J. Steroid Biochem. Molec. Biol. Vol. 43, No. 8, pp. 973–979, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00
Pergamon Press Ltd

MOLECULAR BASIS OF 17α-HYDROXYLASE/17,20-LYASE DEFICIENCY

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Summary—17α-Hydroxylase deficiency is characterized by a defect in either or both of 17α-hydroxylase and 17,20-lyase activities, based on the fact that a single polypeptide *P*450c17 can catalyze both reactions. The clinical manifestations of 17α-hydroxylase/17,20-lyase deficiency seem to be more heterogeneous than expected, varying from the classical type to less symptomatic forms as also observed in 21-hydroxylase deficiency. We have sequenced all eight exons of the *CYP*17 (*P*450c17) gene in DNA from several patients, reconstructed the mutations in a human *P*450c17 cDNA and expressed the mutant *P*450c17 in COS 1 cells to characterize the kinetic properties of 17α-hydroxylase and 17,20-lyase activities. The molecular bases of cases clinically reported as 17α-hydroxylase deficiency have turned out to be complete or partial combined deficiencies of 17α-hydroxylase/17,20-lyase. The elucidation of the molecular basis generally explains the patient's clinical profiles including the sexual phenotype of the external genitalia. In one case clinically reported as isolated 17,20-lyase deficiency, the molecular basis was found to be partial combined deficiency of both activities, somewhat discordant with the patient's clinical profile. Based on the results obtained so far we can predict that those 17α-hydroxylase deficient individuals having a homozygous stop codon in the *CYP*17 gene positioned at the amino terminal side of the *P*450c17 heme-binding cysteine (442) will all have the same phenotype. However those individuals having homozygous missense mutations or those who are compound heterozygotes having a missense mutation in at least one *CYP*17 allele will display their own unique phenotype which clinically will be subtly different from all others.

INTRODUCTION

One of the essential steps for cortisol production is 17α-hydroxylation of pregnenolone or progesterone. 17α-Hydroxylated pregnenolone or progesterone is further converted to dehydroepiandrosterone (DHEA) and Δ^4-androstenedione (precursor for sex steroids) by the 17,20-lyase reaction. 17α-Hydroxylase activity is not separable from 17,20-lyase activity during purification, cloning and expression procedures [1, 2], indicating that a single polypeptide *P*450c17 can catalyze both reactions. Although the full-length human *P*450c17 cDNAs have been isolated from testis [3] and adrenal [3, 4] independently, their structures are identical. While rat *P*450c17 can convert both 17α-hydroxypregnenolone and 17α-hydroxyprogesterone to their respective C19 steroids, bovine and human *P*450c17 can convert only 17α-hydroxypregnenolone to DHEA [5]. The human *P*450c17 gene is a single copy gene [6, 7] and has been mapped to chromosone 10q24-q25 [8]. 17α-Hydroxylase deficiency, originally described by Biglieri *et al.* [9], is an autosomal recessive disorder and a relatively rare cause of congenital adrenal hyperplasia (CAH). Based on the fact that *P*450c17 can catalyze both 17α-hydroxylase/17,20-lyase reactions, cases which have traditionally been reported as "17α-hydroxylase deficiency" should theoretically occur in either of two forms: (1) isolated deficiency of 17α-hydroxylase or (2) combined deficiencies of 17α-hydroxylase/17,20-lyase. However, it is often difficult to distinguish these two forms solely from clinical and biochemical profiles. To date, more than 120 cases of this disease have been reported [10]. While most of them are clinically reported to have complete "17α-hydroxylase deficiency", about 20 cases seem to be partial forms of this enzyme deficiency [10]. On the other hand, a third type of

Proceedings of the First International Symposium on A Molecular View of Steroid Biosynthesis and Metabolism, Jerusalem, Israel, 14–17 October 1991.

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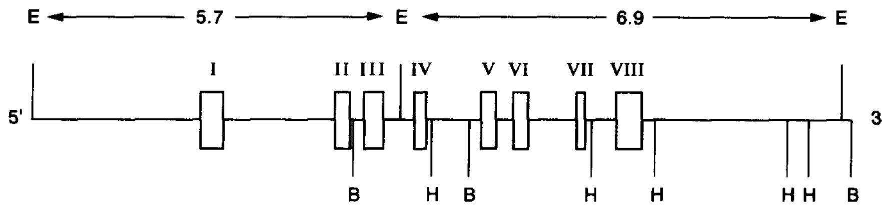

Fig. 1. Schematic diagram of the genomic structure of the human *CYP*17 gene [7]. The shaded boxes represent 8 exons and lines between boxes represent introns. The restriction endonuclease sites indicated are E = *Eco*RI, B = *Bam*HI and H = *Hind* III.

deficiency, isolated "17,20-lyase deficiency" is also described in the literature. This is much less common than "17α-hydroxylase deficiency" and only 14 cases have been reported [10] since it was first described by Zachmann *et al.* [11].

