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Role of Microsomal Steroid Hydroxylases in Δ^7-Steroid Biosynthesis

T. A. Sushko*, A. A. Gilep, A. V. Yantsevich, and S. A. Usanov

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Kuprevicha 5/2, 220141 Minsk, Belarus; fax: +375 (172) 637-274; E-mail: sushko-tatjana@rambler.ru; agilep@iboch.bas-net.by

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Abstract—CYP17 (steroid 17α-hydroxylase/17,20-lyase) is a key enzyme in steroid hormone biosynthesis. It catalyzes two independent reactions at the same active center and has a unique ability to differentiate Δ^4-steroids and Δ^5-steroids in the 17,20-lyase reaction. The present work presents a complex experimental analysis of the role of CYP17 in the metabolism of 7-dehydrosteroids. The data indicate the existence of a possible alternative pathway of steroid hormone biosynthesis using 7-dehydrosteroids. The major reaction products of CYP17 catalyzed hydroxylation of 7-dehydropregnenolone have been identified. Catalytic activity of CYP17 from different species with 7-dehydropregnenolone has been estimated. It is shown that CYP21 cannot use Δ^5-Δ^7 steroids as a substrate.

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Key words: cytochrome P450, CYP17, CYP21, 7-dehydrosteroids, steroid hormone biosynthesis

Steroid hormone biosynthesis pathways vary greatly among different species of vertebrate animals. Differences in catalytic activity and expression profiles of cytochrome P450s involved in steroid hormone biosynthesis supply needs of the particular animal species.

CYP11A1 catalyzes the first, rate-limiting for steroid hormone biosynthesis reaction of conversion of cholesterol to pregnenolone (**P5**) (Fig. 1) [1]. P5 might be further converted by the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase to progesterone (**P4**) or be metabolized by cytochrome CYP17 in the endoplasmic reticulum.

Cytochrome CYP17 (17α-steroid hydroxylase/17,20-lyase) is a key enzyme of steroid hormone biosynthesis that is unique due to its ability to catalyze two independent reactions in the same active center. CYP17 catalyzes either selective 17α-hydroxylation of P5 and P4 with the formation of 17α-hydroxy-derivatives serving as a precursors for mineralocorticoids and glucocorticoids biosynthesis, or 17,20-lyase reaction, where 17α-hydroxyprogesterone (**17OHP4**) and 17α-hydroxypregnenolone (**17OHP5**) are transformed to androstendione (**AND**) and dehydroepiandrosterone (**DHEA**), which are precursors for sex hormone biosynthesis [2, 3] (Fig. 1). The ratio between the 17α-hydroxylase and 17,20-lyase activities is physiologically important and may direct steroid hormone biosynthesis to the production of either corticoids or sex hormones [2]. Cytochrome CYP21 (steroid 21-hydroxylase), in turn, catalyzes the reaction of P4 and 17OHP4 conversion to 11-deoxycorticosterone (**DOC**) and 11-deoxycortisol (**DCS**) accordingly, providing an important step in aldosterone and cortisol synthesis [4]. Cytochrome CYP17 is characterized by the unique ability to differentiate Δ^4-steroids (P4 derivatives) and Δ^5-steroids (P5 derivatives) [5, 6]. There are significant species-specific differences in the metabolism of P4 and P5 [7-15].

Until recently, it was suggested that the main routes of steroid biosynthesis in mammals had been studied in

Abbreviations: ALA, 5-aminolevulinic acid; AND, androstendione; CPR, NADPH-dependent cytochrome P450-reductase; CYP17, steroid 17α-hydroxylase/17,20-lyase; CYP21, steroid 21-hydroxylase; DCS, 11-deoxycortisol; DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone; DTT, dithiothreitol; 7dhC, 7-dehydrocholesterol; 7dhP5, 7-dehydropregnenolone; 7dh17OHP5, 7-dehydro-17α-hydroxypregnenolone; IPTG, isopropyl-β-D-thiogalactopyranoside; K_d, dissociation constant; 17OHP4, 17α-hydroxyprogesterone; 17OHP5, 17α-hydroxypregnenolone; P4, progesterone; P5, pregnenolone; PMSF, phenylmethylsulfonyl fluoride.

* To whom correspondence should be addressed.

