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15-HYDROXYEICOSATETRAENOIC ACID DEHYDROGENASE ACTIVITY IN MICROSOMAL FRACTION OF MOUSE LIVER HOMOGENATE

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Summary. The microsomal fraction of mouse liver homogenate showed NAD(P)-dependent dehydrogenase activity involved in the conversion of 15-hydroxyeicosatetraenoic acid to 15-ketoeicosatetraenoic acid, which was determined quantitatively by HPLC assay. This enzyme, tightly bound to membranes and relatively stable, possessed apparent values of Km of 8.3 μM and Vmax of 2.8 nmoles /mg.min in the oxidation of 15-HETE ,and gave an optimum pH of 9.8. Additionally, the enzyme, not susceptible to the inhibition by indomethacin and showing a similar cosubstrate specificity between NAD and NADP, utilized other hydroxylated eicosanoids as substrates, based on HPLC analyses.

In mammalian cells, arachidonic acid is known to be oxygenated to generate the bioactive metabolites , prostaglandins (1), leukotrienes (2), etc.. Subsequently, 5,15-diHETE (3), 14,15-diHETE and lipoxins (4), which include 15-hydroxyl group commonly, were also reported as bioactive products derived from 15-lipoxygenase-catalyzed oxidation of arachidonic acid.

Whereas the inactivation pathways of prostaglandins and leukotrienes are well defined (5,6), little is known about the metabolism of other 15-hydroxylated eicosatetraenoic acids, 15-HETE, 5,15-diHETE , 14,15-diHETE and lipoxins. Previously (7,8), 15-hydroxyprostaglandin dehydrogenase responsible for the inactivation of prostaglandins by oxidizing the 15-hydroxyl group of prostaglandins was reported to be present in the cytosol fractions of prostate, kidney etc.. Recently (9), 3$\mathcal{L}$-hydroxysteroid dehydrogenase from liver cytosol was also reported to possess the activity to oxidize 15-hydroxy-prostaglandins, although the activity toward the other 15-hydroxylated eicosatetraenoic acids was not investigated. In this study,we show that a microsomal membrane-bound dehydrogenase activity responsible for the oxidation of 15-HETE is determined quantitatively by RP-HPLC assay, and we propose that the enzyme may be involved in the NAD(P)-dependent oxidative metabolism of some 15-hydroxylated eicosanoids.

Abbreviations : PGB_2, prostaglandin B ; 15-HETE , 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid ; 15-KETE , 15-oxo-5,8,11-cis-13-trans-eicosatetraenic acid ; 5,15-diHETE, 5(S),15(S)-dihydroxy-6,13-trans-8,11-cis-eicosatetraenoic acid ; 8,15-diHETE , 8(S),15(S)-dihydroxy-5,11-trans-9,13-cis-eicosatetraenoic acid ; 14,15-diHETE , 14(R),15(S)-dihydroxy-5,8-cis-10,12-trans-eicosatetraenoic acid ; 11-trans-lipoxin A, 5(S),6(S,R),15(S)-trihydroxy-7,9,11,13-trans-eicosatetraenoic acid ; RP-HPLC , reverse phase-HPLC ; TLC , thin layer chromatography.

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Materials and Methods

Hydroxysteroid dehydrogenase (Grade II), soybean lipoxygenase (Type I), NAD, NADP, PGB_2 and arachidonic acid were products of Sigma Chemical Co.. Methanol and ethyl ether were from Aldrich and Tedia Chemical Co., respectively. 15-HETE, 5,15-diHETE, 8,15-diHETE and 11-trans lipoxin A were prepared as described, respectively (10,11). 15-Ketoeicosatetraenoic acid (15-KETE) was non-enzymatically produced as reported (12), and enzymatically prepared by 30 min-incubation of 15-HETE (1 mM) with microbial hydroxysteroid dehydrogenase (10 units) in 30 ml of 0.1 M pyrophosphate buffer, pH 8.9 containing 2 mM NAD, followed by extraction with ether, separation by TLC (petroleum ether : ethyl ether : acetic acid = 50 : 50 : 1), methylation, hydrogenation over PtO_2 and GC/MS analysis by a JEOL JMS-DX spectrometer equipped with a 1 % OV-1 column as described (12). Separately, liver homogenate(1 g) in 30 ml of 0.1 M phosphate buffer, pH 7.0 including 2 mM NAD was incubated with 15-HETE (1 mM) , and the reaction product was analysed as above.

