ON THE RATE-LIMITING STEP IN ω-HYDROXYLATION OF LAURIC ACID.

Ingemar Björkhem and Mats Hamberg

Department of Chemistry, Karolinska Institutet and Department of Medical Chemistry, Royal Veterinary College, Stockholm, Sweden.

Received March 3, 1972

SUMMARY

Incubation of lauric acid with rat liver microsomes and NADPH yielded a mixture of 11-D-, 11-L-, and 12-hydroxylauric acids. 11-L- and 11-D-hydroxylations of 11-$^2H_2$-lauric acid were accompanied by marked isotope effects, indicating that in these reactions splitting of the C-H bond is rate limiting. When 11- and 12-hydroxylations of lauric acid were carried out in D_2O, 12-hydroxylation but not 11-hydroxylation was inhibited, showing that different steps are rate limiting in the two reactions.

The rate-limiting step in hydroxylations catalyzed by the microsomal fraction fortified with NADPH has been defined only in a few instances (1-4). The possibility that breaking of the C-H bond in the substrate is rate limiting in such hydroxylations can be studied with specifically deuterium- or tritium-labeled substrates. Microsomal hydroxylations are known to involve direct elimination of hydrogen from the substrate, and substitution of this hydrogen with deuterium or tritium will lead to a kinetic isotope effect for the over-all reaction, provided the loss of the isotope occurs in a rate-limiting step. Recently (4), it was shown that microsomal 9-hydroxylation (ω2-hydroxylation) of 9-$^2H_2$-decanoic acid involved a marked isotope effect, indicating that breaking of the C-H bond in the substrate is rate limiting in ω2-hydroxylation of decanoic acid. On the other hand, microsomal 10-hydroxylation of 10-$^2H_3$-decanoic acid (ω1-hydroxylation) occurred without isotope effect, showing that breaking of the C-H bond is not rate limiting in this reaction. It was considered of interest to examine if the same situation prevails for ω1- and ω2-

hydroxylation of lauric acid. In contrast to decanoic acid which is hydroxylated in the ω2-position only to a small extent, lauric acid is hydroxylated as efficiently in the ω2- as in the ω1-position (11- and 12-positions respectively) by the microsomal fraction of a rat liver homogenate fortified with NADPH (5). A further reason to study the rate-limiting step in ω-hydroxylation of lauric acid is that lauric acid gives type I spectral changes when mixed with microsomes (6). It has been suggested from the results of studies of microsomal hydroxylations of drugs that the rate-limiting step in microsomal hydroxylations of type I compounds is the reduction of the cytochrome P-450-substrate complex (3). It has also recently been shown that some microsomal hydroxylations which involve reduction of cytochrome P-450-substrate complex as rate-limiting step are inhibited by 20-50% when the hydroxylations are performed in a medium with D_2O (7).

The present work deals with ω2-hydroxylation of lauric acid, specifically labeled with deuterium in the ω2-position, and with the effect of D_2O on ω1- and ω2-hydroxylations of lauric and decanoic acids.

MATERIALS AND METHODS

1-^{14}C-Lauric acid (20 μC/mg) and 1-^{14}C-decanoic acid (2 μC/mg) were obtained from Radiochemical Centre and were purified by silic acid chromatography (4, 5). 11-$^{2}H_2$-Lauric acid was prepared by anodic coupling of sodium 2-$^{2}H_2$-propionate and methyl hydrogen undecadioate followed by silic-acid chromatography and reversed-phase partition chromatography (4). The identity of the material obtained (m.p. 42.5-43.5°, lauric acid, 42.5-43.5°) was established by thin layer chromatography and gas-liquid chromatography (2.2% QF-1, methyl ester) as well as mass spectrometry. The mass spectrometric analysis showed that the material mainly consisted of dideuterated molecules (cf. Table I). The procedure for preparation of the microsomal fraction was the same

TABLE I. Experiments with 11-$^{2}H_2$-lauric acid and mixtures of 11-$^{2}H_2$- and 11-$^{1}H_2$-lauric acids.

