Cytochrome P-450 for 11β- and 18-Hydroxylase Activities of Bovine Adrenocortical Mitochondria: One Enzyme or Two?†

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ABSTRACT: The 11β- and 18-hydroxylase activities of a highly purified cytochrome P-450 from bovine adrenocortical mitochondria have been examined in detail with a view to determining whether the two activities are shown by a single protein or by two distinct proteins. The purified enzyme shows a single N-terminal residue (glutamic acid) and its amino acid composition is reported. Both enzyme activities decay considerably during storage at 4 °C for 11 days and the rates of decay are similar for the two activities. Metyrapone inhibits both activities competitively (K_i = 1.50 and 1.43 μM for 11β- and 18-hydroxylase, respectively). Carbon monoxide inhibits both activities and the ratio $CO:O_2$ for 50% inhibition is similar for the two activities (K = 1.69 and 1.53). A variety of nonspecific inhibitors produce approximately the same inhibition of both activities. Finally, antiserum produced by rabbits to the purified enzyme on double diffusion in agarose gels gives a single band with the purified enzyme. Increasing concentrations of antiserum added to the assay system produce increasing and proportionate inhibition of both activities. The evidence strongly supports earlier suggestions that the two hydroxylase activities occur in a single protein.

Evidence from a number of laboratories has suggested that a single cytochrome P-450 protein is responsible for both the steroid 11β- and 18-hydroxylase activities observed in adrenocortical mitochondria (Bjorkham and Kalmar, 1975; Rapp and Dahl, 1976). The recent finding from this laboratory that these two activities copurify through a procedure which results in the production of a protein yielding a single band on electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate confirms this idea (Watanuki et al., 1977b). The availability of a highly purified 11β- and 18-hydroxylase enzyme has enabled us to extend these studies by detailed examination of the action of various inhibitors on the two enzyme activities, end-group analysis of the protein, loss of both activities on storage, and the effect of antibodies raised against the purified enzyme. The present paper describes the results of these studies which support the idea that the two enzyme activities reside in a single protein.

Experimental Procedure

The relevant methods used in these investigations have been published elsewhere. The preparation of the 11β- and 18-hydroxylase, evidence for its purity, and methods for measurement of the two hydroxylase activities (Watanuki et al., 1977b), together with methods for the preparation of adrenodoxin (Suhara et al., 1972b) and adrenodoxin reductase (Suhara et al., 1972a), have been described. Similarly, the methods used in preparing and examining mixtures of carbon monoxide, oxygen, and nitrogen (Hall et al., 1975), determination of amino acid content (Tilley et al., 1977), preparation of antibodies (Watanuki et al., 1977b), and the sources of chemicals other than those which follow have been published (Shikita and Hall, 1974). Metyrapone[1] was generously provided by Ciba-Geigy Corporation, Summit, N.J.

Kinetic parameters were determined in triplicate under conditions in which the standard error of V_{max} was less than 12% of the value of this constant and those for K_i were less than 17%; these conditions permit calculation of t as described by Cleland (1963, 1967).

Amino acid composition was determined on three different samples of P-450 as described previously (Tilley et al., 1977). Each determination was performed in duplicate at 24 and 72 h of acid hydrolysis. Cysteine was measured by the method of Ellman (1959) and tryptophan was determined by magnetic circular dichroism (Barth et al., 1972). End-group analysis was performed by dansylation and hydrolysis (Gros and Labouesse, 1969) followed by identification of the dansyl amino acid using two-dimensional thin-layer chromatography on polyamide (Weiner et al., 1972).

Results

Amino Acid Composition and End-Group Analysis. Table I shows the amino acid composition of the adrenal 11β- and 18-hydroxylase P-450. This was determined on three separate preparations; in one case the enzyme was not treated prior to hydrolysis, in a second case the protein was subjected to isoelectric focusing in 6 M urea (Tilley et al., 1977), while the third sample was obtained from 12 sodium dodecyl sulfate gels pooled, homogenized, and extracted in sodium bicarbonate (5 mM) containing 0.05% sodium dodecyl sulfate. This pooled sample (500 μg of protein) was subjected to Folch extraction to remove phospholipid (Watanuki et al., 1977b) prior to electrophoresis in sodium dodecyl sulfate gels. Following these treatments the enzyme was subjected to acid hydrolysis. The values in the table are from the third of the above treatments. However, all three samples agreed to the nearest whole number for each residue. Comparison with amino acid composition of side-chain cleavage P-450 (Tilley et al., 1977) reveals striking differences in aspartate plus asparagine and in the aromatic amino acids phenylalanine and tyrosine, both of which are present in considerably higher proportions in the side-chain cleavage enzyme.

