Hereditary Male Pseudohermaphroditism Associated with an Unstable Form of 5α-Reductase

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ABSTRACT The properties of 5α-reductase have been compared in genital skin fibroblasts cultured from five patients from three families (Los Angeles, Dallas, and Dominican Republic) in which hereditary male pseudohermaphroditism has been established to result from deficient conversion of testosterone to dihydrotestosterone.

Despite the fact that 5α-reductase was immeasurable in a homogenate of epididymis removed from one of the Los Angeles patients, 5α-reductase activity was normal in intact fibroblasts and fibroblast extracts from both patients from the Los Angeles family. Although the apparent K_m for testosterone was also near normal, the apparent K_m for NADPH in these mutants is elevated some 40-fold above normal. Furthermore, the enzyme is not protected against denaturation at 45°C by concentrations of NADPH that stabilize normal 5α-reductase, and in intact fibroblasts from these patients (but not from controls), enzyme activity decreases promptly when protein synthesis is inhibited. We conclude that the mutation in this family results in an unstable enzyme.

In contrast 5α-reductase activity in fibroblast extracts from a patient from the Dominican Republic family is similar to that previously described in two members of the Dallas family, namely total enzyme activity is low at the optimal pH for the normal reaction, and the apparent K_m for testosterone is some 20-fold higher than that of the controls. We conclude that the mutations in the Dallas and Dominican Republic families are similar and result in low activity of the enzyme as the result of a decreased affinity for testosterone.

Thus, two distinct types of mutations can produce male pseudohermaphroditism due to deficient dihydrotestosterone formation.

INTRODUCTION

The secretion of testosterone by the fetal testis at a critical time during embryogenesis is essential for the conversion of the sexually indifferent embryo into the male phenotype (1, 2). Testosterone itself is thought to be responsible for the virilization of the Wolffian ducts into epididymis, vas deferens, and seminal vesicles whereas dihydrotestosterone,[1] the 5α-reduced derivative of testosterone, is responsible for the formation of the male external genitalia, urethra, and prostate (3, 4).

Pseudovaginal perineoscrotal hypospadias is an autosomal recessive disorder in which 46, XY males have a distinct form of male pseudohermaphroditism characterized by normal male levels of plasma testosterone and male Wolffian structures but predominantly female external genitalia at birth (5–7), findings that could be explained by deficiency of dihydrotestosterone formation. In keeping with this concept, three affected families in which the disorder appears to result from such a deficiency have been characterized in Dallas (8–11), the Dominican Republic (12, 13), and Los Angeles (14). On the basis of endocrinological studies (all three families), direct biopsy measurements (two families), and studies of cultured skin fibroblasts (one family) it has been demon-

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[1] *Abbreviation and nomenclature used in this paper*: 3α-androstanediol, 5α-androstane-3α,17β-diol; androstanedione, 5α-androstane-3,17-dione; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; CoA, coenzyme A; 5α-reductase, NADPH: Δ^4-3-ketosteroid-5α-oxidoreductase; 17β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid:$NADP^+$ 17-oxidoreductase (EC, 1.1.1.64).

strated that dihydrotestosterone formation in these individuals is deficient, and it has been concluded that the anatomical abnormality is the result of this deficiency.

We have previously characterized the enzymatic abnormality in two subjects from the Dallas pedigree and have demonstrated that the defective dihydrotestosterone formation is the consequence of a deficiency in 5α-reductase activity (10, 11). In the present study we compare the kinetic characteristics of 5α-reductase in skin fibroblasts from five affected members of the three families and conclude that the abnormalities in the Dallas and Dominican Republic families are similar—probably structural mutations that affect the interaction of the enzyme with steroid substrate. In contrast, the abnormality in the Los Angeles family appears to alter the binding of the cofactor NADPH and to result in profound instability of the enzyme.

METHODS

Cell culture. The fibroblast strains used in these experiments were established from explants of foreskin (Table I). The controls include normal subjects, patients with developmental defects of the external genitalia (hypospadias, microphallus, cryptorchidism), and a male with pseudohermaphroditism due to deficiency of the enzyme 17β-hydroxysteroid dehydrogenase. The mutant fibroblasts from patients with deficient dihydrotestosterone formation include strains from two Dallas patients (8), a previously undescribed patient from the Dominican Republic pedigree (012-088) (12, 13), and two patients from Los Angeles (14).

