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Molecular Cloning and Heterologous Expression in *E. coli* of Cytochrome P45017α. Comparison of Structural and Functional Properties of Substrate-Specific Cytochromes P450 from Different Species

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Abstract—To elucidate the nature of substrate specificity and intrinsic mechanism of hydroxylation of steroids, in the present work we carried out molecular cloning and heterologous expression of cDNA for three new forms of cytochrome P45017α from species of the Bovidae family (sheep, goat, and bison), which catalyze 17α-hydroxylation of both progesterone (P4) or pregnenolone (P5) and 17,20-lyase reaction resulting in cleavage of side chain with formation of C_{19}-steroids. Recombinant cytochromes P45017α were expressed in *E. coli* as derivatives, containing a six-His tag at the C-terminal sequence that simplifies purification of the cloned heme proteins using metal-affinity chromatography. Highly purified cytochromes P45017α were used for determination of enzyme activity and specificity in relation to progesterone, pregnenolone, 17α-hydroxyprogesterone, and 17α-hydroxypregnenolone with registration of the kinetics of reaction product formation using HPLC. It is shown that each form of cytochrome P45017α is characterized by a specific profile of enzyme activity and dependence of 17,20-lyase reaction on the presence of cytochrome b_5 in the reaction mixture. The analysis of the activity of the known forms of cytochrome P45017α in view of the data obtained in the present work allows the division of known cytochromes P45017α into three main group: group A (pig, hamster, rat), cytochromes P45017α catalyze the reaction of 17α-hydroxylation of both P4 and P5 steroids and the 17,20-lyase reaction of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone; group B (human, bovine, sheep, goat, and bison), cytochromes P45017α, which have no or have insignificant 17,20-lyase activity in relation to 17α-hydroxyprogesterone; group C (guinea pig), cytochrome P45017α which either has no or has insignificant 17,20-lyase activity on transformation 17α-hydroxypregnenolone to dehydroepiandrosterone.

Key words: cytochrome P45017α, heterologous expression, protein–protein interaction, cytochrome P450

Cytochrome P45017α (the heme protein coded by the *CYP17* gene) is a microsomal type of cytochrome P450 and participates in the reaction of steroid hormone biosynthesis in endoplasmic reticulum membranes of some steroidogenic organs and tissues [1]. Cytochrome P45017α catalyzes the reaction of selective 17α-hydroxylation of pregnenolone (P5) and progesterone (P4) with formation of the corresponding 17α-hydroxy-derivatives, which are precursors in the biosynthesis of glucocorticoid hormones. Cytochrome P45017α also catalyzes the reaction of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone conversion via the 17,20-lyase reaction to androstenedione and dehydroepiandrosterone, respectively, that are intermediate steroids in the biosynthesis of sex hormones. Thus, cytochrome P45017α is a key enzyme in biosynthesis of steroid hormones that determines the direction of reactions either along the pathway of glucocorticoid or sex hormone biosynthesis, and disturbance of the activity of this enzyme results in severe diseases.

Cytochrome P45017α is characterized by unique tissue distribution in humans and animals, ability to "recognize" Δ^4- (progesterone derivatives) and Δ^5-steroids (pregnenolone derivatives) and demonstrate the dependence, mostly for 17,20-lyase reaction, on the presence of cytochrome b_5 in the incubation mixture [2]. Cytochrome P45017α content in different tissues, its catalytic activity, substrate specificity, and dependence on the presence of cytochrome b_5 are important regulatory factors determin-

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ing the direction of reactions in steroidogenesis, their efficiency, and, consequently, the concentration of the main steroid hormones. It is known that besides the indicated reactions, cytochrome P45017α has some extra activities, including, for example, 16α-hydroxylation of progesterone. All this makes cytochrome P45017α an intensively studied object.

Cytochrome P45017α has been purified from different steroidogenic organs (adrenal cortex, testes, ovaries) and species and characterized [3-7]. Cytochromes P45017α from different species show relatively high homology of their amino acid sequences (65-75%), but at the same time they have different types of activity and different dependence on the presence of cytochrome b_5. Recently, some forms of cytochrome P45017α have been cloned and expressed in *E. coli*, allowing the purification of recombinant cytochromes P45017α into highly purified state and the partially characterization of their catalytic properties [8-14]. Analysis of enzyme activity of cytochrome P45017α from different sources indicates the presence of evident species differences. Thus, rat, pig, and hamster cytochrome P45017α catalyzes both 17α-hydroxylation of progesterone and pregnenolone, as well as the 17,20-lyase reaction of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone. However, human and bovine cytochrome P45017α has practically no 17,20-lyase activity with respect to 17α-hydroxyprogesterone, resulting in formation of androstenedione. Finally, guinea pig cytochrome P45017α does not possess 17,20-lyase activity converting 17α-hydroxypregnenolone to dehydroepiandrosterone.

Studies of the structure and function of cytochrome P45017α from different sources, differing in the enzymatic activity profile, give a unique ability to understand the structural bases resulting in the existence of different steroidogenic pathways in different animal species. The factors affecting the ratio of 17α-hydroxylase and 17,20-lyase activities of cytochrome P45017α, are cytochrome b_5 [15-18], phosphorylation of cytochrome P45017α [19], ratio between cytochrome P45017α and NADPH-cytochrome P450 reductase [20], pH of media [21], and some other factors.

Many attempts have been made previously to separate 17α-hydroxylase and 17,20-lyase activities of cytochrome P45017α [22-26]. One of the first approaches in this direction consisted of an attempt to isolate cytochrome P45017α from the tissues of patients with inherited deficiency of 17,20-lyase activity of cytochrome P45017α [22]. Using site-directed mutagenesis, numerous attempts have been made to identify amino acid residues important for manifestation of 17,20-lyase activity by cytochrome P45017α [23-26]. Finally, to gain this aim an approach consisting of construction of chimeric forms of cytochrome P45017α from cytochrome P45017α with different enzyme activity profiles has been used [27, 28].

Besides bifunctional activity, cytochrome P45017α is characterized by unique ability to differentiate Δ^4- and Δ^5-steroids, and of the two types of reaction catalyzed by this heme protein, cytochrome b_5 stimulates, as a rule, only the 17,20-lyase reaction [29, 30]. Moreover, cytochrome P45017α possesses 16α-hydroxylase activity with respect to progesterone [31].

The literature data available on cytochrome P45017α do not allow, however, identification of the sequences specific for enzyme having a particular type of activity. It is necessary to more carefully study the natural changes in the heme protein molecule for the presence of a particular type of activity of cytochrome P45017α. The aim of the present work was to molecular clone 3 new forms of cytochrome P45017α from the species of Bovidae family (sheep, goat, and bison), heterologously express in *E. coli*, and isolate and purify recombinant cytochrome P45017α using metal-affinity chromatography and perform detailed physicochemical characterization.

