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Chimerogenesis in Estimation of Specificity and Pathway Directions for Cytochrome P45017α Catalyzed Reactions

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Abstract—Cytochrome P45017α is a key enzyme in steroid hormone biosynthesis. It catalyzes the reaction of 17α-hydroxylation of progesterone (P4) and pregnenolone (P5) and the 17,20-lyase reaction resulting in side chain cleavage of C_{21} steroids to form C_{19} steroids. Depending on the activity of cytochrome P45017α, steroid hormone biosynthesis pathways are directed either for biosynthesis of mineralocorticoids and glucocorticoids or sex hormones. The formation of sex hormones starts from biosynthesis of androstenedione. Androstenedione formation is a result of two reactions: 17,20-lyase reaction of 17α-hydroxyprogesterone (Δ^4-pathway) and 3β-hydroxysteroid dehydrogenase/Δ^4,Δ^5-isomerase reaction using dehydroepiandrosterone as substrate (Δ^5-pathway). In case of exclusive direction of the 17,20-lyase reaction either through the Δ^4- or the Δ^5-pathway, the formation of sex hormones depends more on specificity and activity of 3β-hydroxysteroid-dehydrogenase/Δ^4,Δ^5-isomerase. Depending on species, the cytochromes P45017α can utilize as a substrate for 17,20-lyase activity Δ^4-steroids, Δ^5-steroids, or both types of steroids. To identify the structural elements of cytochrome P45017α responsible for substrate recognition, in the present work we used exchange of homologous fragments of cytochrome P45017α having different types of activities. We engineered more than 10 different types of chimeric cytochrome P45017α. Chimeric cytochromes P45017α have been expressed in *E. coli* and purified. The expression of chimeric cytochrome P45017α with the point of exchange between exons III and IV results in inability of the recombinant hemeprotein to properly bind heme. The determination of activity of chimeric cytochromes P45017α shows that the structural element responsible for switching activity between Δ^4- or Δ^5-pathway is located in the region of polypeptide chain coded by exons II-V of CYP17 gene.

Key words: cytochrome P450, cytochrome P45017α, CYP17, steroidogenesis, chimeric proteins

Cytochrome P450 plays a key role in living organisms by participating in numerous metabolic reactions of physiologically active compounds. One of the most important functional roles of cytochrome P450 in *Chordata* is its participation in the biosynthesis of steroid hormones from cholesterol. The key enzyme participating in steroid hormone biosynthesis and regulation of the distribution of steroid between different pathways is cytochrome P45017α (17α-steroid-hydroxylase/17,20-lyase). Cytochrome P45017α, a hemeprotein of endoplasmic reticulum membranes, is mostly expressed in gonads and adrenal cortex [1]. The gene coding cytochrome P45017α, CYP17, contains eight exons [2]. Cytochrome P45017α catalyzes two types of reactions: 17α-hydroxylation of Δ^4- (derivatives of progesterone) and Δ^5-steroids (derivatives of pregnenolone) as well as the 17,20-lyase reaction converting 17α-hydroxysteroids (C_{21}-steroids) into precursors of sex hormones (C_{19}-steroids). Cytochrome P45017α can also hydroxylate steroids at positions other than the 17α-position, but the reaction rate of these reactions is much less than the rate of the main reactions [3]. Some of the alternative reaction products formed in the reactions catalyzed by cytochrome P45017α are biologically active compounds and perform important regulatory roles in humans and

Abbreviations: PCR) polymerase chain reaction; SDS) sodium dodecyl sulfate; HPLC) high performance liquid chromatography; DHEA) dehydroepiandrosterone; P5) pregnenolone; P4) progesterone; 17OH-P5) 17α-hydroxypregnenolone; 17OH-P4) 17α-hydroxyprogesterone; AD) androstenedione.

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other animals. Thus, pig cytochrome P45017α catalyzes pheromone (4,16-androstadien-3-one) biosynthesis [4]. Depending on animal species, cytochrome P45017α predominantly uses as substrate for the 17,20-lyase reaction either Δ^4- or Δ^5-steroids. All cytochromes P45017α, depending on the preferred substrate for the 17,20-lyase reaction used by the cytochrome P45017α, can be divided into several groups. Cytochrome P45017α of Δ^4-type (guinea pig) is not able to catalyze the conversion of 17α-hydroxypregnenolone to dehydroepiandrosterone [5, 6]. Cytochrome P45017α of Δ^5-type is not able to catalyze androstenedione formation from 17α-hydroxyprogesterone (humans, *Bovidae*) [7, 8]. The functional properties of bovine cytochrome P45017α are more carefully characterized [9]. Cytochromes P45017α of Δ^4,Δ^5-type catalyze both types of reactions (rodent, pig) [10-12]. It should be stressed that the difference between molecules of androstenedione and dehydroepiandrosterone consists in the presence of a keto- or hydroxyl group at the third position, as well as Δ^4- or Δ^5-position of the double bond in the steroid molecule.

Homology of cytochromes P45017α of the same type, but evolutionarily separated, is 40-70%. At present it seems impossible by using simple alignment of amino acid sequences to localize cytochrome P45017α fragments responsible for demonstration of one or another type of activity. Moreover, the rate of 17,20-lyase reaction of the majority of cytochromes P45017α in a great degree depends on the presence of cytochrome b_5 in the incubation mixture [6, 13]. In gonads, due to relatively high concentration of cytochrome b_5, steroidogenic pathways are mostly directed to sex hormone formation [1].

The engineering of chimeric proteins containing fragments typical for representatives of Δ^4- and Δ^5-types can be used to localize the fragments of polypeptide chain of cytochrome P45017α responsible for recognition of steroid molecule and regulation of corresponding 17,20-lyase reactions. In the present work we created chimeric forms of cytochrome P45017α using cytochromes P45017α having different profiles of enzymatic activity in the 17,20-lyase reaction. The data of the present work indicate that the part of the polypeptide chain of cytochrome P45017α responsible for determination of the preference of the hemeprotein either to Δ^4- or Δ^5-steroids is localized in the amino acid sequence coded by exons II-V of the CYP17 gene.

MATERIALS AND METHODS

Chemicals. In the present work we used isopropyl-1-thio-β-D-galactopyranoside (IPTG), agarose for electrophoresis, and low-melting agarose for electrophoresis from Gibco BRL (USA); yeast extract, peptone, and tryptone from Difco (USA); pregnenolone, Tween-20, Coomassie G-250, isocitrate, isocitrate dehydrogenase, Tris, EDTA, δ-aminolevulinic acid, phenylmethylsulfonyl fluoride (PMSF), NADPH, 17α-hydroxyprogesterone, progesterone, 17α-hydroxypregnenolone, and pregnenolone from Sigma (USA); DEAE-Sepharose from Pharmacia (Sweden); ^{3}H-labeled 17α-hydroxypregnenolone and ^{3}H-labeled pregnenolone from NEN (USA); Bio-Gel HTP from Bio-Rad (USA); Ni-NTA-Agarose from Qiagen (USA).

Enzymes for restriction and modification of DNA were from New England Biolabs (England), Promega (USA), and Boehringer (Germany). Oligonucleotides for PCR were synthesized on an Applied Biosystems oligonucleotide synthesizer (USA).

Vectors and bacterial strains. PCR products were cloned using plasmid pGEM-T (Promega). All manipulations on cloning and expression were carried out using *E. coli* DH-5α strain (Gibco BRL).

Plasmid pSK$^+b_5$, containing cDNA coding full-length microsomal rat liver cytochrome b_5, was generously presented by Professor A. Ito (Kyushi University, Japan). The expression vector pCWori$^+$ was kindly presented by Professor M. R. Waterman (Vanderbilt University, USA).