TABLE I

Identification of 12 Control Fibroblast Strains and 5 Strains from Patients with 5α-Reductase Deficiency

Foreskin fibroblasts	Diagnosis	Age
		yr
Control strains		
311	Normal	0.5
312	Normal	1.5
313	Normal	6
173	Normal	14
39	Normal	25
164	Normal	25
178	Microphallus	3
188	Hypospadias	5
172	Hypospadias	7
171	Hypospadias	8
351	17β-Hydroxysteroid dehydrogenase deficiency	12
183	Undescended testes	25
5α-Reductase mutants		
139 (Dallas 1)	5α-Reductase deficiency	13
123 (Dallas 2)	5α-Reductase deficiency	11
41 (Dominican Republic)	5α-Reductase deficiency	21
71 (Los Angeles 1)	5α-Reductase deficiency	13
74 (Los Angeles 2)	5α-Reductase deficiency	12

The fibroblast cultures were established, stored, and propagated as described (15). All fibroblasts were utilized before the 20th transfer. To grow fibroblasts for the whole cell assay, cells from stock flasks were dissociated with 0.05% trypsin and 0.02% EDTA at 40°C, and 20,000 cells were seeded (day 0) into wells of Linbro plates (16 mm diameter, Linbro Chemical Co., Hamden, Conn.) in 1 ml of Eagle's minimum essential medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 20 mM tricine Cl (pH 7.4–7.5), 24 mM $NaHCO_3$, 1% (vol/vol) nonessential amino acid solution (100×), and 10% (vol/vol) fetal calf serum. On days 3 and 6 the medium was removed and replaced with the same volume of fresh growth medium. To grow fibroblasts for broken cell assays, cells from stock flasks were dissociated with trypsin and EDTA, and 3×10^5 cells were seeded (day 0) into Falcon petri dishes (diameter 8.5 cm, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in 10 ml of Eagle's minimum essential medium supplemented as above. On days 3 and 6 the medium was removed and the same volume of fresh growth medium was added. On day 7, monolayers were either used for a direct assay of 5α-reductase in intact cells (Linbro plates) or harvested for the preparation of cell-free extracts (Falcon petri dishes).

Cell-free extracts were prepared at 4°C. Each monolayer was rinsed twice with 3 ml of Tris-saline (20 mM Tris Cl, pH 7.4, and 0.15 M NaCl) and harvested by scraping into 2 ml of the same buffer. The cell suspension was centrifuged at 800 *g* for 10 min, and the pellet was twice resuspended in 5 ml of Tris-saline and recentrifuged. The pellet was finally resuspended in 10 mM Tris Cl, pH 7.4, and subjected to sonic disruption using a Biosonik III ultrasonic system, Bronwill Scientific (Rochester, N. Y.) (20,000 Hz, 300 W maximum power). Samples were exposed to two 5-s periods of sonication using a needle probe (4 mm diameter) at a power corresponding to the minimum intensity of the instrument (≅50 W). Cell disruption was >95% as estimated by counting in a hemocytometer. The sonicated cell extracts were used directly.

5α-Reductase assay. For the intact monolayer assay, the medium was aspirated from each well on day 7 and replaced with 0.4 ml of serum-free minimum essential medium containing 50 nM [1,2-^{3}H]testosterone (1.76×10^6 dpm) and 0.2 μM [4-^{14}C]dihydrotestosterone (10,000 dpm). The wells were incubated in a CO_2 incubator at 37°C for 2 h, and the steroids were extracted and prepared for chromatography as described (9).

The standard assay mixture for cell-free extracts contained 50 nM [1,2-^{3}H]testosterone (4.4×10^5 dpm), 0.8 μM [4-^{14}C]dihydrotestosterone (10,000 dpm), 3 mM NADPH, 0.1 M Na citrate, 0.1 M Tris Cl (pH 5.5 or 9.0), and enzyme (44–183 μg protein) in a total volume of 0.1 ml. Samples were incubated 1 h at 25°C. Under these conditions rates of activity were proportional to the time of incubation and to the amount of sonicate added. The estimate K_m values for the substrate, concentrations of testosterone were varied from 0.01 to 2.5 μM; for measurement of apparent K_m for cofactor, concentrations of NADPH were varied from 0.0025 to 2.5 mM and the concentration of testosterone was held constant at 0.25 or 0.5 μM. Apparent K_m values were determined from double reciprocal plots using the method of least squares. pH optima were determined over the range of 4.5–9.5 using Tris-citrate buffer. At the end of the incubations reactions were stopped by the addition of 5 vol of chloroform:methanol (2:1). Steroids were then extracted and prepared for chromatography.

