Microsomal Cytochrome P-450 from Neonatal Pig Testis: Two Enzymatic Activities (17α-Hydroxylase and $C_{17,20}$-Lyase) Associated with One Protein†

Shizuo Nakajin, John E. Shively, Pau-Miau Yuan, and Peter F. Hall*

ABSTRACT: Studies have been performed to test the hypothesis that cytochrome P-450 from testicular microsomes consists of a single protein with two enzymatic activities (17α-hydroxylase and $C_{17,20}$-lyase). Three lines of evidence to support the hypothesis were obtained. (1) The enzyme appears to be homogeneous by immunochemical criteria with anti-P-450 IgG (line of identity on immunodiffusion and a single band on immunoelectrophoresis), by demonstration of a single NH_2-terminal amino acid (methionine) and the finding of 16 single amino acids at the NH_2 terminus. (2) Optima for pH and temperature are the same for both enzymatic activities (pH 7.25 and 37 °C), and temperatures between 30 and 44 °C decreased both activities in such a way that the ratio of hydroxylase to lyase was the same at all temperatures tested. (3) A variety of inhibitors affect both activities to the same extent: K_i values for two competitive inhibitors (SU 8000, 0.04 μM; SU 10603, 0.3 μM) are the same for hydroxylase and lyase; partition coefficients for inhibition by carbon monoxide are similar for hydroxylase and lyase (20 ± 2 and 27 ± 3); anti-P-450 (serum and IgG) causes inhibition of both activities to the same extent, and the same is true of a variety of less specific inhibitors. It is concluded that a single heme protein (cytochrome P-450) from microsomes of neonatal pig testis catalyzes two reactions (hydroxylase and lyase) which are sequential steps in the synthesis of androgens by the testis leading to conversion of C_{21} precursors to C_{19} steroid hormones.

We have recently purified a microsomal cytochrome P-450 from neontal pig testis (Nakajin & Hall, 1981). The enzyme catalyzes two distinct reactions involved in the conversion of C_{21} steroids to C_{19} androgens, namely, 17α-hydroxylation and $C_{17,20}$-lyase activity. The enzyme is highly purified, as judged by electrophoresis on polyacrylamide gels with sodium dodecyl sulfate and by immunochemical criteria (Nakajin & Hall, 1981). The two activities copurify through the procedure. These findings suggest that one protein is capable of catalyzing these two distinct reactions. To understand the organization and regulation of the microsomal steps in androgen biosynthesis, it is essential that the nature and number of enzymes involved be determined. We have applied a number of rigorous criteria to test the hypothesis that a single protein is responsible for both the hydroxylase and lyase activities. It will be shown that these criteria support the hypothesis of one enzyme with two activities.

Experimental Procedures

General. The preparation of highly purified testicular microsomal P-450, assays for 17α-hydroxylase and $C_{17,20}$-lyase, and determination of P-450 content are described in a previous paper (Nakajin & Hall, 1981). Methods for studying inhibition of enzyme activity by carbon monoxide have been reported (Hall et al., 1975). Kinetic parameters were measured on three different enzyme preparations under conditions in which standard errors for V_{max} were less than 10% of the value for this constant and those for K_i were less than 12%. These conditions permit calculation of the statistic t as described by Cleland (Cleland, 1963, 1967).

Immunochemical Methods. Preparation of antibodies (Watanuki et al., 1978a,b; Lewis et al., 1977), preparation of IgG from antiserum (Williams & Chase, 1968), immunoelectrophoresis (Weir, 1967), iodination of P-450 (Thorell & Larsson, 1974), and radioimmunoassay (Lewis et al., 1972) were all performed by standard procedures which have been specified elsewhere (Watanuki et al., 1978).

Amino Acid Composition and NH_2-Terminal Sequence. (*i*) *Amino Acid Analysis.* Amino acid compositions were obtained after hydrolysis of proteins (5–20 μg, in duplicate) in 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu & Chang, 1971), for 12, 24, and 48 h in sealed, evacuated tubes at 110 °C. Values for serine and threonine were derived by extrapolation to zero time; values for valine, leucine, and isoleucine reached their maximum levels after hydrolysis for 48 h. Carboxymethylcysteine values were obtained after reduction and alkylation by the method of Hirs (Hirs, 1967). Amino acid and hexosamine analyses were performed on a two-column Beckman 121MB analyzer according to Del Valle & Shively (1979).

