P450 proteins harbouring an iron-containing heme group. The structures of these enzymes complexed with substrates or inhibitors have also been determined highlighting the molecular basis of inhibition (reviewed in [27], in this issue). Importantly, the complex structures also indicated additional moieties that could be introduced into the current inhibitors in order to better exploit the active site, as shown in the case of aromatase [19].

Many steroid-converting enzymes are members of the SDR superfamily and AKR superfamily. These enzymes utilize NAD(P) (H) as the cofactor and, compared with the P450 enzymes, catalyze thermodynamically less-challenging oxidation or reduction of steroid molecules. Often the reaction catalyzed depends on cofactor preference and cellular localization. The SDR family enzymes contain a Rossmann-fold for NAD (P)(H) binding whereas the AKR family adopts a TIM (α/β)₈-barrel fold. Despite the difference in protein folding, both SDR and AKR members share conservation in their catalytic mechanisms [5]. Representatives of these structurally characterized enzymes include the SDR family enzyme 17 β -HSD1, whose structure was the first-determined for any human steroid-converting enzyme [16] and 22 different structure complexes have been reviewed by Thomas and Potter [38]. The structures of the AKR family enzymes 17 β -HSD5 (AKR1C3) (~40 structures determined), 3 α -HSD3 (AKR1C2), and steroid 5 β -reductase (AKR1D1) [11] have also been elucidated. Worthy of note is that AKR1C family enzymes display substrate promiscuity, which is related to the larger steroid binding pocket in these enzymes and the pseudo-symmetric nature of C-19 steroid substrates. The alternative binding modes [28,34] of steroid molecules present in the same active site pocket explains the multispecificity of these enzymes [23,25,2].

Because of the pivotal role of steroid-converting enzymes in a number of diseases, particularly in hormone-dependent cancers, these enzymes have long been targets of drug design. As previously pointed out [38], an interesting approach is to design inhibitors that have two simultaneous targets, *e.g.*, the development of a dual inhibitor of both aromatase and sulfatase to reduce the levels of circulating estradiol [39] as well as dual inhibitors targeting both aromatase and aldosterone synthase (P450 enzyme CYP11B2) to achieve dual effects (anti-cancer and cardiovascular protective effects) [22]. As crystal structures of all these proteins (sulfatase, aromatase, and CYP11B2 [36]) are now available, we anticipate that their structural information will facilitate bifunctional inhibitor design and subsequent optimization.

5. Alternative binding and multiple substrate specificity in steroid-converting enzymes

A frequently observed feature for steroid-converting enzymes is the alternative binding of steroids in the active site pocket, *e.g.* such binding is seen in the narrow substrate tunnel of the 17 β -HSD1 enzyme due to the pseudo-symmetric structures of C19 steroids [14], or on the spatial binding site of 17 β -HSD5 [32], 3 α -HSD3, AKR1C2 [44], and 5 β -reductase, AKR1D1 [11]. The determination of these structures facilitated inhibitor design [33]. In fact, the alternative binding of DHT in 17 β -HSD1 provides a very important synergistic role of the enzyme in breast cancer: in addition to estrogen activation, the enzyme inactivates DHT, the most active androgen, thereby further enhancing the stimulation of breast cancer cell proliferation [1,42].

Steroid hormone molecules contain a hydrophobic cyclopentenophenanthrene ring, with the functional groups that determine steroid hormone function attached at the C3- and C17-carbons. Traditionally it was thought that hydroxysteroid dehydrogenases were regio- and

stereospecific in terms of substrate specificity and product profile. This assumes that one hydroxysteroid dehydrogenase (HSD) will bind a substrate in a preferred orientation. However, we now know that this is not always the case. Some steroids can bind to a particular HSD with two alternative orientations. In one instance they bind the C17 carbon oriented at the active site but in other instances the same HSD can bind this steroid with the C3 carbon oriented at the active site. Rotation of the four rings around their central vertical axis can cause the 3-end to almost superimpose on the 17-end of steroids and vice-versa. The pseudo-symmetric feature of C19-steroids makes it possible for some enzymes to bind a steroid hormone in both the normal and reverse orientations. The alternative binding of some steroid hormones was more recently confirmed by crystallographic and biochemical studies.

The first evidence of alternative binding for steroid hormones in human enzymes came from the crystal structure of h17 β -HSD1 complex with testosterone [14]. The orientation of testosterone in this complex was the reverse of the previously known orientation for estradiol in the enzyme estradiol complex [4,16], with a rotation around the steroid central vertical axis. This structure and related biochemical studies showed that h17 β -HSD1 can bind DHT in both the normal and reverse orientations. In fact, a similar phenomena was seen in *Streptomyces* 3 α -20 β -HSD [15]. Human 17 β -HSD1 can consequently oxidize DHT into A-dione or reduce DHT into 3 β -diol [3].