Compounds	Isotope content		
	% of molecules with		
	0 deuterium	1 deuterium	2 deuterium
11-$^{2}H_2$-Lauric acid incubated	0.6	4.4	95.0
D-11-hydroxylauric acid isolated	2	98	0
L-11-hydroxylauric acid isolated	2	98	0
11-$^{2}H_2$ + 11-$^{1}H_2$-Lauric acid incubated	39.5	2.5	58.0
D-11-hydroxylauric acid isolated	71	29	0
L-11-hydroxylauric acid isolated	73	27	0

as that described previously (4). In incubations with lauric acid, 100 μg of the acid, dissolved in 50 μl of acetone, was added to 1 ml of microsomal suspension (containing about 4 mg of protein) together with 3 μmoles of NADPH in a total volume of 3 ml of 0.1 M potassium phosphate buffer, pH 7.4. Incubations with decanoic acid were performed in the same way, with the exception that 50 μg were used. In some experiments, (cf. Table II) the microsomal fraction was prepared in a medium made up in deuterated water (99.8% pure, Norsk Hydro) instead of water. The incubations were run at 37° for 15 min and were terminated and extracted as described previously (5). Extracts from incubations designed to study the effect of deuterated water were treated with diazomethane and the amount of ω1- and ω2-hydroxylated products was determined with radio-gas chromatography under the conditions described previously (5).

TABLE II. Effects of D_2O on ω1- and ω2-hydroxylation of lauric and decanoic acid.

Substrate	ω 1-Hydroxylated product in		ω 2-Hydroxylated product in	
	H_2O	D_2O	H_2O	D_2O
	% Conversion			
Lauric acid	15.2±0.9	10.1±0.8	10.0±0.7	10.0±0.6
Decanoic acid	40.2±1.3	28.1±0.6	5.2±0.4	6.4±0.5

ω 1- and ω2-Hydroxylated products were assayed as described in Experimental. The results given are the means of 4 experiments with standard deviation of the mean.

Extracts from incubations in which the 11-L- and 11-D-isomers were separated, were subjected to silic-acid chromatography as described previously (4). The hydroxylated products eluted from the column were treated with diazomethane and further purified with thin-layer chromatography (solvent, diethyl ether - petroleum ether, 3:2 (v/v)). The methylated hydroxyacids were treated with (R)-1-phenylethyl isocyanate (4) and the resulting N-(1-phenylethyl) urethane derivatives obtained were subjected to gas-liquid chromatography with a column of 2.2% QF-1 on Gaschrom Q (cf. 4). The content of deuterium in 11-L- and 11-D-hydroxylauric acid isolated from incubations with 11-$^2H_2$-lauric acid or mixtures of 11-$^2H_2$- and 11-$^1H_2$-lauric acid was determined with combined gas chromatography - mass spectrometry. The intensity of the ions at m/e 362 and 363 vs time was recorded with the accelerating voltage alternator equipment of an LKB 9000 instrument. The isotope compositions were calculated from the area of ions 362/363 after correction for the contribution to m/e 363 of the unlabeled derivative (24%) and for the contri-

bution to m/e 362 of the deuterium-labeled derivative (2%). The ion at m/e 362 of unlabeled N-(1-phenylethyl) urethane derivative of methyl 11-hydroxylauric acid is formed by elimination of CH_3 of phenylethyl moiety of the derivative (cf. 4).