MATERIALS AND METHODS

In the present work we used chemicals from following sources: SDS, NADPH, phenylmethylsulfonyl fluoride (PMSF), and Coomassie Blue R-250 from Sigma (USA); Ni-NTA-agarose from Qiagen (USA); Emulgen 913 from Kao Atlas (Japan); agarose, isopropyl-β-D-thiogalactopyranoside (IPTG), and dithiothreitol from Gibco BRL (USA); Bacto-Tryptone, Bacto-Peptone, and Bacto-Yeast extract from Difco Laboratories (USA); Bio-Gel HTP from Bio-Rad (USA). Restriction enzymes and enzymes for DNA modification were purchased from New England Biolabs (England), Promega (USA), and Boehringer (Germany).

Cloning of cDNA for cytochrome P45017α. The procedures for cDNA cloning for cytochrome P45017α from bison (*Bison bison*), goat (*Capra hircus*), and sheep (*Ovis aries*) were similar. The adrenal cortex tissue was immediately frozen in liquid nitrogen and stored at −80°C. The mRNA (poly(A)RNA) was isolated from adrenal cortex using a QuickPrep mRNA reagent kit (Pharmacia, Sweden). Single strand cDNA for cytochrome P45017α was synthesized using universal oligo(dT)12-18 primer and mRNA and Superscript II reverse transcriptase (Gibco BRL).

To amplify the cDNA for cytochrome P45017α, 5′- and 3′-primers were designed based on analysis of conservative sequences identified under alignment of nucleotide sequences of cDNAs coding cytochromes P45017α with known structure. 5′-Primer-(AATGTGGGTGCTCTTGGCTGTC-3′) and 3′-primer (5′-AAGAAGGAATGGTGGGAGAAGG-3′) were synthesized on an Applied Biosystems oligonucleotide synthesizer (USA). Polymerase chain reaction (PCR) was carried out in

incubation mixture (50 μl), containing 50 ng of DNA, 0.5 μM of each primer, 0.2 mM of dNTP (each), 50 mM KCl, 25 mM Tris-HCl buffer, 2 mM $MgCl_2$, and 2.5 IU of Taq DNA polymerase. PCR consisted of 30 cycles and was carried out under following conditions: denature at 94°C for 1 min, anneal at 55°C for 1 min, and synthesize at 72°C for 1.5 min. The products of amplification reaction were cloned to pGEM-T vector (Promega).

To synthesize cDNA for cytochrome P45017α with open reading frame, the following primers were used: 5′-primer (5′-ACTCGAGCCATATGGCTCTGTTATT-AGCTGTCTTTCTGCTCACCC-3′) and 3′-primer (5′-GTCTAGATGGTCGACGAGGTGCTACC-CTCAGCC-3′).

PCR was carried out under conditions described above, using the following temperature regimes: denature at 94°C for 30 sec, anneal at 62°C for 30 sec, and synthesize at 72°C for 2 min. After PCR, the products of amplification of cDNA for cytochrome P45017α were cloned to pGEM-T vector and sequenced to confirm the identity of the amplified sequence to the sequence coding cytochrome P45017α. The plasmids containing cDNA for cytochrome P45017α confirmed by sequencing were subjected to restriction with *Nde*I and *Sal*I restrictases and cDNA for cytochrome P45017α (*Nde*I-*Sal*I fragment) was cloned to expression vector pCW : bov17 : his from which the *Nde*I-*Sal*I fragment was preliminarily removed. For preparative expression, competent *Escherichia coli* DH5α cells were transformed with pCW plasmid, containing cDNA for cytochrome P45017α of each species (for example, pCW : goat17 : his).

Expression, isolation, and purification of cytochrome P45017αHis. Recombinant clones of *Escherichia coli* DH5α containing cDNA encoding cytochrome P45017α were used to inoculate 5 ml of LB (Luria–Bertani) medium containing ampicillin (100 μg/ml), and the culture was incubated at 37°C overnight with continuous shaking. Overnight culture (3 ml) was used to inoculate 1 liter of TB (Terrific Broth) medium, containing thiamine (1 mM), potassium phosphate buffer, pH 7.6 (100 mM), and ampicillin (100 μg/ml) in a 2.8-liter Fernbach flask. The cells were incubated at 37°C under continuous shaking (185 rpm) until the absorbance of cell culture at A_{600}, reached 0.6. After that, IPTG and ampicillin at final concentrations 1 mM and 100 μg/μl, respectively, were added. The cells were incubated at 26°C with shaking (185 rpm) additionally 48 h, then cooled for 1 h at 4°C and collected by centrifugation at 3000*g* for 10 min.

The recombinant cells obtained from 1 liter of incubation mixture (20 g wet weight), were suspended in 40 ml of 50 mM sodium phosphate buffer, pH 7.2, containing 10% glycerol, 0.1 M NaCl, and 0.5 mM EDTA. The cells were centrifuged 10 min at 3000*g* and the pellet was resuspended on ice in 50 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol, 50 μM progesterone, and 0.5 mM PMSF using glass–glass homogenizer. The cells were then frozen at –70°C.

To thawed recombinant cells 40 ml of 50 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol, 50 μM progesterone, 0.5 mM PMSF, and 0.2% Emulgen 913 was added, and the mixture was sonicated 6 times for 30 sec with 1 min intervals. Cytochrome P45017α was solubilized by adding drops of 10% Emulgen 913 to the final concentration of detergent 1% (3 mg detergent per mg protein). At this step it is important to add fresh PMSF to the solubilizate to final concentration 0.5 mM. The suspension was incubated with mixing for 2.5 h at 4°C and centrifuged at 100,000*g* for 30 min. Supernatant was passed preliminarily through a column with DEAE-cellulose (2.5 × 5.0 cm) to remove impurities that are usually not eliminated by metal-affinity chromatography, and the effluent was applied to a Ni-agarose column (1.5 × 10 cm). The column was washed with 10 volumes of 50 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol, 50 μM progesterone, 0.5 mM PMSF, 1.0% Emulgen 913, and 0.3 M NaCl, and cytochrome P45017α was eluted from the column by the same buffer supplemented with 50 mM histidine. The colored fractions were collected, diluted with 20% glycerol, containing 50 μM progesterone, and applied to a column with hydroxyapatite (1.5 × 5.0 cm). The column was washed with five volumes of 80 mM sodium phosphate buffer, pH 7.2, containing 20% glycerol and 0.02% Emulgen 913, and cytochrome P45017α was eluted from the column by increasing phosphate concentration to 300 mM. The final preparation of 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.

Determination of 17α-hydroxylase activity of purified recombinant cytochrome P45017α. Hydroxylation of steroids in a reconstituted system by recombinant cytochrome P45017α was carried out at 37°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$. Highly purified recombinant cytochrome P45017α and recombinant NADPH-cytochrome P450 reductase were added to the incubation mixture at final concentration 0.5 and 1.0 μM, respectively. Recombinant rat cytochrome b_5 (full-length form) [32] was added when necessary to the incubation mixture at final concentration 0.5 μM. Progesterone and pregnenolone were dissolved in ethanol and used at final concentration 50 μM and specific radioactivity ~600,000 cpm. The reaction was started by adding NADPH at final concentration 0.5 mM. Aliquots (0.5 ml) were taken from incubation mixture at chosen time intervals and immediately mixed with 5 ml methylene chloride. The mixture was vigorously mixed and water and organic layers were separated by centrifugation. The organic layer was carefully removed and dried under nitrogen flow. To the pellet, 100 μl of methanol was added and steroids were analyzed on a Waters 840 com-

puterized HPLC chromatograph (Waters, USA) equipped with a 10 μm C_{18} Bondopak column (39 × 300 mm) (Waters), Spectraflow 757 optical flow detector (Waters), and flow radioactivity counter β-RAM (INUS, USA). Steroid metabolites were identified based on the retention time of standards.