The N-terminal residue of 11β- and 18-hydroxylase was found to be glutamic acid.

Loss of Enzyme Activities on Storage. In three preparations

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[1] Abbreviations used are: corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; metyrapone, 2-methyl-1,2-bis(3-pyridyl)-L-propanone.

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TABLE I: Amino Acid Composition of Adrenal P-450 (11β- and 18-Hydroxylase).[a]

Amino acid	Residues/subunit
Asx	35.2
Thr	23.4
Ser	26.2
Glx	45.9
Pro	22.1
Cys[b]	3.6
Gly	30.3
Ala	33.1
Val	32.2
Met	9.2
Ile	21.9
Leu	50.6
Tyr	11.6
Phe	16.9
His	9.9
Lys	21.1
Arg	29.0
Trp[c]	5.4

[a] Residues are expressed per subunit molecular weight of 47 500. [b] Determined spectrophotometrically with 5,5′-dithiobis(2-nitrobenzoic acid) using the method of Ellman (1959). [c] Determined by MCD spectroscopy using the method of Barth et al. (1972).

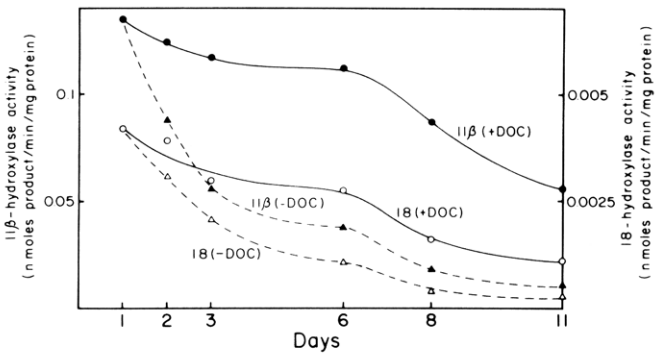

FIGURE 1: Activity of 11β- and 18-hydroxylase activities as a function of duration of storage at 4 °C. The enzyme was examined on the days shown under standard conditions (see Experimental Procedure). The heme content (nanomoles/milligram of protein) did not change with time and the same amount of protein was used in each incubation (78 μg). Products of enzyme activity (corticosterone and 18-hydroxy-11-deoxycorticosterone) were measured by radioimmunoassay with duplicate determinations of zero time values which have been subtracted (0.18 and 0.008 nmol of product/flask for 11β- and 18-hydroxylase, respectively). The values shown are means of triplicate determinations of each activity at each time point. Ranges for these determinations are less than 10% of the values shown in each case. Two samples of P-450 were used for these studies, one stored throughout with deoxycorticosterone (10 μM) and one without addition of this steroid: (●) 11β-hydroxylase; enzyme stored with deoxycorticosterone; (○) 18-hydroxylase; enzyme stored with deoxycorticosterone; (▲) 11β-hydroxylase; enzyme stored without deoxycorticosterone; (△) 18-hydroxylase; enzyme stored without deoxycorticosterone.

of the 11β- and 18-hydroxylase P-450, measurements of both activities were made over a period of 10–14 days following purification of enzyme. A typical study is shown in Figure 1. Preparation of the enzyme required 2 days and on the second of these days (called day 0 in the figure), two samples (5 mL, 0.66 mg of protein/mL for each treatment) of the enzyme were taken and deoxycorticosterone (10 μM) was added to one and the solvent *N,N*-dimethylformamide to the other. On that day and on the other days shown in the figure, aliquots of each sample (with and without deoxycorticosterone) were allowed to come to room temperature before being examined in triplicate under conditions of maximal velocity at 30 °C (Watanuki et al., 1977b). Throughout the preparation and subsequent storage, the enzyme was kept at 4 °C except for the aliquots used to determine enzyme activity. It can be seen that both activities declined during the first week and that deoxycorticosterone delayed this decline with each of the activities. The time courses of decay with and without deoxycorticosterone are similar for both 11β- and 18-hydroxylase activities (Figure 1).