RESULTS

1-^{14}C-Lauric acid was incubated with the microsomal fraction fortified with NADPH and 11-hydroxylauric acid (35-45% of hydroxylated products) and 12-hydroxylauric acid (55-65% of hydroxylated products) were isolated by silic-acid chromatography (4). The identity of the products as 11- and 12-hydroxylauric acid was established by combined

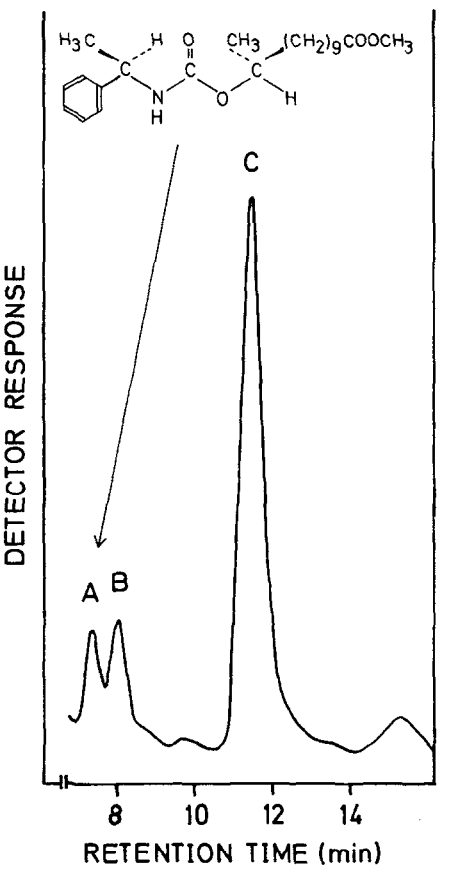

Fig. 1. Partial gas chromatogram of N-(1-phenylethyl) urethane derivative of methyl 11-L-hydroxylaurate (A), methyl 11-D-hydroxylaurate (B) and methyl 12-hydroxylaurate (C) biosynthetized from 11-$^2H_2$ + 11-$^1H_2$-lauric acid (cf. Table I).

gas chromatography - mass spectrometry of methyl esters of trimethylsilyl ethers which showed a fragmentation pattern expected for methyl esters of trimethylsilyl ethers of ω1- and ω2-hydroxylated fatty acids (4, 8). The methyl esters of purified 11- and 12-hydroxylauric acid were converted into N-(1-phenylethyl) urethane derivatives and subjected to gas-liquid chromatography. The N-(1-phenylethyl) urethane derivatives of methyl 11-hydroxylaurate separated into two peaks corresponding to the D- and L-isomers of 11-hydroxylauric acid (cf. Fig. 1). The fragmentation pattern of the N-(1-phenylethyl) urethane derivatives of methyl 11- and 12-hydroxylaurate was the same as that of the corresponding derivatives of methyl 9- and 10-hydroxydecanoate (4). Authentic, optically active 11-hydroxylauric acid has not yet been prepared and analyzed as urethane derivative. However, by analogy with earlier data on gas-liquid chromatographic behaviour of diastereoisomeric urethane derivatives of a number of D- and L-ω2-hydroxyfatty acids and 2-alkanols it seems highly probable, that the derivative of methyl 11-L-hydroxylaurate will appear with a shorter retention time than that of methyl 11-D-hydroxylaurate (4, 9). The enzymatically formed 11-hydroxylauric acid would then be a mixture of 40-44% of the L-isomer and 56-60% of the D-isomer (cf. Fig. 1).

Conversion of 11-$^{2}H_{2}$-lauric acid into 11-D- and 11-L-hydroxylauric acid resulted in loss of one atom of deuterium (Table I). This loss of deuterium was accompanied by an isotope effect, as 11-hydroxylauric acid accounted for less than 10% of the products and 12-hydroxylauric for more than 90% of the products from 11-$^{2}H_{2}$-lauric acid. The presence of an isotope effect in the conversion was further shown by incubation of a mixture of 11-$^{2}H_{2}$-lauric acid and 11-$^{1}H_{2}$-lauric acid. The 11-D and 11-L hydroxylauric acids formed from such mixtures had a ratio labeled molecules to unlabeled molecules which was only about 25-30% of the corresponding ratio in the material incubated (Table I).

