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25-HYDROXYLATION OF VITAMIN D_3 BY A RECONSTITUTED SYSTEM FROM RAT LIVER MICROSOMES

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SUMMARY

Using isotope dilution - mass fragmentography as assay technique, it was shown that highly purified preparations of cytochrome P-450 from rat liver microsomes catalyzed 25-hydroxylation of vitamin D_3 when combined with NADPH-cytochrome P-450 reductase and a phospholipid. The rate of conversion was approximately linear with the amount of cytochrome P-450, and was considerably higher than the rate of conversion obtained with crude liver microsomes. The possibility is discussed that the microsomal fraction contains inhibitors of 25-hydroxylase activity, which may be of regulatory importance in vitamin D_3 metabolism.

The first step in the metabolism of vitamin D_3 to its active form is a 25-hydroxylation. This reaction is catalyzed by both microsomal and mitochondrial fraction of rat liver (1-4). The microsomal as well as the mitochondrial activity is inhibited by carbon monoxide (2,4) and both enzyme systems have been characterized as mixed function oxidases (4,5). In the case of mitochondrial activity, it has been conclusively shown that a species of cytochrome P-450 is involved and that ferredoxin and ferredoxin reductase function as electron carriers (6).

The nature of the microsomal activity has not yet been established and there are several conflicting reports concerning the properties of this enzyme system (1-3). This might in part be due to methodological difficulties. Thus the extent of conversion is very low and different authors have reported conversions varying from about 0.2 fmol/mg protein/min (3) up to about 1000 fmol/mg protein/min (2,4). With the exception of a recent report by Sulimovici et al. (3) there are no previous reports in which linearity has been shown between amount of microsomal protein and enzyme activity. In view of the fact that the system described by Sulimovici et al. had an activity less than 1/10 000 of that of the mitochondria, it can not be completely excluded that mitochondrial contaminants may have been responsible for part of the activity.

Using a highly accurate assay for 25-hydroxylase activity, based on isotope dilution - mass fragmentography, in general we found the microsomal activity to be less than 15% of the mitochondrial activity in homogenates of

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rat liver (7). There was no linearity between enzyme activity and microsomal protein, and the extent of conversion varied widely with different preparations. The varying degree of conversion might be due to inhibitors (7), and a more purified system should be preferred. In preliminary studies, crude preparations of microsomal cytochrome P-450 and NADPH-cytochrome P-450 reductase failed to give significant activity (7). As shown in the present work, however, highly purified microsomal cytochrome P-450 together with NADPH-cytochrome P-450 reductase gave a significant 25-hydroxylase activity towards vitamin D_3. There was at least an approximate linearity between enzyme activity and amount of cytochrome P-450.

EXPERIMENTAL PROCEDURE

Materials. Vitamin D_3 (cholecalciferol), sodium cholate, dilauroylglycero-3-phosphocholine and cofactors were obtained from Sigma Chemical Co. (St. Louis, Mo.). [26-$^2H_3$]25-Hydroxy vitamin D_3 was synthesized as described previously (8). 25-Hydroxy vitamin D_3 was a generous gift from Dr. J. Babcock (The Upjohn Co., Kalamazoo). Octylamine-Sepharose 4B was prepared from CNBr-activated Sepharose 4B (Pharmacia) as described by Cutrecasas (9). Emulgen 913 was obtained from Kao-Atlas (Tokyo) and DEAE-Sepharose CL-6B from Pharmacia (Uppsala, Sweden). Bio-Beads SM-2 and hydroxyapatite were from Bio-Rad Lab. (Richmond, Calif.). Hydroxyapatite was mixed with an equal amount of CF-1 cellulose powder (Whatman) before chromatography. All other chemicals were standard commercial high purity materials.

Methods. Male rats of the Wistar and Sprague-Dawley strain, weighing about 200 g, were used. Phenobarbital, 1 mg per ml of drinking water, was administered for 1 to 2 weeks. Liver homogenates, 33% (w/v), and microsomes were prepared and treated with potassium pyrophosphate as described by van der Hoeven and Coon (10). Cytochrome P-450 (from livers of Wistar rats) was prepared essentially as described by Guengerich (11,12) involving solubilization with sodium cholate, octylamine-Sepharose-hydroxyapatite and DEAE-Sepharose chromatography. Cytochrome P-450 was eluted from the hydroxyapatite column with 80 mM potassium phosphate buffer and applied to a DEAE-Sepharose column equilibrated with 5 mM phosphate buffer. The column was eluted with 5 mM and 35 mM potassium phosphate buffer. The 5 mM phosphate eluate was called cytochrome P-450 fraction A and the 35 mM phosphate eluate was called cytochrome P-450 fraction B. The cytochrome P-450 fractions were treated with Bio-Beads and calcium phosphate gel and dialyzed against 50 mM Tris-acetate buffer pH 7.4, containing 20% (v/v) glycerol and 0.1 mM EDTA.

