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THE ENZYME ACTIVITY OF BOVINE ADRENOCORTICAL CYTOCHROME P-450 PRODUCING PREGNENOLONE FROM CHOLESTEROL : KINETIC AND ELECTROPHORETIC STUDIES ON THE REACTIVITY OF HYDROXYCHOLESTEROL INTERMEDIATES

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SUMMARY Electrofocusing of a highly-purified preparation of bovine adrenocortical cytochrome P-450(scc) showed a single peak of enzyme activity at pH 6.8,when either cholesterol,[20S]-20-hydroxycholesterol,[22R]-22-hydroxycholesterol or [20R,22R]-20,22-dihydroxycholesterol was used as the substrate for the side chain cleavage reaction. The formation of pregnenolone from these hydroxycholesterols was inhibited by [20R,22S]-20,22-epoxycholesterol similarly in a competitive manner and the Ki value for the epoxide was found to be 12-15 μM for all these substrates. When one of the above mentioned substrates was incubated in a concentration sufficient for maximal reaction velocity,the addition of another hydroxycholesterol did not result in further increase of pregnenolone production. These results support the assumption that a single species of enzyme catalyzes all the three steps of the reaction,i.e.,20-hydroxylation,22-hydroxylation and cleavage of carbon chain between carbon-20 and carbon-22.

INTRODUCTION

Cytochrome $P\text{-}450_{scc}$ is an enzyme which catalyzes the formation of pregnenolone from cholesterol and has been isolated from bovine adrenocortical mitochondria as a polymeric protein (1). A subsequent

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study showed that the enzyme performs its catalytic action without dissociating into smaller forms (2). These molecular properties of $P\text{-}450_{scc}$ and the fact that 20- and 22- hydroxycholesterols and 20,22-dihydroxycholesterol occur as intermediates in the reaction (3), have raised the question whether such polymeric species as obtained above is a complex of three different proteins,namely 20-hydroxylase, 22-hydroxylase and 20,22-lyase. However,the electrofocusing experiment and the kinetic studies as reported below demonstrate that the three enzymic activities reside in the same cytochrome.

MATERIALS AND METHODS

Enzyme preparation

Bovine adrenal glands were collected at a slaughter house and stored frozen until use. The glands were thawed at 5°C and mitochondrial pellets were prepared by a conventional method. The mitochondria were disrupted by sonic waves and soluble proteins were removed by ultracentrifugation. The cytochrome was then extracted from the membrane by sodium cholate (4) and purified by a modification of the method reported before (1). Final preparation contained 10-11 nmols of heme (pyridine heme chromogen) per mg of protein (Folin reagent) and showed a sedimentation coefficient of 8.2 S when it was centrifuged on a sucrose density gradient. Adrenodoxin and adrenodoxin reductase were also highly purified according to the described procedures (5,6). These proteins were stored as lyophilized powder (adrenodoxin),in a concentrated solution (reductase) and in 50% glycerol (P-450),all below -20°C.

Compounds

The stereoisomers of 20-hydroxycholesterol,22-hydroxycholesterol, 20,22-dihydroxycholesterol and 20,22-epoxycholesterol were synthesized as reported previously (7,8). Purity of these compounds was examined by thin layer chromatography as well as by gas chromatography as trimethylsilyl ethers. Dimethylformamide was used as the solvent when cholesterol and these compounds were added to the incubation medium.

Electrofocusing

After dialysis against 1 mM Tris-HCl (pH 7.0),an aliquot (2.9 mg protein) of P-450(scc) was subjected to isoelectrofocusing for 48 hrs at 500 V at 5°C in a 110-ml sucrose density gradient containing 2.5% Ampholine (LKB-Produkter AB,Bromma) in the range of pH 3.5-10.

Assay of the enzyme activity

The solutions collected from the isoelectrofocusing column were dialyzed against 10 mM potassium phosphate buffer of pH 7.4 containing 5 mM cysteine and 1 mM EDTA. Aliquots of these solutions were

incubated at 37°C with cholesterol (25 μM) for 2 hrs and with hydroxycholesterols (25 μM) for 30 minutes in the presence of NADPH generating and electron transferring systems as described previously (9). In the kinetic studies, the enzyme mixture was pre-incubated with substrate sterols for 5 minutes at room temperature both in the presence and in the absence of competing analogs. The side-chain cleavage reaction was started by the addition of the NADPH generating system continued for 5 minutes at 37°C and then arrested by the addition of dichloromethane. Under the conditions adopted, the enzyme reaction was linear with time for about 10 minutes at substrate concentrations of 10-25 μM. Pregnenolone thus produced was extracted with dichloromethane and then determined by mass fragmentography with a Shimadzu-LKB-9000S gas chromatograph-mass spectrometer as described in earlier studies (3,9).

