

STEROIDOGENIC ENZYMES: STRUCTURE, FUNCTION, AND ROLE IN REGULATION OF STEROID HORMONE BIOSYNTHESIS

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Summary—In the pathways of steroid hormone biosynthesis there are two major types of enzymes: cytochromes *P450* and other steroid oxidoreductases. This review presents an overview of the function and expression of both types of enzymes with emphasis on steroidogenic *P450*s. The final part of the review on regulation of steroidogenesis includes a description of the normal physiological fluctuations in the steroid output of adrenal cortex and gonads, and provides an analysis of the relative role of enzyme levels in the determination of these fluctuations. The repertoire of enzymes expressed in a steroidogenic cell matches the cell's capacity for the biosynthesis of specific steroids. Thus, steroidogenic capacity is regulated mainly by tissue and cell specific expression of enzymes, and not by selective activation or inhibition of enzymes from a larger repertoire. The quantitative capacity of steroidogenic cells for the biosynthesis of specific steroids is determined by the levels of steroidogenic enzymes. The major physiological variations in enzyme levels, are generally associated with parallel changes in gene expression. The level of expression of each steroidogenic enzyme varies in three characteristics: (a) tissue- and cell-specific expression, determined during tissue and cell differentiation; (b) basal expression, in the absence of trophic hormonal stimulation; and (c) hormonal signal regulated expression. Each of these three types of expression probably represent the functioning of distinct gene regulatory elements. In adult steroidogenic tissues, the levels of most of the cell- and tissue-specific steroidogenic enzymes depend mainly on trophic hormonal stimulation mediated by a complex network of signal transduction systems.

OUTLINE

1. Introduction
2. Enzymes in the Initial Pathways of Steroidogenesis
 - 2.1. *P450*_{scc}
 - 2.2. 3 β -HSD
 - 2.3. *P450*_{c17}
 - 2.4. *P450*_{c21}
 - 2.5. *P450*_{c11} and *P450*_{c18}
 - 2.6. *P450*_{arom}
 - 2.7. 17 β -HSD
 - 2.8. Enzymes that further metabolize steroids
3. Biochemistry and Molecular Biology of Steroidogenic *P450* Systems
 - 3.1. Enzymology of *P450* systems
 - 3.2. Intracellular targeting of the *P450* system enzymes
 - 3.3. Sequence and structural similarities
4. Role of Enzyme Expression in Regulation of Steroidogenesis

- 4.1. Enzyme expression as a determinant of steroidogenic capacity
- 4.2. Methodological issues
- 4.3. Adrenal cortex
- 4.4. Testis
- 4.5. Ovary
- 4.6. Mechanisms of regulation of steroidogenic enzyme levels
- 4.7. Intracellular regulators of steroidogenic enzyme expression

1. INTRODUCTION

The concentrations of steroid hormones in blood fluctuate with a specific periodicity, or in response to physiological or pathological changes, to regulate diverse processes in the body. In mammals there are three endocrine organs that specialize in steroid hormone production: adrenal cortex, ovary, and testis. During pregnancy, placenta develops as an additional major source of steroid hormones. The steroid hormone output of these organs is regulated by specific extracellular hormones and

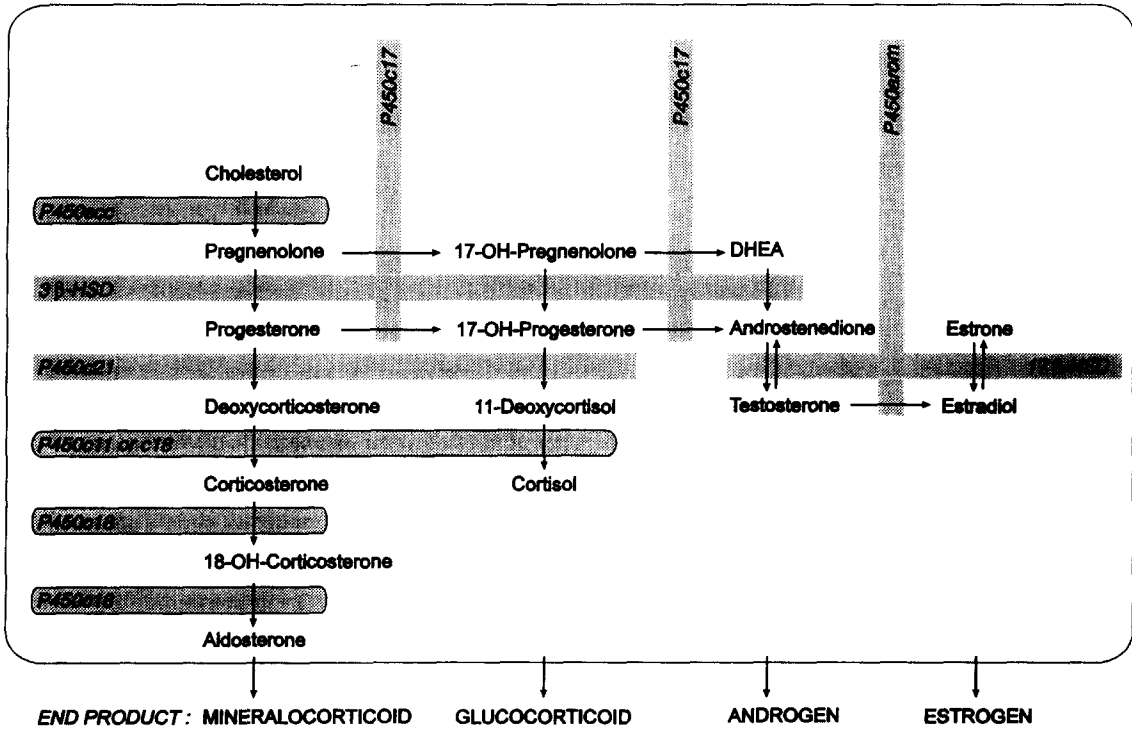


Fig. 1. Pathways of initial steps of steroid hormone biosynthesis in the adrenal cortex and the gonadal cells. The arrows mark substrate conversion to product by the enzyme listed in the shaded area. The enzymes in the shaded area with borders (*P450scc*, *P450c11* and *P450c18*) are located inside the mitochondria. The other enzymes are located in the endoplasmic reticulum. The physiological function of each steroid end product is listed below the frame. The steroids that appear in this figure may be further metabolized within the steroidogenic tissues or in other organs by the enzyme listed in Table 2. DHEA: 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone).

factors that activate a series of intracellular processes that ultimately change the steroid output.

In our current understanding, steroid hormone output depends on the rate of steroid metabolism, i.e. biosynthesis and catabolism [1]. The pathways of steroid hormone biosynthesis start with cholesterol (Fig. 1). Cholesterol is stored in esterified form inside lipid vesicles. Trophic hormones activate a chain of reactions that lead to the hydrolysis of cholesterol esters into free cholesterol, and the transport of cholesterol into mitochondria where it is converted to pregnenolone by *P450scc* [2-4]. After this rate-limiting step, subsequent biosynthetic steps proceed with the flow of substrates through the enzyme systems located in the endoplasmic reticulum and mitochondria. Steroid hormones are hydrophobic molecules that can penetrate biological membranes, and flow into the blood stream after their synthesis without being stored in intracellular vesicles. An increase in the blood levels of steroid hormones is dependent on the continual synthesis and secretion of steroids. Tissue levels of steroids rise transiently after

hormonal stimulation but even these high concentrations are not sufficient to supply enough steroids to increase blood levels [36, 37]. This contrasts with the mode of secretion of peptide hormones and neurotransmitters which accumulate in intracellular vesicles and are secreted in response to specific stimuli.

Thus, steroid hormone output is mainly regulated by events that ultimately affect steroid production through four parameters or processes:

- (1) Steroidogenic enzyme level, determined by transcription, stability and translation of the mRNAs encoding the enzymes.
- (2) Steroidogenic enzyme activity, determined by the conditions of the intracellular milieu, cofactor availability, or the post-translational modification of the enzymes.
- (3) Substrate availability, generally determined by cholesterol mobilization and transport to the mitochondrial *P450scc* which catalyzes the first step in the pathways of steroid biosynthesis.
- (4) Tissue growth, determined by cell division

Table 1. Enzymes that catalyze the initial reaction in the pathways of steroid hormone biosynthesis (see Fig. 1)

Nomenclature		Reaction	Reviews
Enzyme	Gene		
<i>P450</i> type			
<i>P450_{sc}</i>	<i>CYP11A</i>	Cholesterol side chain cleavage (3 successive monooxygenations)	2-4, (5)
<i>P450_{c11}</i>	<i>CYP11B1</i>	C-11 hydroxylation	4, 6, (7-9)
<i>P450_{c18}</i>	<i>CYP11B2</i>	C-18 hydroxylation	6, (7-10)
<i>P450_{c17}</i>	<i>CYP17</i>	C-18 oxidation	
		17 α -Hydroxylation	11, (12)
<i>P450_{c21}</i>	<i>CYP21</i>	C17—C20 bond cleavage	
<i>P450_{arom}</i>	<i>CYP19</i>	C-21 hydroxylation	11, (13, 14)
		Steroid ring-A aromatization (3 successive monooxygenations)	15, 16
Oxidoreductase (dehydrogenase) type			
3 β -HSD	<i>HSD3B</i>	3 β -Hydroxysteroid dehydrogenation	17 (5)
		5-Ene-4-ene isomerization	
17 β -HSD		17 β -Hydroxysteroid dehydrogenation	18-20, (21)
		17-Keto-steroid reduction	

The references in parentheses include description of inborn deficiencies of enzyme. The table includes at most only two reactions for each enzyme, based on the highest amount of products observed in *in vitro* incubations with the enzyme. All the enzymes listed catalyze additional reactions with different stereoselectivities, but at lower yields than the major reactions listed above.

and multiplication, as in the corpus luteum formation.