The effect of deuterated water on 11- and 12-hydroxylation of lauric acid as well as 9- and 10-hydroxylation of decanoic acid is shown in Table II. It is evident that with both substrates ω1-hydroxylation was inhibited by about 30% whereas ω2-hydroxylation was not inhibited.

DISCUSSION

The present work confirms the previous finding that lauric acid is ω2-hydroxylated more efficiently than decanoic acid by the microsomal fraction. In addition, it was shown that the 11-hydroxylauric acid obtained was a mixture containing 40-44% of the 11-L-isomer and 56-60% of the 11-D-isomer. It is evident that the stereospecificity of ω2-hydroxylation is dependent on the chain length of the fatty acid as microsomal ω2-hydroxylation of decanoic acid yields 9-hydroxydecanoic acid which is a mixture consisting of about 25% of the 9-D- and 75% of the 9-L-isomer (4).

The isotope effect in microsomal 11-D- and 11-L-hydroxylation of 11-$^2H_2$-lauric acid was of such a magnitude (10) that it is most likely that splitting of the C-H bond in the substrate is the rate-limiting step in the over-all hydroxylation. The hypothesis that microsomal hydroxylation of type I compounds involves reduction of cytochrome P-450-substrate complex as rate-limiting step (3) is thus not valid for all microsomal hydroxylations. It might be mentioned that microsomal hydroxylation of taurodeoxycholic acid, which also is a type I compound (11), may also involve splitting of the C-H bond in the substrate as rate limiting step (12).

The present work also shows that deuterated water might influence the rate-limiting step in ω1-hydroxylation of lauric and decanoic acids as this hydroxylation was inhibited when the reactions were performed in deuterated water. The deuterated water might influence the rate-limiting step either by affecting protonolysis or hydratisation of the enzyme.

In a recent study the rate-limiting step in several microsomal hydroxylations of steroids was investigated (13). The results of this study led to the suggestion that common features of microsomal hydroxylations in which splitting of the C-H bond is rate limiting are a relatively low sensitivity towards carbon monoxide and a low degree of inhibition in the presence of deuterated water. This concept is supported by the present finding that ω2-hydroxylations of lauric and decanoic acids are not inhibited by deuterated water and the previous finding (5) that these hydroxylations show a relatively low sensitivity towards carbon monoxide.

ACKNOWLEDGEMENT

This work has been supported by the Swedish Medical Research Council (Projects 13X-3141 and 13X-2828). The skilful technical assistance of Miss Irene Ferdman and Miss Kerstin Wahlberg is gratefully acknowledged.

REFERENCES

1. Holzman, J.L., Gram, T.E., Gigon, P.L., and Gillette, J.R., Biochem. J., 110, 407 (1968).
2. Schenkman, J.B., Chem. Biol. Interactions, 3, 306 (1971).
3. Gillette, J.R., Metabolism, 20, 215 (1971).
4. Hamberg, M. and Björkhem, I., J. Biol. Chem., 246, 7411 (1971).
5. Björkhem, I. and Danielsson, Eur. J. Biochem., 17, 450 (1970).
6. Lu, A.Y.H., Junk, K.W. and Coon, M., J. Biol. Chem., 244, 3714 (1969).
7. Holzman, J.L. and Carr, M.L., Life Sci., 9, 1033 (1970).
8. Eglinton, G., Hunneman, D.H. and McCormick, A., Org. Mass Spectrometry, 1, 593 (1968).
9. Hamberg, M., Chem. Phys. Lipids, 6, 152 (1971).
10. Melander, L., Isotope Effects on Reaction Rates, New York (1960) Ronald Press.
11. Hutterer, F., Denk, H., Bacchin, P.G., Schenkman, J.B., Schaffner, F. and Popper, H., Life Sci., 9, 877 (1970).
12. Björkhem, I., Eur. J. Biochem., 18, 299 (1971).
13. Björkhem, I., Eur. J. Biochem., in press (1972).