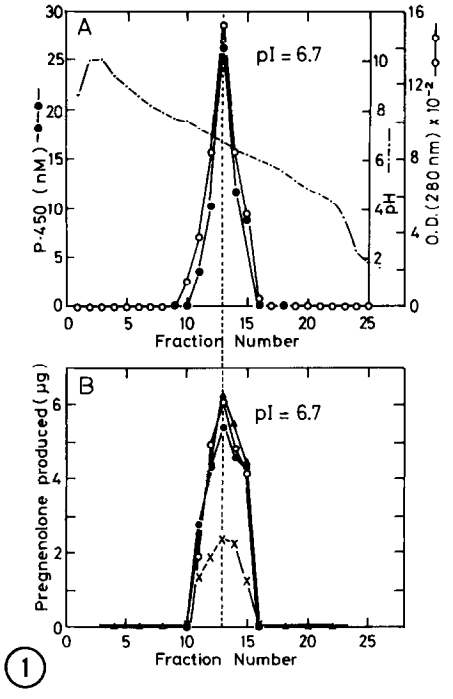
RESULTS

Behavior of the three enzyme activities on isoelectrofocusing

Electrofocusing of the $P\text{-}450_{scc}$ preparation showed a maximum enzymic activity at pH 6.8 (average of 3 experiments) for the production of pregnenolone, regardless whether cholesterol, [20S]-20-hydroxycholesterol, [22R]-22-hydroxycholesterol or [20R,22R]-20,22-dihydroxycholesterol was used as the substrate. Furthermore, the peak of P-450 (as determined by CO difference spectrum) were located at the same position (Fig.1). The results show that the three enzymic activities, i.e. (a) hydroxylation of carbon-20 of cholesterol and [22R]-22-hydroxycholesterol, (b) hydroxylation of carbon-22 of cholesterol and [20S]-20-hydroxycholesterol and (c) cleavage of the chain between carbon-20 and carbon-22 of [20R,22R]-20,22-dihydroxycholesterol, are not separable from each other by isoelectrofocusing.

Competitive inhibition of the side chain cleavage reaction by the 20,22-epoxide

As it has been previously reported, all the isomers of 20,22-epoxycholesterol do not efficiently yield pregnenolone in the enzyme reaction (9,10) but rather powerfully inhibit the side-chain cleavage of cholesterol (9). In the present study, it was revealed that [20R,22S]-20,22-epoxycholesterol inhibits the production of pregnenolone



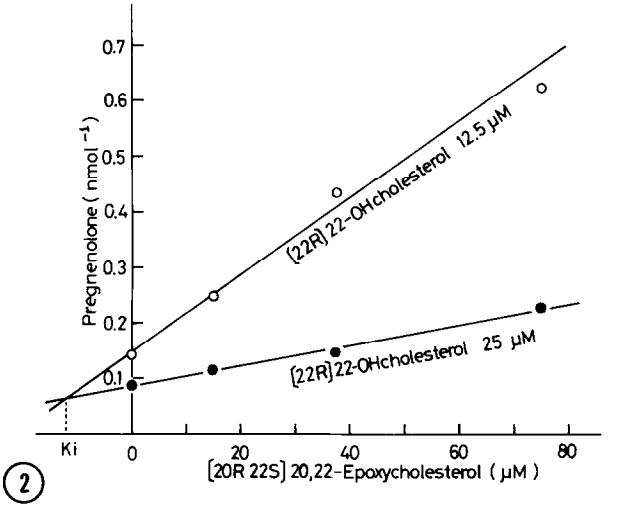


Fig. 1 Electrofocusing of Bovine Adrenal Cytochrome P-450(scc) : (A) Location of proteins as determined by 280 nm light absorption and of P-450 as determined by CO difference spectrum. (B) Location of enzyme activity producing pregnenolone from cholesterol (×), [20S]-20-hydroxycholesterol (○),[22R]-22-hydroxycholesterol (●) and from [20R,22R]-20,22-dihydroxycholesterol (△). Light absorption of the solution was recorded on an automatic spectrophotometer after Ampholites were removed by dialysis. One-seventh of each fraction was used for respective enzyme assay and three-seventh for P-450 determination. The amount of pregnenolone given in the figure is not corrected for total volume of each fraction.