Construction of plasmids for expression of chimeric proteins. To engineer chimeric constructs with the point of exchange at codon 220, the sequence containing cDNA for bovine cytochrome P45017α (P45017α*bov*) was amplified using the following primers:

pCWori$^+$3′ – GACAGCTTATCATCG;

*cav*225*bov* – CTTCCCCAATAAAACCCTGGAAAAGATGAAGGGTTGTGTTC;

pCW5′ – CAGGATCCATCCATCGATGCTTAGG;

*bov*225*cav* – CCTCGGATTTCAGTATACTTCTTCATCTTTTCCATGGCTTTGC;

while cDNA for guinea pig cytochrome P45017α (P45017α*cav*) was amplified using the following primers:

pCWori$^+$3′ – GACAGCTTATCATCG;

*bov*225*cav* – GCAAAGCCATGGAAAAGATGAAGAAGTATACTGAAATCCGAGG;

pCWori$^+$5′ – CAGGATCCATCCATCGATGCTTAGG;

*cav*225*bov* – GAACACAACCCTTCATCTTTTCCAGGGTTTTATTGGGGAAG.

After the first PCR reaction, reaction products were used to synthesize the final sequences of cDNA for chimeras using the following primers: pCWori$^+$3′ –

GACAGCTTATCATCG and pCWori$^+$5′ – CAGGATCCATCCATCGATGCTTAGG.

Reaction products of the amplification reaction were cloned to pGEM-T vector (Promega) and then were recloned into expression vector pCWori$^+$HT. The presence of the desired replacements in the chain of cDNA of cytochrome P45017α was proved by sequencing on an A377 DNA sequencer (Applied Biosystems).

Heterologous expression of chimeric forms of cytochrome P45017α in *E. coli*. Expression plasmids containing cDNA coding cytochrome P45017α or chimeric proteins were used to transform *E. coli* DH5α cells. For preparative expression, overnight culture was diluted 1 : 100 with TBS medium (per 1000 ml: 24 g yeast extract, 12 g tryptone, 2 g peptone, 4 ml glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4), containing 100 μg/ml of ampicillin and were grown up to A_{600} 0.4-0.6 at 37°C and 180 rpm, then expression of cytochrome P45017α was induced by addition of IPTG at final concentration 0.5 mM. The temperature and aeration degree were decreased to 26°C and 120 rpm, respectively, and incubation continued for 48 h.

Expression and purification of recombinant NADPH-cytochrome P450 reductase and cytochrome b_5 from *E. coli*. The procedure for expression and purification of recombinant cytochrome b_5 and NADPH-cytochrome P450 reductase from recombinant *E. coli* did not significantly differ from the procedure previously described [14, 15].

Purification of recombinant cytochrome P45017α from *E. coli*. Cytochrome P45017α was expressed in *E. coli* as derivatives containing 6His-tag in the C-terminal sequence, allowing the use of metal-affinity chromatography for its purification. The membranes prepared after sonication of recombinant bacteria were suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 50 μM progesterone. Cytochrome P450 was solubilized by adding to the membrane suspension 10% Emulgen 913 to final concentration 1% (approximately 3 mg Emulgen 913 per 1 mg protein). The suspension was mixed for 1 h at 4°C followed by centrifugation at 100,000*g* for 30 min. The supernatant containing solubilized cytochrome P45017α was applied on a column with Ni-NTA-Agarose (Qiagen). The column with Ni-NTA-Agarose was washed with 10 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 100 mM glycine, 0.3 M NaCl, 0.2% Emulgen 913, and cytochrome P450 was eluted with the same buffer but containing 50 mM histidine.

The fractions containing cytochrome P45017α were four-fold diluted with 20% glycerol containing 0.1 mM dithiothreitol and 50 μM progesterone and applied to a hydroxyapatite column (1.5 × 10 cm). The column was washed with 5 volumes of 50 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol and 0.1 mM dithiothreitol, and cytochrome P45017α was eluted by increasing concentration of sodium phosphate up to 300 mM. Cytochrome P45017α was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol and 0.1 mM dithiothreitol and stored at –70°C.

Analytical methods. Protein composition of bacterial cells after expression and control of the purity of protein preparations was monitored using SDS-PAGE in 12% gel according to Laemmli [16] using a Mini Protean II instrument (Bio-Rad). Recombinant proteins were immunochemically identified by immunoblotting using polyclonal antibodies prepared against bovine cytochrome P45017α [17]. Endogenous proteolysis of chimeric forms of cytochrome P45017α in recombinant bacterial lysate was estimated using SDS-PAGE in 12% gel with subsequent immunoblotting analysis.

To determine concentration of cytochrome b_5 and NADPH-cytochrome P450 reductase, molar extinction coefficients 117 $mM^{-1}\cdot cm^{-1}$ at 413 nm [14] and 23 $mM^{-1}\cdot cm^{-1}$ at 454 nm [15], respectively, were used. Spectrophotometric measurements were carried out using an Aminco DW2a spectrophotometer modified by Olis (USA). The expression level of cytochrome P45017α in *E. coli* cells and its concentration at all purification steps was monitored by recording reduced carbon monoxide difference spectra. Concentration of cytochrome P450 was determined from sodium dithionite reduced carbon monoxide difference spectra using molar extinction coefficient $\varepsilon_{450\text{-}490}$ 91 $mM^{-1}\cdot cm^{-1}$ [18].

Determination of cytochrome P45017α activity. Catalytic activity of cytochrome P45017α in 17α-hydroxylase and 17,20-lyase reactions were determined in a reconstituted system at 37°C in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. Cytochrome P45017α was added to the incubation mixture at final concentration 0.5 μM. Recombinant rat NADPH-cytochrome P450 reductase was used as a donor of electrons (final concentration 1.0 μM). Radioactive steroids were added together with "cold" steroids dissolved in ethanol at final concentration 50 μM and radioactivity ~100,000 cpm per tube. The reaction was started by adding NADPH at final concentration 0.5 mM and NADPH-regenerating system containing sodium isocitrate and isocitrate dehydrogenase at final concentration 8 mM and 0.1 IU/ml, respectively. Aliquots (0.5 ml) were taken from the incubation mixture at selected time intervals and mixed with 5 ml methylene chloride. The mixture was vigorously mixed and organic and water phases were separated by centrifugation. The organic layer was collected and evaporated under a nitrogen flow. The residual was dissolved in methanol and reaction products were analyzed by HPLC using a Waters 840 apparatus (USA) equipped with Spectraflow 757 optical flow detector and β-RAM radioactive flow counter (INUS, USA) using a 10 μm C_{18} Bondopak column (39 × 300 mm). The reaction products were identified based on the elution time compared to standards.

RESULTS

Comparison of primary structure of different types of cytochromes P45017α. Depending on the ratio of 17α-hydroxylase and 17,20-lyase activities with respect to Δ^4- and Δ^5-steroids, all cytochromes P45017α from different species may be conditionally divided into three main types: Δ^4-type cytochromes P45017α do not have or have very negligible 17,20-lyase activity in conversion of 17α-hydroxypregnenolone into dehydroepiandrosterone; Δ^5-type cytochromes P45017α do not have or have negligible 17,20-lyase activity with respect to 17α-hydroxyprogesterone, converting it to androstenedione; Δ^4,Δ^5-type cytochromes P45017α catalyze reactions of 17α-hydroxylation of both progesterone and pregnenolone as well as 17,20-lyase reaction of oxidation of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone. To determine the structural elements of polypeptide chain conservative for all cytochromes P45017α and for each type of this protein, we analyzed the known primary structures of cytochrome P45017α from different species. Figure 1 shows the comparison of universal amino acid sequences of cytochromes P45017α: cytochrome P45017α of Δ^4-type, cytochrome P45017α of Δ^5-type, and cytochrome P45017α of $\Delta^{4,5}$-type. Each universal amino acid sequence characteristic for cytochromes P45017α of a particular type (Fig. 1) includes all known structures of cytochromes P45017α of this type. The Δ^4-type contains only one cytochrome P450, i.e., guinea pig cytochrome P45017α. The lowest homology for all cytochromes P45017α as well as for cytochromes P45017α of particular types has the polypeptide fragment starting from 190 to 300 amino acids that are coded by exons from III to V of the CYP17 gene. Comparison of the primary structures of known cytochromes P45017α identified the sequences conservative for all known 17α-steroid hydroxylases.