This theme is continued in the AKR family, but extends beyond the pseudo-symmetry of C19 steroids in the narrow binding site of 17 β -HSD1. Human type 3 3 α -HSD: AKR1C2 binds ursodeoxycholate with the C24 carboxylic acid at the catalytic site or O3 of testosterone at the active site [23]. This work predicted that not only the steroid rotate 180° around its central vertical axis but a different face of the steroid would be presented to the cofactor. Recently, DHT was also shown to bind to human 3 α -HSD3 (AKR1C2) in two orientations, which can simultaneously lead to the oxidized product or the reduced product in two monomers from one crystal asymmetric unit, thus enzyme activity was observed at both the C17 and C3 reaction centers [44].

When DHT is bound in a normal orientation — C3 pointing toward the catalytic site, the 3 α -HSD3 showed 3 α activity and can reduce DHT to 5 α -androstane-3 α ,17 β -ol. When it bound DTH in the reverse orientation after a rotation around its central vertical axis and long the steroid faces flip so that the reactive hydroxyl group (C17) points toward the catalytic site, so that 3 α -HSD3 can oxidize DHT to A-dione (5 α -androstane-3,17-dione) [44]. Progesterone (Prog) can also bind to human 3 α -HSD3 in two orientations but with a rotation about the steroid C3–C17 long axis [43]. In both orientations the C20 oxygen atom is in proximity to the catalytic tetrad, however, the alpha and beta faces of the steroid are flipped. In both orientations the steroid C20 ketone groups as well as the distances between the C20 atoms and the C4 position of the nicotinamide ring are incompatible with hydride transfer and represent steroid binding modes before or after the 20 α -hydroxysteroid dehydrogenase reaction.

The binding pocket of 3 α -HSD3 has only one amino acid (Val54) difference from the binding pocket of human 20 α -hydroxysteroid dehydrogenase (human 20 α -HSD; AKR1C1). When this amino acid was mutated to mimic the h20 α -HSD binding pocket (Val54Leu), the

alternative binding was lost in human 3 α -HSD3/V54L and only an orientation that supported 20 α -HSD activity was observed [43,9]. Another study showed that testosterone can bind to human type 5 17 β -hydroxysteroid dehydrogenase (human 17 β -HSD5; AKR1C3) in two orientations—either its O3 or O17 can penetrate the active site [32]. The promiscuity of the AKR1C1, AKR1C2, AKR1C3 and AKR1C4 enzymes towards their steroid substrates was recently reviewed [31].

One determinant of steroid orientation in the HSD binding pocket may be the oxidation state of the cofactor and/or the microenvironment. With an oxidative cofactor like NADP⁺, 17 β -HSD1 prefers to oxidize the C17 position of DHT, whereas with the reductive cofactor NADPH it prefers to reduce the C3 position of DHT to yield 3 β -diol [14]. That is, when 17 β -HSD1 cooperates with an oxidative cofactor/microenvironment, it prefers to bind DHT in a normal C17-orientation, whereas when cooperating with a reductive cofactor/microenvironment 17 β -HSD1 prefers to bind DHT in a reverse orientation. This bifunctional capability may be controlled by whether oxidative or reductive cofactors are used in the above-mentioned HSD-steroid pairs. However, NADPH is the predominant reducing cofactor in the cell and will likely dictate preference for the orientation of steroid binding that favors reduction. A component that is worth considering is that the structures of the reduced and oxidized cofactors are not the same. Reduced cofactors are not aromatic and could assume a boat or chair conformation whereas the oxidized cofactors will be planar. Unfortunately, no structure of a HSD in which the cofactor remains reduced has been determined to test this point further.

The alternative binding observed with some steroid hormones reveals multiple roles of HSD in the complex steroid hormone interactome. It may have arisen from an evolutionary strategy to efficiently recycle steroid hormones through a limited number of enzymes. Moreover, it may play an important physiological role in maintaining a steady-state steroid hormone pool.

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Fig. 1.

Ligand chase experiments from AKR1D1 and the P133R mutant. NADP⁺ is chased from the E·NADP⁺ complex by NADPH. The P133R mutant (red) showed a much faster rate of release of NADP⁺ than the WT enzyme (green). An enzyme-NADP⁺ solution (AKR1D1, 1 μ M; NADP⁺, 5 μ M) from one syringe was rapidly mixed (1:1) with a NADPH solution (100 μ M) from the second syringe in the stopped-flow. In the control experiments, the enzyme solution or the buffer were mixed with the NADPH solution in the stopped-flow (blue). Experiments were performed in 100 mM potassium phosphate pH 7.0. Reproduced with permission from the American Chemical Society, *Biochemistry* (2015) [Epub ahead of print Sep 29].