Development of the RP-HPLC assay for the quantitative determination of 15-KETE was conducted as follows ; various concentrations of 15-KETE were added to 1 ml of 50 mM phophate buffer, pH 7.4 (sample A) and the buffer containing 1 % albumin (sample B). Then, each mixture was acidified with 50 ul of 1 M citric acid , admixed with PGB_2 (2.5 μg) as an internal standard, and then extracted with 5 ml of ether. The extract was concentrated and injected into RP-HPLC system as described (11). The concentration of 15-KETE (λmax at 278 nm, ca. 30,000) (13) was determined from the calibration curve, which was constructed by plotting the peak area ratios versus the concentrations. The percentage recovery was determined by comparing the amounts of 15-KETE extracted from sample A and B.

For enzyme preparation , the frozen mouse liver was homogenized in cold 0.25 M sucrose solution containing 50 mM Tris buffer and 1 mM EDTA. Subcellular fractions were prepared by differential centrifugation (14). The microsomal fraction was sonicated for 30 sec (ARTEK dismembrator), sedimented at 120,000 X g for 30 min , and the pellet was used for the enzyme study. The amount of protein was determined by Lowry method (15).

15-HETE dehydrogenase activity was determined by RP-HPLC assay. Unless otherwise noted, assays were conducted in a final volume of 1 ml containing 50 mM phosphate buffer (pH 7.4), 200 μM 15-HETE and 2 mM NAD. The reaction was started by adding the sonicated microsome, continued for 10 min at 37°C under shaking , and stopped by the addition of 50 μl of 1 M citric acid . The enzyme activity was expressed in nmoles of 15-KETE produced every min, and the kinetic parameters and properties of the dehydrogenase were obtained as reported (14).

To examine the oxidation of the multiple-hydroxylated eicosanoids by dehydrogenases , the dihydroxy acids (200 μM) or lipoxin (10 μM) was incubated with the microsomal preparation (1 mg protein) or microbial hydroxysteroid dehydrogenase (2 units) at 37°C for 30 min in 1 ml of 50 mM Tris buffer, pH 8.8 including 2 mM NAD. The reaction products were extracted as described in RP-HPLC assay except the acidification with 100 μl of 1 M citric acid, and applied onto C_{18} column (11). Separately, the absorbance change at 340 nm during the incubation with hydroxysteroid dehydrogenase was monitored over 30 min at r.t. with a Gilford spectrophotometer.

Results and Discussion

When 15-HETE was incubated with liver homogenate , a more non-polar product (Rf, 0.44) than 15-HETE (Rf, 0.37) appeared as a major product in TLC analysis (12). The product (λ max, 278 nm) , which was also formed from the incubation of 15-HETE with microbial hydroxysteroid dehydrogenase , was methylated with diazomethane, hydrogenated over PtO_2, and then subjected to GC/MS analysis . The characteristic ions were observed at m/e 340 (M), 227 [$\cdot(CH_2)_{12}-CO_2CH_3$] 114 (M - 226) , 99 [$O{=}\dot{C}-(CH_2)_4-CH_3$] and 71 [$\cdot(CH_2)_4-CH_3$], in good agreement with the spectrum as reported (12). The structure of this product was also confirmed by the preparation of standard specimen (12). After having established the structure of the product, we turned to the development of RP-HPLC assay to determine quantitatively the activity of 15-HETE dehydrogenase .

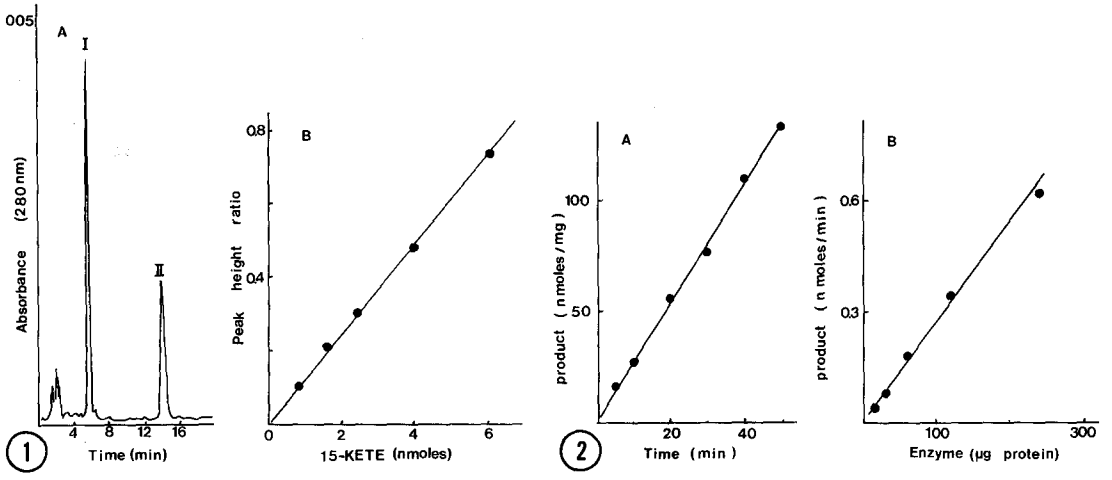

Fig. 1. A) RP-HPLC chromatogram of PGB_2(I) and 15-KETE (II) on the μ Bondapak column (3.9 X 300 mm) , eluted at a flow rate of 1 ml per min with the mobile phase (methanol : water : acetic acid = 75 : 25 : 0.01).
B) Calibration curve for 15-KETE.
Fig. 2. A) Time course of 15-KETE formation with the microsomal fraction.
B) Effect of the enzyme amount on the rate of 15-KETE formation.