(*ii*) *NH_2-Terminal Sequence.* One to four nanomoles of protein was subjected to automated Edman degradations on an updated, modified Beckman 890C sequencer. The instrument is similar to those described by Wittman-Liebold (1973) and Hunkapiller & Hood (1978). Cytochrome P-450 was sequenced untreated or treated with 4-sulfophenyl isothiocyanate (SPITC).[1] Treatment with SPITC was performed in the spinning cup as follows: the sample was dissolved in 0.1% $NaDodSO_4$, dried in the spinning cup, treated with 1 equiv of SPITC per amino group followed by one cycle of Edman degradation starting with delivery of Quadrol and omitting deliveries of PITC and HFBA, and then subjected to regular automated Edman degradation cycles. All samples are retained in the spinning cup by the addition of 6 mg of

† From the Department of Physiology, University of California, Irvine, California 92717 (S.N. and P.F.H.), and City of Hope Research Institute, Duarte, California 91010 (J.E.S. and P.-M.Y.). *Received November 26, 1980.* This investigation was supported by Grant AM-15621 awarded by the National Institute of Arthritis and Metabolism. The amino acid analysis and NH_2-terminal sequencing were supported by Grant 16434 from the National Cancer Institute.

* Address correspondence to this author at The Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545.

[1] Abbreviations used: androstenedione, androst-4-ene-3,17-dione; pregnenolone, 3β-hydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione; $NaDodSO_4$, sodium dodecyl sulfate; SPITC, 4-sulfophenyl isothiocyanate; PITC, phenyl isothiocyanate; HFBA, heptafluorobutyric acid; PTH, phenylthiohydantoin; SU 8000, 3-(6-chloro-3-methyl-2-indenyl)pyridine; SU 10603, γ-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2*H*)-naphthalenone.

0006-2960/81/0420-4037$01.25/0

Table I: Amino Acid Compositions of Cytochromes P-450 from Pig Testes and Rabbit Liver[a]

amino acid	no. of residues/molecule: pig testes	rabbit liver (LM_4)
Asx	47	44
Thr	26	24
Ser	30	31
Glx	53	46
Pro	43	28
Gly	38	38
Ala	38	29
Val	33	32
Met	13	10
Ile	25	22
Leu	62	58
Tyr	14	11
Phe	29	28
Lys	32	26
His	12	11
Arg	30	30
Trp	4	7
Cys[b]	6	6
total	535	482
GlcNAc[c]	2-3	
amide[d]	40	
M_r	59 000	54 200

[a] Data for pig testes (this work) and rabbit liver LM_4 (Haugen & Coon, 1976). [b] Determined as carboxymethylcysteine derivative. [c] Calculated from a maximum value at 48-h hydrolysis. [d] Calculated from average yield of ammonia after correcting for hydrolysis blank.

Table II: Sequencer Analysis of Pig Testes Cytochrome P-450[a]

cycle	amino acid	pmol recovered: SPITC treated	untreated
1	Met	1180	510
2	Trp	670	
3	Val	1270	196
4	Leu	750	101
5	Leu	1230	188
6	Val	1240	141
7	Phe	640	193
8	Phe	690	196
9	Leu	1330	202
10	Leu	1330	133
11	(Ser)	500	
12	Leu	1260	215
13	Thr	750	
14	Tyr	390	
15	Leu	1150	
16	Phe	610	
17	(Lys)	300	
18	(Pro)	550	
19	(?)		
20	(Thr)	350	

[a] The SPITC-treated sample (4.5 nmol) gave an absolute yield of 29% valine at cycle 3, and the untreated sample (1.2 nmol) a yield of 42% at cycle 1. Microsequencing was performed as described by Hunkapiller & Hood (1978).

polybrene which is precycled together with 100 nmol of glycylglycine for four cycles of Edman degradations (Hunkapiller & Hood, 1978). Anilinothiazolinone derivatives of amino acids were automatically converted to their PTH derivatives (Wittman-Liebold et al., 1976), which were separated and identified by high-performance liquid chromatography on a Du Pont Zorbax ODS column with gradient elution with acetonitrile (Hunkapiller & Hood, 1978). Peaks were integrated with a Spectra Physics 4000 integrator which also controlled the gradient elution program of the Waters Associates instrument. Solvents and reagents were purified according to Wittman-Liebold (1973).

Chemicals. Cylinders of various gas mixtures (CO and O_2 with N_2 to 100%) were purchased from Liquid Carbonic, Los Angeles. The composition of each mixture was determined by gas chromatography (Hackney, 1967); values for each cylinder and each gas were found to be within 2% of the value given by the supplier. The two inhibitors of the SU series (SU 8000 and SU 10603) were generously provided by Dr. C. Brownley, Jr., of CIBA Pharmaceutical Co. The sources of other chemicals have been given previously (Nakajin & Hall, 1981; Watanuki et al., 1978a,b; Shikita & Hall, 1974).