Several of these processes may operate concurrently to determine the final steroid hormone output. For example, growth of corpus luteum is accompanied by changes in the expression of steroidogenic enzymes (Section 4.5). Trophic hormones may also activate several of these four processes simultaneously. Whereas, cholesterol supply can be increased within minutes, the induction of enzyme synthesis may require many hours. Hence, these actions of stimulatory hormones have been referred to as 'rapid' vs 'delayed' or 'trophic'. However, the period of initiation of these responses may overlap. For example, brief (30 min) stimulation of adrenocortical cells with ACTH leads to both a rapid increase in steroid secretion and a delayed increase in enzyme synthesis that peaks 36 h later [38].

Among the 4 processes mentioned, this review concentrates on the role of steroidogenic enzyme expression and activity as a determinant of steroidogenic capacity. Studies on the transcriptional regulatory sequences in genes that encode the enzymes are the subject of other reviews in this issue [16, 39-42]. Cholesterol transport is also reviewed in another section of this issue [2, 43].

In the pathways of steroid hormone biosynthesis there are two major types of enzymes: cytochromes *P450* and other steroid oxidoreductases (Fig. 1 and Table 1). Initially this review presents an overview of the function and

expression of both types of enzymes and then expounds on the structure and regulation of steroidogenic *P450*s. Steroid dehydrogenases [17], and *P450*s involved in the biosynthesis of vitamin D and sterols are reviewed in other sections of this issue [44].

The final part of this review on the regulation of steroidogenesis includes a description of the normal physiological fluctuations in the steroid output of adrenal cortex and gonads and provides an analysis of the relative role of the enzyme levels in the determination of these fluctuations (for reviews on placental steroidogenesis see Refs [45, 46]).

2. ENZYMES IN THE INITIAL PATHWAYS OF STEROIDOGENESIS

The initial steps of steroid biosynthesis in steroidogenic tissues are shown in Fig. 1. Activity and expression of the enzymes in different tissues are listed in Tables 1-3. During the past decade, cDNAs for nearly all the

Table 2. A partial list of enzymes that further metabolize steroids

	References
<i>P450</i> type	
<i>P450</i> s that hydroxylate steroids at positions 2 α , 2 β , 6 β , 7 α , 15 α , 15 β , and 16 α	22-29
Oxidoreductase (dehydrogenase) type	
3 α -OH-steroid dehydrogenase	30
5 α -Reductase	31
5 β -Reductase	
11 β -OH-steroid dehydrogenase	8
20 α -OH-steroid dehydrogenase	32
20 β -OH-steroid dehydrogenase	
Conjugating enzyme type	
Sulfotransferase	33
UDP glucuronosyltransferase	34, 35

Table 3. Specificity of expression of steroidogenic enzymes in different cell types in the adrenal cortex, ovary and testis

	Adrenal cortex			Ovary				Testis
	Zona glomerulosa	Zona fasciculata	Theca interna	Granulosa (preantral follicle)	Granulosa (preovulatory follicle)	Luteinized theca (corpus luteum)	Luteinized granulosa (corpus luteum)	Leydig (interstitial tissue)
<i>P450scc</i>	+	+	+	-	+	++ ^a	++	+
3 β -HSD	+	+	+	+	+	++	++	+
<i>P450c11</i>	-	+	-	-	-	-	-	-
<i>P450c18</i>	+	-	-	-	-	-	-	-
<i>P450c17</i>	-	+ ^b	+	-	-	+	-	+
<i>P450c21</i>	+	+	-	-	-	-	-	-
<i>P450arom</i>	-	-	-	-	+	-	+ ^c	-
17 β -HSD	?	+	+	-	-	-	-	+
Major steroid product	Mineralo-corticoid	Glucocorticoid Androgen	Androgen	Estrogen	Progestins Progesterone	Progesterone Androgen	Progesterone Estrogen	Androgen

^a ++, indicates much higher levels only relative to ovarian granulosa cells and not other cells.

^bNot expressed in rat, hamster and rabbit adrenals.

^cExpression dependent on species.

major steroidogenic enzymes have been isolated (Table 4). One of the most important findings that emerge from molecular studies is that almost every steroidogenic enzyme is encoded by only one gene (Table 4). For key enzymes, e.g. *P450scc*, different genes that are specifically expressed in different tissues do not exist. Thus,

a single gene for each enzyme must carry all the signals for its specific regulation in different tissues and cells. A second most important finding is that some of the enzymes, e.g. *P450c17*, *P450c18*, catalyze more than one step in the pathway (Fig. 1). Therefore, it is recommended that the enzymes be referred to with a single

Table 4. Characteristics of the genes and mRNAs of cytochromes *P450* and their electron transfer proteins

	Species	Gene (n)	Chromosome	Gene length (kb)	mRNA length (kb)	Protein length (aa) (Preseq.) ^a	References
<i>Mitochondrial</i>							
<i>Adrenodoxin reductase</i>	Man	1	17	12	2	459 (491)	47-51
	Cow	1	-	6-12	2	460 (492)	47, 52, 53
<i>Adrenodoxin</i>	Man	2 (2) ^b	11 (20, 21)	>20	1, 1.4, 1.7	124 (184)	51, 54, 55
	Cow	1	-	>27	1, 1.4, 1.7	128 (186)	56-59
<i>P450scc</i>	Man	1	15	>20	2	482 (521)	51, 60-62
	Cow	1	-	-	2	481 (520)	63-65
	Pig	-	-	-	-	481 (520)	66
	Rat	1	-	-	2	490 (526)	67, 68
<i>P450c11</i>	Man	1	8	-	-	479 (503)	7, 8, 69, 70
	Cow	2 (2)	-	-	1.8, 4.1	479 (503)	71-74
	Rat	-	-	-	-	475 (499)	6, 75
	Mouse	1	15	7	-	(500)	76
<i>P450c18</i>	Man	1	8	-	-	479 (503)	7, 8, 69, 77
	Rat	-(?)	-	-	-	476 (510)	6, 78
	Mouse	1	15	7	2.6	(500)	76
<i>Microsomal</i>							
<i>P450red.</i>	Human	1	-	-	-	676	79, 80
	Porcine	-	-	-	-	678	81
	Rabbit	-	-	-	2.4	679	82
	Rat	1	-	20	2.7	678	79, 83-85
	Mouse	1	6	-	-	-	79
<i>P450c17</i>	Man	1	10	6.6	1.9	508	51, 86-88
	Cow	1	-	6.6	1.9	509	89, 90
	Rat	1	-	-	1.9	507	91, 92
	Mouse	1	19	-	-	507	93
	Chicken	-	-	-	1.9	508	94
<i>P450c21</i>	Man	1 (1)	6	3.3	2	494-495	13, 95, 96
	Cow	1 (1)	-	3.4	2.2, 2.4	496	97-101
	Mouse	1 (1)	17	3.1	2.2	487	102, 103
<i>P450arom</i>	Man	1	15	>70	2.9, 3.4	503	16, 104-108
	Rat	-	-	-	-	508	109
	Mouse	1	9	-	2.1, 2.5	503	110
	Chicken	-	-	-	4	507	111
	Trout	-	-	-	-	2.6	522

^aThe presequence of the protein includes amino terminal extension signal peptide.

^bThe number of pseudogenes are listed in parentheses.

P450 nomenclature and not with a commonly used "hydroxylase" name that would require the use of two or more hydroxylase names for a single enzyme molecule [25].

The first two major steps in the biosynthesis of steroids are common to all steroidogenic organs: (1) conversion of cholesterol to pregnenolone by *P450_{scc}* inside the mitochondria; and (2) conversion of pregnenolone to progesterone by 3β -hydroxysteroid dehydrogenase (3β -HSD) in the endoplasmic reticulum (Fig. 1). The following steps of steroidogenesis are catalyzed by enzymes expressed only in some steroidogenic cell types (Table 3). The cell-specific expression of these enzymes determines the capacity of each cell to synthesize different steroid hormones (Table 3). The major functions of these enzymes and their sites of expression are listed below.