Fig. 1 illustrates the typical chromatogram of PGB_2 as an internal standard and 15-ketoeicosatetraenoic acid (15-KETE) , which eluted at 6 min and 14 min , respectively. As shown in Fig.2 , a good linear relationship between amount of 15-KETE and the peak height ratio of 15-KETE to internal standard was observed. The range of coefficient of variance for this method was within 5 % for intraday assay. Recovery determined by comparing the yields of 15-KETE added to the albumin-including buffer and to the buffer only ranges between 95 and 98 %. No appreciable degradation of 15-KETE was seen up to 3 days at 4° C .

When the 15-HETE dehydrogenase activity was measured by RP-HPLC assay, the total activity in the frozen liver amounts to 150-250 nmoles/min .

Table 1 demonstrates the distribution of 15-HETE dehydrogenase activity in the three fractions of liver homogenate. Interestingly, it was observed that the enzyme of the microsomal fraction was very stable, whereas the enzyme activities in both cytosol and mitochondrial portions decreased gradually during storage at 4° C and less than 10 % of total activity remained after one day, respectively.

In this investigation , the emphasis was on the microsomal fraction. Attempts to solubilize the enzyme from microsomes using sonication, papain or 0.2 % Triton X-100 were unsuccessful, while microsomal esterase (> 90 %) was easily solubilized by sonication. These observations suggest that the 15-HETE dehydrogenase activity is tightly bound to the membrane. In time course studies (Fig. 2A), the formation of 15-ketoeicosatetraenoic acid increased linearly with the incubation time for at least

Table 1

Subcellular distribution of the 15-HETE dehydrogenase activity in mouse liver homogenate

Fraction	Total Protein	Total Activity (nmoles.min)	Specific Activity (nmoles.min .mg)
Microsome	28.2 (16.8)	47.7	1.69 (2.8)
Mitochondria	16.4	29.1	1.77
Cytosol	36.6	163.4	4.46

Values are the average of triplicate assays, and the parentheses show the specific activity of the sonicated microsomal pellet.

50 min. Fig. 2B demonstrates that the amount of 15-ketoeicosatetraenoic acid produced from 15-HETE by the microsomal fraction increased proportionally to the protein concentration. The effect of 15-HETE concentration on the 15-HETE dehydrogenase is illustrated in Fig. 3A. The apparent Km and Vmax for 15-HETE were determined to be 8.3 μM and 2.8 nmoles /mg.min. No great difference of specificity between NAD and NADP was observed. At a high concentration (200 μM) of 15-HETE , apparent Michaelis constant (Km) for NAD were determined to be 60 μM. Bioconversion of 15-HETE was negligible in the absence of added pyridine nucleotide. The pH dependence of this dehydrogenase was examined using three different buffer system, 0.05 M sodium phophate (7.6 - 8.4), 0.05 M Tris-HCl (8.4 - 9.2) and 0.05 M glycine-NaOH (pH 9.6 - 11.2). The optimal pH of the enzyme for the oxidation of 15-HETE was found to be around 9.8 (Fig. 3B). The properties of the microsomal dehydrogenase seem to be similar to those of prostaglandin dehydrogenase (9). However, this enzyme was not inhibited significantly by 10 μM indomethacin, though indomethacin is known to inhibit the 9-hydroxy-prostaglandin dehydrogenase strongly (9). Besides, the microsomal localization and NAD(P)-specificy

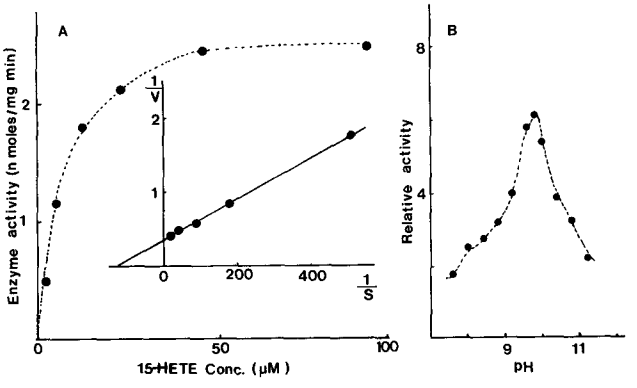

Fig. 3. A) Effect of 15-HETE concentration on the rate of 15-KETE formation. Inset , Lineweaver-Burk plot of 1/15-HETE concentration (mM) against 1/velocity (nmoles / mg.min).
B) Effect of pH on the rate of 15-KETE formation.