10 μg each of authentic testosterone, androstenedione, di-

hydrotestosterone, androstanedione, and 3α-androstanediol was added to the extracts which were then taken to dryness, reconstituted in 20 μl chloroform, and applied to plastic sheets (20 × 20 cm) precoated with silica gel. After chromatography in dichloromethane:ethyl acetate:methanol (85:15:3), the steroids were visualized either by spraying with water or by spraying with anisaldehyde reagent (100 ml glacial acetic acid, 2 ml concentrated H_2SO_4, 1 ml of *p*-anisaldehyde) and heating at 100°C for 15 min. The zone corresponding to dihydrotestosterone was cut and assayed for ^3H and ^{14}C after the addition of 10 ml of 0.4% 2,4-diphenyloxazole in toluene:methanol (10:1). 5α-Reductase activity (picomoles per hour) was calculated from the fraction of total ^3H-radioactivity recovered in the dihydrotestosterone area corrected for the recovery of the [^{14}C]dihydrotestosterone. This method of assessing 5α-reductase with the use of a dihydrotestosterone internal recovery standard is a simplification of our previous technique in which radioactivity recovered in all 5α-reduced steroids was used for the determination of activity (9–11). In nine homogenates the two methods were compared, and the new method on average gave results that were almost identical to the previous method (99.7±3.2% SEM).

Other assays. NADPH-cytochrome *c* reductase was assayed using the method of Masters et al. (16). 3-Hydroxy-3-methylglutaryl coenzyme A (CoA)[1] reductase was measured as described by Brown et al. (17). 17β-Hydroxysteroid dehydrogenase activity was assayed by measuring the conversion of [1,2,6,7-^3H]androstenedione to testosterone, dihydrotestosterone, and 3α-androstanediol at 37°C in Tris-citrate (pH 5.5) using the chromatographic procedure described above; substrate concentrations varied from 0.05 to 10 μM, and NADPH concentrations were from 0.01 to 5.0 mM. NADPH oxidation was measured spectrophotometrically at 340 nm at 25°C in 0.1 M Tris-citrate buffer, pH 5.5, and with 0.5 mg enzyme protein per ml. Protein was assayed by the method of Lowry et al. (18) using bovine serum albumin as the standard.

Materials. The sources of materials used for cell culture and for chromatography have been described (9–11) except that the fetal calf serum was from Grand Island Biological Co. (Grand Island, N. Y.). [1,2-^3H]Testosterone (40 Ci/mmol), [4-^{14}C]dihydrotestosterone (57 mCi/mmol), D,L-3-[glutaryl-3-^{14}C]hydroxy-3-methylglutaryl CoA (7.7 mCi/mmol), and [1,2,6,7-^3H]androstenedione (89 Ci/mmol) were from New England Nuclear (Boston, Mass.). Type III cytochrome *c* was from Sigma Chemical Co. (St. Louis, Mo.). NADPH was from P-L Biochemicals, Inc. (Milwaukee, Wis.).

RESULTS

In the initial studies, 5α-reductase activity was assessed in 12 control fibroblast strains and the mutant strains from the three families (Fig. 1). In intact monolayers, activity in the controls ranged from 2.3 to 110 pmol/h per mg protein, whereas the activity in the two mutants from the Dallas family fell below this level (0.9 and 1.3 pmol/h per mg protein). The activity from the other three patients was in the lower range of normal (5, 4, and 3.9 pmol/h per mg protein). As noted previously (10, 11), discrimination between normals and mutants was more clear-cut when the assay was performed in broken cells at pH 5.5. The normal range at pH 5.5 varied from 2.9 to 156 pmol/h per mg protein, and activity in three of the mutants was clearly lower (0.1, 0.1, and 0.2 pmol/h per mg protein). However, 5α-reductase activity in the extracts from the Los Angeles family (3.5 and 4.0 pmol/h per mg protein) was again within the normal range. As before (10, 11), there was little difference between any of the mutants and controls at pH 9.0.