In "17α-hydroxylase deficiency", the reduced production of cortisol provokes an increase in ACTH. Under the influence of elevated ACTH levels, the patient's adrenals synthesize increased levels of mineralocorticoids such as deoxycorticosterone and corticosterone which give rise to hypertension and hypokalemia. On the other hand, impaired production of sex steroids causes abnormalities of sexual development. Namely, the decrease of androgens including testosterone dramatically affects the sexual phenotype of genetic males because the differentiation of male external genitalia depends upon fetal testosterone production. So in genetic males (46XY) with complete 17α-hydroxylase and/or 17,20-lyase deficiencies, the testis does not produce testosterone *in utero*, resulting in absence of masculinization, although normal Mullerian duct regression takes place because of normal production of Mullerian Inhibitory Factor from the testis [12]. Thus, such patients have a blind vagina, absence of Mullerian structures (fallopian tubes, uterus and upper one third of vagina), lack of sexual hair and female external genitalia. On the other hand, in genetic females (46XX) with complete absence of these activities, because of impaired production of estrogens, the patients have primary amenorrhea and no pubertal development leading to hypoplastic breasts and lack of sexual hair. However, despite the biochemical evidence of "17α-hydroxylase deficiency", some genetic male patients are reported to have normal, infantile or ambiguous external genitalia [10], suggesting some degree of androgen production in these individuals. Likewise, some genetically female patients are reported to have normal or irregular menstruation, suggesting some degree of estrogen production [10]. Thus, the elucidation of the molecular basis of these mutants varying from complete to partial deficiencies will greatly contribute not only to the general understanding of the structure–function relationships in *P*450c17 but also to our understanding of sexual development. In this article, we present a review of the molecular bases of all of the reported cases of *P*450c17 deficiency.

MOLECULAR BASIS OF 17α-HYDROXYLASE AND 17,20-LYASE DEFICIENCY

Broadly speaking, five events are required before a microsomal form of cytochrome *P*450 can fulfil its function: proper anchoring into the microsomal membrane: heme binding; substrate binding; transfer of electrons from NADPH–cytochrome *P*450 reductase, and O_2 binding. Mutations that impair one or more of these events can be considered to be the molecular basis for the defect in *P*450c17 activity. Southern blot analysis of genomic DNA from normal individuals after *Eco*RI digestion and hybridization with a full length human *P*450c17 cDNA [4] shows 5.7 kb band 6.9 kb *Eco*RI fragments, which together contain all 8 exons of the *CYP*17 gene (Fig. 1). All of the patients analyzed so far have shown the normal pattern of Southern blot hybridization, indicating that no large structural alterations exist within the mutant *CYP*17 genes. Thus, in our strategy to reveal the molecular basis in 17α-hydroxylase/17,20-lyase deficiency, the genomic clones containing these *Eco*RI fragments of each patient were obtained by a conventional cloning method and analyzed by complete exonic sequencing [7, 13]. In some cases, the exonic sequence was also analyzed by direct sequencing [14] using polymerase chain reaction (PCR). Mutations can take place in either the structural gene encoding *P*450c17 or in the regulatory sequences that determine the level of gene expression. To date, 10 different mutations in 20 individuals have been identified and all of them are located in the structural gene (Fig. 2).