Determination of 17,20-lyase activity of purified recombinant cytochrome P45017α. Hydroxylation of 17α-hydroxypregnenolone by cytochrome P45017α with formation of dehydroepiandrosterone and 17α-hydroxyprogesterone with formation androstenedione was carried out in a reconstituted system at 37°C in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. Highly purified recombinant cytochrome P45017α containing six histidine residues at the C-terminal sequence was added to incubation mixture at final concentration 0.5 μM. [^{3}H]17α-Hydroxypregnenolone or [^{3}H]17α-hydroxyprogesterone was added together with "cold" steroid, dissolved in ethanol, at the final concentration 50 μM with radioactivity 600,000 cpm per tube. The reaction was started by adding NADPH at final concentration 0.5 mM. Aliquots (0.5 ml) were taken at chosen times from reaction mixture, mixed with 5 ml of methylene chloride, and analyzed by HPLC as described above.

Analytical methods. The concentration of cytochrome P450 was determined spectrophotometrically using molar absorbance coefficient $\varepsilon_{450\text{-}490}$ = 91 $mM^{-1}\cdot cm^{-1}$ [33]. The concentration of cytochrome b_5 was determined from oxidized absolute absorbance spectra of the heme protein using molar absorbance coefficient ε_{413} = 117 $mM^{-1}\cdot cm^{-1}$ [34], while content of heme in preparations of highly purified cytochrome P45017α were determined using the pyridine-heme-chromogen method [33].

Protein concentration was determined using a Micro BCA Protein Assay kit (Pierce, USA). SDS PAGE was carried out using a Protean II (Bio-Rad), as previously described [35]. Protein molecular weight was determined using Protein Molecular Weight Standards (Gibco BRL).

RESULTS

Cloning of cytochrome P45017α from different sources. The strategy for molecular cloning of cytochrome P45017α from steroidogenic tissues of different species belonging to the Bovidae family consisted of the synthesis of the first cDNA strand using reverse transcription with following amplification using degenerated primers, synthesized based on the conservative sequences, identified by alignment of known nucleotide sequences coding the cytochrome P45017α of different species. The products of PCR were cloned to pGEM-T vector and their identity to cytochrome P45017α was confirmed by sequencing. Figure 1 shows the nucleotide sequence of cDNA coding sheep cytochrome P45017α, consisting of ~1600 base pairs with the open reading frame coding the protein consisting of 509 amino acid residues.

Figure 2 shows the alignment of amino acid sequences of goat, sheep, and bison cytochromes P45017α cloned in the present work as well as the known sequences of bovine and human cytochromes P45017α. As follows from this figure, the representatives of the Bovidae family are characterized by a high degree of homology of cytochromes P45017α, which is in the range 95-99% (Table 1). The most interesting is the replacement in bison cytochrome P45017α of conservative cysteine residue Cys235 for tyrosine, threonine residue Thr343 for alanine, and asparagine residue Asn289 for serine. At the same time, more differences become evident when compared to the structure of human cytochrome P45017α, possessing similar with bovine cytochrome P45017α type of activity. The functional and evolutional significance of these replacements is still not clear.

Design of expression vector and heterologous expression of cytochrome P45017α from different species in *Escherichia coli*. To characterize physicochemically and catalytically the cloned cytochromes P45017α, we heterologously expressed them in *Escherichia coli*. For that purpose, cloned cDNAs, coding different cytochromes P45017α, were preliminarily subjected to a second PCR to introduce additional *Nde*I and *Sal*I restriction sites, with subsequent cloning of the *Nde*I-*Sal*I fragment, coding cytochrome P45017α, to expression vector pCWori$^+$ HT. This vector contains an additional fragment before the stop-codon, coding six histidine residues, and was successfully used for expression of human cytochrome P45017α and its purification by metal-affinity chromatography [13]. Figure 3 represents the scheme of expression vector pCWori$^+$P450sheep-HT, which contains cDNA coding sheep cytochrome P45017α, consisting of 509 amino acid residues and six additional histidine residues at the C-terminal sequence.

Figure 4 presents a diagram showing the expression level of cloned cytochromes P45017α in *E. coli* cells. Despite of the high degree of homology between representatives of the Bovidae family, most effectively in *E. coli* cells was expressed sheep and goat cytochromes P45017α, while less effectively bison cytochrome P45017α.

Isolation and purification of recombinant cytochrome P45017α. Insertion of an additional six histidine residues to the C-terminal sequence of cytochrome P45017α has practically no effect on its physicochemical and catalytic properties, but dramatically simplifies the procedure for purification of the recombinant heme protein using metal-affinity chromatography. Figure 5 shows absolute absorbance spectra of highly purified (using metal-affinity chromatography) cytochrome P45017α. Since progesterone was used in

