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CYTOCHROME *P*-450 C_{21scc}: ONE ENZYME WITH TWO ACTIONS: HYDROXYLASE AND LYASE

PETER F. HALL

Department of Medicine, Prince of Wales Hospital, Randwick, University of New South Wales, Kensington, NSW 2033, Australia

Summary—Testis, adrenal, ovary and placenta contain a microsomal cytochrome *P*-450 that is capable of converting progesterone to androstenedione and pregnenolone to dehydroepiandrosterone. This conversion requires 17α-hydroxylation followed by $C_{17,20}$-lyase activity which are both catalyzed by this one protein. Gene cloning and Northern blotting reveal that, at least in man, the same gene is responsible for both testicular and adrenal enzymes. The enzyme was first purified from neonatal pig testis. Both the testicular and adrenal enzymes show a marked preference for the 5-ene substrate (pregnenolone) in keeping with the extensive use of the 5-ene pathway in that species. Affinity alkylation with 17α-bromoacetoxyprogesterone reveals a conserved cysteine at the active site of the enzyme and confirms the conclusion that a single enzyme catalyzes both reactions. Under some circumstances the enzyme catalyzes only 17α-hydroxylation to permit the formation of the C_{21} steroid cortisol. The regulation of lyase activity, i.e. the determination of the extent to which the second activity is expressed, results from the availability of *P*-450 reductase. No doubt the greater concentration of this protein in testicular as opposed to adrenal microsomes (× 3.5) is responsible for the production of androgens in the testis and cortisol in the adrenal. Testicular cytochrome b_5 also specifically stimulates lyase activity and also causes the porcine enzyme to catalyze a new reaction, i.e. Δ^{16}-synthetase, resulting in synthesis of the important pheromone androsta-4,16-dien-3-one from progesterone.

INTRODUCTION

The conversion of progesterone to androstenedione proceeds in two steps, namely 17-hydroxylation followed by $C_{17,20}$-lyase (Fig. 1). Two analogous reactions result in the conversion of the corresponding 5-ene substrate (pregnenolone) to dehydroepiandrosterone [1]. The substrate chosen by the relevant enzyme system varies in different species being largely 4-ene in rat; a mixture of both subtrates is used in other species [1, 2]. It has long been suspected that cytochrome *P*-450 is involved in these reactions and because 17α-hydroxylation determines whether the pathway leads to glucocorticoids or aldosterone and because lyase determines whether the pathway proceeds to C_{21} steroid (cortisol) or to C_{19} sex steroids, the relevant enzyme system is of great interest. Some years ago this laboratory purified a cytochrome *P*-450 from testicular microsomes that catalyzes both the above reactions. This paper describes the properties, structure and regulation of the enzyme.

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SIDE CHAIN CLEAVAGE OF PROGESTERONE

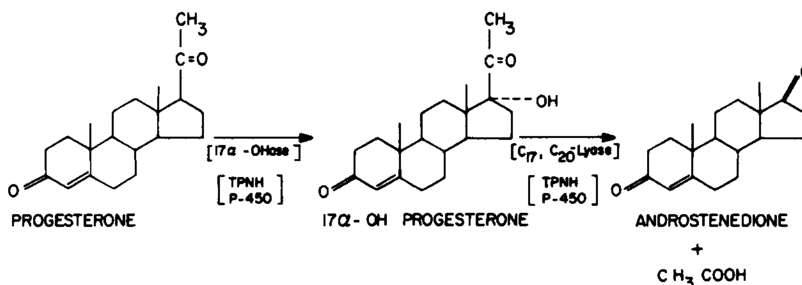

Fig. 1. The conversion of progesterone to androstenedione.

METHODS

The relevant methods for purification and characterization of the enzyme have been reported [3, 4]. In addition procedures for determination of the amino acid sequence [4], photochemical action spectra [5], substrate-induced difference spectra [6], affinity alkylation of the active site [7] and gene cloning [8] have all been published in detail.

RESULTS AND DISCUSSION

One enzyme catalyzes both reactions

When the testicular microsomal *P*-450 was purified to homogeneity we were greatly surprised to discover that the pure enzyme catalyzes both reactions (hydroxylase and lyase) with both substrates (progesterone and pregnenolone) [3, 4]. The enzyme, which will be referred to here as C_{21} side chain cleavage (or C_{21scc}) *P*-450, possesses a single subunit of mol. wt 59,000 and the reduced *P*-450–CO complex shows an absorbance peak at 448 nm. Values for the K_m of the two reactions with progesterone and 17α-hydroxyprogesterone as substrates were 1.5 and 2.4 μM, respectively [6]. The pure enzyme gave a single peak on SDS–polyacrylamide gels [3]. A polyclonal antibody raised against the enzyme gave a line of identity on immunodiffusion against the enzyme and a single line on immunoelectrophoresis (Fig. 2) [4]. The antibody inhibited both enzyme activities with the same relationship between log activity as a function of log concentration of IgG [4]. A single NH_2 terminal methionine was observed [4]. Moreover a variety of agents such as various inhibitors, temperature, pH etc. affected both reactions in the same way so that the ratio of hydroxylase to lyase remained constant with increasing inhibition [4]. These observations argued strongly for a single enzyme catalyzing two reactions.