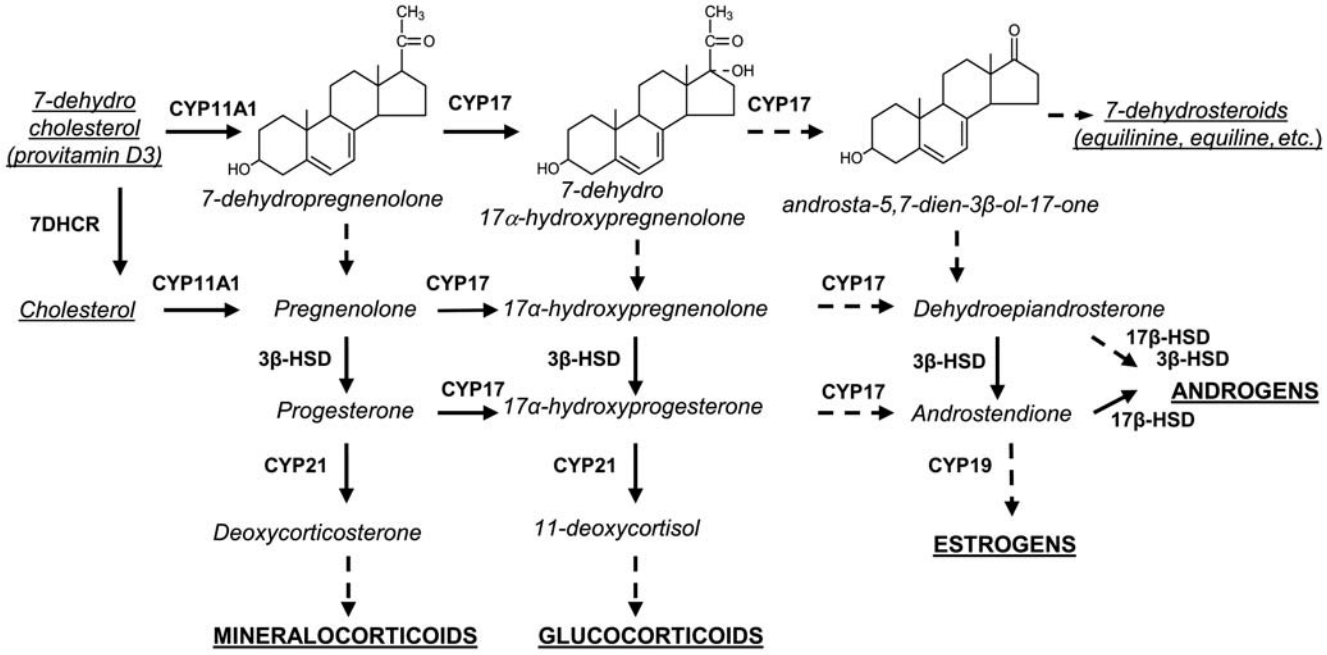

Fig. 1. Scheme of steroid hormone biosynthesis pathway in *Chordata*.

detail and the chemical structures of the final products and intermediates were known. However, investigations of the metabolism of alternative steroids and their physiological significance are now very relevant. For example, it was shown that 7-dehydrocholesterol (**7dhC**) might be effectively metabolized *in vitro* by CYP11A1, which catalyzes the conversion of cholesterol to P5, with the production of 7-dehydropregnenolone (**7dhP5**) [16, 17]. The kinetic parameters of this process are comparable with the oxidation of cholesterol by the same enzyme [17]. This fact indicates the possible existence of an alternative pathway of biosynthesis of steroid hormones with unsaturated bonds in the B-ring, which begins from the conversion of 7dhC by cytochrome CYP11A1.

The presence of conjugated double bonds in positions 5 and 7 of the steroid B-ring is a characteristic feature of 7-dehydrosteroids. The enzyme 7-dehydrocholesterol reductase reduces unsaturated bonds in the B-ring of the molecule and converts 7dhC, which is an intermediate in a cholesterol biosynthesis pathway, to cholesterol. Insufficiency of 7-dehydrocholesterol reductase catalytic activity causes significant decrease or lack of conversion of 7dhC to cholesterol and accumulation of steroidal 5,7-dienes. This pathological condition is described as Smith–Lemli–Opitz syndrome (SLOS) [18]. More than a half of the steroids circulating in a fetus with SLOS are estrogens with unsaturated bonds in the B-ring [19]. This fact suggests the participation of enzymes involved in steroid biosynthesis in the metabolism of Δ^7-steroids in the case of this pathology.

Furthermore, there are indications of the metabolism of steroids with unsaturated bonds in the B-ring not only under pathological conditions, but also under normal conditions. Therefore, a significant amount of 7dhC, which is localized at the plasma membranes of keratinocytes, is accumulated in skin, where the precursors of vitamin D3 is formed from 7dhC by exposure to ultraviolet radiation [20]. Vitamin D3, like vitamin D2, is effectively metabolized by cytochrome CYP11A1 [21, 22]. Evidence for the expression of steroid hormone biosynthesis enzymes such as CYP17 and CYP21 in skin [23] suggests the possibility of subsequent metabolism of 7-dehydrosteroids.