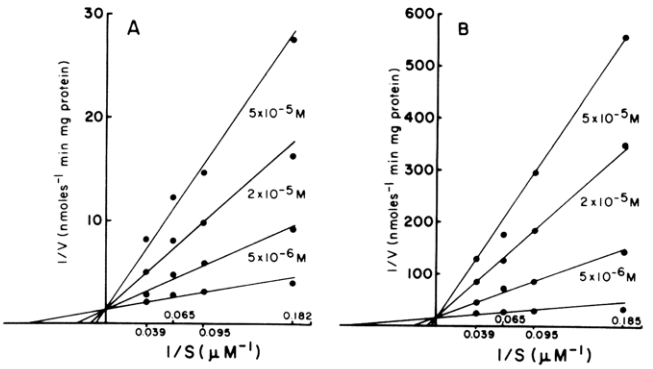

FIGURE 2: Kinetic studies of 11β- (A) and 18-hydroxylase (B) activities without and with various concentrations of the inhibitor metyrapone. P-450 (50 μg) was present in each flask and values at zero time were subtracted from the values shown (see legend to Figure 1). The products of the two enzyme activities (corticosterone and 18-hydroxy-11-deoxycorticosterone) were measured by radioimmunoassay (Watanuki et al., 1977b).

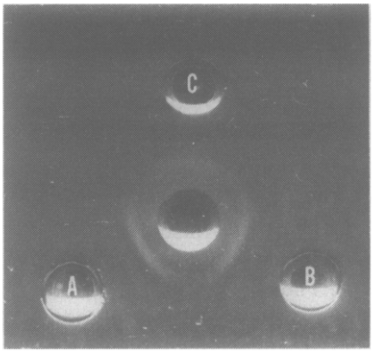

FIGURE 3: Double diffusion of 11β- and 18-hydroxylase enzyme and rabbit antiserum raised against this enzyme. The center well contained anti-P-450 (11β- and 18-hydroxylase enzyme) (100 μL previously concentrated to 20 μL by precipitation with ammonium sulfate (40% saturated) followed by dialysis). The remaining cells contained the following: (A) P-450 11β- and 18-hydroxylase, 20 μg; (B) P-450 11β- and 18-hydroxylase, 20 μg; (C) P-450 11β- and 18-hydroxylase, 10 μg. The halo around each well and the width of the bands result from the aggregation of P-450 under the conditions of double diffusion.

Inhibition by Metyrapone of 11β- and 18-Hydroxylase Activities. Figure 2 shows double reciprocal plots of 11β- and 18-hydroxylase activities of mitochondrial P-450 from bovine adrenal cortex without and with various concentrations of metyrapone and with various concentrations of deoxycorticosterone as substrate. It can be seen that inhibition of both enzyme activities appears to be competitive. Values for K_i are as follows: 11β-hydroxylase, 1.50 mM; 18-hydroxylase, 1.43 mM. Careful examination of inhibition of 11β-hydroxylase by metyrapone with less purified enzyme supports the view that inhibition is competitive (Betz et al., 1975). In the present case, the determinations shown were performed with three different preparations of the enzyme and statistical evaluation of the data shows that V_{max} is not significantly different for each line of a family of curves so that the effect of metyrapone disappears at infinite substrate concentration; this indicates that

TABLE II: Influence of Carbon Monoxide on 11β- and 18-Hydroxylase Activities.[a]

Gas phase (%)			11β-Hydroxylase act. (nmol min^{-1} (mg of protein)$^{-1}$)	K	18-Hydroxylase act. (nmol min^{-1} (mg of protein)$^{-1}$)	K
CO	O_2	N_2				
0	21	79	2.78		0.211	
0	4	96	2.05		0.171	
26.7	3.6	69.7	0.40	1.85	0.027	1.41
18.2	3.7	78.1	0.58	1.92	0.041	1.57
1.9	4.0	94.1	1.54	1.43	0.131	1.59
1.1	4.0	94.9	1.74	1.55	0.146	1.55

[a] The details of methods used to prepare and measure the gas phases (Hall et al., 1975) and the methods for measuring enzyme activities (Watanuki et al., 1977b) have been published elsewhere. K = partition coefficient, i.e. CO:O_2 at 50% inhibition.

TABLE III: Inhibition of 11β- and 18-Hydroxylase Activities by Various Agents.[a]

Treatment	Relative enzyme act. 11β-Hydroxylase	18-Hydroxylase
None	100	100
1 M KCl	0	0
10% MeOH	7.0	5.0
10% EtOH	0.5	0.5
10% acetone	1.5	0
Anti-11β-antiserum	19.0	21.0
Temperature of incubation (°C)		
2	14.8	14.0
20	58.0	55.8
50	0	2.3
Enzyme heated at 30 °C		
For 20 min	26.6	27.4
For 60 min	4.8	6.5

[a] One preparation of P-450 was used for these studies and was incubated for 10 min at 30 °C under standard conditions (Watanuki et al., 1977a,b). Activity without additions (100%) was 2.3 and 0.1 nmol (mg of protein)$^{-1}$ min^{-1} for 11β- and 18-hydroxylase activities, respectively. The various agents shown were pipetted (as the last addition) to flasks prior to incubation. Values shown are means of duplicate determinations.