Results

Amino Acid Composition and NH_2-Terminal Sequence Analysis. Table I shows the amino acid composition of the testicular microsomal P-450 that forms the subject of this study. The composition is based upon results of hydrolysis at three times (12, 24, and 48 h) and quantitation of cysteine as the carboxymethyl derivative. Amide content is based upon the ammonia peak corrected for blank values and shows 40 mol of glutamine plus asparagine out of 100 mol of acids plus amides (Asx + Glx). The testicular P-450 contains similar amounts of cysteine and tryptophan as the liver cytochromes P-450 already reported [Table I, Haugen & Coon (1976), and Botelho et al. (1979)]. The data confirm the finding that this enzyme is a glycoprotein (Nakajin & Hall, 1981), since amino acid analysis reveals three glucosamine residues per molecule. The recovery during amino acid analysis was virtually 100% of the weighed sample. Table I also shows considerable similarity in amino acid composition between the testicular enzyme and one form from rabbit liver called LM_4 (Haugen & Coon, 1976). This similarity extends to P-450 from rat liver (Botelho et al., 1979) and bacterial cytochrome P-450 (Tsai et al., 1971). Moreover, the mammalian and bacterial proteins show almost identical percentages of hydrophobic amino acids (44–46%). These proteins show greatest variation in contents of alanine, leucine, and phenylalanine.

The results of NH_2-terminal sequencing of cytochrome P-450 from porcine testes are shown in Table II. The yield of the single NH_2-terminal amino acid (methionine) was 42% (0.5 nmol from 1.2 nmol sequenced), and since the average yield of NH_2-terminal methionine as the PTH derivative in this laboratory is 70%, a corrected yield of 60% is obtained. Although an excellent yield of the NH_2-terminal amino acid is obtained, the yield of amino acids at subsequent cycles is greatly reduced. A similar problem with other cytochromes P-450 was reported by Botelho et al. (1979) and is probably due to the high solubility of membrane proteins in organic solvents. This problem was overcome by Botelho et al. (1979) and by us by treatment of the cytochrome P-450 with SPITC. This reagent forms derivatives of amino groups with a sulfonic acid function and thus renders the protein less soluble in organic solvents. One complication of this method is a slight decrease in absolute yields of PTH amino acids. In spite of this drawback, an overall increase in yield of all subsequent amino acids is observed, thus allowing one to obtain an extended amino acid sequence (Table II). In no case was evidence for the presence of a second amino acid observed. These results strongly support the existence of a single protein species of high purity in this cytochrome P-450 preparation. A comparison of the sequence of pig testicular cytochrome P-450 with other cytochromes P-450 is shown in Figure 1. Although a number of the species sequenced to date have NH_2-terminal methionine, the overall degree of homology is low. One

Cytochrome	1 5 10 15
Pig Testes P-450	Met-Trp-Val-Leu-Leu-Val-Phe-Phe-Leu-Leu-(Ser)-Leu-Thr-Tyr-Leu-Phe
Rat Liver P-450$_a$	Met-Leu-Asp-Thr-Gly-Leu-Leu-Leu-Val-Val- Ile -Leu-Ala-Thr-Leu-Thr
Rat Liver P-450$_b$	Glu-Pro-Thr-Ile-Leu-Leu-Leu-Leu-Ala-Leu- Leu -Val-Gly-Phe-Leu-Leu
Rat Liver P450$_c$	Ile-Thr-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Glu- Thr -Ala-Ser-Glu-Leu-Leu
Rabbit Liver P-450LM$_2$	Met-Glu-Phe-Ser-Leu-Leu-Leu-Leu-Leu-Ala- Phe -Leu-Ala-Gly-Leu-Leu

FIGURE 1: Comparison of the NH_2-terminal sequences of cytochromes P-450 from pig testes (this work), rat liver (Botelho et al., 1979), and rabbit liver (Haugen et al., 1977).

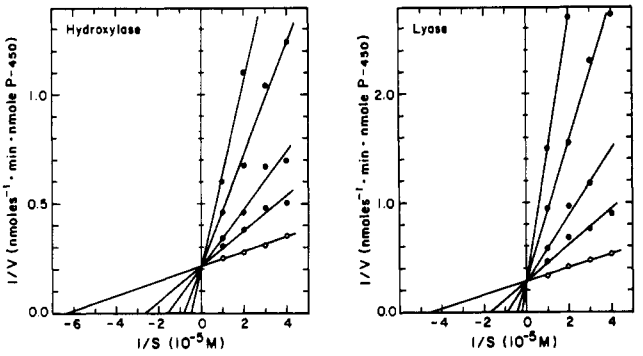

FIGURE 2: Kinetic studies of testicular microsomal P-450. (Left) 17α-Hydroxylation of progesterone without and with various concentrations of SU 8000. (Right) Lyase activity with 17α-hydroxyprogesterone as substrate without and with SU 8000. Enzyme activities were measured under standard conditions as reported in detail elsewhere (Nakajin & Hall, 1981). When inhibitor was used, it was the last addition (in 20 μL of ethanol) before preincubation. The reaction was started by addition of NADPH (Nakajin & Hall, 1981). The concentrations of inhibitor used were 5×10^{-8} M, 1×10^{-7} M, 2.5×10^{-7} M, and 5×10^{-7} M for both activities.

striking feature is the high degree of hydrophobicity observed in all species sequenced.