Increased level of Δ^7-steroids is typical for some animals. High content of estrogens with unsaturated bonds in the B-ring is found in pregnant mares [24]. It seems that estrogens of this type play a role only in a certain stage of ontogenesis [25]. Additionally, it was demonstrated *ex vivo* that 7dhC is effectively converted to 7dhP5 by adrenal glands of various animals [22]. Furthermore, steroidal Δ^5-Δ^7 and Δ^4-Δ^7-dienes are formed in adrenal tissues.

Therefore, the goal of the present study was to elucidate the role of human microsomal cytochrome P450s involved in steroid hormone biosynthesis in the metabolism of Δ^7-steroids. In the present work, the possibility of metabolism of 7-dehydrosteroids is shown for the first time, and the products of the reaction are identified. The features of the metabolism of 7-dehydrosteroids by CYP17 from different species have been studied.

MATERIALS AND METHODS

Materials. The following chemicals were used in the present work: 7-dehydrocholesterol, P4, 17OHP4, P5, 17OHP5, sodium cholate, Triton X-100, NADPH, phenylmethylsulfonyl fluoride (PMSF), aminolevulinic acid (ALA was synthesized in the Laboratory of Lipid Chemistry of the Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus and kindly provided by Prof. M. A. Kisel), 2-hydroxypropyl-β-cyclodextrin, sodium dodecyl sulfate, Coomassie brilliant blue R-250, Hepes, Tris buffer, arabinose, ampicillin, kanamycin, cholesterol oxidase (from *Cellulomonas* species) (Sigma, USA); Ni^{2+}-NTA agarose (Qiagen, USA); Emulgen 913 (Em-913) (Kao Atlas, Japan); agarose, isopropyl-β-D-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) (Gibco BRL, USA); Bacto-Tryptone, Bacto-Peptone, and Bacto-Yeast extract (Difco Laboratories, USA); 7-dehydropregnenolone (Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus), Bio-Gel HTP (Bio-Rad, USA); methylene chloride (Ekos-1, Russia). Restriction enzymes and other enzymes for DNA modification were from New England Biolabs (USA) and Promega (USA).

Expression of CYP17 and CYP21. *Escherichia coli* JM109 competent cells were transformed with plasmid pCWori containing *CYP17A1* (or pTrc99 containing human *CYP21A2*) and plasmid pGro12 containing molecular chaperones GroES–GroEL [26, 27]. Transformed cells were screened on Petri dishes with LB-agar containing ampicillin (100 μg/ml) and kanamycin (40 μg/ml). Overnight culture (3 ml) was used to inoculate 0.5 liter of TB-medium containing 100 mM potassium-phosphate buffer, pH 7.4, rare salt solution, ampicillin (100 μg/ml), and kanamycin (40 μg/ml). The mixture was incubated in a thermostated orbital shaker at 37°C and 180 rpm. After reaching absorbance A_{600} ~ 0.4, protein expression was stimulated by adding IPTG (0.5 mM), also adding ALA (0.65 mM), arabinose (4 mg/ml), ampicillin (100 μg/ml), and kanamycin (40 μg/ml). After 48 h of incubation in the orbital shaker at 26°C and 140 rpm, the cells were cooled for 1 h at 4°C and collected by centrifugation (5000*g*, 10 min). The pellet was suspended in buffer A (50 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol, 0.3 M NaCl) containing 0.5 mM PMSF and 50 μM P4 (1 volume cells/4 volumes buffer). The cells were frozen to the temperature of –73°C.

Isolation and purification of recombinant proteins. Cells were thawed and treated using an Emulsiflex C5 apparatus (Avestin, Canada). Recombinant CYP17 and CYP21 were solubilized from membranes by adding drops of Em-913 to final concentration 1%. The suspension was centrifuged 1 h at 100,000*g* to remove non-solubilized membrane structures. The supernatant was applied to a column with Ni^{2+}-NTA-agarose equilibrated with buffer A. The column was washed with 2-3 volumes of buffer A containing 0.2% Em-913 and then with 10 volumes of buffer A containing 0.2% Em-913 and 100 mM glycine (buffer B). Proteins were eluted from the column with buffer B containing 50 mM histidine and 0.2% Em-913 (0.5% for CYP21). Eluted fractions were applied to a column with hydroxyapatite equilibrated with 10 mM potassium-phosphate buffer, pH 7.4. The column was washed with 10 volumes of 50 mM potassium-phosphate buffer, pH 7.4, containing 20% glycerol, 50 μM P4, 0.2% Em-913 (0.5% for CYP21), and 0.1 mM DTT. The proteins were eluted from the column with 0.3 M potassium-phosphate buffer, pH 7.4, containing 20% glycerol, 50 μM P4, 0.2% Em-913 (0.5% for CYP21), and 0.1 mM DTT. The highly purified heme proteins were stored at –73°C. Highly purified recombinant NADPH-cytochrome P450 reductase (CPR) from *Rattus norvegicus* was heterologously expressed in *E. coli* and purified using affinity chromatography as previously described [28].

