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MOLECULAR GENETIC STUDIES ON THE BIOSYNTHESIS OF ALDOSTERONE IN HUMANS

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Summary—Corticosterone methyl oxidase Type I (CMO I) and II (CMO II) have been postulated to be the enzymes involved in the final two steps of aldosterone biosynthesis in humans. We have isolated human cDNAs for *P*450c11 and *P*450c18 as well as the corresponding genes, *CYP11B1* and *CYP11B2*. Both protein products of these two genes as expressed in COS-7 cells exhibit steroid 11β-hydroxylase activity, but only *P*450c18, a product of *CYP11B2*, carried steroid 18-hydroxylase activity to form aldosterone. These results indicate that *CYP11B2* encodes CMO, the actual catalytic function of which is retained by *P*450c18, a multifunctional enzyme. This conclusion is further supported by the finding that the *P*450c18 gene, *CYP11B2*, is mutated at several different loci in patients deficient in CMO I or II.

INTRODUCTION

In humans, corticosterone methyl oxidase Type I (CMO I) and II (CMO II) have been postulated for a long time as respectively catalyzing the final two steps of aldosterone biosynthesis, i.e. the conversion of corticosterone to 18-hydroxycorticosterone and the subsequent conversion of 18-hydroxycorticosterone to aldosterone [1–3]. Recently, several investigators have claimed, however, that bovine cytochrome *P*450c11 is the enzyme responsible for the synthesis of aldosterone as well as corticosterone [4, 5]. In contrast, it has been demonstrated that rat *P*450c11 does not catalyze the synthesis of aldosterone, but rat $P450_{aldo}$ distinct from rat *P*450c11 is the enzyme responsible for the synthesis of aldosterone [6, 7]. More recently, two types of human cytochrome *P*450c11 related genes (*CYP11B1* and *CYP11B2*) have been isolated and sequenced [8]. *CYP11B1* has been identified as the gene for *P*450c11 [8, 9], and *CYP11B2* has been identified as the gene for *P*450c18 (or aldosterone synthase) [9–12]. In this paper, we present evidence to show that the main functions of *P*450c11 and *P*450c18 lie in the synthesis of glucocorticoids and mineralocorticoids, respectively, and that *P*450c18 is essentially the same protein as CMO, defects of which result in the characteristic biochemical phenotypes of CMO I and II clinical variants.

RESULTS AND DISCUSSION

Figure 1 shows the nucleotide sequence of a cDNA coding for *P*450c18 and its deduced amino acid sequence as compared with those for *P*450c11. Both nucleotide sequences are 93% identical. Also, both amino acid sequences deduced from the nucleotide sequences are 93% identical.

In order to elucidate the functional characteristics of the protein products, *P*450c11 and *P*450c18, encoded by *CYP11B1* and *CYP11B2*, respectively, two expression plasmids (pSV11β and pSVC18) were constructed and steroid hydroxylase activity of these two *P*450s was determined. Figure 2 represents the typical elution profiles of the reaction products when 11-deoxycorticosterone [Fig. 2(A and B)] and 11-deoxycortisol [Fig. 2(C and D)] are respectively used as substrates. As

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Abbreviation: CMO, corticosterone methyl oxidase.