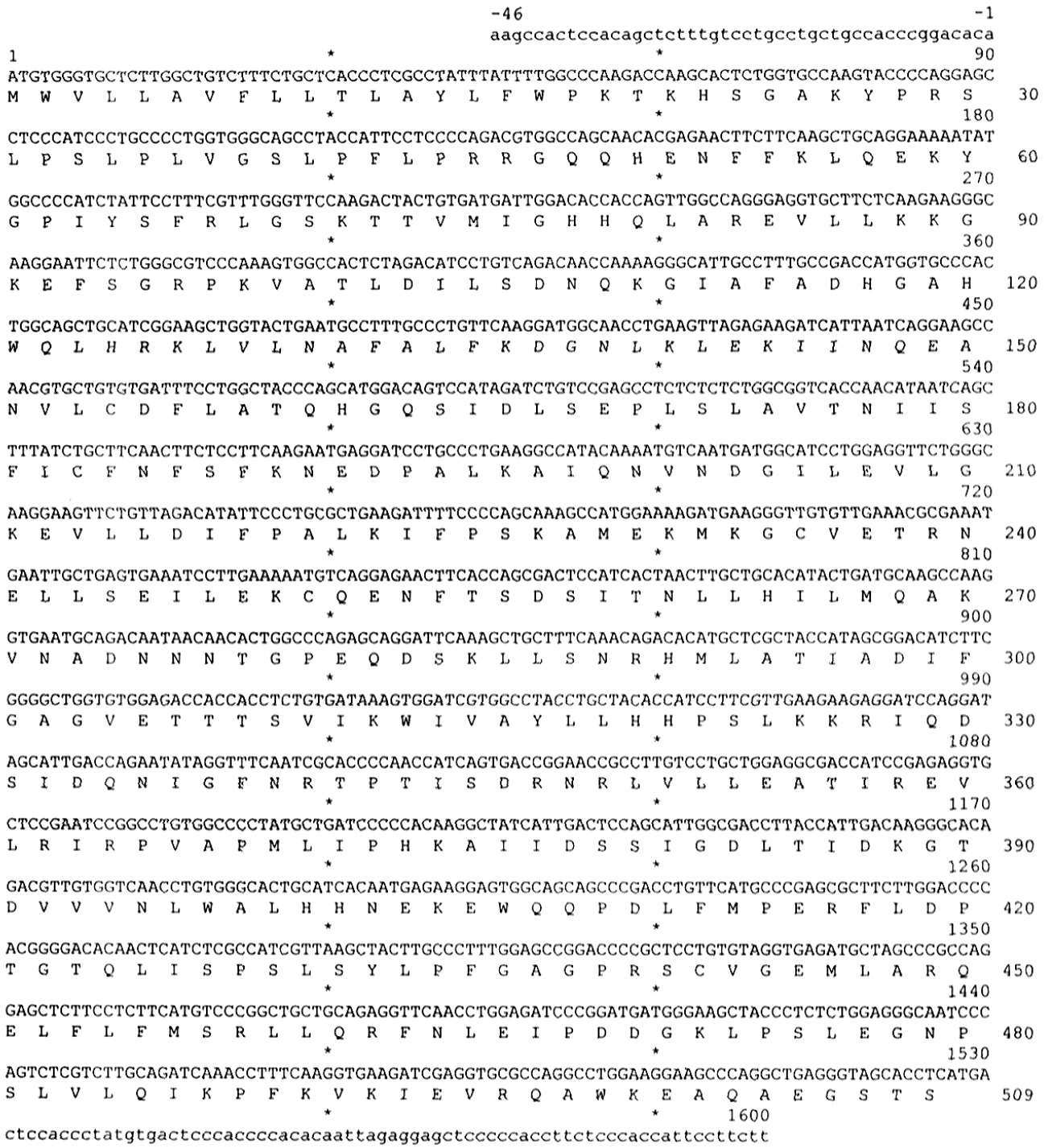

Fig. 1. Coding nucleotide and amino acid sequences of sheep cytochrome P45017α. The numbers over the nucleotide sequence are related to cDNA, while the values from the right side belong to the amino acid sequence.

buffer solutions during cytochrome P45017α purification for its stabilization, the oxidized cytochrome P45017α is predominantly in the high spin state, indicating that practically all purified heme protein is in substrate-bound state. The reduction of cytochrome P45017α with sodium dithionite results in a shift of the absorbance maximum to 412 nm. Further bubbling of reduced heme protein with carbon monoxide is followed by complex formation between reduced cytochrome P45017α and carbon monoxide with absorbance maximum at 449 nm. The absence of absorbance in the region of 420 nm indicates the absence in the prepara-

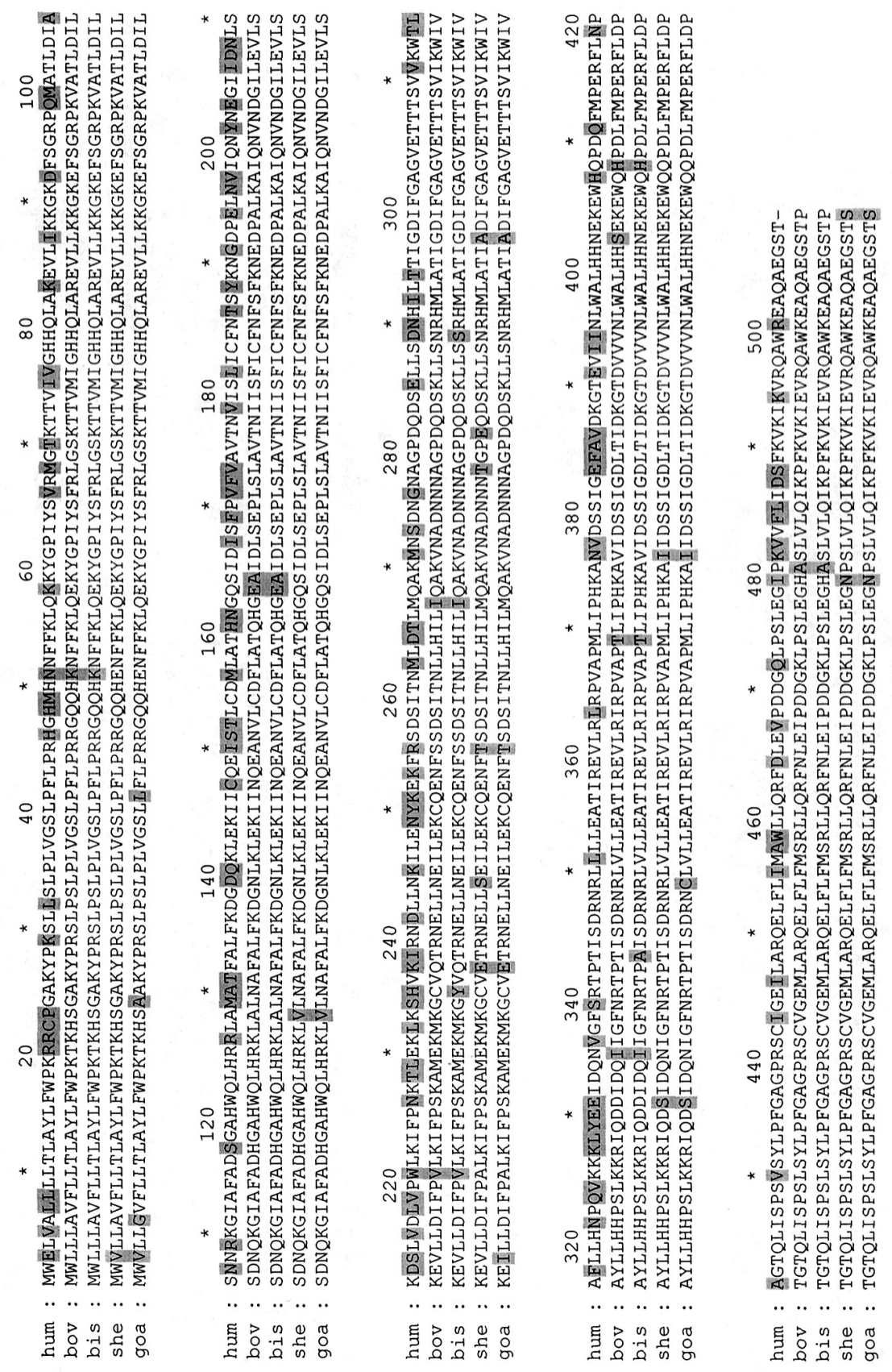

Fig. 2. Alignment of amino acid sequences of cytochromes P45017α from different species, representatives of the Bovidae family: bovine (*Bos taurus*, bov), goat (*Capra hircus*, goa), sheep, (*Ovis aries*, she), bison (*Bison bison*, bis), and human (*Homo sapiens*, hum). Amino acid replacements are indicated on gray background.