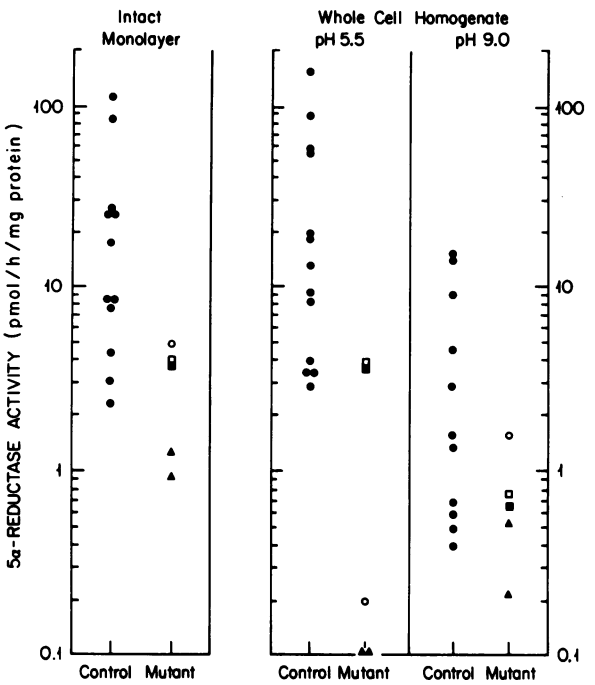

FIGURE 1 5α-Reductase activity in 12 normal and 5 mutant fibroblast strains derived from foreskin. Fibroblasts were grown as described in the text, and on day 7, 5α-reductase was measured both in intact monolayers and in homogenates using 50 nM [1,2-^3H]testosterone. Each point represents the mean of two or three determinations. ●, Controls; □, Los Angeles 1; ■, Los Angeles 2; ○, Dominican Republic; ▲, Dallas 1; △, Dallas 2.

When 5α-reductase was examined in homogenates as a function of pH (Fig. 2), activity in mutant fibroblasts from Dallas and Dominican Republic families was indistinguishable—very low on the acidic side but rising to normal levels above pH 6.0. In contrast, activity in the two Los Angeles strains, like the control (10, 11), exhibited a peak of activity around pH 5.5 as well as activity in the alkaline range.

The kinetic characteristics of the mutants were then examined. When testosterone concentration was varied (Fig. 3 and Table II) fibroblasts from the Dallas and the Dominican Republic exhibited apparent K_m (1.8, 1.5, and 3.4 μM) that were high compared to the normal range of 0.08±0.01 μM SEM. In contrast, the apparent K_m for the two fibroblast strains from Los Angeles were near normal (0.16 and 0.18 μM).

When the concentration of NADPH was varied, the

[2] Abbreviation used in this paper: CoA, coenzyme A.

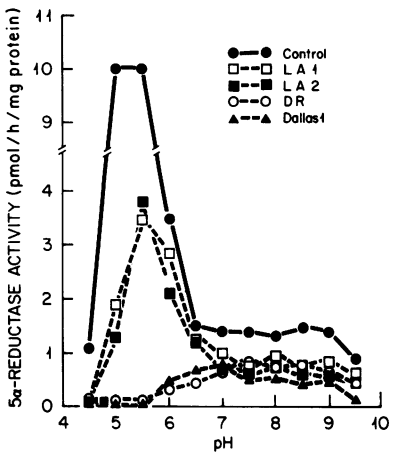

FIGURE 2 5α-Reductase activity in mutant and normal fibroblasts as a function of pH. Cells were grown as described, and on day 7 the cells were homogenized, and 5α-reductase activity was assayed at varying pH as described in the text. The control strain was 178. LA, Los Angeles; DR, Dominican Republic.

situation was different (Fig. 4 and Table II). The apparent K_m for five normal fibroblast strains averaged 40±8.3 μM SEM (range 20–54 μM) whereas the apparent K_m for the two Los Angeles fibroblast strains were higher (1,790 and 900 μM). Measurement of the apparent K_m for NADPH in fibroblasts from the Dallas and Dominican Republic families was technically difficult because of the low rates obtained at lower NADPH concentrations. Nevertheless, the apparent K_m appeared to be intermediate between the normal range and the values observed in the Los Angeles mutants.