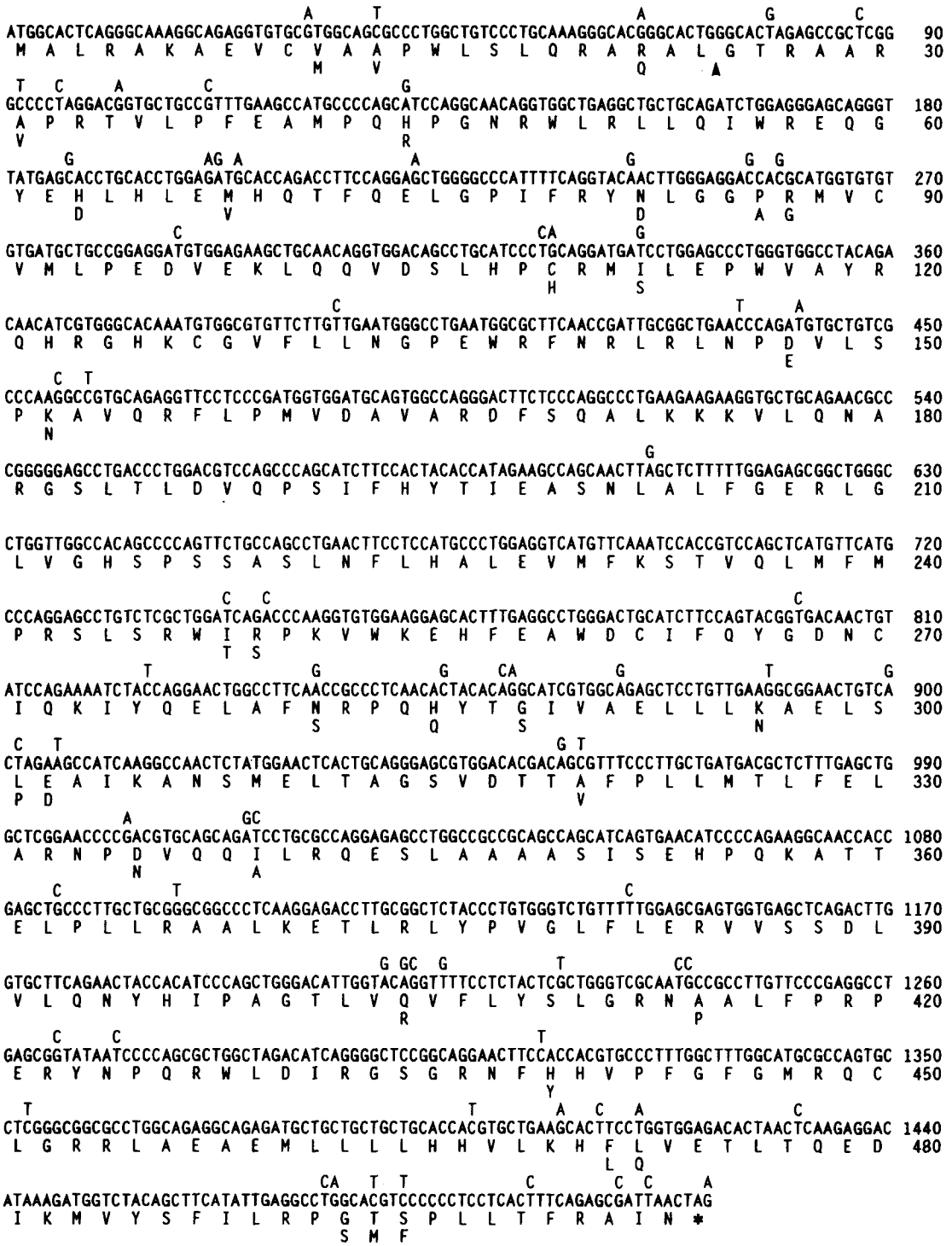

Fig. 1. The nucleotide sequence of the coding region of the cDNA encoding steroid 18-hydroxylase (*P*450c18) and its deduced amino acid sequence. The nucleotides of the cDNA for steroid 11β-hydroxylase (*P*450c11) and its deduced amino acid residues, which are different from those for *P*450c18, are shown above and below each sequence, respectively. An arrowhead indicates the possible cleavage site for precursor polypeptide to form a mature protein. An asterisk denotes the translational termination codon. The complete nucleotide sequence data are available in Genbank/EMBL/DDBJ Nucleotide Sequence Databases under the accession numbers, X54741 and X55764.