These studies were performed with the enzyme from neonatal pig testes because in this species the testis at birth is comprised largely of Leydig cells. The pure enzyme from this source showed a lower K_m and K_d (dissociation constant) for 5-ene as opposed to 4-ene substrates in keeping with extensive use of the 5-ene pathway in pig [6, 9]. Moreover, spectral and binding studies revealed a single binding site for the substrates for hydroxylase and lyase activities of the enzyme. This was confirmed by equilibrium dialysis [6]. In addition the second reaction (lyase) shows powerful forward inhibition by the substrates progesterone and pregnenolone [6]. This inhibition may be important in the regulation of the enzyme at high concentrations of substrate and was observed with microsomes from rat testis [10].

Involvement of cytochrome P-450

The involvement of *P*-450 in 17-hydroxylation is to be expected from what is known about steroid hydroxylation. That the cleavage of a C—C bond might require *P*-450 came as something of a surprise although surprise was hardly justified when one considers earlier work

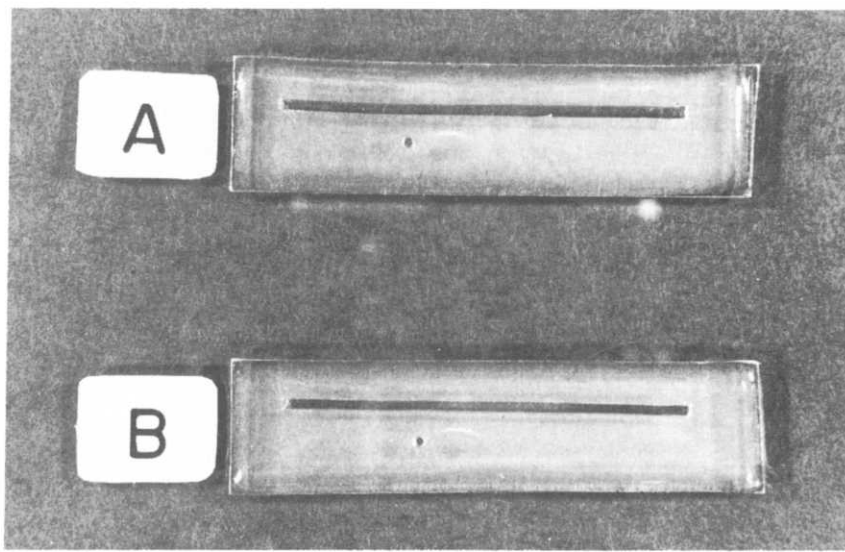

Fig. 2. Immunoelectrophoresis of purified porcine testicular C_{21scc} *P*-450.

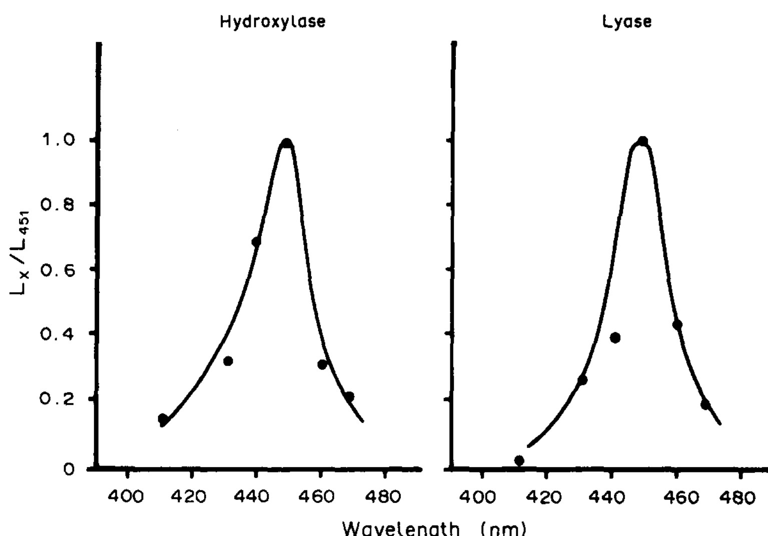

Fig. 3. Photochemical action spectra of hydroxylase and lyase activities of C_{21scc} P-450.

from this and other laboratories showing that the side chain cleavage P-450 catalyzes an analogous reaction in the side chain of cholesterol [11–14]. To demonstrate the involvement of P-450 in both reactions, photochemical action spectra were performed. This procedure revealed that inhibition of both steps by carbon monoxide was reversed specifically by light of wavelength 450 nm (Fig. 3) which demonstrates that the CO complex specifically absorbs light of this wave length. Since this is the hallmark of the action of all cytochromes P-450 we must conclude that both hydroxylase and lyase activities of the C_{21scc} enzyme involve typical P-450 reactions. The mechanism by which monooxygenation can cleave a C—C bond remains to be explored.