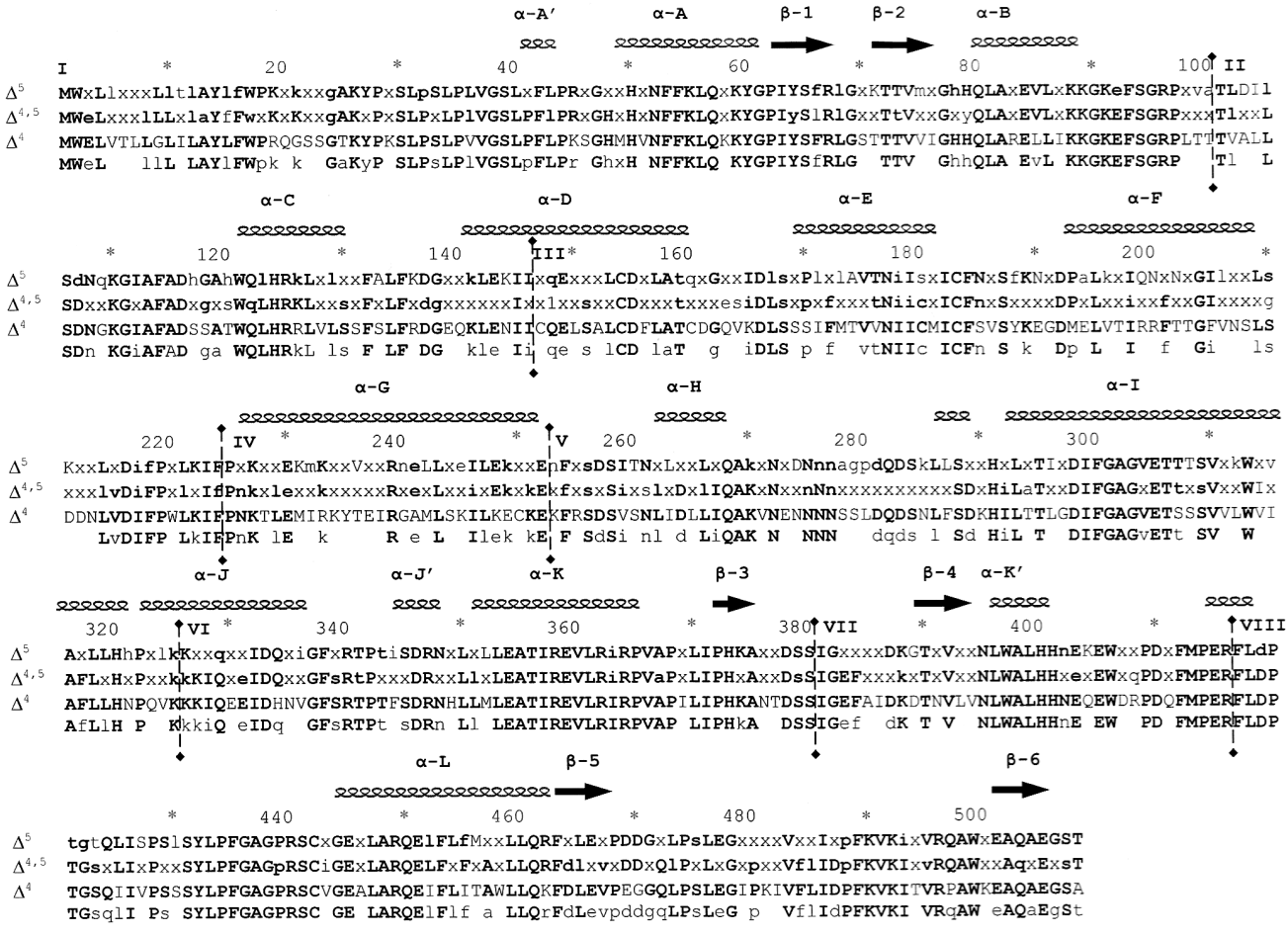

Fig. 1. Alignment of amino acid sequence of cytochromes P45017α demonstrating different types of activity: Δ^5-type, consensus of sequences of human cytochrome P45017α and sequences of cytochrome P45017α from *Bovidae*; $\Delta^{4,5}$-type, consensus of sequences of pig, rat, mouse, and horse cytochromes P45017α; Δ^4-type, guinea pig cytochrome P45017α. The Roman numbers show exons of gene CYP17, coding these fragments of cytochrome P450 structure. Dotted lines separate the junction sites of cytochrome P45017α structure that are coded by different exons of the CYP17 gene. ≋, α-helix; ⟶, β-sheet.

These conservative sequences first of all include the fragments of polypeptide chain of cytochrome P45017α characteristic for all hemeproteins of the cytochrome P450 superfamily. Strictly specific for cytochrome P45017α proved to be the polypeptide chain fragments corresponding to the third quarter of the cytochrome P45017α molecule, i.e., fragments of polypeptide chain participating in coordination of the heme group (heme-binding fragment) and formation of the heme pocket (Fig. 1).

Alignment of amino acid sequences of Δ^4-type cytochromes P45017α or cytochromes P45017α that are able to catalyze hydroxylation reaction with respect to Δ^4- and Δ^5-steroids (Fig. 1) reveals amino acid sequence characteristic only for a particular type cytochrome P45017α. Since there is only one known Δ^4-type cytochrome P45017α, guinea pig cytochrome P45017α, at the moment it is impossible to identify conservative sequence characteristic for this type of cytochrome P45017α. In Fig. 1, symbol "x" indicates variability of this amino acid residue in other representatives of this type cytochrome P45017α. The upper-case letters are assigned to the strictly conservative amino acid residues in all three types of cytochrome P45017α, while the lower-case letters show amino acid residues conservative only for the structures of particular type cytochromes P45017α. From Fig. 1 it follows that the most conservative for all types of cytochrome P45017α is the N-terminal sequence (α-helices A, B, and C and β-sheets 1 and 2) and C-terminal fragment (α-helices K, K′, L, heme-binding peptide, and β-sheets 3 and 4). The most variable among all type cytochromes P45017α are α-helices D, E,