Inhibition of 17α-Hydroxylase and Lyase Activities by SU 8000 and SU 10603. Figure 2 shows double-reciprocal plots of the 17α-hydroxylase and $C_{17,20}$-lyase activities of testicular microsomal P-450 without and with various concentrations of SU 8000. It can be seen that inhibition of both activities appears to be competitive. Statistical evaluation of the data in Figure 2 and of similar studies performed with two different preparations of the enzyme showed that V_{max} for each enzyme activity is not significantly different for each of a family of curves with different concentrations of inhibitor, so that the effect of SU 8000 disappears at infinite substrate concentrations. It can, therefore, be concluded that inhibition is competitive. Moreover, plots for various concentrations of SU 8000 showing slope as a function of inhibitor concentration gave straight lines for both activities. Finally, the K_i values with SU 8000 (0.04 and 0.03 μM, respectively) are not significantly different for the two activities, hydroxylase and lyase ($p > 0.7$). Similar studies were performed with the related inhibitor SU 10603, with qualitatively similar results (data not shown); values for K_i were 0.3 and 0.4 μM. Again, statistical evaluation showed that this difference is not significant ($p > 0.7$).

Inhibition of 17α-Hydroxylase and Lyase Activities by Carbon Monoxide. As reported for microsomal preparations (Betz et al., 1976), carbon monoxide inhibits both enzymatic activities of testicular microsomal P-450, and Table III shows that the partition coefficient (k), i.e., the ratio of $CO:O_2$ required to produce 50% inhibition, is similar for the two activities; the differences seen are less than those observed between other cytochromes P-450 (Conney et al., 1968) and presumably could represent responses by two active sites on one protein.

Activities of Hydroxylase and Lyase: Optima for pH and Temperature. It was observed that optima for pH and temperature are the same for the two activities, namely, 7.25 for pH and 37 °C for temperature (data not shown). Figure 3 shows the influence of heat on the two enzyme activities when the enzyme was kept at various temperatures for 5 min before assay of enzyme activities. It is seen that the two activities decline as a function of temperature in a parallel fashion. When the values shown were transformed to log/log plots, linear relationships were observed for each activity as a function of temperature. The two regression lines showed the same slope within the limits of $p > 0.7$. Evidently the ratio hydroxylase/lyase remains constant as both enzyme activities decline on heating.

Table III: Inhibition of Testicular Microsomal P-450 by Carbon Monoxide

gas phase[a]		17α-hydroxylase		$C_{17,20}$-lyase	
CO	O_2	(nmol/min)/ nmol of P-450	K^b	(nmol/min)/ nmol of P-450	K^b
0	21	4.17		2.80	
0	4.08	3.41		1.81	
4.1	4.02	3.38		1.91	
39.9	3.95	2.28	20.5	1.33	27.3
80.8	3.74	1.61	19.2	1.00	26.4

[a] Gas phase included N_2 to 100% in each case. [b] K, partition coefficient; i.e., $CO:O_2$ at 50% inhibition of enzyme activity. The relevant methods are given under Experimental Procedures except that the amount of P-450 used was 70 pmol with 0.66 unit of reductase, substrate was present at a concentration of 10 μM, and incubation was for 10 min.

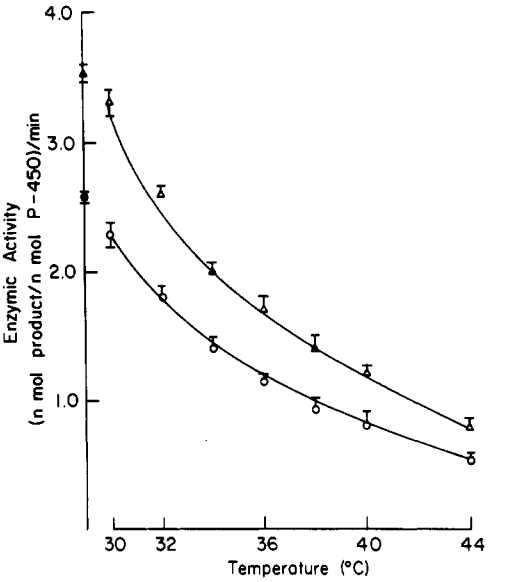

FIGURE 3: Influence of temperature on the two enzymatic activities of testicular microsomal P-450. Samples of purified testicular P-450 were heated for 5 min at the temperatures shown before being subjected to the standard assay procedure (Nakajin & Hall, 1980). Values shown are means and ranges of triplicate determinations. (Triangles) 17α-Hydroxylase; (circles) lyase; (solid symbols) enzyme at 0 °C before incubation.

Actions of Various Agents on 17α-Hydroxylase and $C_{17,20}$-Lyase Activities of Testicular P-450. The relative effects of a variety of agents on the two enzymatic activities of the P-450 from testicular microsomes were examined (acetone, methanol, temperature of incubation, etc.). Although the extent of inhibition varied considerably from agent to agent, the effect for each agent on the two enzymatic activities was qualitatively the same and quantitatively remarkably similar in all cases (not shown).