TABLE II.
Comparison of 5α-Reductase Kinetics in Control and Mutant Cell Strains

Foreskin cell strains	Apparent K_m	
	Testosterone	NADPH
	μM	
5α-Reductase mutants		
Dallas 1	1.8	252
Dallas 2	1.5	249
Dominican Republic	3.4	97
Los Angeles 1	0.16	1760
Los Angeles 2	0.18	900
Five controls	0.08±0.01 SEM	40±8.3 SEM

The apparent K_m for NADPH were then determined for other NADPH-dependent enzymes in control and Los Angeles fibroblasts (Table III). There was no significant difference in the apparent K_m for cytochrome *c* reductase, 3-hydroxy-3-methylglutaryl CoA reductase, or 17β-hydroxysteroid dehydrogenase despite the fact that the apparent K_m of 5α-reductase for NADPH was 40-fold greater in Los Angeles fibroblasts than in the control. No difference was noted in the disappearance of NADPH in homogenates of normal (strain 39) or Los Angeles 2 fibroblasts (1.0 and 0.8 nmol/min per mg protein, respectively).

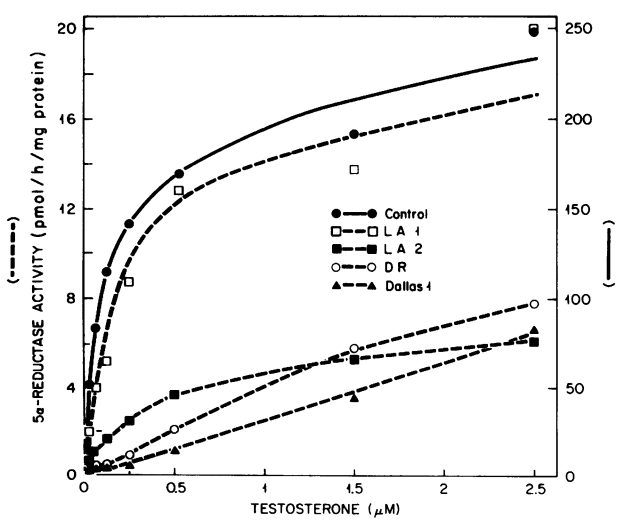

FIGURE 3 5α-Reductase activity as a function of testosterone concentration. Cells were grown as described in the text. On day 7 the cells were homogenized, and 5α-reductase was assayed at varying concentrations of testosterone. The control strain was 39. LA, Los Angeles; DR, Dominican Republic.

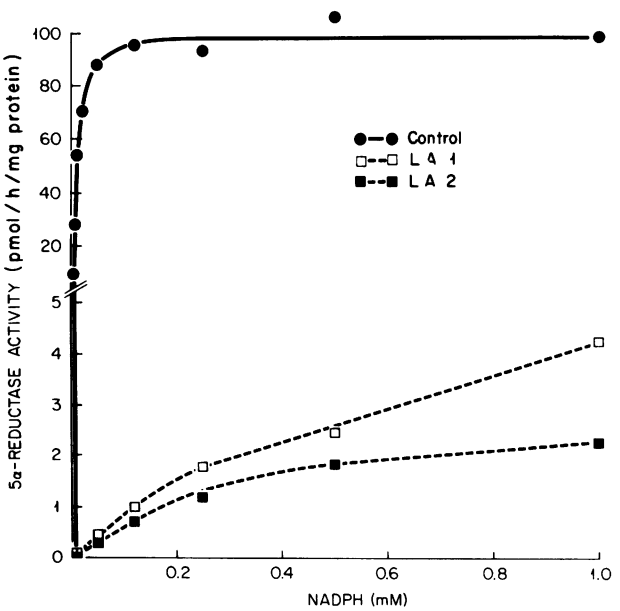

FIGURE 4 5α-Reductase activity as a function of NADPH concentration. Cells were grown as described in the text. On day 7 the cells were homogenized, and 5α-reductase was assayed at varying concentrations of NADPH. The control strain was 39. LA, Los Angeles.

TABLE III
Comparison of NADPH Kinetics in Control and Los Angeles Cell Strains

Enzyme	Apparent K_m			
	NADPH		Substrate other than NADPH*	
	Control	Los Angeles 2	Control	Los Angeles 2
	μM			
5α-Reductase	20	800	0.06	0.18
NADPH cytochrome *c* reductase	5	9	—	—
3-Hydroxy-3-methylglutaryl CoA reductase	10	10	19.4	25.6
17β-Hydroxysteroid dehydrogenase	4,200	5,000	2.3	5.0

The control was cell strain 171.
* The substrates used were as follows: [^{3}H]testosterone for 5α-reductase, [^{14}C]3-hydroxy-3-methylglutaryl CoA for 3-hydroxy-3-methylglutaryl CoA reductase, and [^{3}H]androstenedione for 17β-hydroxysteroid dehydrogenase.