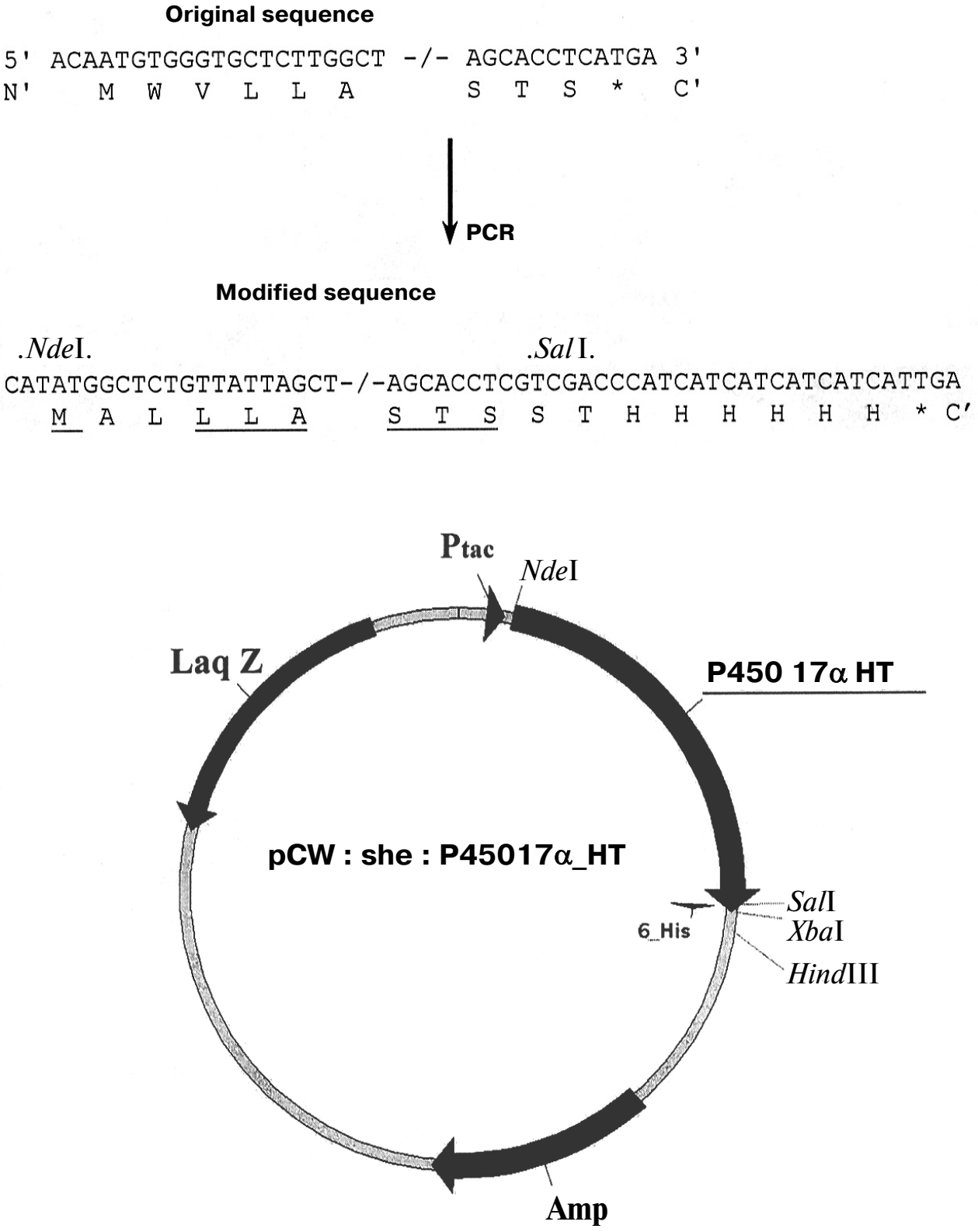

Fig. 3. Construction of expression plasmid for heterologous expression in *E. coli* of sheep cytochrome P45017α.

Table 1. Identity of cytochromes P45017α from different species

Source of cytochrome P450	Number of cysteine residues	*Bos taurus* a.a./nucl.	*Ovis aries* a.a./nucl.	*Capra hircus* a.a./nucl.	*Bison bison* a.a./nucl.
Bos taurus	5		95.5/96.8	95.3/96.8	99.2/99.4
Ovis aries	5	95.5/96.8		98.2/99.0	95.1/96.5
Capra hircus	6	95.3/96.8	98.2/99.0		94.9/96.5
Bison bison	4	99.2/99.4	95.1/96.5	94.9/96.5	

Note: a.a., identity of amino acid residues; nucl., identity of nucleotides.

tion of highly purified cytochrome P45017α of an inactivated form of cytochrome P450—cytochrome P420.

Catalytic activity of recombinant cytochrome P45017α from different species. Cytochrome P45017α possesses at least two types of activity—17α-hydroxylase and 17,20-lyase activities. Besides, the substrate of cytochrome P45017α depending on species may be either Δ^4- (derivatives of progesterone), or Δ^5-steroids (derivatives of pregnenolone). Table 2 presents the values of catalytic activity of highly purified cytochromes P45017α cloned in the present work, determined in a reconstituted system with respect to pregnenolone and progesterone. All cloned cytochromes P45017α possess 17α-hydroxylase activity with respect to both pregnenolone and progesterone. At the same time, progesterone is the most effective substrate for cloned cytochrome P45017α. The highest activity with both steroids is shown by sheep cytochrome P45017α. At the same time, strong differentiation is observed in the activity of cloned cytochrome P45017α with respect to substrate for the 17,20-lyase reaction. Thus, although 17α-hydroxypregnenolone is an excellent substrate for all cytochromes P45017α of the

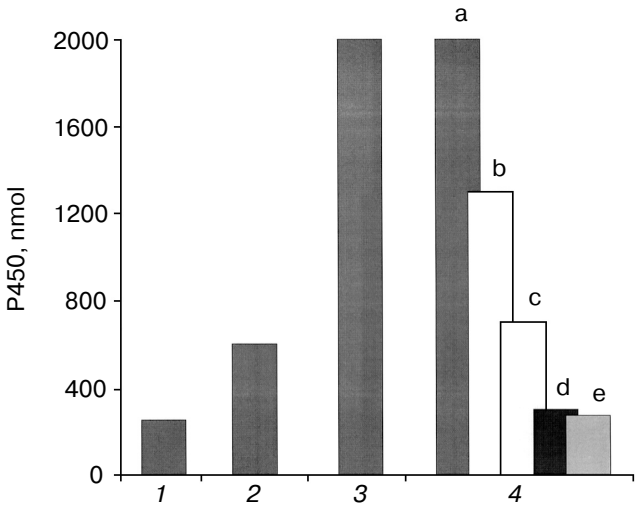

Fig. 4. Level of heterologous expression of cytochrome P45017α from bison (*1*), bovine (*2*), sheep (*3*), and goat (*4*) in nmol cytochrome P450 per liter of culture and the yield of cytochrome P45017α in the process of its isolation and purification: cytochrome P45017α content in the cells (*a*), after sonication of recombinant cells (*b*), after solubilization (*c*), after metal-affinity chromatography (*d*), and after absorption chromatography on hydroxyapatite (*e*).

