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Unique property of liver mitochondrial P450 to catalyze the two physiologically important reactions involved in both cholesterol catabolism and vitamin D activation

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The cDNA for vitamin D 25-hydroxylase in rat liver mitochondria was transfected in COS cells in order to confirm our previous postulation that both 5β-cholestane-3α,7α,12α-triol 27-hydroxylation and vitamin D 25-hydroxylation are catalyzed by a common enzyme. As a result it was found that both enzyme activities could be reconstituted from the solubilized extract of mitochondria of these cells, NADPH, NADPH-adrenodoxin reductase and adrenodoxin, giving unequivocal evidence that the two enzyme activities are catalyzed by a common enzyme.

Vitamin D 25-hydroxylase; Cytochrome P450; 5β-Cholestane-3α,7α,12α-triol

1. INTRODUCTION

Through more than 20 years of research we have established that the P450 that hydroxylates 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, which are the intermediates in the conversion of cholesterol to bile acids, at position 27 [1-12] does exist in liver mitochondria. Meanwhile, Björkhem and Holmberg found an activity catalyzing vitamin D_3 25-hydroxylation in liver mitochondria, an important reaction which should be carried out before vitamin D_3 is converted into its active form, 1α,25-dihydroxyvitamin D_3 [13,14]. Since then a question has arisen whether these two P450s are identical or not. Recently, we have purified both enzymes to a homogeneity based on their catalytic activities [12,15]. To our surprise, both preparations revealed the enzyme activity of the other [16]. Since vitamin D 25-hydroxylation and cholestanetriol 27-hydroxylation are esentially different reactions, we considered at first that it was due to a contamination of the other enzyme. However, further studies based on enzyme kinetics as well as chromatographic properties led us to postulate that the two enzyme activities are catalyzed by a common active site of a common enzyme protein [16,17].

For further confirmation of this postulation we isolated a cDNA for liver mitochondrial P450 using the polyclonal antibodies against the enzyme purified by vitamin D 25-hydroxylation activity [18] and expressed the cDNA in COS cells to test whether mitochondria of the transfected cells can catalyze both enzyme activities.

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2. MATERIALS AND METHODS

5β-[^{3}H]Cholestane-3α,7α,12α-triol was synthesized as described elsewhere [12] and 1α-hydroxyvitamin D_3 was purchased from Duphar BV (Weesp, The Netherlands). Adrenodoxin and NADPH-adrenodoxin reductase were prepared according to the method of Suhara et al. [19,20]. The cDNA (1.9 kbp) for vitamin D 25-hydroxylase was obtained as described in a previous paper [18] and was ligated into the expression vector pSVL in *Sma*I site (designated as pSVL25). Simian COS 7 cells were transfected by the DEAE-dextran method [21]. To assay the activity of both cholestanetriol 27-hydroxylase and vitamin D 25-hydroxylase in vitro, cells were harvested 72 h after transfection, frozen at −80°C and then thawed to homogenize them with an equal volume of 0.25 M sucrose buffer (pH 7.4). Mitochondria were obtained by differential centrifugation and solubilized by 10% cholate. Reconstitution of the enzyme activities was performed as described previously, using NADPH, adrenodoxin and NADPH-adrenodoxin reductase as electron donors [12,15]. The solubilized mitochondrial extract was incubated with 5β-[^{3}H]cholestane-3α,7α,12α-triol or 1α-hydroxyvitamin D_3 (a derivative of vitamin D_3 synthesized for therapeutic use for vitamin D deficiency and is the preferred substrate for vitamin D 25-hydroxylase to vitamin D_3 since the turnover number toward the former is 8-10-fold higher than toward the latter). The product of the former was confirmed by thin layer chromatography [12]. The product of the latter was analyzed by both straight and reversed phase high performance liquid chromatographies (HPLC) as described previously [15]. For further identification of the product another straight phase solvent system (dichloromethane/propanol, 2:19) was also used to differentiate the product from a possible peroxidation byproduct, 10-oxo-19-nor-25-hydroxyvitamin D_3 [22]. Western blotting was performed by the method of Towbin et al. [23].