Analytical methods. Cytochrome P450 concentration was determined spectrophotometrically using coefficient $\varepsilon_{450\text{-}490} = 91\ mM^{-1}\cdot cm^{-1}$ for the absorbance of the complex of reduced hemoprotein with CO [29]. The CPR concentration was determined from the absorption spectrum using molar extinction coefficient $\varepsilon_{456} = 21.4\ mM^{-1}\cdot cm^{-1}$ [30]. Protein homogeneity was determined electrophoretically using SDS-PAGE.

Interaction of cytochrome P450 with substrate induces changes in conformation of the active center of the molecule and leads to the transition from six-coordinated low-spin heme-iron species towards five-coordinated high-spin complex [31] that is visualized by increase in absorbance at wavelength 390 nm and decrease in absorbance at 420 nm. These changes are referred to as the type I spectral response. Titrations with the steroid substrates were conducted in 50 mM potassium-phosphate buffer, pH 7.4. Each cuvette contained 1 μM of protein solution. Ligands, dissolved in ethanol, were added to the final concentration of 50 μM. The same volume of ethanol was added to the control cuvette. The titration was stopped if light scattering increased and the baseline changed as a result of decrease in solubility of the steroid with increase in concentration. When the dissociation constant (K_d) was calculated, ligands with values more than 25 μM were not further used. The maximal concentration of steroid (50 μM) was used to determine the fullness of the enzyme active center occupation with the steroid. The K_d value was calculated using SigmaPlot:

$$A = (\Delta A_{max}/2E_t)\cdot(S + E_t + K_d) - \sqrt{(S + E_t + K_d)^2 - (4\cdot S\cdot E_t)}$$

[32, 33],

where A – amplitude of the spectral response, A_{max} – amplitude of the spectral response under saturating concentration of ligand, E_t – total protein concentration, S –

total ligand concentration, K_d – dissociation constant of the enzyme–substrate complex.

Determination of CYP17 catalytic activity. The 17α-hydroxylase activity of the recombinant CYP17 was determined in a reconstituted system containing CYP17 and CPR. Aliquots of concentrated proteins (0.5 μM CYP17 and 1 μM CPR) were mixed and incubated at room temperature for 5 min. The steroid substrates were added to the reaction mixture to final concentration 50 μM and incubated 10 min at temperature 37°C. The reaction was started by adding NADPH to final concentration 0.25 mM. Aliquots (0.5 ml) were taken from the incubation mixture at selected time intervals. The reaction was stopped by adding 5 ml of methylene chloride. The mixture was vigorously shaken, and the layers were separated by centrifugation at 5000*g* for 10 min. The aqueous layer was carefully removed, and the organic layer was dried under a flow of argon at temperature 40°C. The dry residue was dissolved in 100 μl of methanol and analyzed using an Accella HPLC equipped with diode-array and mass-spectrometer detector LCQ-Fleet (ThermoSci, USA).

Determination of CYP21 catalytic activity with 7dhP5. The catalytic activity of CYP21 was determined in a reconstituted system containing CYP21 and CPR. Activity was measured as described above for the CYP17 in a system containing 0.05 μM P450 and 0.1 μM CPR at temperature 37°C.

RESULTS

Estimation of parameters of interaction of CYP17 with steroid substrates. Cytochromes P450 involved in steroid hormone biosynthesis are characterized by high substrate specificity. A distinctive feature of cytochromes P450 is a characteristic change in spectral properties of the heme protein occurring on ligand binding. Substrate binding induces type I spectral response, which is characterized by decrease in absorbance at 420 nm and increase in absorbance at 390 nm [31]. The experiment for estimation of catalytic activity of CYP17 was preceded by comparative study of the binding of the different substrates to CYP17. Spectral studies show that *H. sapiens* CYP17 has a characteristic type I spectrum resulting from increase in high-spin enzyme content occurring on interaction with steroid substrates. The maximal value of K_d was determined for the reaction products (AND and DHEA). It was established that *H. sapiens* CYP17 has high affinity towards 7dhP5 ($K_d = 1.75 \pm 0.25$ μM) (Fig. 2), comparable with the affinity of CYP17 to natural steroid substrates (Table 1). Therefore, we have shown that 7dhP5 might