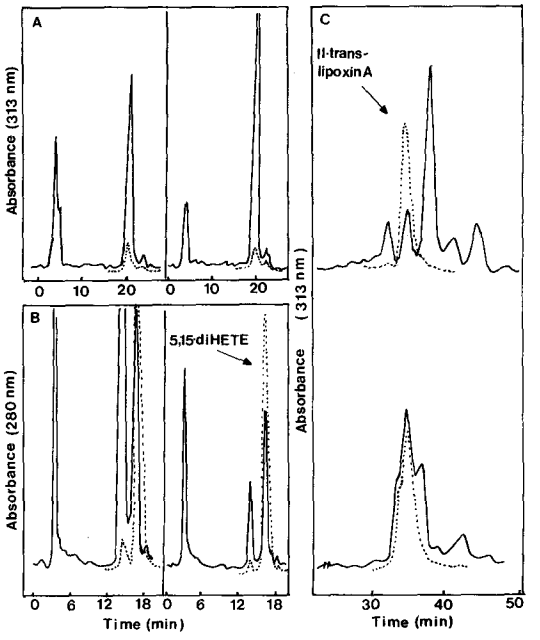

Fig. 4. A) RP-HPLC profile of products derived from the oxidation of 8,15-diHETE by dehydrogenases. Left, oxidation by hydroxysteroid dehydrogenase (——) and nonenzymatic oxidation (-----). Right, oxidation by microsomal dehydrogenase (——) and nonenzymatic oxidation (-----). Non-enzymatic oxidation was performed in the absence of NAD or with boiled enzyme. HPLC was carried out with μ Bondapak column (3.9 X 300 mm), eluted at a flow rate of 1 ml with a mobile phase (methanol : water : acetic acid = 65 : 35 : 0.01).
B) RP-HPLC profile of products formed from enzymatic oxidation of 5,15-diHETE. Experimental procedures are the same as described above.
C) RP-HPLC profile of products formed, the oxidation of 11-trans-lipoxin A. Upper, oxidation by hydroxysteroid dehydrogenase (——). Downer, oxidation by microsomal dehydrogenase (——) and non-enzymatic oxidation with boiled enzyme (-----). HPLC was performed with a Resolve column (3.9 X 150 mm), eluted with a mobile mixture (methanol : acetonitrile : water : acetic acid = 100 : 100 : 270 : 0.2) at 1 ml/min.

of this enzyme are in contrast to the cytosolic localization and NAD-specificity of 15-hydroxy-prostaglandin dehydrogenase from placenta (7). Based on these observations, it is assumed that the 15-HETE dehydrogenase of the microsomal fraction was different from prostaglandin dehydrogenase. To see whether the microsomal dehydrogenase can oxidize other 15-hydroxylated eicosatetraenoic acids, the various eicosanoic acids were incubated with the microsomal enzyme , and then the reaction products were subjected to RP-HPLC analysis (Fig. 4 A,B and C). The microsomal dehydrogenase-catalyzed oxidation of these eicosanoic acids was evaluated by comparison with the oxidation of the respective acid by the microbial hydroxysteroid dehydrogenase, which exhibited the time-dependent increment of absorbance at 340 nm during the incubation with the respective hydroxylated acid.

Fig. 4 demonstrates that there is a similarity between the HPLC profiles of oxidation products generated from the incubation of the respective acid with two different enzymes , suggesting that the microsomal dehydrogenase oxidizes the hydroxylated eicosanoic acids in the same way as does

the hydroxysteroid dehydrogenase, although the positional selectivity in the oxidation of hydroxyl groups of the hydroxylated eicosanoic acids was not established. It is thus conceivable that the microsomal dehydrogenase-catalized oxidation pathway may correspond to one of the metabolic pathways of the bioactive multiple-hydroxylated eicosatetraenoic acids. In a separate experiment, it was found unexpectedly that PGB_2 was not utilized by microsomal dehydrogenase, whereas it was easily oxdized by microbial hydroxysteroid dehydrogenase.

Based on these results , it is supposed that the microsomal dehydrogenase , which can be determined quantitatively by RP-HPLC assay , is one of the enzymes involved in the oxidative metabolism of 15-hydroxylated eicosatetraenoic acids other than prostaglandins.

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