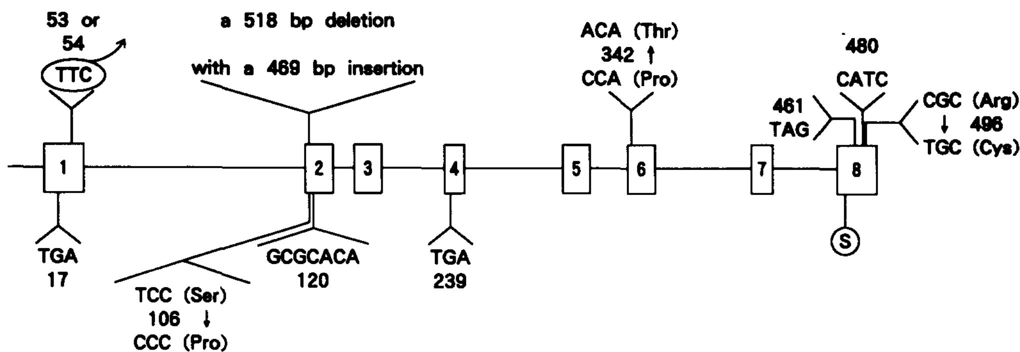

Fig. 2. Schematic representation of the human *CYP*17 gene showing the position of mutations reported to date (Refs [7, 13–15, 17–21 and 26]). The numbered boxes represent the exons; the lines between them represent the introns. The circle noted in exon 8 represents the cysteine (442) required for heme binding by *P*450c17.

Table 1 is a brief summary of the clinical profiles and molecular defects of these patients.

Complete combined 17α-hydroxylase and 17,20-lyase deficiencies

Five kinds of mutations which completely abolish *P*450c17 activity have been identified (cases 1–17 in Table 1). In two Canadian patients (cases 1 and 2), a homozygous mutation comprising a 4 base duplication (CATC) at codon 480 was found in exon 8 of their *CYP*17 genes [7, 15]. The 4 base duplication alters the reading frame, leading to a carboxy terminal sequence that is completely different from that of normal *P*450c17. In addition, the mutant sequence is 3 amino acids shorter than the normal sequence because it contains in-frame stop codon. Expression studies in COS 1 cells of the mutant *P*450c17 cDNA construct containing this mutation showed absence of both 17α-hydroxylase and 17,20-lyase activities (Table 2), which was compatible with the biochemical findings regarding steroid levels in serum and *in vitro* enzyme assays using a testis from patient ML. It is not certain how the alteration of the amino acid sequence in the carboxy terminal region extinguishes *P*450c17 activity. One possible speculation for the mechanism may be the impaired interaction between this mutant protein and NADPH–cytochrome *P*450 reductase since the alteration decreases the number of basic amino acid residues. The reduction of positive charges in the carboxy-terminal region may electrostatically influence the association with carboxylate residues of the microsomal *P*450 reductase [16]. Although

Table 1. Summary of clinical profiles and molecular defects in which the structure of the *CYP*17 gene has been analyzed

Case	Family	Residence	Karyotype	Social sex	Mutation	Reference
1 (ML)	A	Canada	46XY	F	4-bp duplication (480)	7
2 (BD)	B	Canada	46XY	F	Same as case 1	15
3	C	Netherlands	46XY	F	Same as case 1	14
4	C	Netherlands	46XY	F	Same as case 1	14
5	D	Netherlands	46XX	F	Same as case 1	14
6	E	Netherlands	46XX	F	Same as case 1	14
7	E	Netherlands	46XX	F	Same as case 1	14
8	F	Netherlands	46XY	F	Same as case 1	14
9	G	Netherlands	46XY	F	Same as case 1	14
10	H	Netherlands	46XY	M	Same as case 1	14
11 (JY)	I	Japan	46XX	F	Trp → End (17)	13
12 (JK)	J	Japan	46XY	F	7-bp duplication (120)	17
13 (DG)	K	Italy	46XY	F	Deletion and Insertion (Exon 2-3)	18
14 (DA)	K	Italy	46XX	F	Same as case 13	18
15 (DM)	K	Italy	46XX	F	Same as case 13	18
16	L	Guam	46XY	F	Ser-106 → Pro	19
17	M	Guam	46XY	F	Same as case 16	19
18 (JG)	N	Japan	46XX	F	Deletion of Phe (53 or 54)	20
19 (DL)	O	Canada	46XY	M	(1) Arg-239 → End (2) Pro-342 → Thr	21
20 (IS)	P	Swiss	46XY	F	(1) Gln-461 → End (2) Arg-496 → Cys	26

Case 10 is a heterozygous patient for the mutation and thus may have a different mutation on the other *CYP*17 allele. All cases except 19 and 20 are homozygous for their respective mutations.