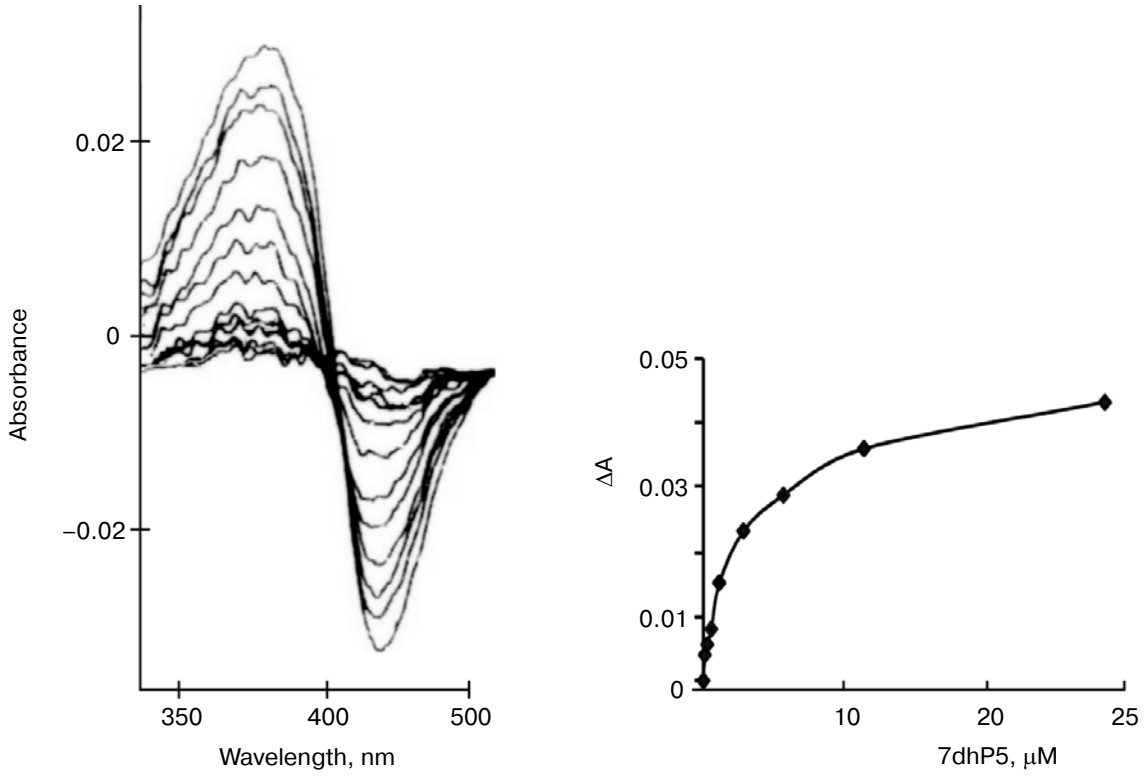

Fig. 2. Spectral changes induced by 7dhP5 binding to the active center of CYP17. The titration was conducted in 50 mM potassium-phosphate buffer, pH 7.4. Each cuvette contained 1 μM of CYP17.

Table 1. Interaction of human CYP17 with steroid substrates

Substrate	ΔA_{max}	K_d, μM
P5	0.07	0.86 ± 0.12
17OHP5	0.05	0.63 ± 0.05
P4	0.05	1.92 ± 0.09
17OHP4	0.02	2.32 ± 0.35
7dhP5	0.05	1.75 ± 0.25
DHEA	–	–
AND	–	–

Note: The titrations were conducted in 50 mM potassium-phosphate buffer, pH 7.4. Each cuvette contained 1 μM CYP17. Ligand concentration was changed in the range of concentration 0.1-50 μM by the addition of the ligand dissolved in ethanol.

effectively bind to the substrate recognition site and to the active site of CYP17.

Catalytic activity of CYP17 with 7dhP5. Efficient binding demonstrated by spectral methods does not always mean that a substance can be effectively metabolized by the enzyme. We evaluated the catalytic activity of the recombinant highly purified human CYP17 using 7dhP5 as a substrate in a reconstituted system. It was found that CYP17 efficiently metabolizes 7dhP5 (Fig. 3). Mass spectrometric analysis identified the first product with maximal quantity as 7-dehydro-17α-hydroxypregnenolone (**7dh17OHP5**), and the second product as androsta-5,7-dien-3β-ol-17-one (**7dhDHEA**) (Fig. 3). These results indicate site-specific hydroxylation of either Δ^4- and Δ^5-steroids, or Δ^7–Δ^5-steroids by *H. sapiens* CYP17.