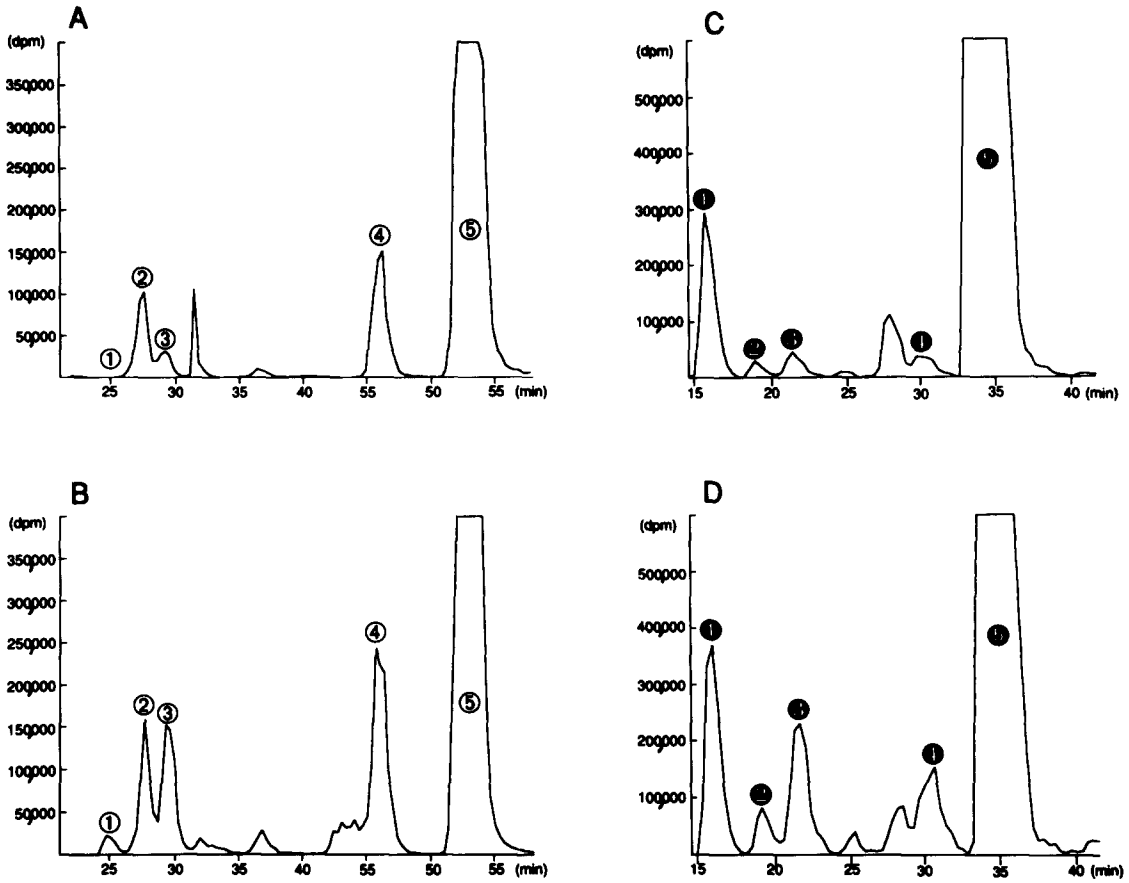

Fig. 2. Elution profile on HPLC of the reaction products formed in the presence of *P*450c11 or *P*450c18 as expressed in the mitochondria of COS-7 cells. Steroid hydroxylase activity of *P*450s was measured as described previously [11, 14]. The reaction was performed in the presence of *P*450c11 (A, C) or *P*450c18 (B, D) using 11-deoxycorticosterone (A, B) or 11-deoxycortisol (C, D) as a substrate. Radioactive products were analyzed by reverse-phase HPLC on a Wakosil 5C18 column (4.6 × 250 mm) with a mobile phase of aqueous 60% methanol at a flow rate of 0.3 ml/min. In A and B, peaks **1**, **2**, **3**, **4** and **5** correspond to those of aldosterone, 19-hydroxy-11-deoxycorticosterone, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone, and corticosterone, respectively. In C and D, peaks **1**, **2**, **3**, **4** and **5** correspond to those of 19-hydroxy-11-deoxycortisol, 18-oxocortisol, 18-hydroxycortisol, 18-hydroxy-11-deoxycortisol, and cortisol, respectively.