2.1. *P450_{scc}*

This enzyme catalyzes the first and rate-limiting step in the biosynthesis of steroid hormones [2–4, 113, 114]. It converts cholesterol to pregnenolone in three successive monooxygenations (hydroxylations at C-22, followed by C-20, and finally cleavage of the C-20,22 bond). Hydroxylated intermediates of cholesterol bind very tightly to *P450_{scc}* and do not show significant dissociation from the enzyme [4]. In contrast, the final product pregnenolone has a dissociation constant 40 to 600-fold higher than those of intermediates, facilitating its release from the enzyme [4]. Purified *P450_{scc}* can efficiently catalyze side chain cleavage of cholesterol sulfate, and 6β -hydroxylation of deoxycorticosterone [115, 116]. Yet, it is not known whether these reactions represent a major activity *in vivo*.

P450_{scc} is expressed in all three zones of the adrenal cortex [117, 118]. In the testis it is found in the steroidogenic Leydig cells [119]. In the ovary it is expressed in the theca interna; its expression in the granulosa cells depends on the stage of growth of the follicle as discussed in Section 4.5. In addition to steroidogenic tissues the expression of *P450_{scc}* has been detected in the brain [120], but at levels that are more than an order of magnitude lower (Table 5).

2.2. 3β -HSD

This enzyme has two major catalytic activities which in concert convert 3β -hydroxy-5-ene steroids into 3-keto-4-ene (Table 1). In contrast to steroidogenic *P450*s each of which is encoded by a single gene, in the human, rat and mouse

Table 5. The concentration of mitochondrial *P450_{scc}* in steroidogenic tissues and brain

Tissue	Concentration (pmol/mg protein)*	Reference
Adrenal cortex	400	121
Corpus luteum	80–400	121, 122
Ovary	<5	121, 122
Placenta	<50	123
Testis Leydig cells	—	119
Brain	<10	124

*The values shown are per total tissue protein. The concentrations for placenta and brain were calculated assuming that the mitochondrial fraction represents 20% of the total cellular protein.

genomes there are at least 2–3 homologous genes encoding 3β -HSDs that share 80–94% sequence identity within each species [17]. Two types of 3β -HSDs from human and rat can use either pregnenolone, 17-OH-pregnenolone or dehydroepiandrosterone (DHEA) as substrates [17]. The different types have different K_m values for the same substrates. However, each type of enzyme can use pregnenolone or DHEA as substrates with similar K_m values [17]. Rat type I 3β -HSD also shows 17β -HSD activity with 5α -androstane steroids but not with estradiol, estrone, androstenedione, or testosterone [125].

In the human adrenal cortex and gonads, but not placenta, only type II 3β -HSD is expressed, whereas in the rat both type I and II are found in these tissues [17]. Immunohistochemical studies reveal localization of 3β -HSD in the same steroidogenic cells as *P450_{scc}* in all three zones of the adrenal cortex, in the interstitial Leydig cells of the testis, but not in seminiferous tubules, in the theca interna of the ovary and in corpora lutea [126]. Immunostaining failed to detect 3β -HSD in rat granulosa cells [126] even though these cells display high 3β -HSD activity converting pregnenolone to progesterone even before the induction of *P450_{scc}* [127]. In contrast to steroidogenic *P450*s, 3β -HSD activity is present in a wide range of tissues [17].

2.3. *P450_{c17}*

This enzyme catalyzes two key reactions: (a) 17α -hydroxylation of C21 steroids; and (b) cleavage of the C17–C20 bond of C21 steroids. The 17α -hydroxylation is a required step in cortisol biosynthesis, whereas the C17–C20 bond side chain cleavage is essential for the biosynthesis of androgens (Fig. 1). Genetic deficiencies affecting either one or both activities have been characterized [12].

Immunohistochemical analysis shows that *P450_{c17}* is present in the zona fasciculata and reticularis but not in zona glomerulosa of the porcine adrenal [128]. This is consistent with the

role of zona glomerulosa as the site of mineralocorticoid biosynthesis where 17 α -hydroxylation is not required (Fig. 1). *P450c17* is not expressed in the rat adrenal cortex; consequently corticosterone, and not cortisol, is the major glucocorticoid in this species and in some other rodents [129]. However, *P450c17* is expressed in guinea pig adrenal cortex [130–134].

In the ovary, *P450c17* is expressed in theca interna cells [135, 136]. Antibody or cDNA probes show very low expression of *P450c17* in granulosa cells of humans and rats [135–137]. Thus, theca interna cells can synthesize androgens, but granulosa cells which produce estrogens are dependent on androgen precursor supply from theca interna [138]. This process is called the two cell hypothesis of follicular estrogen production [138].

The fact that *P450c17* catalyzes two reactions, poses a conundrum: What determines the relative flow of steroids through glucocorticoid and androgen pathways (see Fig. 1)? Adrenal cortex capacity for androgen synthesis changes during puberty [139]. These pathways are probably developmentally regulated by changes in the function of the zona reticularis. In Leydig cells, in the absence of *P450c21* there is no alternative pathway but to androgen production (see Fig. 1).

The C17—C20 bond cleavage activity of *P450c17* depends on the concentration of electron transfer protein *P450* reductase, and it can be increased to the level of the 17 α -hydroxylation activity [11]. Thus, changing levels of this reductase or cytochrome *b₅*, may affect the ratio of the two activities [11, 140]. Different steroidogenic tissues appear to have different levels of these proteins [11]. The two activities of *P450c17* are also strongly dependent on steroid concentrations [141, 142]. Thus, the intracellular concentrations of substrates and products probably also play a role in regulating the relative rates of these activities [141, 142].

The substrate specificity of *P450c17* varies with species [129]. *P450c17* from all species examined can hydroxylate C-17 of both pregnenolone and progesterone, although the K_m for these two substrates can differ by 10-fold in some species [11, 91, 143–145]. Whereas, the rat *P450c17* can convert both 17-OH-pregnenolone and 17-OH-progesterone into DHEA and androstenedione, respectively [91, 143], the human and bovine *P450c17* can cleave the C17—C20 bond of 17-OH-pregnenolone but not of 17-OH-progesterone [91, 144, 145]. Thus, the substrate

specificity of *P450c17* determines whether androgen biosynthesis proceeds mainly through pregnenolone or progesterone as shown in Fig. 1. The formation of androgen may be catalyzed without the 17 α -hydroxylated substrate leaving the enzyme [141].

2.4. *P450c21*

This enzyme catalyzes an essential step in the synthesis of gluco- and mineralocorticoids (Fig. 1). Congenital adrenal hyperplasia which results from an inborn deficiency of this enzyme is a common genetic disorder [5, 13, 14]. Deficiency in the *P450c21* hydroxylation step, channels the steroid biosynthetic pathway in the direction of androgen production, resulting in gluco- and mineralocorticoid deficiency and excessive virilization [5, 14].

P450c21 is expressed in all three zones of the adrenal cortex [146]. Apart from the adrenal cortex, *P450s* with steroid 21-hydroxylation activity have been found in other tissues, e.g. liver [147]. However, these *P450s* show little similarity to the steroidogenic *P450c21* and represent products of other genes [25, 147]. Immunohistochemical studies using anti-*P450c21* antibody revealed a cross reacting protein only in the distal tubules of the bovine kidney, which is the site of the mineralocorticoid action in the kidney [146]. However, it is not known whether this protein is the product of the same gene that encodes the adrenal *P450c21*. Examination of the kidney by *in situ* hybridization using a specific *P450c21* probe could resolve this question.

2.5. *P450c11* and *P450c18*

These two enzymes are also uniquely expressed in the adrenal cortex [6, 148]. In the human and rat adrenal, *P450c11* is expressed in the zona fasciculata which specializes in glucocorticoid production under mainly ACTH regulation. Whereas *P450c18* is expressed in the zona glomerulosa and is regulated by the renin-angiotensin system [6–8, 149–151]. The human and rat *P450c18* can catalyze aldosterone formation, but *P450c11* cannot [6–8]. Two bovine isozymes of *P450c11* expressed in COS-7 cells can catalyze both corticosterone and aldosterone production [152]. However, it is not known whether these enzymes function in both zones, or whether there is a bovine *P450c18* gene that is specifically expressed in zona glomerulosa.

With 11-deoxy substrates, both *P450c11* and *P450c18* also catalyze 19-hydroxylation but at

less than 10% of 11 β -hydroxylation activity [7]. *P450c11* can also hydroxylate androstenedione resulting in the formation of 11 β -hydroxylated androgens [e.g. 134]. Steroid 11 β -hydroxylation activity has been detected in the gonads of some species [153], but it is not known whether this reflects a low expression of *P450c11* in these tissues or the activity of another enzyme.