The final cytochrome P-450 fractions A and B contained 11 and 18 nmol of cytochrome P-450 per mg of protein respectively. Cytochrome P-450 was determined as described by Omura and Sato (13) and protein was determined according to Lowry et al. (14). On SDS-polyacrylamide gel electrophoreses (15,16) cytochrome P-450 fraction A gave two protein bands corresponding to apparent molecular weights of 46,000 and 50,000. Cytochrome P-450 fraction B gave only one band, corresponding to an apparent molecular weight of 50,000. NADPH-Cytochrome P-450 reductase was prepared from livers of Sprague-Dawley rats according to Yasukochi and Masters (17) as described previously (18) and had a specific activity of 55 units per mg of protein. Reductase activity was assayed according to Masters et al. (19) at 30°C using 0.3 M potassium phosphate buffer. One unit corresponds to reduction of 1 umol of cytochrome c per minute. The NADPH-cytochrome P-450 reductase preparations used were homogenous upon electrophoresis. 100,000 x g Supernatant fraction was prepared in 0.1 M Tris-Cl buffer, pH 7.4, using livers of untreated Sprague-Dawley rats.

Incubations were performed at 37°C for 20 or 30 min with 50 μg of vitamin D_3 dissolved in 10 μl of acetone with 0.75 or 1.5 nmol of cytochrome P-450, 3 units of NADPH-cytochrome P-450 reductase, 25 μg of dilauroylglycero-3-phosphocholine and 1.5 μmol of NADPH in a total volume of 1.5 ml of 0.1 M Tris-Cl buffer pH 7.4. Cytochrome P-450, NADPH-cytochrome P-450 reductase and dilauroyl-3-phosphocholine were preincubated at 20°C for 3 min, vitamin D_3 and buffer were then added and the mixture was preincubated at 37 °C for 1 min. After that the reaction was started by addition of NADPH. The incubations were terminated by addition of 3 ml of toluene and 73 ng of $\left[26\text{-}^2H_3\right]$25-hydroxy vitamin D_3 was added as internal standard. The incubations were extracted and the product purified and derivatized as described previously (7). The first channel of the multiple ion detector was focused on the ion at m/e 586 and the second on the ion at m/e 589. The ion at m/e 586 corresponds to the molecular ion in the mass spectrum of the t-butyldimethylsilyl/trimethylsilyl derivative of unlabeled 25-hydroxy vitamin D_3 and the ion at m/e 589 corresponds to the corresponding ion in the mass spectrum of derivative of deuterium labeled 25-hydroxy vitamin D_3. The amount of unlabeled 25-hydroxy vitamin D_3 was calculated from the ratio between the peak at m/e 586 and the peak at m/e 589 using a standard curve (6,7).

RESULTS

Incubation of vitamin D_3 with a reconstituted system consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase, NADPH and a synthetic phospholipid resulted into a significant conversion into 25-hydroxy