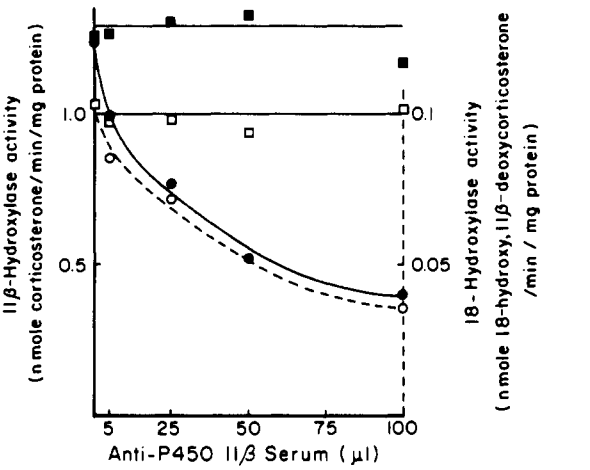

FIGURE 4: The effect of anti-P-450 antiserum on 11β- and 18-hydroxylase activities of adrenal cytochrome P-450. Rabbit antiserum to the purified 11β- and 18-hydroxylase was added in the amounts shown, to flasks containing the necessary components for measuring these two enzyme activities (Watanuki et al., 1977b). Antiserum was the last addition to the flasks, which were kept on ice until incubation began. The details of the assay are given in the above reference. Normal rabbit serum was added to control flasks. At 50 μL of antiserum, values for open and closed circles coincide. The range of duplicate determinations was less than 5% of the values shown in each case: (●) 11β-hydroxylase + anti-11β- and 18-hydroxylase; (○) 18-hydroxylase + anti-11β- and 18-hydroxylase; (■) 11β-hydroxylase + normal rabbit serum; (□) 18-hydroxylase + normal rabbit serum.

inhibition is competitive in this system. Moreover, plots for metyrapone showing slope as a function of inhibitor concentration gave straight lines for both activities. Finally, the K_i values with metyrapone were not significantly different for the two activities ($P > 0.7$).

Inhibition of 11β- and 18-Hydroxylase Activities by Carbon Monoxide. As expected, carbon monoxide inhibits both enzyme activities and Table II 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 variations seen are within the limits of experimental error.

The Actions of Various Agents on 11β- and 18-Hydroxylase Activities. Table III shows the relative effects of a variety of nonspecific agents on the two enzyme activities of the cytochrome P-450 described in this paper. Although the extent of inhibition produced by the agents shown varies considerably, it is remarkable how similar the responses of the two enzyme activities are to each of the conditions tested.

Inhibition of 11β- and 18-Hydroxylase Activities by Rabbit Antiserum. Figure 3 shows the result of double diffusion in agarose gels between rabbit antiserum prepared against highly purified 11β- and 18-hydroxylase from bovine adrenocortical mitochondria and the purified enzyme itself. The gel shows a single band indicating that the enzyme is immunochemically homogeneous. This antiserum inhibits both 11β- and 18-hydroxylase activities of the enzyme (Figure 4). The inhibition, expressed as percentage of the control (no antiserum) activity, is approximately the same for 11β- and 18-hydroxylase activities at each concentration of antiserum tested.

Discussion

The findings reported in this paper lend strong support to the idea that 11β- and 18-hydroxylase activities in adrenocortical mitochondria are properties of a single protein. Firstly, the purified enzyme shows a single band on sodium dodecyl sulfate gels (Watanuki et al., 1977b) and gives a single N-terminal amino acid on dansylation (glutamic acid); if the protein contains two separate peptides and if both chains possess a free N-terminal amino acid, they must possess the same N-terminal amino acid and the protein subunits must be of very similar molecular weights. Secondly, both enzyme activities decay on storage at 4 °C at rates which show no evidence of differential loss of the 11β- and 18-hydroxylase functions. The present findings confirm earlier reports that deoxycorticosterone decreases the rate of loss of enzyme activity at 4 °C (Takemori et al., 1975; Watanuki et al., 1977b) but again both activities disappear at similar rates in the presence and absence of this substrate (Figure 1).