Regulation of lyase activity

Obviously the biosynthetic consequences of the activity of the hydroxylase–lyase depend upon whether one or both reactions are catalyzed. In adrenal gland lyase activity is kept to a minimum so that the principal product of adrenal microsomes is 11-deoxycortisol which gives rise to cortisol. In testicular microsomes lyase activity is expressed with the formation of C_{19} androgens to the exclusion of C_{21} steroids (Fig. 4). After excluding competition between lyase and 21-hydroxylase (by means of anti-21-hydroxylase which completely inhibited this enzyme without altering lyase activity [15]), we observed with the pure P-450 that lyase activity increased relative to hydroxylase as a function of the concentration of the electron carrier P-450 reductase [16]. A similar effect was seen with microsomes from the adrenal cortex which acted more like testicular microsomes (i.e. high lyase activity) when exogenous reductase was added (Fig. 5). On the other hand, testicular microsomes showed much less lyase activity i.e. behaved more like adrenal microsomes when

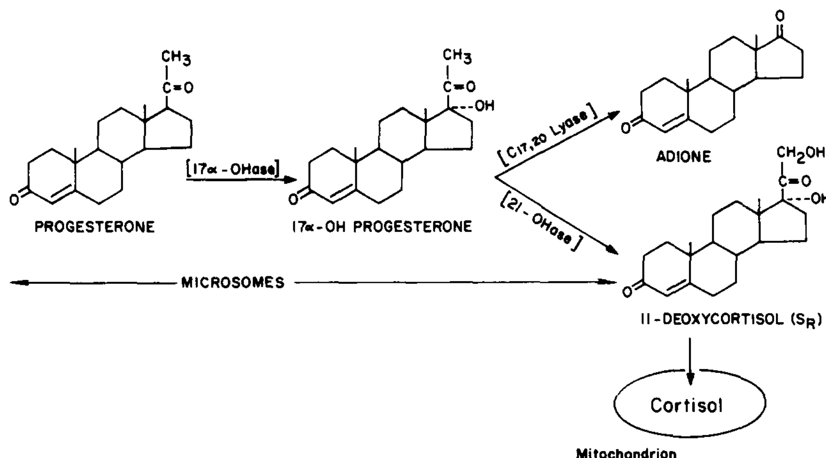

Fig. 4. Alternative synthetic pathways from 17α-hydroxyprogesterone.

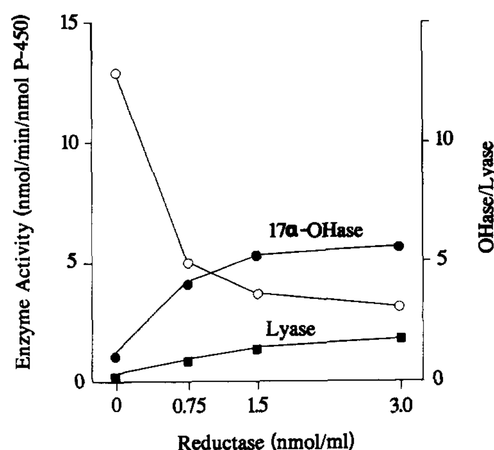

Fig. 5. The influence of reductase on hydroxylase and lyase activities of adrenal microsomes.

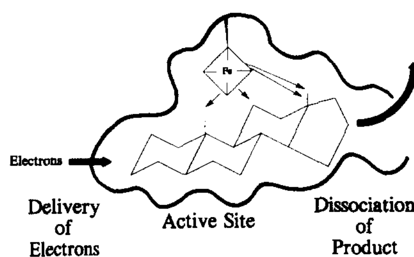

Fig. 6. Diagram showing two factors in the regulation of multistep reactions catalyzed by a cytochrome P-450.

anti-reductase was added [15]. Moreover, it was found that testicular microsomes contain approx. 3.5 times as much reductase activity as adrenal microsomes [15].