2.6. *P450arom*

P450arom catalyzes the conversion of testosterone into the 17 β -estradiol (Fig. 1 and Table 1). In the ovary *P450arom* is expressed in granulosa cells which is the major site of estrogen production in females [136, 154] (see Section 4.5). However, this enzyme is widely expressed in many tissues besides gonads, e.g. adipocytes, breast, central nervous system, skin and placenta [15, 16]. The gene encoding *P450arom* is the longest amongst steroidogenic *P450* genes (Table 4). This gene is also unique among *P450* genes in having alternative promoters that are utilized in a tissue-specific manner [16].

2.7. 17 β -HSD

This enzyme is also referred as 17-ketosteroid reductase. It catalyzes the reversible conversion of the 17-keto and 17 β -hydroxy groups in androgens and estrogens, including androstenedione, DHEA, and 17 β -estradiol. The direction of the reaction depends on the substrate and cofactor [18–20]. For the conversion between androstenedione and testosterone, the porcine testicular 17 β -HSD prefers NADPH rather than NADH ($K_m = 11$ vs 177 μ M) [155]. There are multiple 17 β -HSD isozymes with androgen or estrogen specificity [18–20]. The isozyme from the porcine testis also displays 20 α -HSD activity at about a tenth of the 17 β -HSD activity [155].

A 17 β -HSD has been purified from placenta, and a cDNA and two in tandem homologous genes have been cloned and sequenced [19, 156]. This enzyme catalyzes the interconversion of 17 β -estradiol and estrone, and can also use androgen substrates [19]. Its K_m for 17 β -estradiol is 10 μ M vs 250 μ M for testosterone [19]. Thus, this enzyme is specific for estrogens and not androgens. Its cDNA hybridizes relatively weakly to a few bands on a testis RNA blot [156]. One of these may represent a homologous mRNA that encodes a different enzyme with specificity for androgens rather than estrogens. Testicular 17 β -HSD deficiency is a rare genetic

disorder, but is observed frequently in an inbred Arab population [21]. In affected individuals androstenedione to testosterone conversion is markedly impaired [21]. The cloning of the gene that encodes the androgenic 17 β -HSD may be useful in elucidating the molecular defect in this disease.

Immunohistochemical studies using an antibody against a testicular 17 β -HSD indicate that the enzyme is expressed in the testis in Leydig cells of the interstitial tissue, and in the ovary, in the theca interna, but not in granulosa cells or corpus luteum [157]. This is consistent with androgen synthesis in testicular Leydig cells and in ovarian theca interna. Both androgenic and estrogenic 17 β -HSD activities have been observed in a wide range of tissues [20]. These activities probably do not represent the androgenic isozyme. In testicular 17 β -HSD deficiency, 17 β -HSD activity is defective only in the testis but appears normal in other tissues [21], thus supporting other findings that there are multiple isozymes with 17 β -HSD activity [18–20].

2.8. Enzymes that further metabolize steroids

The enzymes noted above catalyze the initial steps in steroidogenesis. The end products of these reactions can be further metabolized by other enzymes (Table 2) within the cell of production or after transport to cells in the same or other organs. Some properties of these enzymes are outlined below:

- (1) Steroid metabolism by some of these enzymes may result in the formation of a steroid product with potent biological activity, as in the case of conversion of testosterone to 5 α -dihydrotestosterone by 5 α -reductase, or inactivation of the steroid, as in the case of liver hydroxylation and conjugation of steroids, or inactivation of cortisol by 11 β -HSD (see refs in Table 2).
- (2) In contrast to the initial steroidogenic enzymes which are encoded by a single gene (Table 4), the cloning of cDNAs for some of these enzymes revealed large families of genes with many members (see refs in Table 2). The enzymes encoded by these genes have similar sequences, but different substrate specificities.
- (3) While most steroidogenic enzymes have strict substrate specificities, the enzymes that further metabolize steroids generally

have broader substrate specificities. For example, 3 α -HSD and 20 α -HSD can metabolize both C19 (androstane) and C21 (pregnane) steroids. Similarly, some conjugating enzymes can accommodate a broad range of steroid structures. The stereoselectivities of some enzymes may also be broader: different *P450*s can hydroxylate a steroid at several positions [28] and 20 α -HSD also shows 3 α -HSD activity [32].

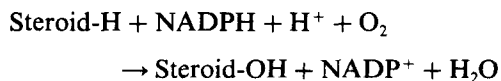
- (4) Whereas the expression of most enzymes in the initial pathways (Fig. 1) is limited to steroidogenic tissues, enzymes of further metabolism (Table 2) are expressed in a broad range of tissues besides steroidogenic organs. For example, 3 α -HSD, and 5 α -reductase activities are present in many peripheral tissues as well as all areas of the central nervous system including white matter [158, 159].

3. BIOCHEMISTRY AND MOLECULAR BIOLOGY OF STEROIDOGENIC *P450* SYSTEMS

3.1. Enzymology of *P450* systems

As noted above, most of the reactions in the pathways of steroid biosynthesis are catalyzed by *P450* type enzymes. *P450*s are found in nearly all tissues of the vertebrates where they catalyze different metabolic or biosynthetic reactions generally involving small hydrophobic molecules. The steroidogenic *P450*s resemble other *P450*s in many biochemical properties. All cytochromes *P450* are *b* type cytochromes wherein the environment of the heme forms the active site of the enzyme that binds the substrate and O₂ [22, 23]. CO competes with O₂ for binding to this site and thus can inhibit enzymatic activity. The name *P450* derives from the distinct absorption maximum at 450 nm of the reduced and CO bound form of the enzyme.

The reactions catalyzed by steroidogenic *P450*s are either a single hydroxylation (mono-oxygenation) at a specific position on the steroid molecule, or a series of consecutive monooxygenations which result in C—C bond cleavage or aromatization of the steroid ring A (Table 1). Each *P450*-catalyzed hydroxylation has the following stoichiometry:



This reaction consumes two electrons, a H⁺, and O₂, one atom of which is incorporated into the substrate, while the second is reduced to H₂O (hence mono-oxygenation). The electrons are transferred from NADPH to cytochrome *P450* by specific electron carrier proteins. In the mitochondrial *P450* systems this function is performed by two proteins: adrenodoxin reductase, which is an FAD containing flavoprotein, and adrenodoxin, which is a ferredoxin type iron-sulfur protein [3, 4, 47, 113, 114, 160]. The microsomal *P450* systems are dependent on *P450* reductase which is a flavoprotein with FAD and FMN as cofactors [22, 23, 47, 161]. In a catalytic cycle, the mitochondrial *P450* accepts one electron at a time from adrenodoxin, and the microsomal *P450* from the FMN of *P450* reductase. Thus, the cofactor FMN fulfills a role similar to that of adrenodoxin in the mitochondrial *P450* systems as a single electron acceptor and donor [161] (Fig. 2). Some microsomal *P450*s can also accept the second electron from cytochrome *b*₅ [11, 22, 140].

All eukaryotic *P450*s are membrane associated enzymes. The amino termini of both the microsomal *P450*s and *P450* reductase invariably contain a 20–30 residue long highly hydrophobic segment which anchors these enzymes to the lipid bilayer of the endoplasmic reticulum, while their main body remains in the cytoplasmic side [162, 163, 163a] (Fig. 2). Mitochondrial *P450*s are located on the matrix side of the inner mitochondrial membrane [117, 118, 164]. The mitochondrial *P450*s behave as highly hydrophobic proteins, but contrary to the microsomal *P450*s they lack a hydrophobic amino terminal sequence that could be predicted as a membrane spanning segment [71, 165, 166]. Thus, the region that anchors the mitochondrial *P450*s to the membrane remains unidentified. Both adrenodoxin reductase and adrenodoxin are easily solubilized proteins, and do not contain a highly hydrophobic segment [47]. However, immunocytochemical studies show that these proteins are also associated with the inner mitochondrial membrane [167], most likely mainly by ionic interactions.

Generally the reductase component of *P450* systems is present at much lower concentrations than the *P450*s inside cells [121, 168]. This low molar ratio of reductase to *P450*, and kinetic studies of enzyme–enzyme interactions indicate that the *P450* system enzymes do not form static complexes on the membrane and are independently mobile [22, 113, 160].