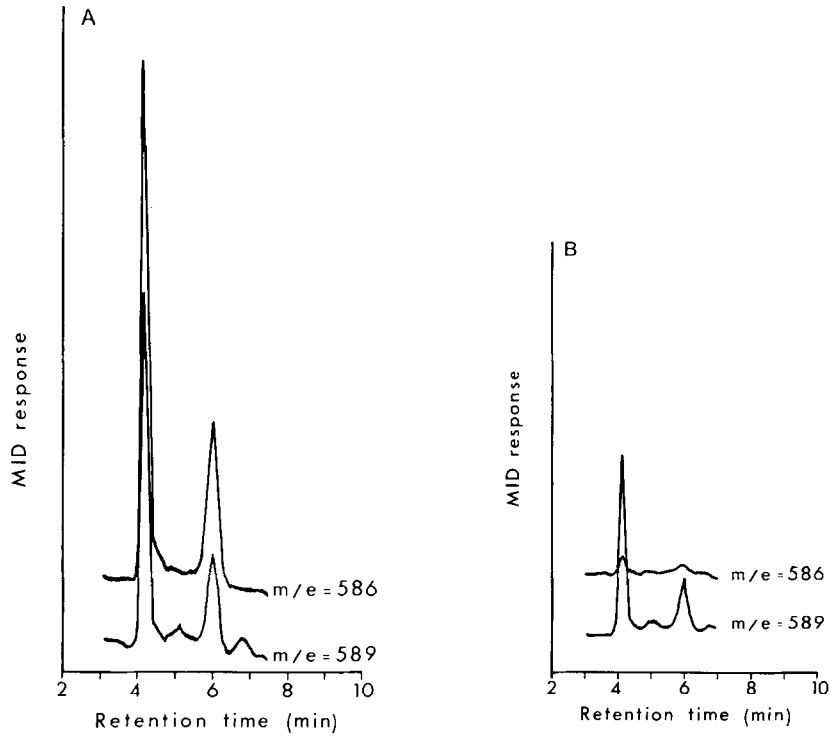

Fig. 1. MID recordings at m/e 586 and m/e 589 of the trimethylsilyl/t-butyldimethylsilyl derivative of incubation extract of vitamin D_3 with the complete reconstituted system (A) and the reconstituted system minus NADPH-cytochrome P-450 reductase (B). A fixed amount of $^2H_3$-labeled 25-hydroxy vitamin D_3 (73 ng) had been added to the incubation mixture prior to extraction (cf. Experimental Procedure).

vitamin D_3. Thus mass fragmentographic analysis of derivative of an extract of such an incubation, to which deuterium labeled 25-hydroxy vitamin D_3 had been added, gave two significant peaks in the tracing of the ion at m/e 586 (Fig. 1A). The two peaks obtained correspond to the pyro- and isopyro form of derivative of 25-hydroxy vitamin D_3. The two peaks obtained in the tracing of the ion at m/e 589 corespond to the deuterium labeled internal standard. Analysis of an extract of a corresponding incubation to which no NADPH-cytochrome P-450 reductase had been added, gave no significant peaks in the tracing at m/e 586 (Fig. 1B).

The exact quantity of unlabeled 25-hydroxy vitamin D_3 formed in the different incubations could be calculated from the ratio between the peak height at m/e 586 and the peak height at m/e 589, with use of a standard curve (cf. Experimental Procedure and ref. 6).

In order to ascertain the identity of the product, some incubations were analyzed by tracing of the ions at m/e 131 and m/e 134. The results obtained

Table 1.

Reconstitution of rat liver vitamin D_3 25-hydroxylase activity. The complete system contained: cytochrome P-450 0.75 nmol (fraction A) or 1.5 nmol (fraction B), NADPH-cytochrome P-450 reductase 3 units, phosphatidylcholine 25 µg, NADPH 1.5 µmol. Standard incubation conditions were used (cf. Methods).

System	Cytochrome P-450 fraction A	Cytochrome P-450 fraction B
	pmol formed per incubation	
Complete	108 (205)+	73
Minus NADPH-cytochrome P-450 reductase	3 (10)+	13
Minus cytochrome P-450	18	1
Minus NADPH	10	8
Minus phosphatidylcholine	93	20

+The figure within parentheses represents another experiment using the same conditions.

were almost identical with those obtained by tracing the ions at m/e 586 and m/e 589.

Table 1 summarizes different experiments with cytochrome P-450 fractions A and B. The degree of conversion was higher with A than with B. No significant formation of product was seen when cytochrome P-450, NADPH-cytochrome P-450 reductase or NADPH was omitted. The phospholipid had a stimulatory effect when using cytochrome P-450 fraction B but not when using cytochrome P-450 fraction A.

Addition of 100,000 x g supernatant fluid from a liver homogenate of an untreated rat reduced the enzyme activity of the reconstituted system with more than 50% (results not shown in Tabe).

Fig. 2 shows that the rate of conversion was at least approximately linear with the amount of added cytochrome P-450 fraction A (up to 0.5 nmol) and cytochrome P-450 fraction B (up to 0.5 nmol).

DISCUSSION

The identification of the product as 25-hydroxy vitamin D_3 is based on identical properties in thin-layer and gas chromatography and on the fact that a mass spectrum of the t-butyldimethylsilyl/trimethylsilyl ether contained the characteristic ions at m/e 586 (molecular ion) and at m/e 131 (cleavage between C_{24} and C_{25} in a C_{27}-steroid side chain containing an TMSiO-group at

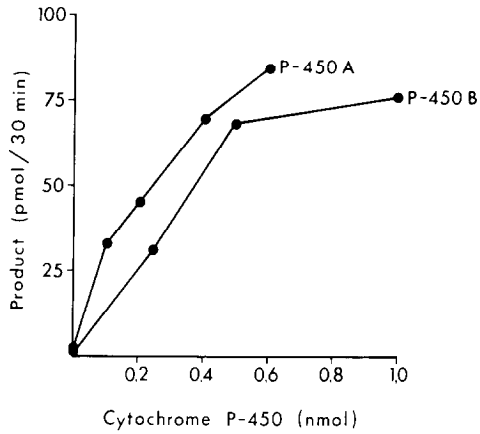

Fig. 2. Effect of amount of added cytochrome P-450 (cytochrome P-450 fraction A and B) on the rate of 25-hydroxylation of vitamin D_3 by the reconstituted system.