These studies draw attention to the important part played by the rate of reduction of P-450 in determining the extent of the second of two consecutive reactions catalyzed by a single P-450. It is well known among the drug metabolizing cytochromes P-450 that one enzyme can catalyze more than one reaction with a given substrate and even at a given carbon atom of one substrate. Any monooxygenase activates molecular oxygen and inserts one atom of the activated oxygen on a carbon (or other atom) close enough in the active site to the heme iron. The promiscuous activity of cytochromes P-450 described above can be understood as the result of rapid reduction of P-450 relative to dissociation of substrate (or product) from the active site. If the flow of electrons is high relative to the rate of dissociation of product (or intermediate as the case may be), the chances of a second attack by the enzyme will be great (Fig. 6).

A similar case has recently been reported in the third monooxygenase reaction in the synthesis of aldosterone [16]. These observations would suggest that the rate of synthesis of the electron carriers in a particular organ may be regulated in relation to the production of P-450 enzymes so that appropriate levels of reductive drive are available to the P-450. In this way the enzyme can catalyze one or both reactions as required for the pathway in question [17].

The enzymes from testis and adrenal

Meanwhile the analogous enzyme was isolated from porcine adrenal and found to be very similar to the testicular enzyme [18, 19]. It is interesting to notice that one conservative substitution (thr→ser, adrenal→testis) occurs in the first 16 NH_2 terminal amino acids [19]. At this time we must consider that minor strain differences exist between the slaughterhouse and farm pigs used as the source of porcine enzymes from adrenal and testis, respectively, accounting for minor differences in the amino acid sequences in the two enzymes. Certainly when the enzyme was cloned from human DNA and the RNA from adrenal and testis compared by Northern blotting, no differences were seen between the RNA from the two organs [8].

The active site

Clearly the active site of such an ambidextrous enzyme is of great interest since it appears capable of catalyzing two such different types of reactions, i.e. hydroxylation and cleavage of a C—C bond. Using 17α-bromoacetoxyprogesterone we were able to inhibit the enzyme and to establish that this substrate analogue competes with the natural substrate (progesterone) in binding to the active site [7]. The analogue causes increasing non-competitive inhibition with time as it forms covalent bonds with more and more molecules of enzyme. As a result competitive inhibition gives way to non-competitive as the duration of incubation increases. On a time scale of $t_{1/2}$ in the range of 1–4 h the enzyme undergoes irreversible inactivation. As evidence that the analogue is at the active site of the enzyme, inactivation by the analogue is prevented by excess substrate. When such studies were performed with C_{21scc} P-450, 17α-bromoacetoxyprogesterone derivatized a specific cysteine in a highly conserved region of microsomal cytochromes P-450 near the NH_2 terminus [7]. This region is at, or close to, the substrate-binding site. A point of some importance emerged from these studies when it was found that the inactivation of both hydroxylation and lyase activities followed the same time-course. Clearly the analogue did not dis-

tinguish between the two activities. Moreover the substrates for the two activities (i.e. progesterone and 17α-hydroxyprogesterone) protect both activities (hydroxylase and lyase) against inactivation by the analogue. It appears, then, that the two activities are catalyzed not only by one enzyme but by one active site. Other analogues in which the bromoacteoxy side chain is attached to different carbon atoms of progesterone are now being used to extend these studies by identifying other amino acids in the active site.

Influence of cytochrome b_5

It is well known that the activity of microsomal cytochromes P-450 is influenced by cytochrome b_5 which can, in some cases, stimulate and in other cases inhibit specific reactions of drug metabolism [20–22]. In other cases b_5 appears to have no effect on the enzyme activity of a given P-450. The role of b_5 has been considered to involve electron transport from NADH to a reductase (cytochrome b_5–P-450 reductase) and thence to P-450. Moreover it has been suggested that this transport provides the second electron required to enable P-450 to activate oxygen [23]. The involvement of b_5 has been studied by comparing the activity of P-450 with excess NADPH to the activity of the enzyme with excess NADPH plus excess NADH. However b_5 greatly stimulates the activity of pure C_{21scc} in the absence of b_5-P-450 reductase and NADH [24]. Clearly b_5 is capable of direct interaction with P-450_{scc}. The physiological importance of this action remains uncertain [19,20]. A more striking effect of b_5 on P-450 C_{21scc} is seen in a qualitative change in the activity of the pure enzyme from porcine testis when incubated with the enzyme. Not only does b_5 increase both hydroxylase and lyase activities (lyase to a greater extent than hydroxylase) but also causes the production of 16-ene C_{19} steroids i.e. from progesterone the steroid androstadienone (androsta-4,16-dien-3-one) and the corresponding 5-ene-3β-ol from pregnenolone [25]. These compounds are among the male pheromones of the pig [26]. Such pheromones are not formed by testes of other species commonly used for laboratory studies so that a comparison of the action of b_5 on C_{21scc} P-450 from several species, e.g. beef and sheep, may prove rewarding.

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