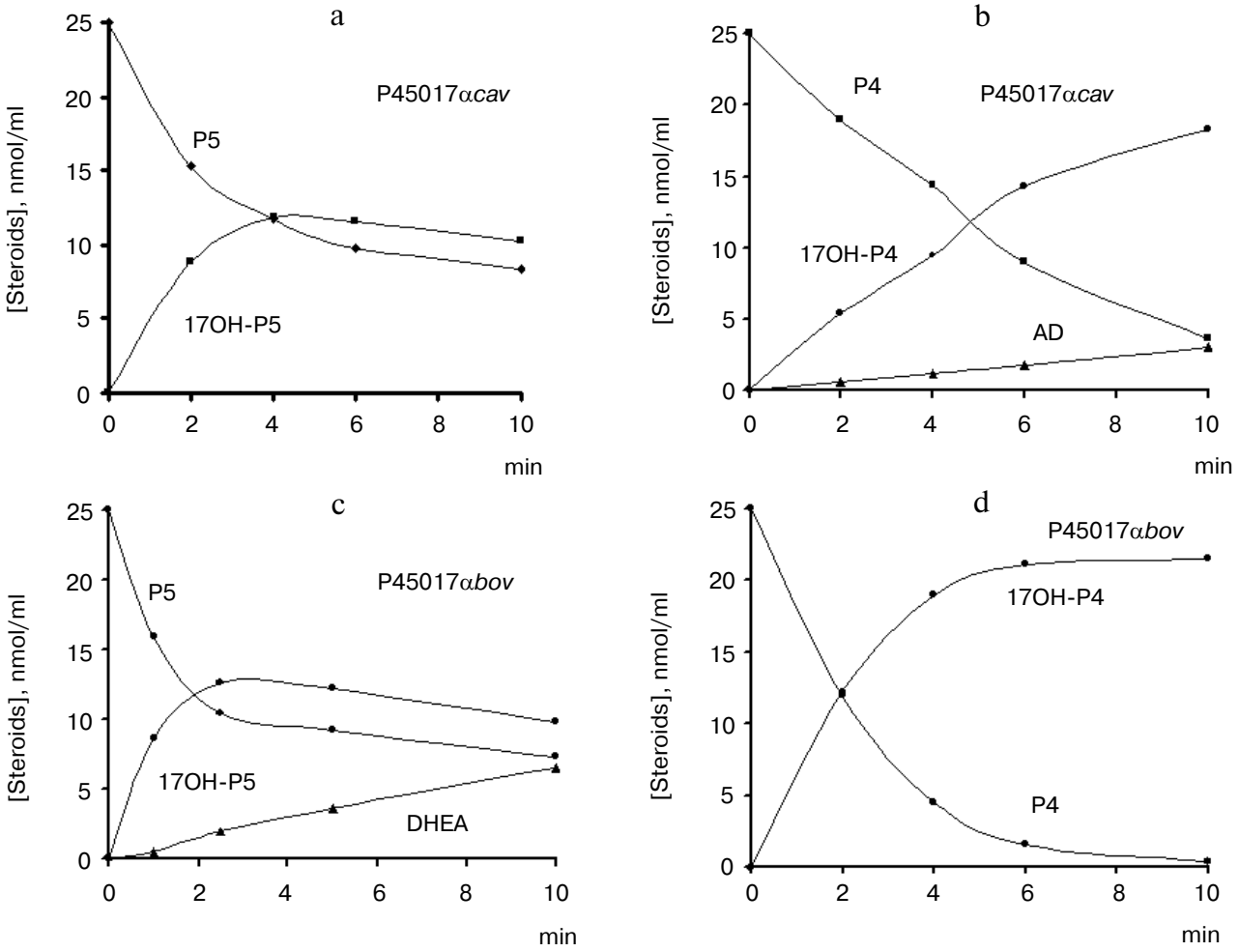

Fig. 2. Kinetics of product formation in the reactions catalyzed by guinea pig cytochrome P45017α (P45017α*cav*) (a, b) and bovine cytochrome P45017α (P45017α*bov*) (c, d). a, c) Metabolism of pregnenolone (Δ^5-type); b, d) metabolism of progesterone (Δ^4-type). Designations: P4, progesterone; 17OH-P4, 17α-hydroxyprogesterone; AD, androstenedione; P5, pregnenolone; 17OH-P5, 17α-hydroxy-pregnenolone; DHEA, dehydroepiandrosterone. Reaction mixture contained 0.5 μM cytochrome P45017α, 1 μM rat NADPH-cytochrome P450 reductase, 0.5 μM cytochrome b_5, 50 μM steroid, and 0.5 mM NADPH in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and NADPH-regenerating system (isocitrate–isocitrate dehydrogenase). Temperature, 37°C.

Catalytic properties of different forms of cytochrome P45017α

P45017α	17α-Hydroxylase activity, min^{-1}		17,20-Lyase activity, min^{-1}	
	formation of 17αOH-P5	formation of 17αOH-P4	formation of DHEA	formation of AD
P45017α*bov*	17	28	0.3	0
P45017α*cav*	6	3.5	0	0.3
bov-100-*cav*	13.8	5.1	0	0.64
cav-100-*bov*	7.3	8.4	0.35	0
bov-300-*cav*	—	0.39	—	0
cav-300-*bov*	—	0.24	—	0
bov-400-*cav*	16	9.6	0.4	0
cav-400-*bov*	2.3	3.1	0	0.05
*cav*100*bov*300*cav*	1.72	2.4	0.07	0
*bov*100*cav*300*bov*	1.34	0.73	0	0.15

Note: Reaction conditions: 0.25 μM cytochrome P45017α, 0.5 μM NADPH-cytochrome P450 reductase, 0.5 mM NADPH in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, NADPH-regenerating system, 37°C. Time intervals: 0, 1, 5, 30 min. Activity of cytochrome P45017α is expressed as nmol reaction product per 1 nmol cytochrome P45017α per 1 min.

F, G, and H. It should be stressed that exactly α-helices F and G participate in formation of substrate access channel to the active site of cytochrome P45017α.

Catalytic properties of Δ^4- and Δ^5-type cytochromes P45017α. Bovine cytochrome P45017α (P45017α*bov*) and guinea pig cytochrome P45017α (P45017α*cav*) were expressed in *E. coli* cells, purified using metal-affinity chromatography, and used as standards of cytochromes P45017α of Δ^5- and Δ^4-types, respectively. Figure 2 shows the kinetic profiles of reactions catalyzed by guinea pig cytochrome P45017α (Fig. 2, a and b) and bovine cytochrome P45017α (Fig. 2, c and d) using pregnenolone (Fig. 2, a and c) or progesterone (Fig. 2, b and d) as the substrate. The catalytic properties of recombinant heterologously expressed cytochromes P45017α are compatible with the activity of analogous enzymes purified from the natural sources. Bovine cytochrome P45017α catalyzes the conversion of pregnenolone to 17α-hydroxypregnenolone. After accumulation of the reaction product, 17α-hydroxypregnenolone, at the ratio to substrate approximately 5 : 1, one can see the formation of the product of the second (17,20-lyase) reaction, dehydroepiandrosterone (Fig. 2c and table). When using progesterone as substrate, bovine cytochrome P45017α converts it to 17α-hydroxyprogesterone, but the 17,20-lyase reaction does not proceed further (Fig. 2d and table). Approximately 15% of substrate is converted during 15 min of incubation to the secondary derivatives. Among secondary derivatives, there are some steroids that can be separated from standard C_{19}-steroids only by HPLC. Guinea pig cytochrome P45017α effectively catalyzes the hydroxylation reaction for both progesterone as well as pregnenolone resulting in corresponding 17α-hydroxy-derivatives (Fig. 2, a and b), but the next 17,20-lyase activity of this enzyme is realized only in the case of 17α-hydroxyprogesterone as the substrate (Fig. 2b). During incubation, one can see the some consumption of the 17α-hydroxylation reaction product, 17α-hydroxypregnenolone (Fig. 2a), but the products of these secondary reactions are steroids that significantly differ from dehydroepiandrosterone. Both cytochromes P45017α have comparable rates in conversion of pregnenolone (P5) and progesterone (P4) giving corresponding 17α-hydroxy-derivatives. A dramatic difference is only observed in the ability of the two cytochromes P45017α to catalyze 17,20-lyase reaction of 17α-hydroxylated derivatives of pregnenolone and progesterone (Fig. 2).

Engineering of plasmid constructs for expression of chimeric forms of cytochrome P45017α. The approaches used to construct different types of chimeric cytochromes P45017α are illustrated in Fig. 3. To achieve the interprotein exchange of the first 100 amino acids, we used internal restriction site *Eco*RI. For chimerogenesis between amino acids 300 and 400 of cytochrome P45017α, we used restriction sites *Bsa*I and *Mbi*I, respectively. To create chimeric proteins with point of exchange at amino acid residue 220 of cytochrome P45017α, we used PCR. The first step in the preparation of the chimeras consisted of amplification of the necessary region of cDNA by using primers located in the region of crossing and primers located close to the start or stop-codon. The PCR products were used further for amplification of the full-length chimeric cDNA for cytochrome P45017α using primers located close to the start or stop-codon.