C_{25} (20)). The formation of both the pyro- and isopyro form of the derivative in the gas chromatography is another specific evidence for identity. In view of the very small amounts of product, it was not possible to obtain a full mass spectrum of the isolated material. From the above findings, however, it is highly unlikely that the product could be anything else than 25-hydroxy vitamin D_3.

There was a requirement for three components of the reconstituted system: cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH. The phospholipid had a significant stimulatory effect only when using the most homogenous of the two preparations of cytochrome P-450. Bhattacharyya and DeLuca have reported an almost absolute requirement of cytosol for microsomal 25-hydroxylase activity (1). Addition of cytosol to the present reconstituted system had an inhibitory effect on the activity. Lack of stimulatory effect by the cytosol on microsomal 25-hydroxylase activity has been reported also in some other publications (3,6,7).

The maximal activity obtained in the present purified system (about 5 pmol/nmol cytochrome P-450/min) was much higher than the activity obtained with crude microsomes (7), and was about 1/4 of the maximal activity obtained in a reconstituted system containing mitochondrial cytochrome P-450 (6). It should be borne in mind, however, that it was not ascertained that all conditions were optimal for assay in the present work. Experiments designed to define the optimal conditions are now in progress.

In contrast to most previous work with crude microsomes, there was an approximate linearity between amount of cytochrome P-450 and extent of conversion. In the evaluation of the results obtained (Fig. 2, Table 1), it must

be pointed out that the present method does not allow measurement of 25-hydroxy vitamin D_3 in amounts less than about 5-10 pmol, and that the precision is rather low in the determination of amounts lower than about 20 pmol.

In addition to vitamin D_3, the present preparations of cytochrome P-450 are also able to catalyze 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol (Hansson and Wikvall, unpublished observation, cf. ref. 21). Thus it is possible that the species of cytochrome P-450 involved in these hydroxylations is the same as that involved in 25-hydroxylation of vitamin D_3. It may be mentioned, however, that 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol by crude microsomes is not affected by rachitis (Björkhem and Holmberg, unpublished observation). According to DeLuca et-al. microsomal 25-hydroxylation of vitamin D_3 is stimulated by the rachitic state (1).

Interestingly, the rate of 25-hydroxylation of vitamin D_3 was much higher with the purified cytochrome P-450 fractions than with the crude microsomal fraction (cf. ref. 7). It seems likely that there are inhibitor(s) in the crude microsomal fraction, which are removed in the purification procedure. It can not be excluded that these inhibitor(s) are of regulatory importance, and this possibility is at present under study.

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REFERENCES

1. Bhattacharyya, M.H. and De Luca, H.F. (1974). Arch. Biochem. Biophys. 160, 58.

2 Delvin, E.E., Arabian, A. and Glorieux, F.H. (1978). Biochem. J. 172 417.

3. Sulimovici, S., Roginsky, M.S., Duffy, J.L. and Pfeifer, R.F. (1979). Arch. Biochim. Biophys. 195, 45.

4. Björkhem, I. and Holmberg, I. (1978). J. Biol. Chem. 253, 842.

5. Madhok, T.C., Schnoes, H.K. and DeLuca, H.F. (1978). Biochem. J. 175, 479.

6. Pedersen, J.I., Holmberg, I. and Björkhem, I. (1979). FEBS Lett. 98, 394.

7. Björkhem, I. and Holmberg, I. J. Biol. Chem. - in press.

8. Björkhem, I. and Holmberg, I. (1976). Clin. Chim. Acta 68, 215.

9. Cuatrecasas, P. J. (1970). J. Biol. Chem. 245, 3059.

10. van der Hoeven, T.A. and Coon, M.J. (1974). J. Biol. Chem. 249, 6302.

11. Guengerich, F.P. (1977). J. Biol. Chem. 252, 3970.

12. Guengerich, F.P. (1978). J. Biol. Chem. 253, 7931.

13. Omura, T. and Sato, R. (1969). J. Biol. Chem. 239, 2370.

14. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R. (1951). J. Biol. Chem. 193, 265.

15. Laemmli, U.K. (1970). Nature 227, 680.

16. Haugen, D.A., van der Hoeven, T.A. and Coon, M.J. (1975). J. Biol. Chem. 250, 3567.

17. Yasukochi, Y. and Masters, B.S.S. (1976). J. Biol. Chem. 251, 5337.

18. Hansson, R. and Wikvall, K. (1979). Eur. J. Biochem. 93, 419.

19. Masters, B.S.S., Williams Jr., C.H. and Kamin, H. (1967). Methods Enzymol. 10, 565.

20. Suda, T., DeLuca, H.F., Schnoes, H. and Blunt, J.W. (1969). Biochem. Biophys. Res. Commun. 35, 182.

21. Björkhem, I., Danielsson, H. and Wikvall, K. (1976). J. Biol. Chem. 25, 3495.