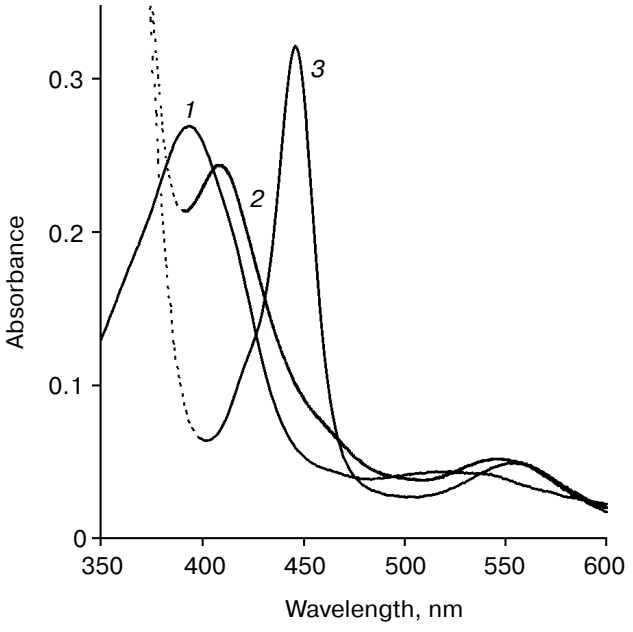

Fig. 5. Absolute absorption spectra of highly purified cytochrome P45017α. Oxidized (*1*), sodium dithionite reduced (*2*), and carbon monoxide complex of reduced cytochrome P45017α (*3*). Concentration of cytochrome P45017α was 2.6 μM.

Table 2. Catalytic activity of recombinant cytochromes P45017α (nmol product/nmol cytochrome P45017α per min)

Source of P45017α	17OH-hydroxy-lation	17,20-lyase reaction	
		$-b_5$	$+b_5$
Pregnenolone			
Bos taurus	17	0.95	4.6
Ovis aries	20	1.2	6.2
Capra hircus	16	0.9	5.1
Bison bison	10	0.6	1.7
Progesterone			
Bos taurus	25	traces	traces
Ovis aries	69	traces	traces
Capra hircus	60	traces	traces
Bison bison	20	traces	traces

Bovidae family, and is effectively transformed by these heme proteins to dehydroepiandrosterone (which is in agreement with earlier results), the 17α-hydroxyprogesterone is practically not hydroxylated by these cytochromes P45017α to androstenedione. In the presence of cytochrome b_5 in the incubation mixture 17,20-lyase activity of cytochrome P45017α with respect to 17α-hydroxypregnenolone is dramatically increased (4-5 times), while cytochrome b_5 does not dramatically affect the 17,20-lyase activity of cytochrome P45017α with respect to 17α-hydroxyprogesterone. The data indicate that the cloned in the present work goat, sheep, and bison cytochromes P45017α (representatives of the Bovidae family) are characterized by similar with bovine cytochrome P45017α profile of activity, possessing 17α-hydroxylase activity with respect to pregnenolone and progesterone, but able to catalyze 17,20-lyase reaction only with respect to 17α-hydroxypregnenolone. This means that in the representatives of these species, biosynthesis of sex hormones mostly goes via the Δ^5-pathway, and the main precursor of sex hormones in these animals is dehydroepiandrosterone. However, the precise sequence of enzymatic conversions is still unclear.

DISCUSSION

In steroidogenic organs and tissues cholesterol with participation of the cytochrome P450-dependent monooxygenase system is transformed to pregnenolone—the main precursor of all steroid hormones. It is commonly accepted that pregnenolone is further transported into endoplasmic reticulum membranes where with participation of 3β-hydroxysteroid-dehydrogenase/Δ^5-,Δ^4-isomerase and cytochrome P45017α it is converted first to 17α-hydroxy-derivatives of pregnenolone and progesterone, and then to the main precursors of sex hormones—dehydroepiandrosterone and androstenedione, respectively. Cytochrome P45017α, having both 17α-hydroxylase and 17,20-lyase activities, regulates the directions of the main pathways of steroid hormone biosynthesis, playing an important role in support of homeostasis. Thus, if the predominant activity is only 17α-hydroxylase activity of cytochrome P45017α, the biosynthesis of steroid hormones is directed mainly to the biosynthesis of glucocorticoids. However, if the predominant activity is 17,20-lyase activity of cytochrome P45017α, biosynthesis of steroid hormones is mainly directed to biosynthesis of sex hormones. This picture becomes much more complicated since in dependence on animal species, biosynthesis of steroid hormones is carried out predominantly either via Δ^5-, or Δ^4-pathways, and cytochrome b_5 stimulates only the second of two reactions catalyzed by cytochrome P45017α.

The alignment of amino acid sequences of cytochromes P45017α with different types of activity do not, however, allow to precisely predict localization in the polypeptide chain of amino acid residues that are responsible for regulation of direction of biosynthetic pathways. The finding of evolutionarily close forms may result in discovery of cytochromes P45017α, similar based on amino acid sequence, but differing based on their functional parameters. In the present work, in order to study the variability of cytochrome P45017α structures among closely related species, we selected the Bovidae family that comprises a huge number of representatives available for such kind of studies. Bovine cytochrome P45017α is the most studied of all known cytochromes P45017α. Moreover, in GeneBank there is nucleotide sequence of coding region of cDNA for sheep cytochrome P45017α also belonging to this family. This sequence is 93% homologous to bovine cytochrome P45017α. However, all our attempts at heterologous expression of this sequence in *Escherichia coli* cells resulted in, as a rule, in the synthesis of apoprotein that was unable to properly bind heme. In this connection, we performed our own cloning of sheep cytochrome P45017α and discovered two amino acid replacements with respect to the nucleotide sequence deposited in GeneBank—Ser210Gly and Asn464Tyr. The replacement of the single nucleotide in corresponding codons results in dramatic changes in the folding of the cytochrome P45017α. The other cytochromes P45017α at position 210 contain either Ser, or Gly. Residue Asn464 is a strictly conservative amino acid among cytochromes P45017α with known structure and therefore, it replacement for tyrosine appear to result in dramatic changes of the cytochrome P45017α molecule skeleton.

In the present work we cloned and expressed in *Escherichia coli* cells several unknown forms of cytochrome P45017α from animals of the Bovidae family

to try to understand the structural basis for manifestation of the multifaceted activity of this heme protein and to determine the profile of the activity of reactions catalyzed by this heme protein. The cDNA coding cytochrome P45017α was prepared by reverse transcription of mRNA with following amplification of single strand cDNA using degenerate primers, designed based on analysis of conservative sequences found on alignment of amino acid sequences of cytochromes P45017α of known structure. Cloned cytochromes P45017α from the animals of the Bovidae family demonstrate high degree of homology (95-99%) when compared both by their amino acid sequence as well as nucleotide sequence of cDNA coding these heme proteins (Table 1).

The use in the present work of pCWori$^+$ vector, previously proved to be very successful for heterologous expression of bovine and human cytochrome P45017α [13, 34], as well as its modification to insert an additional histidine residues at the C-terminal sequence of cytochrome P45017α and application of metal-affinity chromatography for its purification allowed us to purify and characterize recombinant forms of cloned in the present work cytochromes P45017α. Less effectively expressed in *Escherichia coli* cells bison cytochrome P45017α, ~250 nmol per liter of culture, which is much lower than expression of bovine cytochrome P45017α, ~600 nmol per liter of culture. This difference in expression level is difficult to explain since the two cytochrome P45017α are characterized by a high degree of homology. At the same time, we could reach rather high expression level for goat and sheep cytochrome P45017α, up to 2000 nmol per liter of culture. This fact together with simplified procedure of metal-affinity chromatography for purification of recombinant heme protein makes these cytochromes P45017α very useful for structure–function studies. Highly purified cytochrome P45017α has typical absorption spectra characteristic for high-spin substrate-bound cytochrome P450 (Fig. 5).