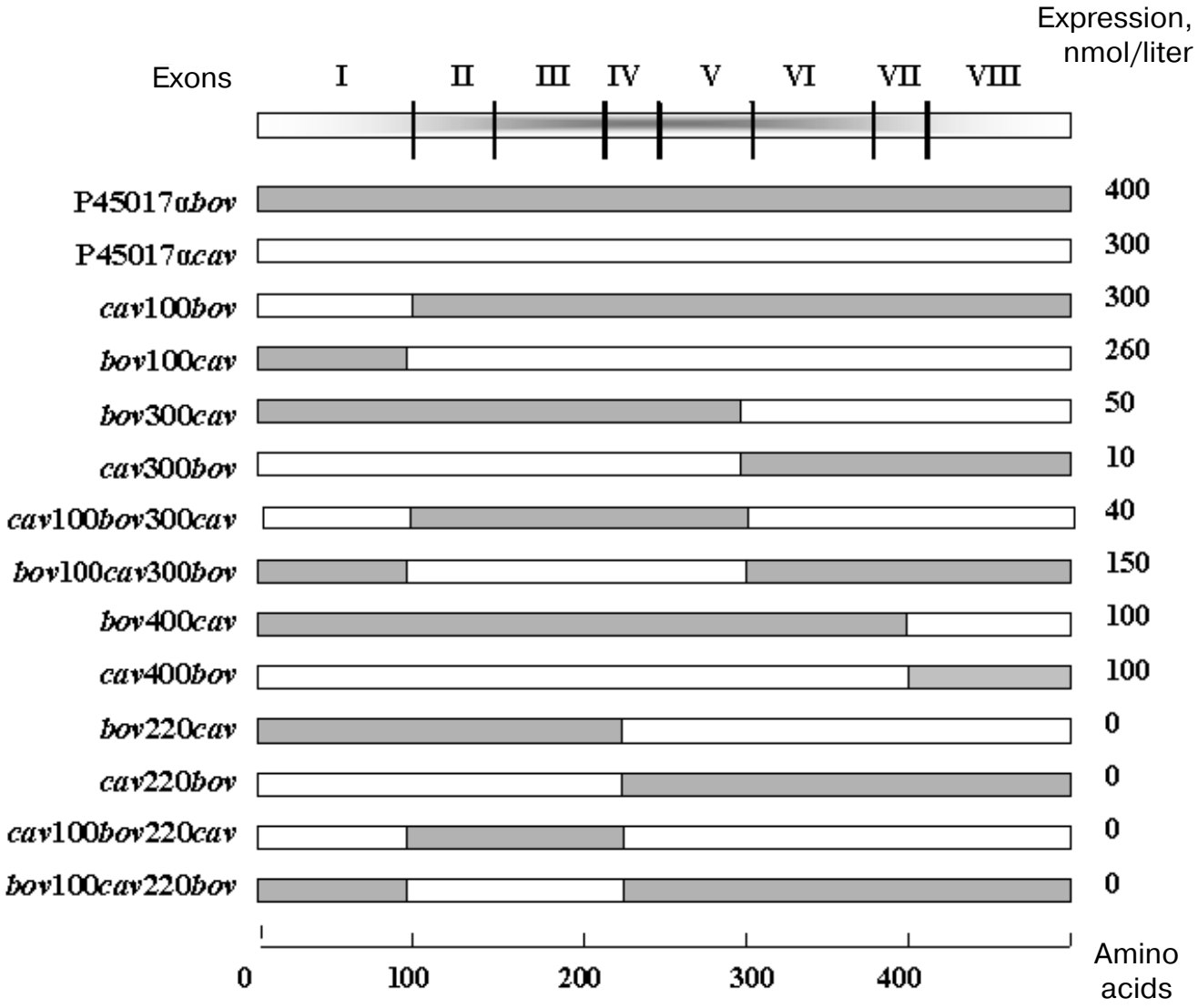

Fig. 3. Scheme showing types of chimeric cytochromes P45017α engineered in the present work. For each type of chimera, the expression level in *E. coli* cells was estimated from the characteristic spectrum of sodium dithionite-reduced CO-complex of hemeprotein.

Expression of chimeric cytochrome P45017α in *E. coli* cells. The chimeric cytochromes P450 expressed in bacterial cells were detected by the presence of immunochemically reactive proteins using antibodies against cytochrome P45017α. All chimeric proteins expressed in *E. coli* interact with antibodies prepared against bovine cytochrome P45017α indicating the retention by the chimeric proteins of intrinsic antigenic structure characteristic for cytochrome P45017α.

SDS-PAGE and immunochemical analysis using immunoblotting (data not presented) revealed the presence of proteolytically modified fragments of cytochrome P45017α in a case of some chimeric forms, which appear to be are already formed directly in the *E. coli* cells. Depending on the type of chimeric cytochrome P45017α, different degree of proteolytic modification is observed, indicating some changes in structural organization of chimeric cytochrome P45017α. In this connection, every expressed chimeric cytochrome P45017α was checked for their ability to bind carbon monoxide in the reduced state as a criterion of correctness of the folding of the chimeric hemeprotein.

Spectral properties of chimeric forms of cytochrome P45017α. The reduced difference CO-spectra of recombinant wild type cytochrome P45017α and spectra of chimeric forms of cytochrome P45017α are characterized by absorbance maximum at 450 nm (Fig. 4). Wild type recombinant cytochrome P45017α shows a characteristic absorption spectrum with maximum of absorbance in the Soret region close to 450 nm. However, some chimeric forms of cytochrome P45017α, and especially those with point of exchange near amino acid residue 220, did not

show characteristic for cytochrome P450 carbon monoxide spectrum (data not shown), indicating the inability of such chimeras to bind heme properly. This appears to be connected with major structural rearrangements in the protein backbone of cytochrome P45017α arising from disturbances in the chimeric hemeprotein molecule folding.

Some of the chimeric cytochromes P45017α demonstrate in the reduced carbon monoxide difference spectrum an absorbance with maximum at 420 nm (Fig. 4). The content of cytochrome P420 in different chimeric forms of cytochrome P450 depends on the type of chimeric hemeprotein, from 0 up to 60%. This suggests that exchange of the structural elements of the N-terminal sequence of cytochromes P45017α with different types of activity results in some changes in microenvironment of cytochrome P45017α heme group and stability of the protein.

Catalytic properties of the chimeric cytochromes P45017α. To assess catalytic properties of chimeric cytochromes P45017α, we measured 17α-hydroxylase and 17,20-lyase activities of all chimeras with respect to Δ^4- and Δ^5-type steroids (table).

The exchange of the first 100 (N-terminal) amino acid residues between two cytochromes P45017α having different types of activity has practically no effect on the activity profile, which is determined by the major part of the chimeric cytochrome P45017α (table). The replacement of the last 100 C-terminal amino acid residues of cytochromes P45017α having different types of activities also has no significant effect on the activity profile (table).

Replacement of the second third of the cytochrome P45017α structure results in significant decrease in the expression level and stability of the chimeric cytochrome P45017α (Figs. 3 and 4). Under these conditions more stable was chimeric cytochrome P45017α in which the first two thirds of the amino acid sequence were represented by the sequence of bovine cytochrome P45017α. This type of chimerogenesis also results in significant decrease in the activity of chimeric hemeprotein, but at the same time the relation to the first or second type of cytochrome P45017α is determined by the origin of the N-terminal sequence of the cytochrome P45017α molecule (table).