Thirdly, the purified enzyme shows inhibition of both hydroxylase activities by metyrapone. In each case, inhibition is competitive and values for K_i are not statistically different (11β-hydroxylase, 1.50 μM; 18-hydroxylase, 1.43 μM). Fourthly, the partition coefficient (K) is similar for the two activities when the enzyme is incubated in various concentrations of carbon monoxide (Table II). Presumably metyrapone acts at a substrate-binding site while carbon monoxide binds specifically to heme. It would be surprising if neither of these inhibitors could distinguish (qualitatively or quantitatively) between two separate proteins. Fifthly, other less specific inhibitors which presumably alter the tertiary and/or quarternary structures of the protein also affect both activities to the same degree within the limits of experimental error (Table III). Once again these substances, which produced widely varying degrees of inhibition, fail to discriminate between the two enzymatic activities.

This evidence makes it extremely probable that we are dealing with a single peptide chain with two hydroxylase activities. A sixth line of evidence supports this view. The immune system of the rabbit does not distinguish two proteins since antiserum raised against the pure enzyme causes proportionate inhibition of the two activities. Although two proteins injected together would cause the rabbit to make two antibodies and each antibody would presumably inhibit only the corresponding enzyme activity, it is unlikely that various concentrations of such an antiserum would inhibit the two activities in the same proportion when inhibition is measured as a function of antibody concentration (Figure 4). Moreover, double diffusion in agarose shows a single band formed between the enzyme and the antiserum. These observations further support the view that the immune system has not distinguished two proteins in the present preparation of 11β- and 18-hydroxylase.

One feature of the enzyme which deserves emphasis is the poor 18-hydroxylase activity seen with corticosterone as substrate although corticosterone is generally regarded as the normal substrate for 18-hydroxylation in vivo (e.g., Ayres et al., 1960). It would also appear that the zona fasiculata synthesizes considerable amounts of 18-hydroxy-11-deoxycorticosterone (Sheppard et al., 1963). We have observed that only about 10% of the corticosterone produced by 11β-hydroxylation of deoxycorticosterone is converted to 18-hydroxy-11-deoxycorticosterone (Watanuki et al., 1977a,b) in confirmation of an earlier observation with a less purified enzyme (Bjorkham and Kalmar, 1975). It may be that there are two enzymes in the adrenal, one for the substrate deoxycorticosterone which accounts for the production of 18-hydroxy-11-deoxycorticosterone by the zona fasiculata and a second in the zona glomerulosa responsible for the synthesis of aldosterone. In this event, we have presumably isolated the enzyme of the fasiculata reticularis. Alternatively, the properties of the enzyme may have been modified on removal of the cytochrome P-450 from a membrane environment. In any case this issue is currently being approached with separated glomerulosa and fasiculata reticularis in this laboratory. Moreover, the knowledge that one protein is capable of both activities may provide a useful clue in considering possible mechanisms of the two reactions.

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References

Ayres, P. J., Eichhorn, J., Hechter, O., Saba, N., Tait, J. F., and Tait, S. A. S. (1960), *Acta Endocrinol. 33,* 27.

Barth, G., Voelter, W., Bunnenberg, E., and Djerassi, C. (1972), *J. Am. Chem. Soc. 94,* 1293.

Betz, G., Tsai, P., and Weakley, R. (1975), *Steroids 25,* 791.

Bjorkham, I., and Kalmar, K. (1975), *Eur. J. Biochem. 51,* 145.

Cleland, W. W. (1963), *Nature (London) 198,* 463.

Cleland, W. W. (1967), *Adv. Enzymol. 29,* 1.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7,* 463.

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

Rapp, J. P., and Dahl, L. K. (1976), *Biochemistry 15,* 1235.

Sheppard, H., Swenson, R., and Mowles, T. F. (1963), *Endocrinology 73,* 819.

Shikita, M., and Hall, P. F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1441.

Suhara, K., Ikeda, Y., Takemori, S., and Katagiri, M. (1972a), *FEBS Lett. 28,* 45.

Suhara, K., Takemori, S., and Katagiri, M. (1972b), *Biochim. Biophys. Acta 263,* 272.

Takemori, S., Suhara, K., Hashimoto, S., Hashimoto, M., Sato, H., Gomi, T., and Katagiri, M. (1975), *Biochem. Biophys. Res. Commun. 63,* 588.

Tilley, B. E., Watanuki, M., and Hall, P. F. (1977), *Biochim. Biophys. Acta 488,* 1330.

Watanuki, M., Granger, M., and Hall, P. F. (1977a), *J. Biol. Chem.* (in press).

Watanuki, M., Tilley, B. E., and Hall, P. F. (1977b), *Biochim. Biophys. Acta 483,* 236.

Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247,* 3242.