shown in Fig. 2(A), aldosterone is not formed at all in the presence of *P*450c11, but is formed exclusively in the presence of *P*450c18 as shown in Fig. 2(B). The amounts of other 18-hydroxycompounds formed from 11-deoxycorticosterone or corticosterone in the presence of *P*450c18 are much higher than those formed in the presence of *P*450c11. In contrast, 18-oxocortisol appears to be formed even in the presence of *P*450c11 [Fig. 2(C)], but the amounts of other 18-hydroxyderivatives formed from 11-deoxycortisol or cortisol in the presence of *P*450c18 are again much higher than those formed in the presence of *P*450c11.

In order to determine whether 18-oxocortisol is actually formed in the presence of *P*450c11, the radioactive fractions corresponding to peak 2 in Fig. 2(C and D) were collected and subjected to purity analysis by periodic acid oxidation. As presented in Fig. 3(A), 75% of the radioactive compound formed in the presence of *P*450c18 was converted to an etiolactone and a 17-ketosteroid which were directly derived from 18-oxocortisol, but negligible amounts of both derivatives were formed when the radioactive compound of peak 2 formed in the presence of *P*450c11 was subjected to the analysis [Fig. 3(B)]. This fact indicates that 18-oxocortisol is not actually formed in the presence of *P*450c11. Table 1 summarizes the steroid hydroxylase activity of *P*450c11 and *P*450c18 when 11-deoxycorticosterone, 11-deoxycortisol, corticosterone, or cortisol is used as a substrate. On the basis of the data in this table, it is reasonable to conclude that *P*450c11

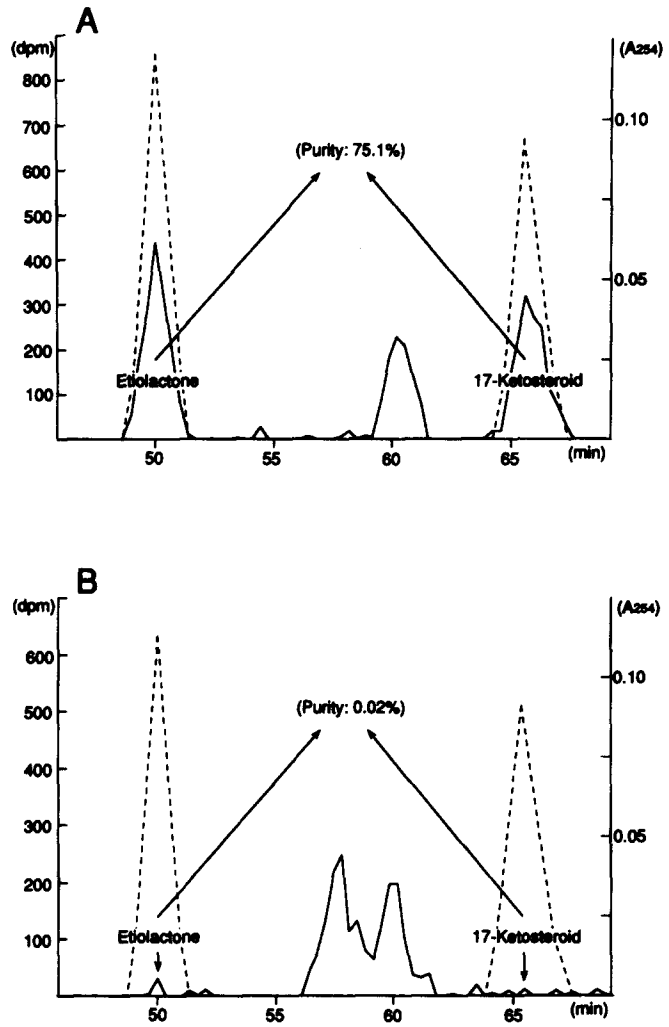

Fig. 3. Analysis of the purity of 18-oxocortisol formed in the presence of *P*450c18 or *P*450c11 as determined by periodic acid oxidation. The radioactive fractions corresponding to peak 2 in Fig. 2(C and D) were collected and subjected to periodic acid oxidation at room temperature for 14 h. The reaction mixture was analyzed by HPLC using a mobile phase of aqueous 45% methanol at a flow rate of 0.3 ml/min. A solid peak in this figure represents the radioactive peak and a dotted peak corresponds to the peak of an authentic etiolactone or an authentic 17-ketosteroid.