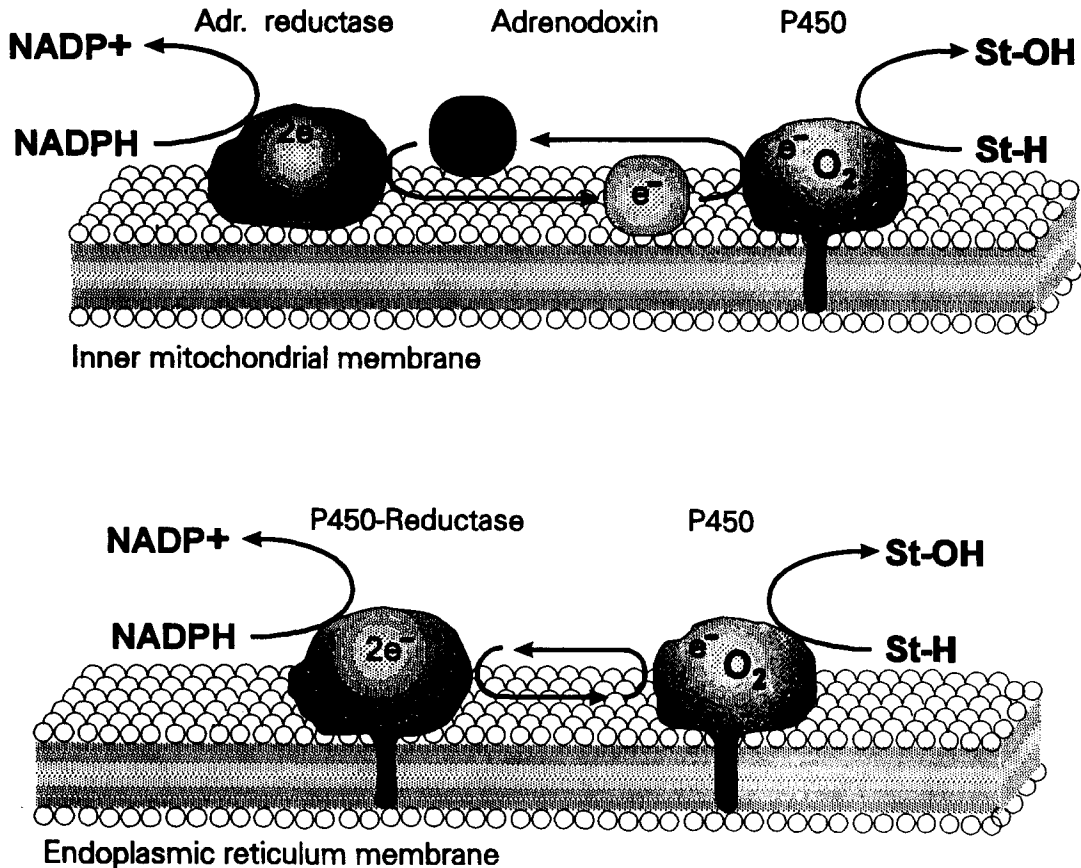


Fig. 2. Models for the membrane organization of the mitochondrial and microsomal *P450* systems. The mitochondrial system faces the matrix (inner) side of the membrane. The microsomal system enzymes face the cytosol and both are anchored to the membrane by hydrophobic amino terminal domains. The membrane binding region of the mitochondrial *P450*s is not known and is depicted in analogy to the microsomal *P450*s.

3.2. Intracellular targeting of the *P450* system enzymes

The mitochondrial *P450*s, adrenodoxin reductase and adrenodoxin are synthesized in precursor forms with an amino terminal extension of 30–60 residues that is absent in the mature enzymes (Table 4). These amino terminal signal peptides are necessary for the specific entry of the enzymes into mitochondria [169–171], and are cleaved by proteases upon entry of the precursor into the mitochondria [172–175]. In bovine adrenal cortex, *P450*_{scc} and adrenodoxin are processed by two distinct proteases [173, 175]. The sequences of the extensions of these proteins share little or no similarity, are not hydrophobic, and invariably contain several basic residues (Arg and Lys) which are essential for the transfer of at least some of these proteins into mitochondria [169–171]. In these respects, these proteins resemble some other mitochondrial proteins [176]. The specific association of the mitochon-

drial precursor proteins with the mitochondrial membrane may be mediated by the polar heads of cardiolipin, a phospholipid that is specifically present in the inner mitochondrial membrane [177]. The bovine mitochondrial *P450*s can be imported only into mitochondria isolated from steroidogenic tissues, whereas the precursor of adrenodoxin which has a wider tissue distribution, does not show this tissue specificity [175–178]. The sequence or structural features responsible for this tissue specificity of transfer have not yet been identified.

The role of the mitochondrial targeting signals has also been examined using expression plasmids containing cDNAs with truncated or modified signal peptide sequences. Mature bovine adrenodoxin reductase and adrenodoxin sequences expressed in yeast (heterologous expression) were found in the cytosolic fraction because of the lack of the mitochondrial signal peptide [179]. Whereas, with a spliced yeast mitochondrial cytochrome *c* oxidase subunit

signal peptide sequence, the expressed proteins were directed into the mitochondrial fraction [179].

Unlike the mitochondrial *P450*s, the microsomal *P450*s and *P450* reductase do not have an extension peptide or basic residues in the amino termini (Table 4). The hydrophobic amino terminus of the microsomal *P450*s determines the membrane entry and association of these proteins [162, 163a]. Wild type *P450* reductase expressed in yeast is normally incorporated into the microsomes [180]. However, truncation of its hydrophobic amino terminus produces an enzyme that remains soluble in the cytosolic fraction [180]. Thus, this segment appears to be the only segment that anchors the protein to the microsomal membrane.

3.3. Sequence and structural similarities

The biochemical similarities between cytochromes *P450* noted above indicated some degree of structural similarity between these enzymes. The precise sequence similarities were established after the sequencing of the cDNAs for each of these enzymes. Comparison of the sequences of these enzymes revealed the following relationships [25]:

- (1) The microsomal steroidogenic *P450*s share <40% sequence similarity with each other. Thus each *P450* represents a distinct family with generally a single member. In contrast, the liver microsomal *P450*s, which are involved in the metabolism of endo- and xenobiotics, belong to large gene families which include many members with >50% sequence similarity [25].
- (2) The three mitochondrial *P450*s, namely *P450_{scc}*, *P450_{c11}* and *P450_{c18}*, share sequence similarity along their entire length [7, 8, 71].
- (3) The mitochondrial and microsomal *P450*s show sequence similarity only around a limited region in the heme binding segment close to the carboxy termini of the enzymes [181].
- (4) The microsomal *P450_{c17}* and *P450_{c21}* share sequence similarity in their amino terminal regions and carboxy terminal halves.
- (5) *P450_{c17}* and *P450_{c21}* share <30% overall sequence identity with the liver microsomal *P450*s. Thus, the liver microsomal and steroidogenic *P450*s represent distinct

but evolutionarily related families of genes.

- (6) The cDNA sequences of most of the human and bovine steroidogenic *P450* system enzymes have been determined (Table 4). The homologous enzymes in these two species share >70% sequence similarity.
- (7) The flavoprotein reductase components of the mitochondrial and microsomal *P450* systems show no sequence similarity and belong to different families of oxidoreductases [47, 52].
- (8) The analysis of the sequence of the mitochondrial adrenodoxin reductase led to the definition of a new large class of oxidoreductases that share an NADP binding site consensus sequence [47, 52]. These analyses also led to the discovery of a sequence difference between NAD and NADP binding sites [52, 182].

Determination of the 3-D structure of eukaryotic *P450*s has been hindered by the difficulties inherent in the crystallization of hydrophobic membrane bound proteins. However, the crystal structure of a bacterial *P450_{BM-3}* which shares significant sequence homology with eukaryotic microsomal *P450*s has been determined [183]. This structure can serve as a model to understand the structure of other *P450*s. The 3-D structure of *P450_{cam}* from *Pseudomonas putida* was elucidated earlier [184]. Although the structures of *P450_{BM-3}* and *P450_{cam}* share similar structural domains, the only significant sequence similarity between *P450_{cam}* and the eukaryotic *P450*s is observed around the heme binding region. There were many attempts to align and match the full sequence of *P450_{cam}* with eukaryotic *P450* sequences. However, the elucidation of the structure of *P450_{BM-3}* showed that these previous alignments did not correctly match different structural domains of the molecules (J. A. Peterson and S. S. Boddupalli, personal communication).

Currently the structure of many *P450*s that metabolize steroids is being investigated by expressing modified cDNAs, chimeric cDNAs that include parts of different *P450*s [e.g. 185], and fused cDNAs that include both *P450* and reductase [e.g. 186], in bacteria and yeasts. These studies are defining the role of specific regions and residues involved in substrate binding, enzymatic activity and interaction with membrane and other molecules. Some of these

are reviewed in this issue [24, 26]. The expression of modified *P450s* is also being used to examine the deleterious effects of genetic mutations on the enzymatic activity of *P450s* [187].

4. ROLE OF ENZYME EXPRESSION IN REGULATION OF STEROIDOGENESIS

Early studies based on measurements of enzyme activities indicated that some changes in blood levels of steroids may result from changes in the levels of specific steroidogenic enzymes ([129] for a historical view see Ref. [188]). However, assays of steroid levels in blood or *in vitro* steroid conversion in tissue homogenates may reflect many different effects other than enzyme levels. The purification of steroidogenic enzymes, production of antibodies and isolation of cDNAs provided refined tools to examine the role of steroidogenic enzymes in regulation of steroidogenesis.