Studies of species-specificity of catalytic activity of CYP17 with 7dhP5. To study species-specificity of the catalytic activity of CYP17s from different organisms with 7dhP5, the catalytic activity of *H. sapiens* CYP17, *E. caballus* CYP17, and *C. porcellus* CYP17 demonstrating different types of activity (Δ^5, Δ^{4-5}, and Δ^4, accordingly) with 7dhP5 was analyzed (Table 2). It was found that *H. sapiens* CYP17 and *E. caballus* CYP17 efficiently metabolize 7dhP5, catalyzing either the 17α-hydroxylase or 17,20-lyase reactions. CYP17 from *C. porcellus* belonging to Δ^4-type of activity accomplishes only 17α-hydroxylation of 7dhP5, and products of 17,20-lyase activity cannot be detected in the reaction mixture. Therefore, the presence of the unsaturated bond in the steroid B-ring does not influence the steroid recognition in the active center of CYP17.

Cytochrome b_5 influences catalytic activity of CYP17. Analysis of the catalytic activity in the presence of cytochrome b_5, which is a natural effector of CYP17, demonstrates that cytochrome b_5 predominantly stimulates 17,20-lyase activity of the enzyme in the reaction with "classical" steroids and does not influence significantly the reaction with Δ^7–Δ^5 steroids.

Catalytic activity of CYP21 with 7dhP5. Cytochrome CYP21 catalyzes important steps in steroid hormone biosynthesis. Investigation of the possibilities of biosynthesis of corticosteroids with a double bond in the 7,8 position is of particular interest. In the present study, the catalytic activity of CYP21 with 7dhP5 was analyzed to elucidate whether Δ^7–Δ^5-steroids might serve as precursors in corticoid hormone biosynthesis. Despite the fact that CYP21 predominantly hydroxylates Δ^4-steroids, we have suggested that 7dhP5 might be used as a substrate due to changes in conformation occurring because of the presence of an unsaturated bond in the B-ring. Analysis of the 3D structure of CYP21 [34] showed that there is no spatial restriction for 7dhP5 metabolism. However, analysis of the enzymatic activity of CYP21 in the reconstituted system containing CPR as electron donor demonstrates that CYP21 cannot metabolize 7dhP5. Presumably, the presence of the keto group in the 3 position of the steroid molecule is the most important factor for binding to CYP21. Further studies are needed to investigate the possibility of metabolism of Δ^7-steroids by cytochrome CYP21.

Table 2. Enzymatic activity of CYP17 from different species with 7dhP5

CYP17	Enzymatic activity, min^{-1}					
	17α-hydroxylase		17,20-lyase			
	P5	7dhP5	17OHP5		7dh17OHP5	
			$-b_5$	$+b_5$	$-b_5$	$+b_5$
H. sapiens	2.4	5.2	0.28	1.2	1.4	1.8
C. porcellus	6	1.2	–	–	–	–
E. caballus	15.5	6.2	2.7	10.5	1.9	3.1