Cytochrome b_5 plays an important role in regulation of the 17,20-lyase activity of cytochrome P45017α *in vivo* [8, 19]. In the presence of cytochrome b_5 in the incubation mixture the rate of 17,20-lyase reaction is significantly increased, while 17α-hydroxylase activity of cytochrome P45017α is practically unchanged. In some organisms, cytochrome b_5 stimulates biosynthesis of steroid pheromones, which also involves the participation of cytochrome P45017α. The presence of cytochrome b_5 in incubation mixture containing bovine or guinea pig cytochrome P45017α does not result in to the appearance of the additional reaction products, but cytochrome b_5 significantly increases the rate of 17,20-lyase reactions

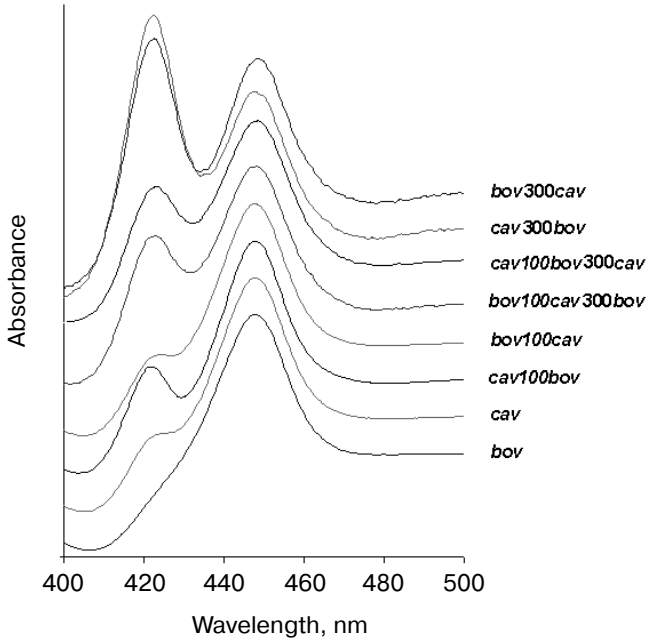

Fig. 4. Reduced difference CO-spectra of different chimeric forms of cytochrome P45017α. Absorption spectra of sodium dithionite reduced carbon monoxide complex of cytochrome P450 in 50 mM Tris-HCl buffer, pH 7.5. Concentration of cytochrome P45017α, 1 μM.

(Fig. 5, P45017α*bov* and P45017α*cav*). The activity of cytochrome P45017α in the reaction of 17α-hydroxylation of progesterone or pregnenolone is highly dependent on the purity of the cytochrome P45017α preparation and the presence mostly in this preparation of cytochrome P420, while the ratio of the rates of 17,20-lyase and 17α-hydroxylase reactions and as a result, the functional activity of hemeprotein, is completely determined by the structure of cytochrome P45017α, as well as the presence in the incubation mixture of specific effectors such as cytochrome b_5 or steroid analogs.

Cytochrome b_5 significantly stimulates the rate of 17,20-lyase reactions catalyzed by chimeric cytochromes P45017α (Fig. 5). However, addition of cytochrome b_5 to the incubation mixture does not confer to Δ^5-type cytochromes P45017α the ability to transform 17α-hydroxyprogesterone into androstenedione, and in the reactions catalyzed by Δ^4-type cytochromes P45017α the ability to transform 17α-hydroxypregnenolone into dehydroepiandrosterone (Fig. 5). These data indicate that cytochrome b_5 binding does not affect the degree of specificity and selectivity of cytochrome P45017α with respect to position of double bond in 17α-hydroxy-derivatives of the steroid molecule.

Chimerogenesis of cytochrome P45017α in the middle part of the molecule with the exchange point at position of amino acid residue 220 (the beginning of α-helix

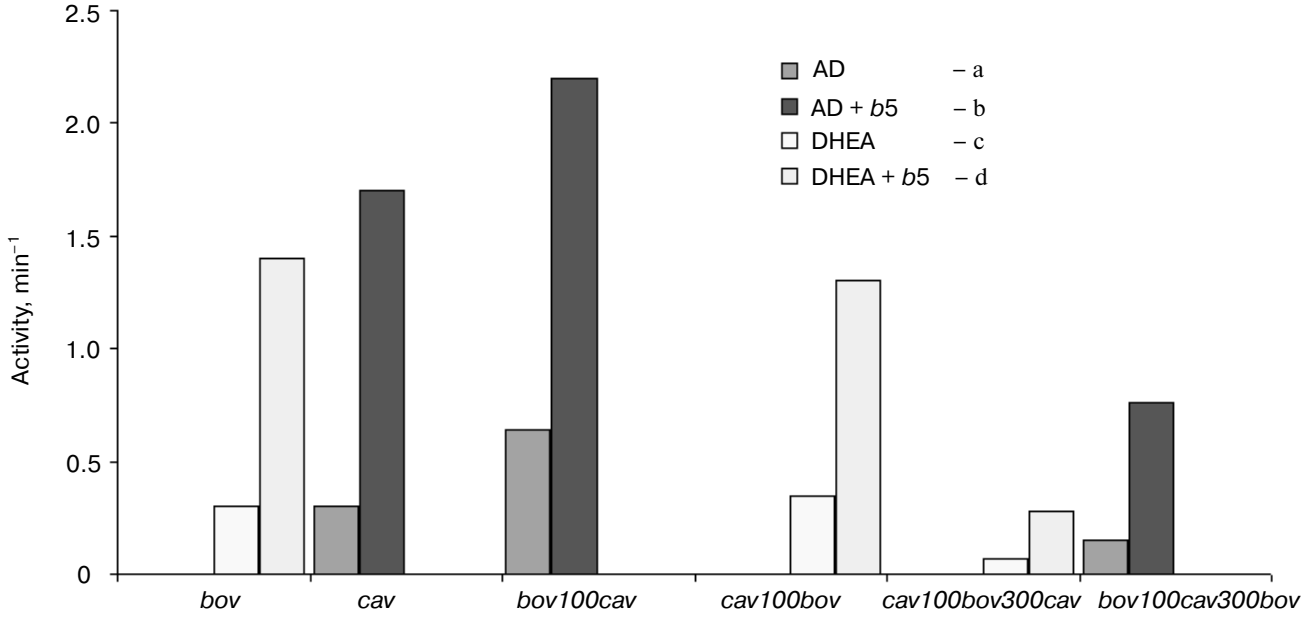

Fig. 5. Effect of cytochrome b_5 on 17,20-lyase activity of cytochrome P45017α: a) formation of androstenedione without addition of cytochrome b_5; b) formation of androstenedione in the presence of cytochrome b_5; c) formation of dehydroepiandrosterone without addition of cytochrome b_5; d) formation of dehydroepiandrosterone in the presence of cytochrome b_5. Reaction mixture contained 0.5 μM cytochrome P45017α, 1 μM NADPH-cytochrome P450 reductase, 0.5 μM cytochrome b_5, 50 μM steroid, 0.5 mM NADPH in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and NADPH-regenerating system. Temperature, 37°C.

G) results in expression of apo-cytochrome P45017α which is unable to correctly bind heme, since the chimeric cytochrome P45017α does not have a characteristic absorption maximum at 450 nm in the presence of sodium dithionite and carbon monoxide. On testing 17α-hydroxylase and 17,20-lyase activities using membranes from recombinant bacteria containing chimeric cytochrome P45017α of this type, we failed to detect substrate consumption or product formation, which indicates severe disturbances in the hemeprotein folding. Both these facts indicate that under such type of chimerogenesis the two parts arising from cytochromes P45017α with different enzymatic activity the profiles are incompatible due to differences in the primary structure and attempts for their joint assembly does not result in formation of spatial structure characteristic for cytochrome P45017α. Thus, based on the results of the experiments described above it is possible to make the following conclusions.

First, the fragment of polypeptide chain responsible for interaction with the molecule of steroid substrate and determining the preference for one or another pathway of oxidation is localized in the region between amino acids from 100 to 300 of the cytochrome P45017α molecule.

Second, equal parts of cytochromes P45017α belonging to different species and possessing different enzymatic activities with respect to Δ^4- and Δ^5-steroid can not always be interchanged, indicating different ways of protein folding of various types of cytochrome P45017α molecules.

Third, the fact that using chimerogenesis we failed to engineer chimeric cytochrome P45017α with both types of activity with respect to Δ^4- and Δ^5-steroids and to form chimeric cytochrome P45017α that does not have 17,20-lyase activity but catalyzes only the 17α-hydroxylase reaction suggests overlapping of structural elements of cytochrome P45017α that determine the nature of the cytochrome P45017α as either one or the other type.

Finally, the profile of enzymatic activity of chimeric forms of cytochrome P45017α as well as the spectrum of the secondary reaction product formed, as a rule, corresponds to the type of hemeprotein with which the similarity of direction of the 17,20-lyase reaction is observed.