Fig. 2 Competitive Inhibition of Pregnenolone Producing Activity of Cytochrome P-450(scc) by [20R,22S]-20,22-epoxycholesterol : Substrate,[22R]-22-hydroxycholesterol (12.5 μM,25 μM). Incubation,5 min. All tubes were equalized with regard to the amount of dimethylformamide (20 μl).

even from any of mono- or di-hydroxycholesterol and that the inhibition occurs in a competitive manner in all cases. Figure 2 shows a typical example of competition between the inhibitor epoxide and the substrate hydroxycholesterol. A similar value of Ki was obtained for this epoxide isomer against other hydroxycholesterol derivatives as well (Table 1).

Table 1

Consistency of the Ki value of [20R,22S]-20,22-Epoxycholesterol in its Competition with Hydroxycholesterol Intermediates of the Cytochrome $P\text{-}450_{scc}$ Reaction.

Substrate	Ki
[20S]-20-Hydroxycholesterol	12.1 μM *
[22R]-22-Hydroxycholesterol	12.4
[20R,22R]-20,22-Dihydroxycholesterol	13.5

* The averages of two determinations conducted in the same manner as shown in Fig. 1.

Mixed substrate study

A semi-quantitative mixed substrate method (11) was used as the third attempt to distinguish the three enzyme activities of the $P\text{-}450_{scc}$ preparation. A separate preliminary study had shown that a maximum velocity of pregnenolone production was achieved at substrate concentrations of about 20 μM in the cases of cholesterol, [20S]-20-hydroxycholesterol and [22R]-22-hydroxycholesterol, while the reaction velocity was not leveled off even at 50 μM in the case of [20R,22R]-20,22-dihydroxycholesterol (data not shown). As it is shown in Fig. 3-A, however, there was no additional increase of pregnenolone formation when either [20S]-20-hydroxycholesterol or [22R]-22-hydroxycholesterol of saturating concentrations was incubated simultaneously with [20R,22R]-20,22-dihydroxycholesterol. Furthermore,a mixture of 20-hydroxycholesterol and 22-hydroxycholesterol did not give rise to a greater production of pregnenolone than when these compounds were

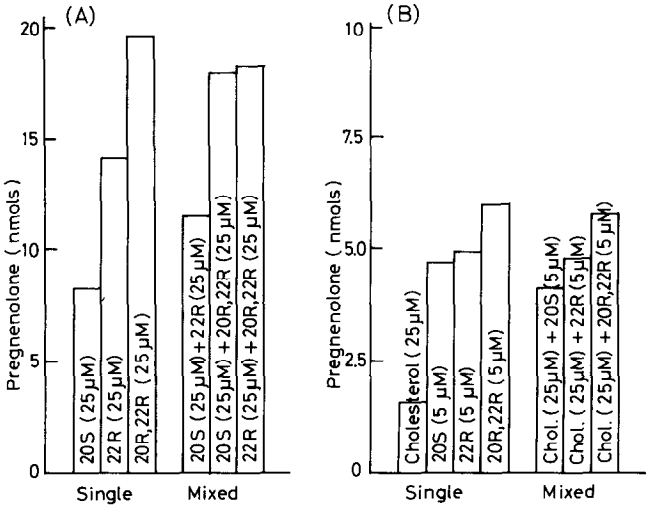


Fig. 3 Effect of Simultaneous Incubation of Two Substrates in the Pregnenolone-Producing Reaction of the Cytochrome P-450 (scc) : (A) Combinations of two hydroxycholesterols. (B) Combinations of cholesterol and hydroxycholesterols. All tubes were equalized with regard to dimethylformamide (20 μl). Note the scale of the ordinate of (A) is one-half of that of (B).

incubated separately. Similarly,when cholesterol of a saturating concentration was incubated with hydroxycholesterols of non-saturating concentrations,there was no further increase of pregnenolone production over the level attainable by hydroxycholesterol alone (Fig.3-B).