these 2 Canadians are ostensibly unrelated, they had a common background in their religion. They were members of the Mennonite religious sect. Since it is known that different groups of Mennonites emigrated to different parts of Canada in the last century, these patients are probably distant relatives of one another. More interestingly, this 4 base duplication has been found not only in these 2 Canadians, but also in another 8 individuals from 6 families (cases 3–10 in Table 1) residing in the Friesland region of The Netherlands [14]. Since the Mennonite Churches derive their name from Meno Simons, an early leader of this sect in Friesland, this 4 base duplication presumably appeared within the Friesian population prior to emigration of the Mennonites from The Netherlands. To date, this mutation is the most widely distributed cause of 17α-hydroxylase deficiency in the world. The next 4 mutations to be described are also examples of complete combined deficiencies of both activities (Table 1). JY (case 11 in Table 1) is karyotypically and phenotypically a female who has hypertension and primary amenorrhea. The sequence analysis revealed that the patient has a stop codon (TGA) at amino acid position 17, which leads to the production of a very truncated protein [13]. JK (case 12 in Table 1) is karyotypically a male but phenotypically a female. This patient has a 7 base pair (bp) duplication at amino acid position 120 in exon 2, which leads to a frame shift and subsequently a premature stop codon at position 157 [17]. These 2 patients are homozygous for their respective mutations. In an Italian family having 1 46XY (case 13) and 2 46XX (cases 14 and 15) siblings, a homozygous mutation consisting of a 518 bp deletion combined with an insertion of 469 bp foreign DNA containing an in-frame stop codon has recently been identified in the region spanning exon II and III [18]. This is the first example of a large-scale mutation in the CYP17 gene. Interestingly, a part of the inserted sequence is highly homologous with a portion of Chicken Marek's disease herpes virus DNA, suggesting a sporadic viral infection involving an ancestor of the patient. Since these three examples of premature stop codons take place at the amino terminal side of the heme binding sequence in *P*450c17, the presence of these mutations leads to absence of a functional *P*450c17 protein in the adrenal cortex and gonads. In addition to these mutations, a single amino acid substitution from serine to proline at amino acid position 106, which completely extinguishes *P*450c17 activities, has also been reported in 2 Guamanian patients having female phenotype [19] (cases 16 and 17 in Table 1).

Partial combined 17α-hydroxylase/17,20-lyase deficiency

The next two cases described are examples of partial combined deficiencies of both activities. JG (case 18 in Table 1) is a Japanese who is genetically and phenotypically a female. She has irregular menstruation, suggesting some degree of estrogen production, although she is reported to be sexually infantile. Sequence analysis revealed a homozygous deletion of the phenylalanine codon (TTC) at either amino acid 53 or 54 in exon 1 [20]. On the other hand, DL (case 19 in Table 1) is a 46XY Canadian male. He is a male pseudohermaphrodite with ambiguous external genitalia, suggesting some degree of testosterone production. The patient was found to be a compound heterozygote, carrying two different inherited mutant alleles in the *P*450c17 gene [21]. One allele contains a stop codon (TGA) in place of arginine (CGA) at amino acid position 239 in exon 4. Because this stop codon resides at the amino terminal side of the heme binding sequence, the resultant truncated protein is totally nonfunctional. The second allele contains a missense mutation, namely a change from proline (CCA) to threonine (ACA) at amino acid position 342 in exon 6. So it is clear that only one *P*450c17 allele containing this mutation is responsible for the total 17α-hydroxylase/17,20-lyase activity in this individual. The expression study of the mutant cDNA constructs containing a change from proline to threonine at 342 or phenylalanine deletion at either 53 or 54 led to the production of the same amount of immunodetectable *P*450c17 protein as found upon expression of the normal *P*450c17 protein. Under the same amount of immunodetectable *P*450c17 protein, both mutants showed partial activities of both 17α-hydroxylase and 17,20-lyase relative to those of wild type. From the comparison based on initial velocities measured for catalysis of progesterone and 17α-hydroxypregnenolone, respectively, both 17α-hydroxylase and 17,20-lyase activities of DL were found to be $<42\%$ of those of wild type, while those of JG were found to be <23 and $<5\%$ of those of wild type, respectively (Table 3). Thus, the enzymatic properties of both mutants are clearly different. In short, while $P450c17^{Pro\rightarrow Thr(342)}$ showed parallel re-