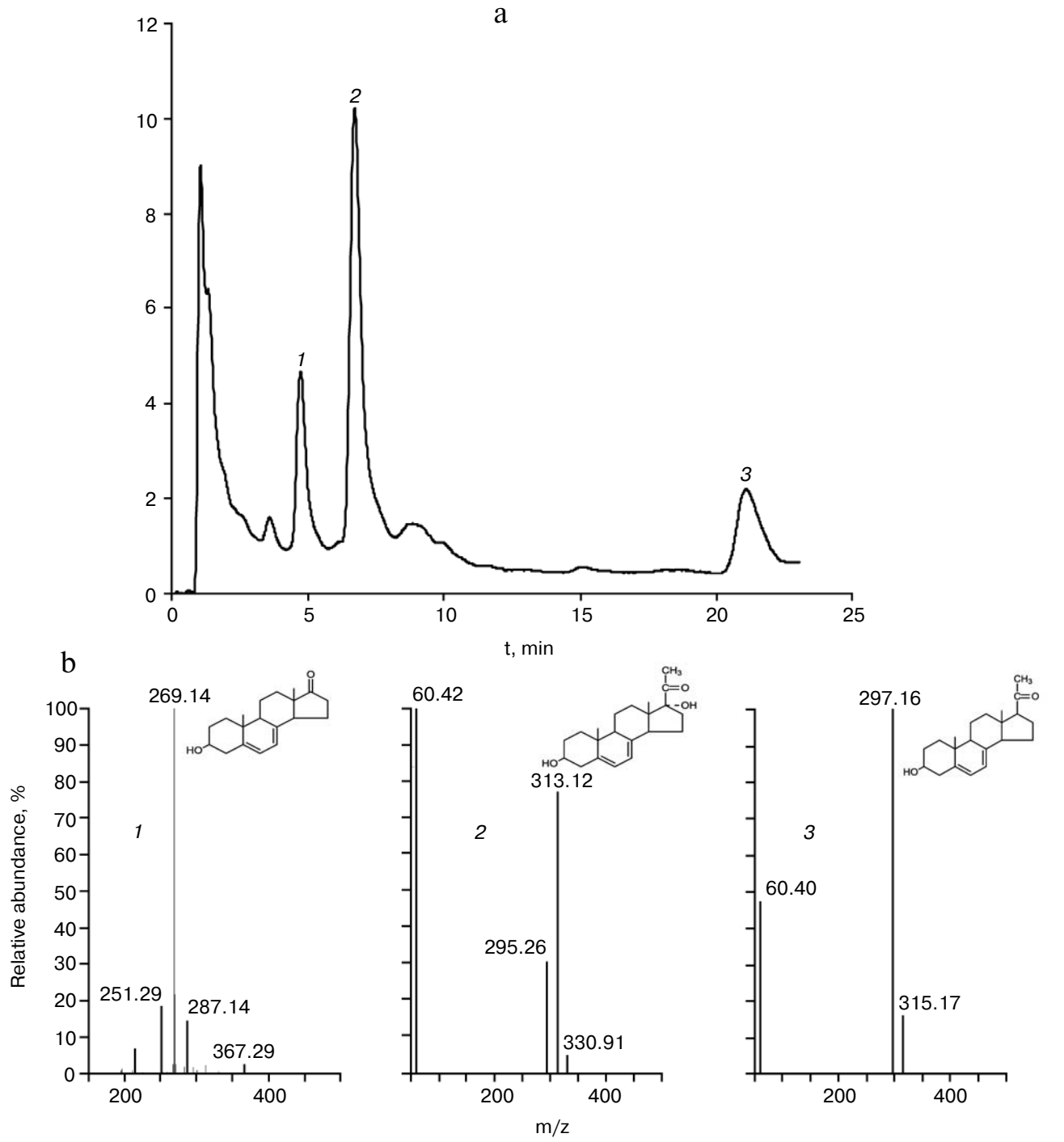

Fig. 3. a) HPLC profile of products formed during metabolism of 7dhP5 by cytochrome CYP17; b) mass spectra of the main reaction products: *1*) 7dhDHEA, m/z ($[MH]^+$) = 287; *2*) 7dh17OHP5, m/z ($[MH]^+$) = 331; *3*) 7dhP5, m/z ($[MH]^+$) = 315.

DISCUSSION

It has been previously postulated that CYP17 is a highly specific enzyme having a small active center that restricts the spatial orientation of substrates [35]. Despite this fact, apart from the catalysis of reactions with "classical" substrates, the possibility of involvement of CYP17 in the metabolism of alternative substrates has been shown [36-42] (Fig. 4).

Recently, investigations of Δ^7-steroid biosynthesis have become very intense. The Δ^7-steroids are predominant for certain species. In humans, Δ^7-steroids can be detected in large amounts in certain pathologies. Their accumulation might cause numerous disorders including