DISCUSSION

It is noted that $P\text{-}450_{scc}$ behaved as a neutral protein (pI = 6.8) upon isoelectrofocusing. The neutral nature of this P-450 should be compared with the acidic nature of its ancillary electron-carrier adrenodoxin (pI = 5.0)(12) as well as with the basic nature of adrenodoxin reductase (pI = 8.9)(13).

In the present study,20-hydroxylase,22-hydroxylase and 20,22-lyase activities were not separated from each other by isoelectrofocusing. This observation supports the hypothesis that the three

enzyme activities reside in one enzyme molecule. As the isoelectro-focusing presently employed might not be effective enough for splitting aggregated molecules,other independent proofs of the one enzyme three step hypothesis were pursued. Thus,the inhibition study of [20R,22S]-20,22-epoxycholesterol upon the hydroxycholesterols showed that the epoxide was competing for the enzyme (subunit or active site) in the same way with all the substrates,as indicated from the similar Ki value obtained. Although the possibility could not be excluded that three different enzymes have coincidentally similar affinity for the epoxide,it seems more probable that there is a common reaction site in a single enzyme catalyzing the three succesive steps. The third line of evidence for the one enzyme-three-step hypothesis was brought by measuring the velocity of reaction on a mixture of two substrates compared with that on each present singly (Fig.3-A,3-B). For example,if one enzyme (subunit or active site) had performed hydroxylation of cholesterol to provide the hydroxylated intermediate to another adjacent enzyme (subunit or active site), the incubation of a mixture of cholesterol and hydroxycholesterol should have yielded a greater amount of pregnenolone than the incubation of hydroxycholesterol only,because the capacity of pregnenolone producing system was not saturated with regard to hydroxycholesterol. The results observed above were not due to the limitation of the electrons supply,because adrenodoxin,adrenodoxin-reductase,NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were all added in large excess. Another probable interpretation of the results in the mixed substrate study is that the enzyme activity of hydroxylase moiety is depressed by the impact of binding of 20,22-dihydroxycholesterol to lyase moiety of the complex or vice versa. However,based on the above mentioned results of the isoelectro-focusing experiment and the kinetic inhibition study,it appears that

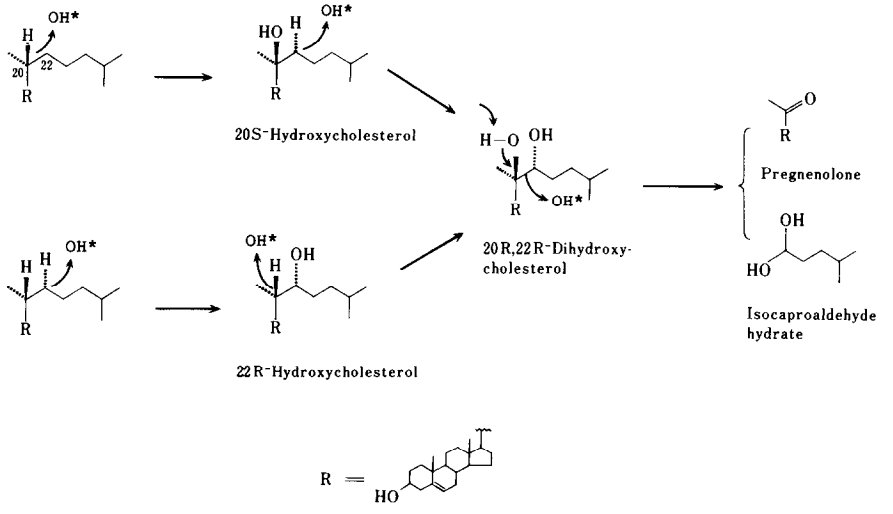


Fig. 4 Hypothetical Mechanism of Side-Chain Cleavage of Cholesterol by Bovine Adrenal Cytochrome P-450 (scc).

cholesterol and hydroxycholesterols are all competing at the same site of the enzyme. If this supposition is correct, the mechanism of cleavage of the carbon-carbon bond of the dihydroxylated compound must resemble the mechanism of hydroxylation at carbon-20 and carbon-22.

Accordingly, we propose in Fig.4 a hypothetical mechanism of side-chain cleavage of cholesterol, involving three consecutive electrophilic attacks of an activated species of oxygen (OH*).

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