The capacity of NADPH to stabilize the normal and Los Angeles enzymes against denaturation was next examined (Fig. 5). In the absence of NADPH, both the normal and mutant enzymes were rapidly inactivated at 45°C so that by the end of 20 min <5% of the starting activity remained. Either 1 or 3 mM NADPH stabilized the normal enzyme so that 80% of starting activity remained after 20 min. Neither concentration of NADPH significantly protected the Los Angeles 1 enzyme against heat denaturation. Similar results were obtained with the other Los Angeles mutant, whereas the temperature stability of the Dallas mutant was similar to that of the controls (data not shown).

The stability of the 5α-reductase in intact monolayers was then examined (Fig. 6). After fibroblasts approach confluency there is little change in the activity of 5α-reductase over the ensuing 3 days. When 1 mM cycloheximide was added after 7 days of culture (time 0) there was no appreciable effect on the enzyme activity in cells from controls and Dallas and Dominican Republic patients. In contrast, activity in the Los Angeles cells fell within 6 h to approximately one-third of that in the noncycloheximide-treated cells and to 10% by 25 h after adding the cycloheximide.

When intact cells were incubated with cycloheximide overnight and then homogenized and assayed for 5α-reductase, activity again virtually disappeared in the Los Angeles mutants (Table IV), indicating that the loss of activity after the addition of cycloheximide was not due to a loss of NADPH.

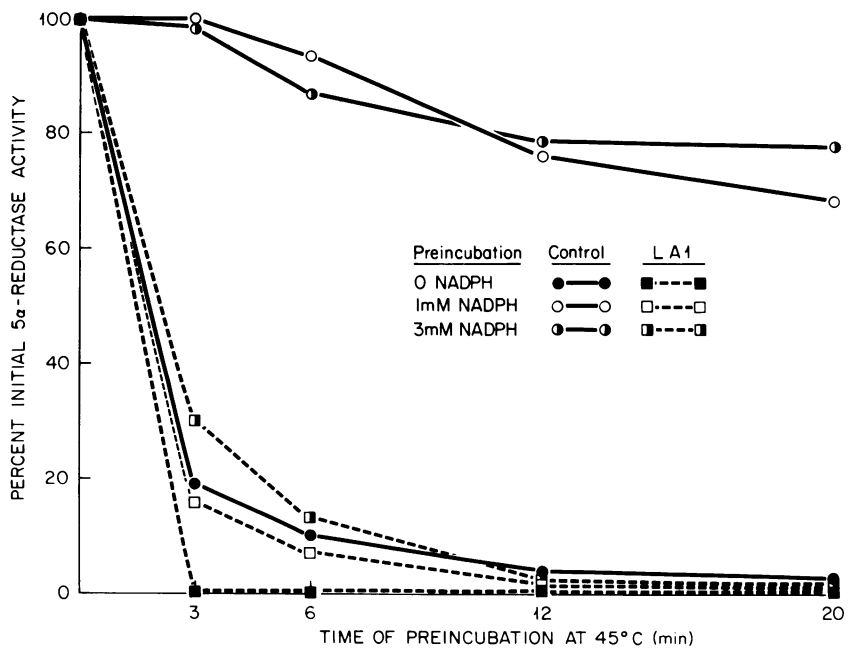

FIGURE 5 Effect of NADPH on the denaturation of 5α-reductase at 45°C. Cells were grown as described in the text and homogenized on day 7. The homogenate was divided into three portions and added to equal volumes of 10 mM Tris, pH 7.4, containing 0, 2, or 6 mM NADPH. The mixtures were then incubated at 45°C, and aliquots were removed at varying times and assayed for 5α-reductase at 25°C for 1 h as described in the text. The control strain was 188. LA, Los Angeles.