Recent studies in this field have been guided generally by the following questions: (a) What is the tissue and cell distribution of steroidogenic enzymes? (b) Is there a relationship between the blood levels of steroids and the tissue levels of steroidogenic enzymes? (c) Which extracellular factors and hormones regulate the levels of steroidogenic enzymes? (d) What chains of molecular events are activated by hormones and other extracellular factors to ultimately change steroidogenic enzyme levels? (e) Are the changes in the levels of the enzymes a result of changes in the transcription, stability or translation of the mRNAs encoding the enzymes? (f) What are the gene sequences responsible for tissue and hormone specific regulation of the genes encoding the enzymes?

Current research on questions (a–e) is summarized below, citing mainly studies that utilized antibody and cDNA probes to measure enzyme and mRNA levels.

4.1. Enzyme expression as a determinant of steroidogenic capacity

Recent research established that the repertoire of enzymes expressed in a steroidogenic cell matches exactly the cell's capacity for the biosynthesis of specific steroids (Table 3). Thus, steroidogenic capacity is regulated mainly by tissue- and cell-specific expression of enzymes, and not by selective activation or inhibition of enzymes from a larger repertoire.

In adrenocortical and testicular tissue, the repertoire of enzymes remains constant during

adult life. Thus, while the *levels* of steroids secreted from these two organs show temporal fluctuations, the major types of steroids do not change (Section 4.3). In contrast, in ovarian follicles the repertoire of enzymes in granulosa cells changes during the final stages of follicle development (Table 3). Consequently, the ovarian steroid output shows persistent major changes in both the *major types and the levels* of the steroids secreted during the female reproductive cycle.

Rapid hourly fluctuations in steroid output cannot reflect changes in enzyme levels, and probably generally result from regulation of substrate cholesterol transport. The levels of steroidogenic enzymes, like most other proteins, are determined by the balance of *de novo* synthesis and normal metabolic degradation. For steroidogenic *P450s* both of these processes are slow, *P450s* are highly stable proteins. *In vitro* studies indicate a half life of 24–42 h [189]. *In vivo* studies indicate an even longer half life of several days [190, 191]. The mRNAs encoding *P450sc*, *P450c11*, *P450c17*, and *P450c21* are also stable with half lives ranging from 5 to 30 h [192].

4.2. Methodological issues

Histochemical techniques are the methods of choice to determine tissue and cellular localization of enzymes using anti-enzyme antibodies, or cDNA probes for *in-situ* hybridization [e.g. 146]. Histochemical techniques can also be used to investigate changes in enzyme levels for a qualitative assessment. However, quantitation of enzyme or mRNA level generally requires the use of protein or RNA blots which are probed using antibody or cDNA probes, respectively [e.g. 38, 121]. These methods can be applied to tissue samples directly. Yet, as the investigations proceed deeper into the molecular level of regulation, they require the use of *in vitro* systems of cultured cells.

Although culture conditions represent a drastic departure from *in vivo* conditions, steroidogenic cells in culture maintain many of their physiological responses. Yet, to avoid detachment from the physiology of steroid hormones, in the common use of *in vitro* systems it is important to consider the following questions: (a) Do the culture conditions inhibit or modify normal physiological responses? (Factors like substratum for cell growth [193], cell density [194, 195], medium and atmosphere composition [121, 196] have been shown to affect the

responses of steroidogenic cells.) (b) Does the blood or local level of the examined factor show changes that parallel the physiological variation in the steroid levels? (c) Is the concentration of the factor within physiological or pharmacological range? (d) Do the *in vitro* changes in steroidogenic capacity and enzyme levels reflect physiological changes in steroid and enzyme levels?

4.3. Adrenal cortex

The blood levels of the major steroid products of all three zones of adrenal cortex, vary in a circadian pattern in response to trophic hormones specific for each tissue [197–200]. Steroidogenic enzyme levels cannot vary drastically within 12 h (see Section 4.1). Thus, circadian variations cannot result from changes in the levels of the enzymes, and most probably reflect hormonal stimulation of steroid biosynthesis via increased cholesterol transport to mitochondria. This assumption is supported by the observations that in rats the diurnal increase in plasma corticosterone is associated with a diurnal decrease in adrenal cholesteryl esters that serve as the precursor of adrenal steroids [200]. In man, the patterns of circadian variation of some adrenal androgens are different from that of cortisol, indicating that these may be regulated mainly by factors different from ACTH [199].

The blood levels of adrenocortical steroids rise rapidly in response to stress conditions that stimulate ACTH secretion [201]. After ACTH injection to human subjects, both plasma cortisol and aldosterone levels increase several fold within 1 h [139, 202]. However, the plasma level of ACTH, after injection, highly correlates with cortisol, but not aldosterone levels, indicating that while cortisol secretion is mainly determined by ACTH, aldosterone secretion is affected by multiple factors secondary to ACTH stimulation [202]. The hypothalamus–pituitary–adrenal system can also be stimulated by cytokines, e.g. interleukin-1, produced by the cells of the immune system [203]. Rapid increases in steroid secretion in response to ACTH or other factors, probably as a rule, result from stimulation of steroidogenesis via increased cholesterol mobilization and transport into mitochondria [2].

The activation of the hypothalamus–pituitary–adrenal axis is vital to cope with stress and disease [201]. Under continued stress, the constant stimulation of the adrenal cortex can enhance its capacity to secrete glucocorticoids by increasing the levels of key steroidogenic

enzymes. Blood glucocorticoid levels are elevated many fold in certain disease states [204, 205], while adrenal androgen levels may be suppressed [205]. Moreover, during acute illness, the responsiveness of the adrenal cortex to ACTH stimulation increases, indicating enhanced steroidogenic capacity probably due to increased steroidogenic enzyme levels (Hanukoglu A., Fried D. and Hanukoglu I., unpublished observations). ACTH treatment increases the activities of certain adrenal steroidogenic enzymes in rodents [133, 206], and changes the cellular fine morphology [207]. Extensive ACTH exposure initially stimulates cellular hypertrophy of adrenocortical cells without causing cell proliferation [208]. Thus, elevations in blood glucocorticoid levels during acute disease probably do not represent adrenal tissue growth.

Plasma levels of glucocorticoids do not differ between males and females [139]. In bovine adrenal cortex, the levels of *P450_{scc}*, *P450_{c11}*, and their electron transfer proteins are similar in different animals and show no sex difference [121]. In guinea pig adrenal cortex the levels of *P450_{c17}* and *P450_{c21}* are also similar in both sexes [131]. In inbred mice, strain differences have been observed in the levels of steroidogenic enzymes in both adrenal cortex and testis [209]. Some *P450*s in the guinea pig adrenal cortex that are immunologically related to the liver *P450*s, and *5 α* -reductase in the rat adrenal cortex, show sexual dimorphism and are affected by sex steroids [131, 210]. These findings indicate that sex steroids do not have a major influence on the initial steroidogenic pathway of the adrenal cortex, but determine later steps of steroid metabolism in these species. Certain steroid metabolizing enzymes in the liver are also expressed in a sexually dimorphic pattern [28, 29]. This dimorphism may have developed to deal with the exposure of each sex to a different steroid spectrum.

Daily fluctuations in the levels of ACTH are not immediately associated with changes in enzyme levels; yet, this daily intermittent stimulation of the adrenal cortex is essential for the maintenance of the normal levels of the steroidogenic enzyme in these tissues. In hypophysectomized animals, the activities of many steroidogenic enzymes decrease greatly [17, 129, 190, 191], in addition to many other cellular changes [e.g. 208]. If the duration of trophic hormone deprivation is not prolonged, these changes can be reversed by the administration of trophic hormones [190, 191].

The secretion of aldosterone from zona glomerulosa is regulated mainly by the renin-angiotensin system and blood sodium and potassium levels [6–8, 149–151]. Angiotensin II can rapidly stimulate aldosterone secretion from glomerulosa cells by increasing cholesterol mobilization similar to ACTH stimulation of the zona fasciculata [2]. However, the intracellular transduction systems involved in this rapid stimulation are different from that of ACTH [151, 211].

In rats fed a low sodium or high potassium diet, plasma aldosterone levels increase to maintain a normal level of plasma electrolytes [150]. The maintenance of this diet for several days, enhances the activity, the protein level, and the mRNA level of *P450scc* and *P450c18* while the same parameters for *P450c11* remain without a significant change [149, 150]. These results establish that constant stimulation of zona glomerulosa activity enhances its steroidogenic capacity by increasing specific enzyme levels in zona glomerulosa and not in zona fasciculata [149, 150].