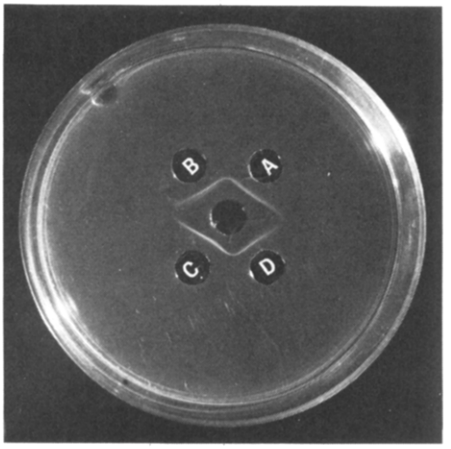

FIGURE 4: Double diffusion in agarose gels of anti-P-450 IgG and crude P-450 (cholate extract) (Nakajin & Hall, 1980). The center well contained IgG (30 μL) and the surrounding wells contained the following: (A and C) P-450 highly purified (2 μg of protein); (B and D) cholate extract (150 μg of protein).

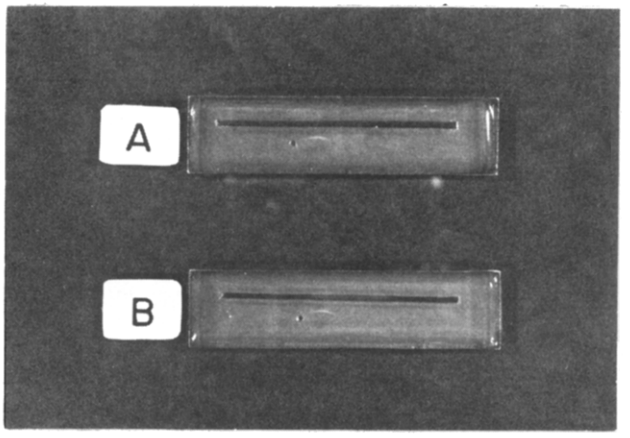

FIGURE 5: Immunoelectrophoresis of testicular microsomal P-450. The well contained a sample of P-450 (13 μg of protein), and the troughs contained anti-P-450 antiserum (20 μL) (A) and anti-P-450 IgG (24.3 mg/mL) (B). The sample was prepared by removing Emulgen and dialyzing against phosphate buffer containing glycerol (40% w/v) as described elsewhere (Nakajin & Hall, 1980). The anode was to the right of the figures. Electrophoresis was performed on agarose (1% w/v) coated slides at 7 mA per plate for 7 h at 4 °C. Following electrophoresis, diffusion was allowed to occur at room temperature overnight.

Reaction of Testicular Microsomal P-450 with Antibodies. Figure 4 shows the result of double diffusion in agarose gels between IgG prepared from anti-P-450 serum and cholate extract on the one hand and purified enzyme on the other hand. The gel shows a single band formed between both antigens and the antibody. Moreover, the bands for cholate extract and pure enzyme touch without overlap—the so-called line of identity. The cholate extract is a crude precursor of the purified enzyme (Nakajin & Hall, 1981). Figure 5 shows a single band on immunoelectrophoresis between enzyme and the same two preparations of antibody (antiserum and IgG prepared from antiserum). A sensitive radioimmunoassay has been developed for this P-450 with the IgG (not shown).

Effect of Anti-P-450 on Enzyme Activity. It can be seen from Figure 6 that IgG prepared from the anti-P-450 antiserum (rabbit) inhibits both enzymatic activities of the testicular P-450, in contrast to preimmune IgG, which was without demonstrable effect. Moreover, in each case, 50% inhibition of both activities is seen at the same concentration of anti-P-450, and the general shapes of the inhibition curves are virtually identical for the two reactions. Three experiments

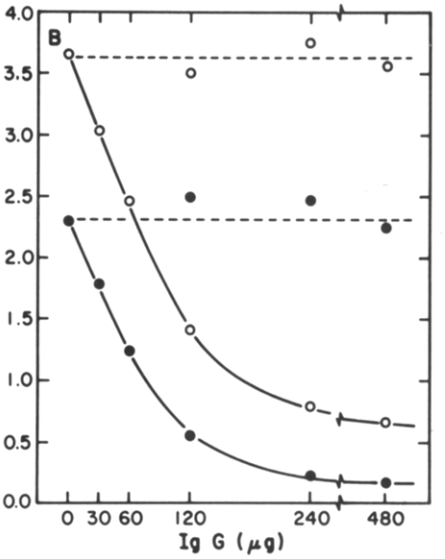

FIGURE 6: Effect of anti-P-450 IgG on the enzymatic activities of testicular microsomal P-450. Enzyme activities were measured under standard conditions except that the last addition prior to incubation was the IgG. Final volume of incubation was 2.0 mL. (○) Hydroxylase; (●) lyase; (---) control (preimmune IgG); (—) antibody (IgG).

like that shown in Figure 6 were performed, and mean values for the two enzyme activities were transformed to log/log plots. The transformed values were linear with respect to concentration of antibody. The two regression lines showed the same slope ($p > 0.6$). Evidently, the ratio hydroxylase/lyase remains constant in spite of progressive inhibition of both activities by antibody. In three separate determinations using different batches of enzyme, the concentrations of IgG required to produce 50% inhibition were for hydroxylase 41.2 ± 8 and for lyase 35.6 ± 9 μg/mL of medium. Similar results were observed with unpurified antiserum (not shown).