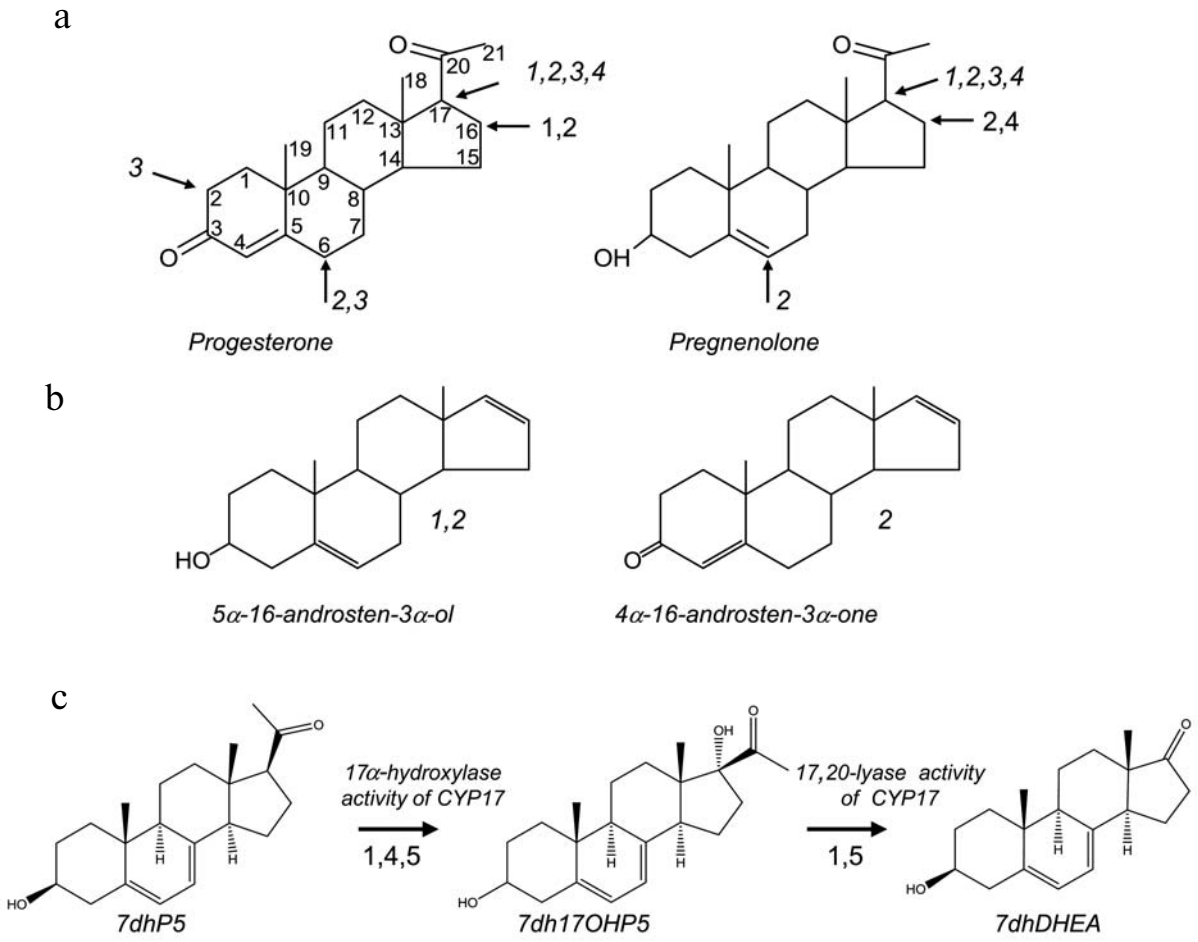

Fig. 4. a) Alternative positions of steroid hydroxylation by CYP17; b) 16-en synthase activity of CYP17. Species: *1*) CYP17 *H. sapiens*; *2*) CYP17 *S. scrofa*; *3*) CYP17 *B. taurus*; *4*) CYP17 *C. porcellus*; *5*) CYP17 *E. caballus*; c) scheme of conversion of 7dhP5 catalyzed by CYP17 *H. sapiens*.

abnormalities in the functioning of the nervous system. Furthermore, wide use of estrogen replacement therapy containing steroids with unsaturated bonds in the B-ring in correction of menopausal disorders is an important factor that stipulates the interest in the metabolism of Δ^7-steroids. It has been demonstrated that long usage of drugs containing equiline and equilinine are risk factors for endometrial and breast cancer. Currently, the functional importance of Δ^7-steroid circulating in the human organism is not elucidated. There are indications that Δ^7-steroids might be substrates for CYP11A1 [16, 17]. In the present work, the possibility of the subsequent metabolism of steroids with unsaturated bonds in the B-ring by the microsomal steroid hydroxylases CYP17 and CYP21, which catalyze key steps of corticoid and sex hormones biosynthesis, has been studied.

The data obtained in the present work indicate the possibility of involvement of CYP17 in an alternative pathway of biosynthesis of estrogen with unsaturated bonds in the B-ring using 7-dehydropregnenolone as a precursor; the products of the reaction were identified using high performance liquid chromatography coupled with mass spectrometry. The 7dh17OHP5 product of the 17α-hydroxylase reaction is consequently converted to 7dhDHEA by the 17,20-lyase reaction (Fig. 4). It has been shown that addition of "classical" substrate P5 leads to decrease in catalytic activity of CYP17 with 7dhP5. This fact can be explained by the effect of competitive inhibition.

The species specificity of this reaction has been studied. It has been determined that the presence of an unsaturated bond in the B-ring does not influence steroid recognition in the active center of CYP17. The CYP17s from different species belonging to different types of enzymatic activity (Δ^5, Δ^{4-5}, Δ^4), metabolize Δ^5–Δ^7-steroids in the same way as "classical" Δ^5-steroid substrate.

We have shown that cytochrome CYP21 cannot metabolize Δ^5–Δ^7-steroids; this fact indicates the major significance of the structure of the A-ring of CYP21 substrates.

Our results have important fundamental significance in updating information about steroid metabolism by CYP17. The possibility of CYP17 metabolizing steroid derivatives with two unsaturated bonds in the B-ring indicates the likely metabolism of steroid hormones of this type. Investigations of possible involvement of other enzymes of steroid hormone biosynthesis pathways in metabolism of such types are needed.

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