Table 2. $P450 17\alpha$ activity of transfected COS 1 cells

	P4→17OHP4,		P5→17OHP5→DHEA			17OHP5→DHEA	
Mock	80.1	1.4	77.2	1.3	1.4	67.2	0.9
WT	18.8	50.0	11.5	53.0	14.9	29.5	38.9
DL	41.9	31.4	26.5	43.8	7.4	53.0	16.5
JG	62.8	17.1	51.7	25.0	3.0	70.6	2.5
IS-1	71.4	8.3	65.4	13.2	3.2	63.7	2.3
IS-2	80.2	1.6	73.2	2.3	1.4	65.7	0.9
ML	80.4	1.5	72.9	1.0	1.4	65.2	0.9

The activities were measured by TLC [20]. The data are expressed as percent of total radioactivity. Substrate concentration was 1 μM. Mock, WT, DL, JG, IS-1, IS-2 and ML represent no plasmid, pCMV17α-H (wild type), pCMVDL17α-H (containing a mutation of Pro-342 to Thr), pCMVJG17α-H (containing a deletion of Phe at 53 or 54), pCMVIS-1 17α-H (containing a mutation of Arg-496 to Cys), pCMVIS-2 17α-H (containing a mutation of Gln-461 to Stop), and pCMVML17α-H (containing a 4 bp duplication at 480). P4, progesterone; 17OHP4, 17α-hydroxyprogesterone; P5, pregnenolone; 17OHP5, 17α-hydroxypregnenolone.

duction of both 17α-hydroxylase and 17,20-lyase activities, $P450c17^{\Delta Phe(53 \text{ or } 54)}$ showed greater reduction in 17,20-lyase activity relative to 17α-hydroxylase activity. It is not certain how these particular mutations reduce both 17α-hydroxylase/17,20-lyase activities. As for $P450c17^{\Delta Phe(53 \text{ or } 54)}$, both K_m and V_{max} for 17α-hydroxylation of progesterone were reduced by a factor of 2 and 3–4, respectively [20]. Based on sequence alignments [22], the missing phenylalanine corrsponds to the location near the carboxy-terminal end of the A-helix in the bacterial $P450$cam, the only $P450$ for which the tertiary structure is known. Perhaps shortening this helix affects the protein folding resulting in the observed altered kinetic properties. Furthermore, the specific 17α-hydroxylase activity of this mutant in microsomes was 3-fold less than that observed in intact cells, indicating that the structure of $P450c17^{\Delta Phe(53 \text{ or } 54)}$ was dramatically altered upon disruption of COS 1 cells [20]. On the other hand, proline at 342 is relatively highly conserved among $P450$s although it is not an invariant amino acid. This proline 342 in $P450$c17 aligns with proline 283 of $P450$cam. The role of this proline is not known. Table 4 is a summary of the activities of each individual. These data are based on the assumption that the total function of each individual correlates with the simple sum of the activities carried by two alleles. About 20% normal 17,20-lyase activity appears to be necessary for partial virilization of male external genitalia. We do know that a 46XY individual (JK), expected to have no $P450$c17 activity because of the homozygous presence of a premature stop codon has female external genitalia. In addition, the father of this patient, a heterozygous carrier for the premature stop codon and therefore expected to have 50% of normal $P450$c17 activity is presumably a normal male. Thus, the threshold 17,20-lyase activity necessary for changing the sexual phenotype of the external genitalia from female to ambiguous is 0–20%, while that for changing from ambiguous to normal is 20–50%. In genetic females, the threshold activity of 17,20-lyase for menstruation seems to be lower than expected since only 5% normal 17,20-lyase activity leads to irregular menstruation. But, of course, we must keep in mind that other epigenetic or nongenetic factors such as individual variation in levels of synthesis of the mutant enzyme and different rates of catabolism or excretion of steroids may also modify the clinical profiles, since it has been demonstrated that a common genetic defect is not always associated with the same clinical profile in 21-hydroxylase [23] or 11β-hydroxylase deficiency [24].

Table 3. Initial rates of reaction for 17α-hydroxylase and 17,20-lyase by transfected COS 1 cells

	17α-Hydroxylase (P4→17P4) pmol/h/dish	17,20-Lyase (17OHP5→DHEA) pmol/h/dish
WT	205 (100)	80 (100)
DL	77 (38.5)	33.0 (41.3)
JG	45.4 (22.7)	3.8 (4.7)

17α-Hydroxylase and 17,20-lyase activities were determined by the conversion of progesterone (P4) to 17α-hydroxyprogesterone (17OHP4) and of 17α-hydroxypregnenolone (17OHP5) to DHEA, respectively. 17,20-Lyase activity of JG was so slow that initial rate of lyase activity was calculated only by data from 24 h time point. The values in parentheses indicate the relative activity of mutants DL and JG when the activity of WT (wild type) is assigned 100%.