Studies of catalytic activity of cloned cytochromes P45017α confirmed that they belong to the group of cytochrome P45017α, having 17,20-lyase activity only with respect to Δ^5-steroids (Table 2). At the same time, 17α-hydroxylase activity of the cloned cytochrome P45017α is much higher with respect to progesterone than to pregnenolone. The sheep and goat cytochrome P45017α have maximal activity both in 17α-hydroxylase and 17,20-lyase reactions, being suitable objects to study this group of enzymes. Alignment of amino acid sequences of cloned cytochrome P45017α indicates a high degree of homology, the differences between the two heme proteins being mostly connected with structurally less organized fragments of cytochrome P45017α, which is predominantly represented by the loops. It is interesting that the pair of sheep and goat cytochromes P45017α are much more homologous between each other than the pair of bovine and bison cytochromes P45017α.

At the same time, the alignment of the primary structures of cytochromes P45017α, having different type of enzyme activity with respect to Δ^5- and Δ^4-steroids (Fig. 6), indicates much more significant differences in the primary structure of these heme proteins in the fragments that represent structurally and functionally important elements of cytochrome P45017α. Thus, the most evident differences between the structures of cytochrome P45017α from different species are observed in the region of amino acid residues 120-200, 200-300, and 320-350. Figure 7 shows the structure of human cytochrome P45017α, for which recently the model for tertiary structure was built using homology molecular modeling [36, 37].

It is necessary to stress that despite the total relative homology of cytochromes P45017α from the Bovidae family, we have found differences in the number of cysteine residues in cytochromes P45017α from this family. The goat cytochrome P45017α has an additional cysteine residue, Cys349, located in the region responsible for interaction with cytochrome b_5 [23]. This can be used to carry out studies on localization of functional elements of this cytochrome P45017α using cysteine-specific fluorescence reagents.

The significant differences in the structure of guinea pig and pig cytochrome P45017α from one side and other cytochromes P45017α from the other side are observed in the region of α-helix A (Figs. 6 and 7), while between guinea pig and pig cytochrome P45017α as well as human cytochrome P45017α in the region of α-helix B. Large differences in the structure of guinea pig and pig cytochrome P45017α and representatives of the Bovidae family are found in the region between residues 130 and 300, which form important structural elements such as α-helices C, D, E, F, I, and H, as well as β-sheets 3 and 5. When compared with cytochrome P45017α representatives of the Bovidae family, guinea pig and pig cytochrome P45017α have evident differences in the structure in the region of α-helices I, J, and L (Fig. 6).

Of significant interest in investigation of the mechanism of cytochrome P45017α catalyzed reactions is the uncoupling of 17α-hydroxylase and 17,20-lyase reactions. Thus, it was shown, for example, neutralization of the charge of residues Lys89, Arg347, and Arg358, forming positively charged site on the proximal surface of cytochrome P45017α, which is thought to be responsible for interaction with cytochrome b_5, results in selective loss of 17,20-lyase activity by cytochrome P45017α without any significant loss of its 17α-hydroxylase activity [23, 37]. All cloned in the present work cytochromes P45017α from the Bovidae family contain conservative residues Arg347 and Arg358, which indicates the principal importance of these amino acid residues in formation of the tertiary structure of cytochrome P45017α.

Thus, the data obtained here indicate that while the differences in amino acid sequence of cloned in the pres-

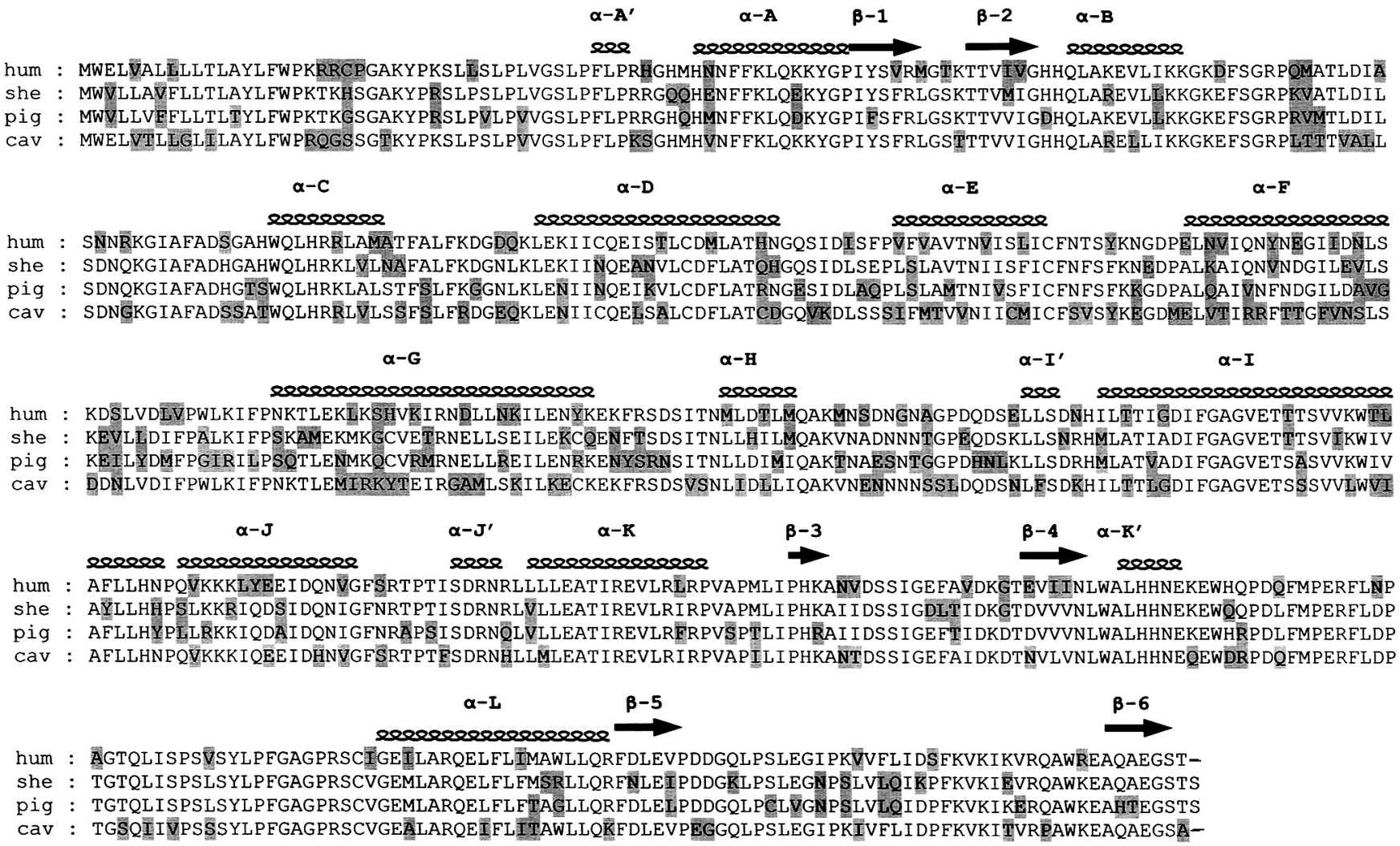

Fig. 6. Alignment of amino acid sequences of cytochrome P45017α from different species: human (*Homo sapiens*, hum), sheep (*Ovis aries*, she), pig (*Sus scrofa*, pig), and guinea pig (*Cavia porcellus*, cav). indicates α-helix, and → indicates β-sheets. Amino acid replacements are indicated by gray background.