4.4. Testis

Similar to adrenocortical steroids, the plasma levels of the testicular steroids fluctuate in response to diurnal variations in trophic hormone gonadotropin secretion [197]. Studies on hypophysectomized animals indicate that this daily intermittent stimulation by gonadotropins is necessary for the expression of steroidogenic enzymes [190, 191, 212]. Yet, the degree of this dependence varies for different enzymes [212]. In different strains of inbred mice the steroidogenic capacity of the Leydig cells is highly correlated with the levels of *P450scc* [209].

In hypogonadal mice with a defective gonadotropin releasing hormone gene, and consequently undetectable levels of LH and FSH, the activities of most steroidogenic enzymes, except 3β -HSD, are very low or undetectable in the testis [213]. LH treatment of these mice increases the testicular enzyme activities within 10 days. These results provide additional evidence for the dependence of steroidogenic enzyme expression on gonadotropin stimulation, and reveal that exposure to gonadotropins during ontogeny is not essential for the development of this capacity [213].

In contrast to adrenal cortex, stress and pathological conditions often disrupt the function of the hypothalamic-pituitary-testicular axis by suppressing LH [214]. However, there may also

be effects directly on the testicular function [214]. *In vivo* immune activation of macrophages that neighbor Leydig cells in the interstitial tissue was found to decrease the level of *P450c17* in Leydig cells of mice to 10% of control values [215]. *In vitro* treatment of Leydig cells with interleukin-1 similarly decreased *P450c17* expression [215]. Interferon- γ also inhibits Leydig cell steroidogenesis and hCG induced enzyme expression [216]. Thus, cytokines and other factors released from immune cells may affect testicular steroidogenic output by suppressing the expression of enzymes essential for androgen synthesis.

4.5. Ovary

In contrast to the adrenal cortex and testis, the blood levels of ovarian steroid hormones change greatly during the female reproductive cycle, which can last many days. The estrous cycles of rodents and ruminants, and the menstrual cycles of primates are characterized by stages of ovarian follicle development, ovulation, corpus luteum formation and subsequent luteolysis and regression in the absence of pregnancy. The lengths of these stages vary greatly among species, yet, the pattern of steroid hormone levels during the reproductive cycle of most mammalian species shows certain common characteristics: (1) a sharp rise in estradiol levels prior to the ovulatory surge of LH. (2) An increase in progesterone levels after ovulation that is sustained during the luteal phase or ensuing pregnancy (human [217], monkeys [218–220], cow [221], rat [222]). The studies, summarized below, indicate that these changes result from major changes in the levels of the steroidogenic enzymes during the development of the ovarian follicle.

The rise in estradiol levels prior to ovulation is dependent on LH/FSH stimulation of the final stages of preovulatory follicle development [134]. As noted above, estradiol biosynthesis by *P450arom* in granulosa cells is dependent on androgen precursor from the theca interna (Table 3). Thus, to increase estradiol biosynthesis, the following changes would be expected to occur in the follicle: a rise in theca cell enzymes responsible for androgen biosynthesis, e.g. *P450scc* and *P450c17*, and a rise in granulosa cell *P450arom*. Studies on rats indeed showed that gonadotropin stimulation greatly enhances *P450scc* levels in thecal and interstitial tissue within 24 h, while granulosa cells remain without detectable *P450scc* [223]. The level of

P450c17 is correlated with the developmental stage/size of preovulatory follicles [135]. *In vitro* studies with human and rat granulosa cells showed that gonadotropin stimulation greatly increases *P450arom* mRNA, protein level and activity [109, 224]. These studies establish that the preovulatory rise of estradiol is a result of enhancement of expression of specific steroidogenic enzymes in a defined population of cells in the preovulatory follicle.

The increase in blood progesterone levels after ovulation, results from two concomitant processes: growth of the corpus luteum, and an increase in steroidogenic enzymes. Progesterone biosynthesis is dependent on two enzymes *P450scc*, and 3β -HSD (Fig. 1). The expression of these enzymes is greatly enhanced in luteal cells (cow [121, 225–227], pig [122], rat [17, 135, 228, 229]). Expression of *P450scc* in rat granulosa cells is initiated with a delay after expression in theca cells, and takes place close to the time of LH surge [223, 229]. After ovulation, the theca and granulosa cells become the progenitors of small and large luteal cells of the corpus luteum, while this cell lineage continues to be reflected in the functioning of the two types of luteal cells [230–232, 291]. The ovulatory surge of LH is associated with the disappearance of *P450c17* expression in bovine [225] and rat [135], but not in human corpora lutea. The factor(s) that mediate this suppression may be different from LH, because in cultured luteinizing rat follicles the activity of *P450c17* is maintained with or without LH [135, 154]. The continued expression of *P450arom* for estrogen synthesis in the corpus luteum varies among species [109, 138, 230].

In the corpus luteum, the total amount of *P450scc* can increase about 100-fold over the levels found in the ovary (Table 5). This is accompanied by increases in the levels of the electron carriers adrenodoxin reductase and adrenodoxin [121, 122]. In contrast, the level of the microsomal *P450* reductase shows no significant change [225]. The increases in the levels of *P450scc* and 3β -HSD are correlated with increases in their mRNAs, indicating enhanced transcription of the respective genes [226, 227]. The correlation of the levels of the three mitochondrial *P450scc* system proteins [121] indicate that the genes of these enzymes may share certain common regulatory sequences which remain to be identified. The change in the levels of these enzymes is probably directly initiated by gonadotropins

which have been shown to also enhance their expression in granulosa cells in culture, ([154]; human [137, 233, 234], bovine [235], porcine [236, 237], rat [229, 238], chicken [238a]. However, after this initial turning-on, the expression of these genes can continue in luteal cells in the absence of gonadotropin stimulation [68, 154, 229].

One interesting aspect of steroidogenic enzyme expression in the developing follicle is the vectorial change that is initiated by the gonadotropins. During the final stages of maturation of the rat preovulatory follicle, *P450scc* is expressed first at the periphery of the follicle in theca interna cells and then in granulosa cells and finally, just before ovulation, in cumulus cells [223, 241]. The sequential expression of *P450scc* in the different follicular cells could result from a gradient of gonadotropin concentration or responsiveness in the follicle. In the rat preovulatory follicle, the concentration of LH receptors shows a steep gradient decreasing from the periphery of the follicle to its center [242, 243]. Immunohistochemical and *in situ* hybridization studies revealed that LH receptors are expressed only in granulosa cells of preovulatory follicles, but not in small follicles [291, 292]. Thus, the expression of *P450scc* in granulosa cells is closely associated with the induction of LH receptors in these cells.

The steroidogenic response of follicular cells to gonadotropins may be modulated by other endocrine and paracrine factors, e.g. ovarian steroids, inhibin, activin, and various peptide factors [138, 244, 245]. Among these, estradiol and insulin-like growth factors (IGF) have been shown to have a strong synergistic effect with gonadotropins on the induction of *P450scc* in granulosa cells from some species [67, 236, 246]. In human granulosa cells, IGF-II and *P450scc* mRNAs are coordinately regulated [233]. IGFs probably function mainly as paracrine factors, mediating some actions of trophic hormones, as they are secreted by ovarian cells, and can act locally on granulosa cells that have receptors for IGF [245]. Vasoactive intestinal peptide was shown to induce *P450scc* in rat granulosa cells though to a very minor degree as compared to FSH [247].

Ovarian cells possess a system of cholesterol mobilization similar to adrenocortical cells [237, 248, 249]. Thus, gonadotropin stimulation of cholesterol mobilization is required in addition to effects on enzyme expression.

4.6. Mechanisms of regulation of steroidogenic enzyme levels

The studies summarized above indicate that the level of expression of each steroidogenic enzyme varies in three characteristics:

- (a) Tissue- and cell-specific expression, determined by processes that switch genes on or off during tissue and cell differentiation, fixing the fate of the regulation of the gene in successive lineages of the cell.
- (b) Basal expression, determined by intracellular processes in the absence of trophic hormonal stimulation.
- (c) Hormonal signal regulated expression, determined by hormonal stimuli that affect the expression of the gene positively or negatively.

Each of these three types of expression probably represent the functioning of specific gene regulatory elements.

As noted above, in adult steroidogenic tissues, the levels of most of the cell- and tissue-specific steroidogenic enzymes depend mainly on trophic hormonal stimulation, independent of whether the cell's repertoire of enzymes remains constant (as in the adrenal cortex and testis) or changes as a result of tissue growth (as in the ovarian follicle) (see Section 4.1). In contrast, during ontogenic development, the same trophic hormones may not be necessary for the initial expression of enzymes [213, 250].