Isolated 17,20-lyase deficiency

Case IS (case 20 in Table 1) is a 46XY individual having female external genitalia. Interestingly, she was clinically diagnosed as having isolated 17,20-lyase deficiency [25], which is, however, limited to the Δ^4-pathway only. Sequence analysis revealed that this

Table 4. Relative comparison of 17α-hydroxylase and 17,20-lyase activities among normal individuals and patients

	17α-Hydroxylase	17,20-Lyase	Ext. genitalia
Normal (46XY)	100	100	Male
Father of JK (46XY)	50	50	Male
DL (46XY)	20	20	Ambiguous
JK (46XY)	0	0	Female
			Menstruation
Normal (46XX)	100	100	Regular
JG (46XX)	23	5	Irregular
JY (46XX)	0	0	None

These data are based on the result of the expression studies in COS 1 cells. The total activity of the normal individual is given as 100%. The activity of the father of JK is presumed to be 50%.

patient was also a compound heterozygote having different mutant alleles of the *P*450c17 genes [26]. Both mutations were found in exon 8. One allele contains a missense mutation from arginine (CGC) to cysteine (TGC) at amino acid position 496. The second allele has a stop codon (TAG) in place of glutamine (CAG) at position 461. Because this stop codon is located 19 amino acids to the carboxy-terminal side of the heme-binding cysteine, the resultant protein is shortened by 48 amino acids but nevertheless might bind heme. Results of expression studies in COS 1 cells using cDNA constructs coding the two mutations, IS-1 (Arg496 → Cys) and IS-2 (Gln461 → Stop) are summarized in Table 2. By Western blot analysis, the protein containing $P450c17^{Pro \rightarrow Stop(461)}$ was smaller than wild type *P*450c17 as expected. $P450c17^{Arg \rightarrow Cys(496)}$ showed partial 17α-hydroxylase and 17,20-lyase activity, while $P450c17^{Gln \rightarrow Stop(461)}$ showed slight or no 17α-hydroxylase activity and was completely inactive in the 17,20-lyase assay. From the comparison based on the initial velocity catalyzing progesterone, 17α-hydroxylase activity of $P450c17^{Arg \rightarrow Cys(496)}$ was found to be 9.8% of wild type (14.0 vs 143 pmol/h/dish). 17,20-Lyase activity of $P450c17^{Arg \rightarrow Cys(496)}$ was around 4% of wild type based on the 24 h result in Table 2. The absence of activity of $P450c17^{Gln \rightarrow Stop(461)}$ could result from the inability of the truncated protein to bind heme or from the absence of key residues in the C-terminal 48 amino acids. The mechanism for the reduction in activities in $P450c17^{Arg \rightarrow Cys(496)}$ is not clear at present. While this patient has clinically isolated 17,20-lyase deficiency in the Δ^4-pathway only, *in vitro* studies using COS 1 cells showed marked reduction in both 17α-hydroxylase and 17,20-lyase activities even with Δ^5-substrates. This result would explain the patient's phenotypic profile of female external genitalia considering that even 20% of normal 17,20-lyase activity causes partial masculinization of external genitalia.

CONCLUSIONS

Ten different mutations found in the *CYP*17 structural genes of 20 individuals having impaired *P*450c17 activities are reviewed. The molecular bases generally explain patient's clinical profiles. However, it is often not possible to explain the biochemical basis of reduced or absent *P*450c17 activities. In order to obtain a deeper understanding of the mechanism whereby each mutation affects the *P*450c17 activities, a better expression system than COS 1 cells that will permit biophysical analysis of each mutant *P*450c17 protein with respect to heme binding, substrate binding, and interaction with *P*450 reductase are essential. Such analysis using an expression system in *E. coli* is currently under investigation.

Acknowledgements—The authors appreciate greatly the important contribution of Drs M. Kagimoto, K. Kagimoto, J. S. D. Winter, N. Matsui, A. Shibata, S. Suzuki, K. Hashiba, J. R. Pratt, M. Zachmann, R. Ahlgren, A. Biason and F. Mantero. The support of Grant 1084 from The March of Dimes Birth Defects Foundation and USPHS Grant GM 37942 is greatly appreciated.

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