DISCUSSION

To understand the mechanism of strictly selective steroid hydroxylation and the nature of the dual activities of cytochrome P45017α, several methodological approaches have been used in recent times: 1) studies of the naturally occurring mutations resulting in disturbance of the functional properties of the enzyme; 2) site-directed mutagenesis of amino acid residues whose functional importance are expected or predicted theoretically; and 3) homologous modeling of the active site of cytochrome P45017α [10]. The use of genetic engineering, directed mutagenesis, and heterologous expression of recombinant proteins allows estimation of the functional impor-

tance of separate elements of the structure of the protein molecule. There are many manifestations of inherited diseases connected with insufficient activity of cytochrome P45017α.

The most commonly observed are diseases with complete absence of both 17α-hydroxylase and 17,20-lyase activities of cytochrome P45017α, which are caused by disturbance of biosynthesis and folding of the polypeptide chain or replacements of some amino acid residues of cytochrome P45017α in the structural elements critically important for enzyme functioning [20-22]. Widely distributed are natural mutations resulting in inability of cytochrome P45017α catalyze the 17,20-lyase reaction, but preserving the 17α-hydroxylase activity of the hemeprotein [23]. Such type mutations were found either in the region that is thought to be the substrate-binding center or in the site of cytochrome P45017α that is supposed to be responsible for interaction with cytochrome b_5.

Cytochromes P45017α from various species have sequence homology 40-98% depending on evolutionary distance between the organisms (Fig. 1). All cytochromes P45017α based on the preferable substrate for the 17,20-lyase reaction can be divided into three main types. The single representative of Δ^4-type cytochrome P45017α is guinea pig cytochrome P45017α. Several cytochromes P45017α from different organisms are of the Δ^5-type, including human cytochrome P45017α. Homology of amino acid sequence of guinea pig cytochrome P45017α and Δ^5-type cytochromes P45017α is 56-70% (Fig. 1).

One of the most studied Δ^5-type cytochromes P45017α is bovine cytochrome P45017α. To localize the fragments of the polypeptide chain responsible for determination of substrate specificity of cytochrome P45017α in the 17,20-lyase reaction, in the present work we engineered a set of chimeric hemeproteins (Fig. 3) containing fragments of polypeptide chain of both guinea pig cytochrome P45017α (Δ^4-type) as well as fragments of bovine cytochrome P45017α (Δ^5-type). It should be stressed that in contrast to that pair of cytochromes P45017α, under alignment of amino acid sequences of cytochromes P45017α from other chordates, one can see many breaks or inserts, which might result in inadequate interpretation of the homology of primary and secondary structures of the hemeproteins. The point of the polypeptide chain where exchange of cytochrome P45017α structure has been carried out corresponds to the region of junctions of the fragments of polypeptide chain coded by adjacent exons of the CYP17 gene. The fragments of polypeptide chain in which chimerogenesis was carried out correspond to the following amino acid residues of cytochrome P45017α: 96, 220, 300, and 400.

The exchange of some homologous fragments of cytochromes P45017α of two types results in formation of chimeric hemeproteins that are unable to bind heme correctly (Fig. 3) but having immunochemical properties similar to cytochrome P45017α. The most critical proved to be the replacements performed in the point of exchange between exons III and IV.

Guinea pig cytochrome P45017α and bovine cytochrome P45017α evolutionarily originate from the same ancestor. The alignment of their amino acid sequences does not show any additional inserts in homologous fragments besides several additional amino acids in the C-terminal sequence of the cytochrome P45017α molecule (Fig. 1). However, exchange of homologous fragments is not always possible, which indicates not only differences in the primary structure of various cytochromes P45017α but also differences in spatial organization of the protein molecules. The amino acid sequence of cytochrome P45017α that determines the direction of biosynthetic pathways to form the precursors of sex hormones is located in the region corresponding to the sequence coded by exons II-V of the CYP17 gene.

The use of chimerogenesis to identify fragments of the polypeptide chain of cytochrome P450 responsible for demonstration of catalytic properties of the enzyme was previously described to study different types of cytochrome P450. Thus, engineering of a chimeric construct by exchange of the first 146 amino acid residues of rat cytochrome P4501A2 with the homologous fragment of rat cytochrome P4501A1 results in a hemeprotein possessing catalytic properties of cytochrome P4501A2 [24]. The reverse replacement of the first 143 residues of cytochrome P4501A1 with analogous fragment of rabbit cytochrome P4501A2 results in synthesis of cytochrome P450 with functional properties of cytochrome P4501A1 [25]. The replacement of the first 268 residues of rat cytochrome P4501A2 with homologous fragment of rat cytochrome P4501A1 results in synthesis of inactive enzyme [24]. Previously, chimerogenesis was also used to study cytochrome P45017α [3, 26]. There have been attempts to create chimeric cytochrome P45017α from human and bovine cytochrome P45017α, which are both Δ^5-type cytochromes P45017α. Human cytochrome P45017α has much higher steroid 16α-hydroxylase activity compared to bovine cytochrome P45017α. Three points corresponding to codon 100 (common *Xba*I restriction site), 175 (common *Pst*I restriction site), and 451 (common *Sac*I restriction site) were used for exchange [3]. The expression of chimeric proteins was carried out in Cos1-cells. It is found that the region responsible for demonstration of catalytic activity is located between amino acid residues 175 and 451. The part of the chimeric cytochrome P45017α with region of exchange at the position of codon 175 proved to be catalytically inactive.

Recently, a similar approach was used to create chimeric proteins between representative of Δ^5-type cytochrome P45017α (human cytochrome P45017α) and a representative of $\Delta^{4,5}$-type cytochrome P45017α (rat cytochrome P45017α) [26]. To change the homologous

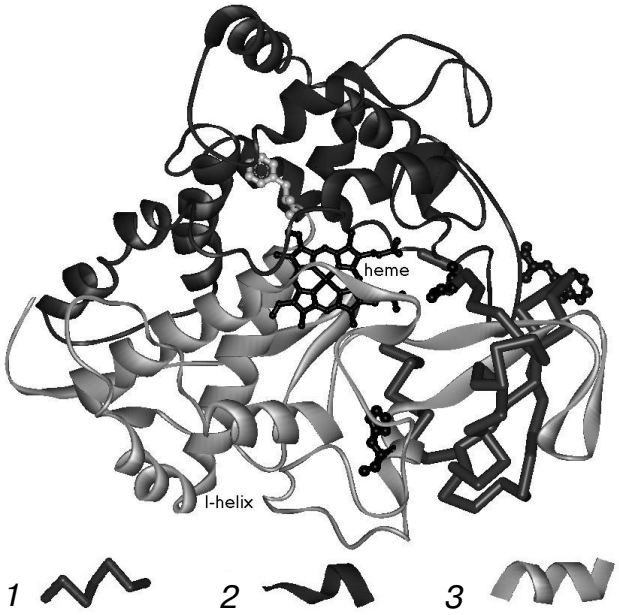

Fig. 6. Three-dimensional structure of cytochrome P45017α with indication of the region of polypeptide chain (aa 100-300) responsible for selectivity with respect to Δ^5- or Δ^4-steroids in the 17,20-lyase reaction. *1*) aa N′-100; *2*) aa 100-300; *3*) aa 300-C′. Cytochrome P450 coordinates (2c17) were taken from the Protein Data Bank.

fragments, the authors used random chimerogenesis. This method is based on the ability of bacterial cells in the process of transformation by linearized plasmid to perform the internal recombination of plasmid fragments with similar nucleotide sequence. As a result, some chimeras with point of exchange at positions 86, 301, 350, 414, and 478 were prepared. It was found that on the replacement of the first 86 amino acid residues of human cytochrome P45017α with homologous fragments of rat cytochrome P45017α, the chimeric hemeprotein preserve the catalytic properties of the human cytochrome P45017α. Similar exchanges but at positions 301, 350, 414, or 478 resulted in chimeric hemeproteins possessing catalytic properties of rat cytochrome P45017α. It was shown that using exchange points at positions 301 or 414 dramatically decrease the stability of the cytochrome P450. Consequently, the region responsible for conversion of Δ^5-type cytochrome P45017α to $\Delta^{4,5}$-type may be localized between amino acid residues 86 and 301. These results [26] are in agreement with the data obtained in the present work characterizing chimerogenesis between cytochromes P45017α of Δ^4- and Δ^5-types.