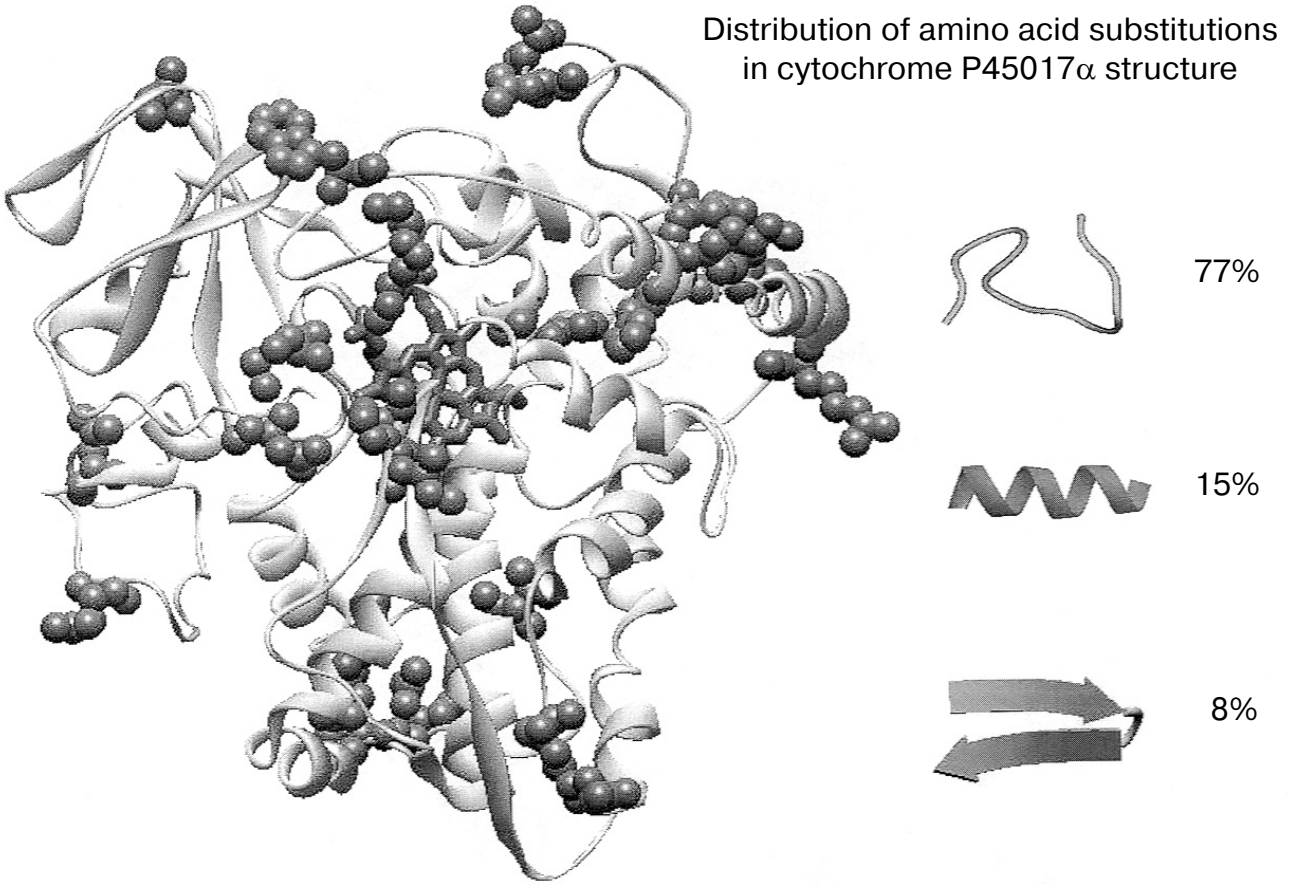

Fig. 7. Theoretical model of the tertiary structure of human cytochrome P45017α with indication of structural elements mostly subjected to replacement in a frame of one animal family. Coordinates for human cytochrome P45017α were taken from Protein Data Bank (PDB). The code for human cytochrome P45017α is 2c17.

Table 3. Catalytic activity of recombinant cytochrome P45017α (nmol product/nmol cytochrome P45017α per min)

Source of P45017α	Type of activity					
	17α-hydroxylase		17,20-lyase			
	pregnenolone	progesterone	17α-hydroxypregnenolone		17α-hydroxyprogesterone	
	$-b_5$	$-b_5$	$-b_5$	$+b_5$	$-b_5$	$+b_5$
Bos taurus	17	25	0.95	4.6	0	0
Ovis aries	20	69	1.2	6.2	0	0
Capra hircus	16	60	0.9	5.1	0	0
Bison bison	10	20	0.6	1.7	0	0
Homo sapiens	2.4	5.3	0.3	1.4	0	0
Cavia porcellus	6	3.5	0	0	0.3	1.7
Sus scrofa	6.0	7.8	0.15	0.8	0.4	1.25
Rattus norvegicus	8.1	6.2	0.6	0.8	0.5	2.2

ent work cytochromes P45017α, representatives of the Bovidae family, are usually negligible and affect mostly non-structured fragments of cytochrome the P45017α molecule (Figs. 6 and 7), the differences between cytochromes P45017α having different activity profiles and belonging to different taxon units are much more evident and are related to important structural elements that participate in formation of tertiary structure of the heme protein. It is necessary to stress that if we take into account the predicted theoretical model of tertiary structure of cytochrome P45017α [37], the amino acid residues of cytochrome P45017α representatives of the Bovidae family, which are more frequently replaced, are localized mostly on the surface of the cytochrome P45017α molecule (Fig. 7).

The analysis of enzymatic activities of cytochromes P45017α with known structure (Table 3) and taking into account results obtained in the present and previous studies [5, 17, 24, 26] allows the division of all known cytochrome P45017α into three main groups: $\Delta^{4,5}$-type (pig, hamster, rat) cytochrome P45017α catalyses 17α-hydroxylation of progesterone and pregnenolone and 17,20-lyase reaction of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone; Δ^5-type (human, bovine, sheep, goat, bison) cytochromes P45017α do not have or possess negligible 17,20-lyase activity with respect to 17α-hydroxyprogesterone converting it to androstenedione; Δ^4-type (guinea pig), cytochrome P45017α that does not have or possesses negligible 17,20-lyase activity with respect to 17α-hydroxypregnenolone converting it to dehydroepiandrosterone.

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