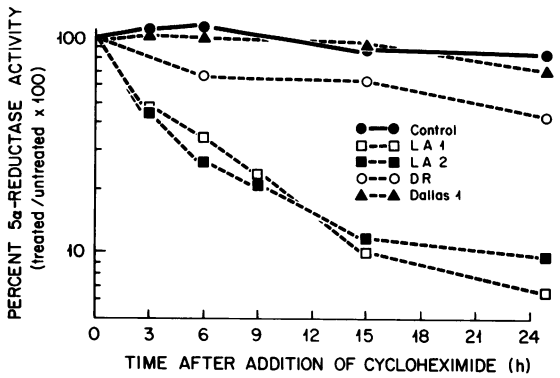

FIGURE 6 Stability of 5α-reductase in intact cells. Cells were grown in small Linbro plates as described in the text. On day 7 cycloheximide was added to half the wells of each cell strain to make the final concentration 1 mM. 5α-Reductase activity was assayed using the whole cell at 0, 3, 6, 9, 15, and 25 h after the addition of cycloheximide. The control strain was 188. LA, Los Angeles; DR, Dominican Republic.

DISCUSSION

5α-Reductase activity has previously been studied in fibroblasts from the two affected male pseudohermaphrodites of the Dallas family (9–11). Enzyme activity was shown to be low both in intact monolayers and in homogenates at pH 5.5, whereas activity in homogenates was normal in the more alkaline range (9–11). We have now performed comparative studies of the enzyme in these two individuals and three patients from two additional pedigrees in which the diagnosis of 5α-reductase deficiency has been substantiated. The disorder in one of these families (Dominican Republic) appears to be similar to that previously characterized in the Dallas family (although not quite so severe), namely low activity at pH 5.5 but normal activity at pH 9.0 with a markedly elevated apparent K_m for testosterone as compared to controls. We conclude that the mutation in these two families probably results in an altered enzyme with reduced affinity for testosterone.

TABLE IV
Effect of Cycloheximide on 5α-Reductase Activity

	5α-Reductase		
	Without cycloheximide		With cycloheximide
Cell strain	Time 0	22 h	22 h
	pmol/h/mg protein		
Control	9.3	13.7	10.7
Los Angeles 1	5.5	3.7	0.1
Los Angeles 2	2.5	2.6	0.1
Dallas 1	0.1	0.1	0.1
Dominican Republic	0.1	0.2	0.1

Cells were grown as described in the text. On day 7 one-third of the dishes were harvested, homogenized, and assayed for 5α-reductase at pH 5.5 as described. Cycloheximide was added to half of the remaining dishes of each cell strain at a final concentration of 1 mM, and after 22 h of additional incubation, all cells were harvested, homogenized, and assayed for 5α-reductase. The control strain was 178.

The disorder in the third family (Los Angeles) is different. 5α-Reductase activity is normal both in intact cells from these patients and in homogenates at acid and alkaline pH. Moreover, the apparent K_m for testosterone is similar to controls, whereas the apparent K_m for NADPH is elevated some 40-fold. The altered K_m is accompanied by an increased instability of the enzyme as indicated by (*a*) a rapid disappearance of activity after inhibition of protein synthesis with cycloheximide and (*b*) lack of protection by NADPH against heat denaturation (19).

The mechanism by which the altered enzyme causes the pseudohermaphroditism is unknown. Since NADPH levels in most tissues appear to be in the range of 500 μM or less (20), it is possible that failure of production of dihydrotestosterone (for example in embryonic tissues) might be due to insufficient cofactor to satisfy binding requirements of the mutant enzyme. If this were the sole explanation for the defective virilization, however, it would not explain the fact that activity was undetectable in homogenates of epididymis removed at surgery from one of the Los Angeles patients (14). It is possible that if other tissues had been examined, more normal activity would have been found, but the fact that the activity in an androgen target tissue such as epididymis was undetectable raises the possibility that instability of the enzyme might result in low activity in the steady state in some tissues. The factors that regulate 5α-reductase activity in different tissues are poorly understood; if however low activity in vivo can result from an unstable enzyme the implication is that the rate of synthesis cannot be regulated to compensate for the rapid turnover. A precedent for such a defect exists in the Sardinian variant of glucose-6-phosphate dehydrogenase deficiency in which the mutant enzyme has diminished affinity for NADP and increased heat lability (21). In affected individuals the enzyme activity is absent in erythrocytes and detectable but low in leukocytes. A similar relationship could exist between the 5α-reductase of epididymis and that of other tissues (including the cultured fibroblast).

Thus, we propose that low 5α-reductase activity in the Dallas and Dominican Republic families results from the synthesis of an abnormal enzyme with poor affinity for steroid substrate. In the Los Angeles family, low activity in tissues in the steady state might result because the enzyme is inherently unstable.

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