In addition to studies directed to exchange of homologous fragments of different cytochromes P450, catalytic functions of this hemeprotein have also been studied by site-directed mutagenesis. The replacement of the single amino acid residue Phe209Leu in mouse cytochrome P4502A4 results in transformation of coumarin 7-hydroxylase activity into testosterone 15α-hydroxylase activity [27].

Studies of catalytic properties of cytochrome P45017α using site-directed mutagenesis indicate that replacement of Phe343, which is present in rodent and guinea pig, for Ile343, which is characteristic for human and bovine cytochrome P45017α, results in significant decrease in the level of 17,20-lyase activity with respect to 17α-hydroxyprogesterone [10]. Based on these results it was concluded that this amino acid residue plays an important role in determination of the direction of the enzymatic reaction. These results, however, do not agree with the data obtained in the present work, since the amino acid residue in position 344 is located outside the regions determining substrate specificity (between SRS4 {288-315} and SRS5 {371-381}, see below).

There are several theoretical models of three-dimensional structure of cytochrome P45017α [10, 28]. The substrate-binding pocket of cytochrome P45017α, according to these models, is specially limited by the heme group, which forms a concave substrate-binding site, and α-helix I, which lies close to the angle of heme ring, by β-sheets 3, 4, and 5, and the sites adjoining to α-helices B, C, and K of the cytochrome P450 molecule (Fig. 6).

Based on the contemporary thoughts on the general organization of cytochrome P450 (Fig. 6), it is possible to conclude that in the mechanism of the function of preferable use of a particular type of steroid molecule the following structural elements of cytochrome P45017α are not involved: terminal part of the α-helix I and all of α-helix L, as well as the fragments adjoining β-sheets 3, 4, and 5, α-helices B and K of the hemeprotein molecule, involved in formation of substrate-binding site.

Theoretical analysis of conservative sequences of cytochromes P450 with known structure revealed structural elements of cytochrome P450 that participate in binding and coordination of substrate in the active site (Substrate Recognition Site, SRS) [29]. Independent of specific distribution of separate elements of the secondary structure for every cytochrome P450 and the presence of intrinsic exon–intron structure for each cytochrome P450, there are at least five of six SRS [29-32]. Approximation of theoretical data on distribution of SRS from cytochrome P450 CYP2 family on the sequence of cytochrome P45017α suggests the following distribution of SRS-domains in the structure of cytochrome P45017α: SRS1 {101-125}, SRS2 {205-213}, SRS3 {237-244}, SRS4 {288-315}, SRS5 {371-381}, SRS6 {484-490}. The experimental data obtained in the present work indicate the possible participation in the fine regulation of preferable use as a substrate of a particular type of steroid molecule the following substrate-recognition sites: SRS1, SRS2, SRS3, and SRS4. Substrate recognition sites SRS5 and SRS6 appear to be not involved in binding and fixation of steroid molecule and are outside regions that

affect selectivity of the cytochrome P45017α with respect to substrate to be hydroxylated.

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REFERENCES

1. Hanukoglu, I. (1992) *J. Steroid Biochem. Mol. Biol.*, **43**, 779-804.
2. Bhasker, C. R., Adler, B. S., Dee, A., John, M. E., Kagimoto, M., Zuber, M. X., Ahlgren, R., Wang, X. D., Simpson, E. R., and Waterman, M. R. (1989) *Arch. Biochem. Biophys.*, **271**, 479-487.
3. Lorence, M. C., Trant, J. M., Clark, B. J., Khyatt, B., Mason, J. I., Estabrook, R. W., and Waterman, M. R. (1990) *Biochemistry*, **29**, 9819-9824.
4. Nakajin, S., Takahashi, M., Shinoda, M., and Hall, P. F. (1985) *Biochem. Biophys. Res. Commun.*, **132**, 708-713.
5. Beaudoin, C., Lavallee, B., Tremblay, Y., Hum, D. W., Breton, R., de Launoit, Y., and Belanger, A. (1998) *DNA Cell Biol.*, **17**, 707-715.
6. Shinzawa, K., Kominami, S., and Takemori, S. (1985) *Biochim. Biophys. Acta*, **833**, 151-160.
7. Swart, P., Estabrook, R. W., Mason, J. I., and Waterman, M. R. (1989) *Biochem. Soc. Trans.*, **17**, 1025-1026.
8. Lee-Robichaud, P., Wright, J. N., Akhtar, M. E., and Akhtar, M. (1995) *Biochem. J.*, **308** (Pt. 3), 901-908.
9. Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5597-5601.
10. Buczko, E., Koh, Y. C., Miyagawa, Y., and Dufau, M. L. (1995) *J. Steroid Biochem. Mol. Biol.*, **52**, 209-218.
11. Kitamura, M., Buczko, E., and Dufau, M. L. (1991) *Mol. Endocrinol.*, **5**, 1373-1380.
12. Nakajin, S., Hall, P. F., and Onoda, M. (1981) *J. Biol. Chem.*, **256**, 6134-6139.
13. Ishii-Ohba, H., Matsumura, R., Inano, H., and Tamaoki, B. (1984) *J. Biochem. (Tokyo)*, **95**, 335-343.
14. Chudaev, M. V., Chernogolov, A. A., and Usanov, S. A. (1997) *17th Int. Conf. Biochem. Mol. Biol.*, pp. 24-29.
15. Shen, A. L., Porter, T. D., Wilson, T. E., and Kasper, C. B. (1989) *J. Biol. Chem.*, **264**, 7584-7589.
16. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
17. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.
18. Omura, T., and Sato, R. (1964) *J. Biol. Chem.*, **239**, 2370-2378.
19. Katagiri, M., Kagawa, N., and Waterman, M. R. (1995) *Arch. Biochem. Biophys.*, **317**, 343-347.
20. Fardella, C. E., Hum, D. W., Homoki, J., and Miller, W. L. (1994) *J. Clin. Endocrinol. Metab.*, **79**, 160-164.
21. Lam, C. W., Arlt, W., Chan, C. K., Honour, J. W., Lin, C. J., Tong, S. F., Choy, K. W., and Miller, W. L. (2001) *Mol. Genet. Metab.*, **72**, 254-259.
22. Monno, S., Ogawa, H., Date, T., Fujioka, M., Miller, W. L., and Kobayashi, M. (1993) *J. Biol. Chem.*, **268**, 25811-25817.
23. Geller, D. H., Auchus, R. J., and Miller, W. L. (1999) *Mol. Endocrinol.*, **13**, 167-175.
24. Sakaki, T., Shibata, M., Yabusaki, Y., and Ohkawa, H. (1987) *DNA*, **6**, 31-39.
25. Pompon, D. (1988) *Eur. J. Biochem.*, **177**, 285-293.
26. Brock, B. J., and Waterman, M. R. (2000) *Arch. Biochem. Biophys.*, **373**, 401-408.
27. Lindberg, R. L., and Negishi, M. (1989) *Nature*, **339**, 632-634.
28. Auchus, R. J., and Miller, W. L. (1999) *Mol. Endocrinol.*, **13**, 1169-1182.
29. Gotoh, O. (1992) *J. Biol. Chem.*, **267**, 83-90.
30. Loughran, P. A., Roman, L. J., Aitken, A. E., Miller, R. T., and Masters, B. S. (2000) *Biochemistry*, **39**, 15110-15120.
31. Podust, L. M., Stojan, J., Poulos, T. L., and Waterman, M. R. (2001) *J. Inorg. Biochem.*, **87**, 227-235.
32. Roussel, F., Khan, K. K., and Halpert, J. R. (2000) *Arch. Biochem. Biophys.*, **374**, 269-278.