mainly participates in the synthesis of glucocorticoids such as cortisol whereas *P*450c18 participates in the synthesis of mineralocorticoids such as aldosterone. Figure 4 represents the metabolic pathways for the synthesis of glucocorticoids and mineralocorticoids in humans. It should be emphasized that CMO I and II, which catalyze reaction steps **6** and **7** in the figure, exactly correspond to *P*450c18.

We further isolated two types of genomic DNAs corresponding to the genes for *P*450c11 and *P*450c18 from normal individuals, CMO I or II deficient patients, and established that these two genomic DNAs are identical to *CYP11B1* and *CYP11B2*, respectively [8, 12]. Of particular interest is the observation that the nucleotide sequences of the promoter regions of both genes are quite different (identity: 48%) although those for their exons are 93% identical. In coincidence with this observation, promoter activity of both genes as analyzed by CAT assay is also remarkably different. Further analysis [13–15] has indicated that the *P*450c18 gene (*CYP11B2*) is mutated at several different loci in patients deficient in CMO I or II. On the basis of these results, Table 2 summarizes the role of *P*450c11 and *P*450c18 as related to CMO I and II in the human adrenal cortex.

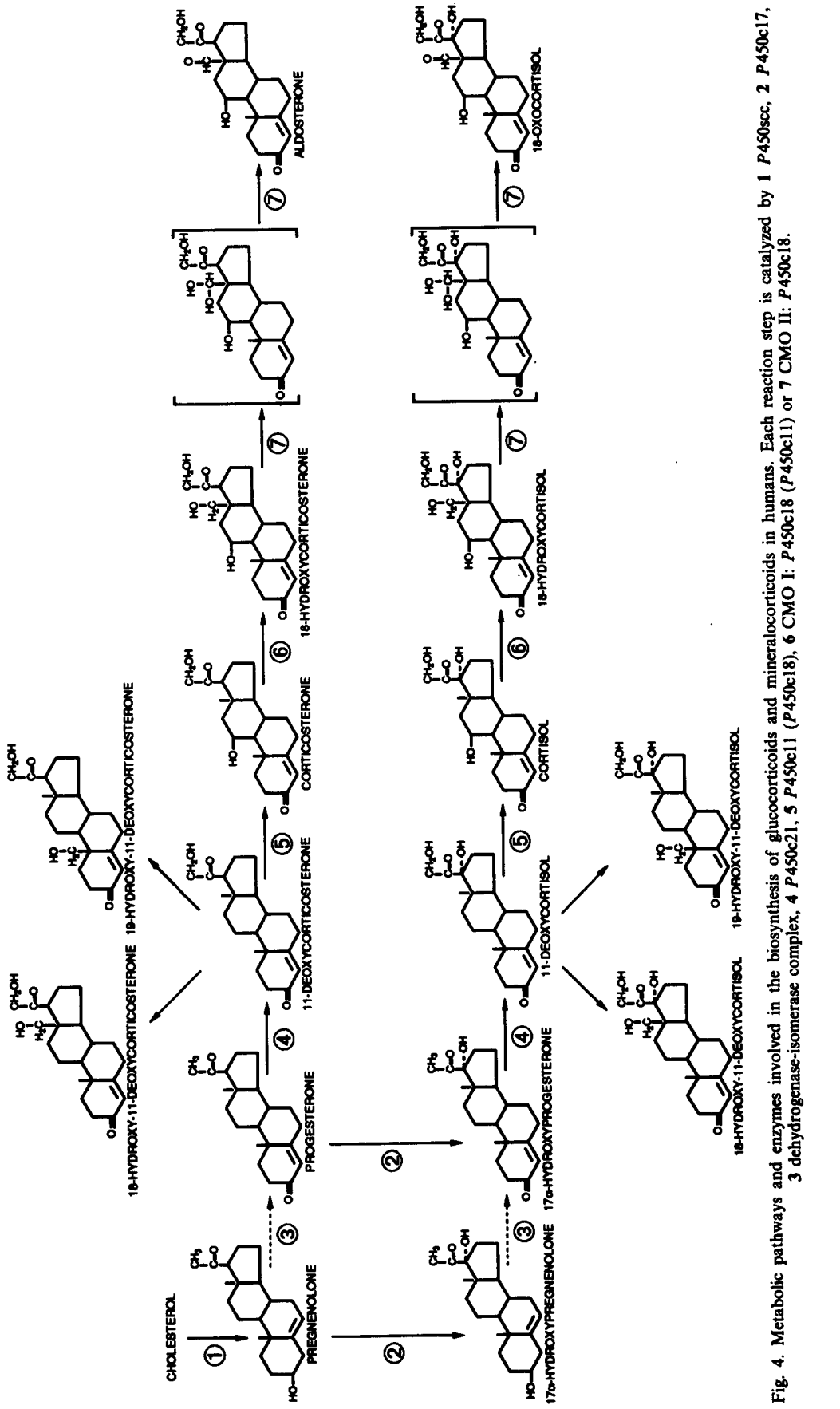

Fig. 4. Metabolic pathways and enzymes involved in the biosynthesis of glucocorticoids and mineralocorticoids in humans. Each reaction step is catalyzed by 1 *P*450scc, 2 *P*450c17, 3 dehydrogenase-isomerase complex, 4 *P*450c21, 5 *P*450c11 (*P*450c18), 6 CMO I: *P*450c18 (*P*450c11) or 7 CMO II: *P*450c18.