3. RESULTS AND DISCUSSION

The results are as shown in Table I. The solubilized mitochondrial fraction of pSVL25-transfected cells revealed both 5β-cholestane-3α,7α,12α-triol 27-hydroxylation and vitamin D 25-hydroxylation activities. The mitochondrial fraction obtained from control cells

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Table I

Expression of 5β-cholestane-3α,7α,12α-triol 27-hydroxylase and vitamin D 25-hydroxylase in COS 7 cells

Exp. no.		5β-Cholestane-3α,7α,12α,27-tetrol formed (A) (pmol/min/mg of protein)	1α,25-Dihidroxy-vitamin D_3 formed[a] (B) (pmol/min/mg of protein)	Ratio of the two enzyme activities (A/B)
1	pSVL25-COS	114	2.33	49
	Control-COS	6.7	0.17	39
2	pSVL25-COS	80.2	1.68	48
	Control-COS	7.9	0.21	38

Mitochondrial fraction was prepared from COS cells 72 h after transfecting the recombinant (pSVL25) or non-recombinant (control) expression vector. Reconstitution mixture contained solubilized mitochondria, NADPH-adrenodoxin reductase, adrenodoxin and NADPH and either substrate.

[a]Vitamin D 25-hydroxylase activity was assayed using 1α-hydroxy vitamin D_3 as substrate

transfected with only pSVL vector produced more than 10-fold lower amounts of products. The product of 1α-hydroxyvitamin D_3 showed the same retention time as that of the authentic sample both on straight and reversed phase high performance liquid chromatographies and the product of cholestanetriol showed the same R_f value as that of 5β-cholestane-3α,7α,12α,27-tetrol on thin layer chromatography. The ratio of the two hydroxylation activities was similar to those observed with rat liver mitochondria and the purified enzyme [17]. Western blotting analysis of the expressed protein in the transfected cells (Fig. 1, lane 2) showed that a single protein band (M_r 52 500) reactive with anti-vitamin D 25-hydroxylase antibodies was observable at the position corresponding to the purified vitamin D 25-hydroxylase (lane 3), whereas in control cells no such immunoreactive band was observable (lane 1). Since no other band reacting with the antibodies was observed, the expressed protein seemed to have been wel processed to be converted into the mature form.

During the course of our study, Dahlbäck and Wikvall [24] purified rabbit liver mitochondrial P450 based on absorption spectra and noticed that the preparation revealed 5β-cholestanetriol 26 (or 27)-hydroxylation activity together with very low vitamin D_3 25-hydroxylation activity. Since the turnover number of rabbit liver mitochondrial vitamin D_3 25-hydroxylase was unknown and the vitamin D_3 25-hydroxylation activity was very low, it was not possible to establish whether it was due to contamination by vitamin D_3 25-hydroxylase or not. Subsequently, Andersson et al. [25] isolated a cDNA encoding rabbit liver mitochondrial cholestanetriol 26 (or 27)-hydroxylase and expressed it in COS cells. However, nothing is described about the vitamin D_3 25-hydroxylation activity of the expressed protein. Meanwhile, Dahlbäck [26] observed that a monoclonal antibody raised against cholestanetriol 26 (or 27)-hydroxylase was able to bind only cholestanetriol 26 (or 27)-hydroxylase and to decrease the activity of 26 (or 27)-hydroxylation of 5β-cholestane-3α,7α,12α-triol, whereas it did not decrease vitamin D_3 25-hydroxylation activity. She thus suggested that different species of liver mitochondrial P450s are involved in the two hydroxylations. The reason of the discrepancy is presently unknown.

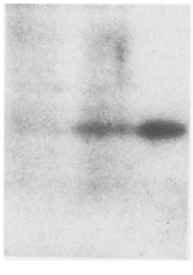

Fig. 1. Western blotting of the protein expressed in COS cells transfected with pSVL25. Mitochondrial proteins were subjected to the SDS-polyacrylamide gel electrophoresis and first incubated with the antibodies to rat liver mitochondrial vitamin D 25-hydroxylase and then visualized by incubating with ^{125}I-anti-mouse antibodies. Lane 1, control cells transfected with only pSVL vector (42 μg); Lane 2, transfected cells (42 μg); Lane 3, the purified rat liver mitochondrial vitamin D 25-hydroxylase (1.3 μg).

In conclusion, we have now obtained unequivocal evidence that a single protein expressed from a single cDNA for vitamin D 25-hydroxylase reveals the two enzyme activities, vitamin D_3 25-hydroxylation and 5β-cholestane-3α,7α,12α-triol 27-hydroxylation. Since these two reactions are functionally different, this may provide an example of broad functional specificity of cytochrome P450. This may be an additional versatility of cytochrome P450, which is already well-known for its versatility of showing broad substrate specificity and broad regio-selectivity.

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