Discussion

It is well-known that two proteins can copurify to give the false appearance of one protein with two enzymatic activities. This is especially likely as the result of hydrophobic interactions between membrane-bound proteins. When it has been determined that one protein is capable of catalyzing two distinct reactions, the question arises as to whether there is one active site or two; presumably the greater the difference between the nature of the two reactions catalyzed, the greater the likelihood of two different active sites—other things being equal.

Apart from the obvious need to decide between these various alternatives in any enzyme, the present case involves several considerations that emphasize the importance of reaching such a decision. In the first place, a single active site would presumably not readily release the intermediate, i.e., the product of 17α-hydroxylation, before it serves as substrate for the lyase. This will reduce the number of alternative pathways between pregnenolone and testosterone and will, in turn, be important in considering the relative preponderance of the Δ^4 or Δ^5 pathway (Hall, 1970; Eik-Nes & Kekre, 1963; Tamaoki & Shikita, 1966; Chubb & Ewing, 1979). Similarly, the number of enzymes will be important in considering the organization of the steroidogenic microsomal vesicle; for example, the preferred pathway from pregnenolone to testosterone may be determined by the arrangement of the enzymes within the microsomal vesicle. In the second place, it appears that down-regulation of LH (decreased response to a second injection) is at least partly attributable to inhibition of $C_{17,20}$-lyase activity (Ciggoraga et al., 1979). The mechanism of this

interesting phenomenon is not understood, but the means by which the gonadotrophin can inhibit lyase without inhibiting 17α-hydroxylase is of considerable importance. In the third place, the genetic control of steroidogenesis must be capable of explaining extensive 17α-hydroxylation with little lyase activity in the adrenal cortex which, in some species, produces 17α-hydroxy-C_{21} steroids (e.g., cortisol) (Samuels, 1960).

Three lines of evidence point to a single protein catalyzing 17α-hydroxylation and $C_{17,20}$-lyase activity: evidence of homogeneity, similarities in certain characteristics of the two activities, and similar effects of inhibitors on the hydroxylase and lyase activities. The case for homogeneity rests upon electrophoresis in polyacrylamide gels (Nakajin & Hall, 1981), immunochemical evidence (including immunoelectrophoresis) (Figures 4 and 5), the finding of a single NH_2-terminal amino acid (methionine), and the amino acid sequence from the NH_2 terminus.

A different approach to the question of one enzyme or two is provided by examining the effect of temperature of the two enzymatic activities. The fact that the decline in both activities was similar so that the ratio hydroxylase/lyase remains unchanged (Figure 3) argues strongly for a temperature-dependent change in a single protein molecule. A variety of inhibitors of the enzymatic activities provides a third approach to this question. K_i values for SU 8000 and those for SU 10603 are the same for the two enzymatic activities (see Results). A very different form of inhibition of the activities, namely, the use of antibody, showed that anti-P-450 IgG fails to distinguish the two enzymatic activities (Figure 6). A variety of inhibitory influences also affect both enzymatic activities to about the same extent (Results). It seems clear that one cytochrome P-450 catalyzes both 17α-hydroxylation and $C_{17,20}$-lyase activity.

These findings differ from an earlier report by Betz and co-workers (Betz et al., 1976), who concluded from studies based on rat testicular microsomes that 17α-hydroxylation and cleavage of the C_{17}–C_{20} bond were catalyzed by two different enzymes. These workers observed differences in the inhibition by carbon monoxide and that by metyrapone on the two activities. The reasons for the difference between these findings and those reported here are not clear at present. Apart from possible differences between rat and pig, problems associated with the use of microsomes may be important. A number of heme proteins are present in these organelles which might combine with carbon monoxide. Moreover, even with the purified enzyme, some difference in the partition coefficients for carbon monoxide was observed (Table III). In any case, this approach cannot distinguish between one enzyme with two active sites and two separate enzymes. Again, the work of Samuels and colleagues in rat testicular microsomes has revealed unexpected complexities in the partition of lipophilic substances between the cytoplasm and the microsomal enzymes in question (Matsumoto & Samuels, 1969). In this connection, it is interesting to notice the similarities in the behavior of the two enzyme activities (hydroxylase and lyase) in microsomes from chicken testis reported by Purvis and co-workers (Purvis et al., 1973). The results of these authors would certainly be compatible with a single enzyme catalyzing the two reactions.