Table 1. Steroid hydroxylase activity of mitochondria in COS-7 cells transfected with pSV11β or pSVC18

Substrate	Products	Hydroxylase activity derived from pSV11β (pmol/mg protein)	Hydroxylase activity derived from pSVC18 (pmol/mg protein)
11-Deoxycorticosterone	18-Hydroxy-11-deoxycorticosterone	17.8	35.5
	19-Hydroxy-11-deoxycorticosterone	10.2	14.9
	Corticosterone	458.0	430.3
	18-Hydroxycorticosterone	3.3	18.7
	Aldosterone	<0.02	2.2
11-Deoxycortisol	18-Hydroxy-11-deoxycortisol	1.5	6.4
	19-Hydroxy-11-deoxycortisol	7.4	10.5
	Cortisol	429.4	417.0
	18-Hydroxycortisol	1.2	7.8
	18-Oxocortisol	<0.02	1.8
Corticosterone	18-Hydroxycorticosterone	1.7	11.2
	Aldosterone	<0.02	1.0
Cortisol	18-Hydroxycortisol	0.8	4.3
	18-Oxocortisol	<0.02	0.4

Hydroxylase activity of *P*450s expressed in COS-7 cells was measured as described previously [9, 12]. Values represent the amount of each product formed in a 20-min incubation with [^{3}H]deoxycorticosterone, [^{3}H]deoxycortisol, [^{3}H]corticosterone or [^{3}H]cortisol as a substrate. Amounts of 18-oxocortisol were determined on the basis of its purity as analyzed by periodic acid oxidation to form an etiolactone and a 17-ketosteroid as described in the text and Fig. 3.

Table 2. Role of *P*450c11 and *P*450c18 in the human adrenal cortex

Gene	Enzyme	Function	Inborn errors
CYP11B1	Steroid 11β-hydroxylase (*P*450c11)	Glucocorticoid synthesis (Cortisol)	11β-Hydroxylase deficiency
CYP11B2	Steroid 18-hydroxylase (*P*450c18)	Mineralocorticoid synthesis (Aldosterone)	CMO I and II deficiencies

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