In adult tissues, trophic hormones may affect steroidogenic enzyme synthesis via effects on transcription, stability or translation of the mRNAs that encode the enzymes. The major physiological variations in enzyme levels (as in ovarian follicular and adrenal glomerulosa cells, see above) are associated with parallel changes in mRNA levels. The dependence of enzyme synthesis on transcriptional induction is most conspicuous in ovarian granulosa cells during follicle growth (see Section 4.5). *In vitro* studies provide further evidence that trophic hormones can enhance the transcription of genes encoding steroidogenic enzymes: after trophic hormonal stimulation of steroidogenic cells in culture, mRNA levels for steroidogenic enzymes generally increase within a few hours, and reach peak values at 6–12 h (adrenal cortex [38, 39, 90, 99, 132, 147, 234, 248], ovary [68, 153, 224, 229, 235–239] testis [40, 119, 251]).

In a study that measured both *P450_{scc}* system enzyme and mRNA levels (using antibody and

cDNA probes, respectively), enzyme levels increased only between 6–12 h after ACTH stimulation of adrenocortical cells, a time when mRNA levels had already reached their peak values [38]. Thus, the increase in enzyme level lags behind the mRNA level by 6–12 h [38]. When the mRNA levels start to drop, the levels of the enzymes do not show an accompanying decrease, probably reflecting the fact that the enzymes have much longer half lives than the mRNAs (Section 4.1). The time course and magnitude of induction of mRNAs encoding different enzymes vary significantly, indicating differential regulation of the genes (see Ref. [38]).

The reason for the relative slowness of the induction of the steroidogenic enzyme mRNAs is currently not known. The mechanism of this induction is probably different from that of other genes that can be induced rapidly, within <1 h [252]. In some steroidogenic cells, the induction of *P450* mRNA synthesis can be inhibited by cycloheximide, implicating a requirement for the synthesis of a specific protein that may be necessary for the induction [250, 253, 254]. This effect has not been consistently observed in all cases of steroidogenic *P450* induction [255, 256]. Trophic hormones ACTH, angiotensin II, and hCG induce rapidly (in <1 h) the transcription of genes encoding *c-fos* and *jun-B* in steroidogenic cells [257]. These 'primary response' gene products function as transcription factors [258], and may be involved in the regulation of expression of steroidogenic enzymes.

Enzyme levels may also be affected by a change in the stability or translation rate of the mRNAs that encode them. In bovine adrenocortical cells in culture, ACTH was observed to increase the stability of *P450_{scc}* mRNA 5-fold but had no effect on *P450_{c11}*, *P450_{c17}*, and *P450_{c21}* mRNAs [192]. In *in vivo* studies with guinea pigs, chronic ACTH treatment increases the activities of steroidogenic enzymes, without a significant effect on the levels of the mRNAs encoding the enzymes [133]. This suggests that under this chronic treatment, ACTH may have a post-transcriptional effect on the mRNAs encoding steroidogenic enzymes [133].

4.7. Intracellular regulators of steroidogenic enzyme expression

Trophic hormones regulate the function of steroidogenic cells by binding to specific receptors on the cell surface and activating intracellular signal transduction systems [259–263]. Trophic hormone receptors that have been

characterized to-date belong to the superfamily of G-protein linked receptors with seven hydrophobic segments embedded in the cell membrane [264, 265]. The interaction of hormone bound receptor with specific G-proteins [266] activates some membrane bound enzymes, e.g. adenylate cyclase and phospholipases, causing a transient increase in intracellular levels of second messengers such as cAMP, cGMP, inositol triphosphate, Ca^{2+} , and diacylglycerol. These small regulatory molecules transmit the hormonal signal inside the cell by activating protein kinases, phospholipases, or by directly interacting with other proteins. For example, cAMP activates protein kinase A, and Ca^{2+} and diacylglycerol activate protein kinase C [259–262]. An additional signal transduction system involves tyrosine kinases, which are generally activated by growth factors, but also may be involved in the actions of some hormones [263]. Some of these different signaling systems may be activated in concert to modulate the cellular response by a complex crosstalk network [259–262]. The ultimate cellular response to hormones is generally effected by protein kinases, which phosphorylate proteins and affect their function [259–262].

In examining possible molecules that may mediate the effects of trophic hormones, the following questions are posed: (1) Does the agent have the same effect as the hormone? (2) Does the trophic hormone increase the levels of the agent at doses similar to those required for the cellular response? (3) Are the time courses and durations of action of the agent and the hormone similar? (4) Does inhibiting the action of the agent inhibit the action of the hormone? Based on some of these criteria, the following agents have been suggested as mediators of rapid hormonal activation of steroidogenesis via cholesterol mobilization: cAMP, cGMP, inositol triphosphate, Ca^{2+} , diacylglycerol and arachidonic acid [211, 267–281].

In both adrenocortical and gonadal cells, cAMP has been viewed as the main mediator of trophic hormone (ACTH and LH/FSH, respectively) induction of steroidogenic enzymes, based on the following observations: (a) these hormones stimulate adenylate cyclase and increase intracellular cAMP (refs in previous paragraph); (b) treatment of cultured cells for > 24 h with cAMP analogs, or other agents (e.g. forskolin, cholera toxin) that increase intracellular cAMP, induces expression of steroidogenic enzyme mRNAs to the same levels as hormonal stimulation (refs in Sections 4.3–4.5); (c) a mutation

in cAMP activated protein kinase A alters enzyme inducibility in a mouse adrenocortical cell line [282].

The criterion of similar duration of action (see above) was examined only in a recent study that compared the effects of ACTH and cAMP [38]. Normally, ACTH is secreted in pulses with a circadian pattern and the blood level of ACTH shows a peak that lasts only a few hours [198]. The intracellular levels of cAMP peak even more rapidly, within 5–10 min after hormonal stimulation, and then gradually decrease [38, 283]. In an experiment designed to mimic this physiological pattern, adrenocortical cells were exposed to ACTH, cAMP analogs, or forskolin, for only short periods, and the cells were harvested after 12–36 h to assay enzyme and mRNA levels. Whereas, a short exposure (30–60 min) of cells to ACTH induced the enzymes and their mRNAs, cAMP induction required treatment of cells continuously for nearly 24 h [38]. These findings question the physiological relevance of experiments using prolonged (24 h) stimulation with cAMP analogs, and suggest that trophic hormone action is not mediated solely through cAMP but uses additional signal transduction systems.

The cAMP pathway of enzyme induction may be modified by the activation of other signaling systems. As noted in Section 4.5, IGF-I has a synergistic effect with gonadotropins on the induction of *P450scc* in granulosa cells. Similarly, in secondary cultures of adrenocortical cells the induction of 11β - and 21 -hydroxylases by cholera toxin, but not of 17α -hydroxylase, is dependent on IGF-I [284, 285]. A tyrosine kinase inhibitor, tryphostin, arrests FSH or cAMP induced synthesis of *P450scc* in granulosa cells, suggesting that a tyrosine kinase may be involved in the pathway of signal transduction downstream from cAMP [286].

Besides protein kinase A, both adrenocortical and gonadal cells possess protein kinase C [287, 288]. The concurrent activation of protein kinase A and C pathways, (using agents that increase cAMP, and phorbol ester, TPA, respectively) generally has been observed to suppress expression of steroidogenic enzymes in both adrenocortical cells [61, 284, 289] and a granulosa cell line where *P450scc* can be induced by cAMP analogs [167, 290]. In the granulosa cells, TPA shows no effect by itself, but severely inhibits induction of steroidogenesis when added with forskolin [287]. TPA does not inhibit forskolin elevation of cAMP levels, indicating that

TPA prevents the action, but not the synthesis of cAMP [287]. The effect of TPA on P450c21 expression in adrenocortical cells depends on the mitotic activity of the cells: in non-dividing cultures it is without effect, whereas in cultures with high mitotic activity it induces P450c21 at low concentrations (0.3–3 nM TPA), and inhibits expression at a higher concentration [285]. Even extensive exposure to ACTH does not stimulate adrenocortical cell proliferation *in vivo* [208]. Thus, effects in cultures undergoing mitosis probably do not reflect the normal function of the adult adrenal cortex. The inhibitory effect of protein kinase C activation may be part of a homeostatic mechanism.

To summarize the current results it can be concluded that while cAMP pathway may be part of the mechanism of steroidogenic enzyme gene induction by trophic hormones, many parts of the puzzle are still missing. There are lines of evidence indicating that the same pathway is not mediating both rapid cholesterol mobilization and slow enzyme induction. The pathways for these distinct processes may have a common origin but we do not yet know where and how they diverge. Elucidation of the mechanism of enzyme induction requires an understanding of how brief pulses of trophic hormones can exert long lasting effects [e.g. 38]. These actions may be mediated by a cascade of short-lived mediators (e.g. cAMP → protein kinase A → protein phosphorylation → primary response gene induction → cellular response), or alternatively by as yet unidentified long-lasting mediators that after initial hormonal stimulation maintain a metabolic memory in the cell. Future research coming from two directions, investigating events from the cell membrane towards the cell nucleus on the one hand, and from gene regulatory elements towards the cytoplasmic regulatory factors on the other, gives hope to provide the answers.

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