The present studies do not distinguish between one active site or two. However, the heme content of the enzyme is less than one heme group per protein subunit. Yet, the values are like those initially reported for hepatic P-450 (Levin et al, 1974) and would be reasonable for one heme group (and hence one active site) per protein subunit—especially since heme is lost during purification (Nakajin & Hall, 1981); two sites would require twice as much heme if both reactions utilize heme. Against the idea of one active site are observations by Samuels and co-workers, who reported some exchange between exogenous 17α-hydroxyprogesterone and the enzyme-bound intermediate generated by the enzyme from progesterone (Samuels & Matsumoto, 1974). This finding suggested the movement of an intermediate from one active site (17α-hydroxylase) to another (lyase). Moreover, the two reactions appear so different in nature as to suggest the need for two sites. The decision between these alternatives requires further investigation.

The main conclusion from these studies, namely, that one cytochrome P-450 catalyzes two reactions, brings this enzyme into line with the two mitochondrial cytochromes P-450, namely, side-chain cleavage, which catalyzes three different reactions (Takagi et al., 1975), and 11β-18-hydroxylase (Watanuki et al., 1978a,b), which catalyzes two reactions. In none of these enzymes has the number of active sites been rigorously determined. The first step in this direction for the testicular microsomal enzyme will be to determine whether heme is necessary for the lyase activity as in the case of side-chain cleavage. Studies to this end are at present being performed in this laboratory.

Acknowledgments

We acknowledge the expert technical assistance of Russell Blacher for amino acid analysis and Ursino Del Valle and Mark Levy and David Hawke for NH_2-terminal sequencing.

References

Betz, G., Tsai, P., & Weakley, P. (1976) *J. Biol. Chem. 251*, 2839.

Botelho, L. H., Ryan, D. E., & Levin, W. (1979) *J. Biol. Chem. 254*, 5635.

Chubb, C., & Ewing, L. L. (1979) *Am. J. Physiol. 237*, E247.

Ciggoraga, S. B., Dufau, M. L., & Catt, K. J. (1979) *J. Biol. Chem. 253*, 4297.

Cleland, W. W. (1963) *Nature (London) 168*, 463.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1.

Conney, A. H., Levin, W., Ikeda, M., Kuntzman, R., Cooper, D. Y., Rosenthal, O. (1968) *J. Biol. Chem. 243*, 3912.

Del Valle, U., & Shively, J. E. (1979) *Anal. Biochem. 96*, 77.

Duque, C., Morisaki, I., Nobuo, I., & Shikita, M. (1978) *Biochem. Biophys. Res. Commun. 82*, 179.

Eik-Nes, K. B., & Kekre, M. (1963) *Biochim. Biophys. Acta 78*, 449.

Hackney, J. D. (1967) *Aspen Emphysema Conf. 10*, 413.

Hall, P. F. (1970) in *The Testis* (Johnson, A. D., Gomes, W. R., & Van Denmark, V. L., Eds.) Vol. II, pp 1–17, Academic Press, New York.

Hall, P. F., Lee Lewes, J., & Lipson, E. D. (1975) *J. Biol. Chem. 250*, 2283.

Haugen, D. A., & Coon, M. J. (1976) *J. Biol. Chem. 251*, 7929.

Haugen, D. A., Armes, L. G., Yasinobu, K. T., & Coon, M. J. (1977) *Biochem. Biophys. Res. Commun. 77*, 967.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 197.

Hunkapiller, M. W., & Hood, L. E. (1978) *Biochemistry 17*, 2124.

Levin, W., Ryan, D., West, S., & Lu, A. Y. H. (1974) *J. Biol. Chem. 240*, 1747.

Lewis, J. E., Nelson, J. C., & Elder, H. A. (1972) *Nature (London), New Biol. 239*, 214.

Lewis, J. E., Carmack, C. E., Yamamoto, R., & Granger, G. A. (1977) *J. Immunol. Methods 14*, 163.

Liu, T.-Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842.
Matsumoto, K., & Samuels, L. T. (1969) *Endocrinology 85*, 402.
Nakajin, S., & Hall, P. F. (1981) *J. Biol. Chem.* (in press).
Purvis, J. L., Canick, J. A., Latif, S. A., Rosenbaum, J. H., Hologgitas, J., & Menard, R. H. (1973) *Arch. Biochem. Biophys. 159*, 39.
Samuels, L. T. (1960) *Metabolism of Steroid Hormones in Metabolic Pathways* (Greenberg, D. M., Ed.) Vol. 1, Chapter 11, p 431, Academic Press, New York.
Samuels, L. T., & Matsumoto, K. (1974) *Endocrinology 9*, 55.
Shikita, M., & Hall, P. F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1441.
Takagi, Y., Shikita, M., & Hall, P. F. (1975) *J. Biol. Chem. 250*, 8445.
Tamaoki, B., & Shikita, M. (1966) *Eur. J. Steroids 1*, 351.
Thorell, J. I., & Larsson, I. (1974) *Immunochemistry 11*, 203.
Tsai, R. L., Gunsalus, I. C., & Duk, K. (1971) *Biochem. Biophys. Res. Commun. 45*, 1300.
Watanuki, M., Granger, G. A., & Hall, P. F. (1978a) *J. Biol. Chem. 253*, 2927.
Watanuki, M., Tilley, B. E., & Hall, P. F. (1978b) *Biochemistry 17*, 127.
Weir, D. M. (1967) *Handbook of Experimental Immunology*, p 655, Blackwell Scientific Publications, Oxford.
Williams, C. A., & Chase, M. W. (1968) *Methods in Immunology*, p 154, Academic Press, New York.
Wittman-Liebold, B. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1415.
Wittman-Liebold, B., Graffunder, H., & Kohls, H. (1976) *Anal. Biochem. 75*, 621.

Changes in the Substrate Specificities of an Enzyme during Directed Evolution of New Functions†

Barry G. Hall

ABSTRACT: Wild-type *ebg* enzyme, the second β-galactosidase of *Escherichia coli* K12, does not permit growth on lactose. As part of a study of the evolution of new enzymatic functions, I have selected, from a *lacZ* deletion strain, a variety of spontaneous mutants that grow on lactose and other β-galactoside sugars. Single point mutations in the structural gene *ebgA* alter the enzyme so that it hydrolyzes lactose or lactulose effectively; two mutations in *ebgA* permit galactosylarabinose hydrolysis, while three mutations are required for lactobionic acid hydrolysis. Wild-type *ebg* enzyme and 16 functional mutant *ebg* enzymes were purified and analyzed kinetically to determine how the substrate specificities had changed during the directed evolution of these new functions. The specificities for the biologically selected substrates generally increased by at least an order of magnitude via increased V_{max} and decreased K_m for the substrate. These changes were very specific for the selected substrate, often being accompanied by decreased specificities for other related substrates. The single, double, or triple substitutions in the enzymes did not detectably alter the thermal stability of *ebg* enzyme.

The *ebg* system of *Escherichia coli* K12 is being used as a model to study the acquisitive evolution of new metabolic functions. Beginning with a strain of *E. coli* K12 bearing a large deletion within the *lacZ* (β-galactosidase) gene, I have selected a series of spontaneous mutant strains that have evolved the ability to utilize lactose and other β-galactoside sugars as sole carbon and energy sources (Hall & Hartl, 1974; Hall, 1976a,b, 1977, 1978; Hall & Zuzel, 1980). The spontaneous mutations occur in the *ebg* (evolved β-galactosidase) operon located at 66 min on the *E. coli* map (Hall & Hartl, 1975; Bachmann & Low, 1980). The operon is under negative control by the product of the tightly linked *ebgR* gene (Hall & Hartl, 1975). Mutations in *ebgR* are important to the evolutionary process (Hall & Clarke, 1977) but are outside the scope of this paper. The structural gene of interest is *ebgA* which specifies a 120 000 molecular weight polypeptide that is the subunit of the homohexameric *ebg* β-galactosidase (Hall, 1976a). The wild-type enzyme is designated *ebg*°, "o" standing for original (Hall, 1976a). *ebg*° enzyme has a very low activity toward lactose, and even constitutive strains, which synthesize nearly 5% of their soluble protein as *ebg*° enzyme, fail to grow on lactose or on the other β-galactoside sugars discussed in this paper (Hall & Hartl, 1975; Hall, 1978).

Spontaneous single point mutations in *ebgA* alter the properties of *ebg* enzyme so that it hydrolyzes lactose well enough to permit growth (Hall, 1977). I have isolated a large series of spontaneous *ebgA* mutants that have evolved the ability to utilize lactose (4-*O*-β-D-galactopyranosyl-D-glucose), lactulose (4-*O*-β-D-galactopyranosyl-D-fructose), galactosylarabinose (3-*O*-β-D-galactopyranosyl-D-arabinose), or lactobionic acid (4-*O*-β-D-galactopyranosyl-D-gluconic acid). The mutant strains have been classified according to the β-galactoside sugars which they can use and according to their growth rates on those sugars (Hall, 1978; Hall & Zuzel, 1980).

Class I mutants carry a single point mutation in region I of the *ebgA* gene (Hall & Zuzel, 1980). They are isolated by selecting for lactose utilization, and they grow rapidly on lactose while failing to utilize lactulose, galactosylarabinose, or lactobionic acid (Hall, 1978). Class II mutants carry a single point mutation in region II of the *ebgA* gene, about one

† From the Microbiology Section U-44, Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268. *Received December 18, 1980.* This work was supported by National Institutes of Health Grant AI14766. B.G.H. is the recipient of Research Career Development Award 1 KO4 AI00366 from the National Institute of Allergy and Infectious Diseases.

0006-2960/81/0420-4042$01.25/0