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NADH-DEPENDENT CINERULOSE REDUCTASE IN RAT LIVER MICROSOMES

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Summary: In the course of studies on the metabolism of a new antitumor anthracycline antibiotic, aclacinomycin A, the new keto reductase which catalyzes the reduction of keto group of L-cinerulose of aclacinomycin A to L-rhodinose was found in rat liver microsomal membrane. The enzyme requires NADH for the reduction and showed optimum pH at 7.0. Km value for aclacinomycin A, 2.1 x 10^{-5} M and the concentration of NADH need to half maximal activity, 6.2 x 10^{-5} M were obtained. The activity was potently inhibited by detergents, such as Triton X-100, sodium deoxycholate and sodium dodecyl sulfate.

A new anthracycline antibiotic, aclacinomycin A is produced by Streptomyces galilaeus MA144-M1 (ATCC 31133), and consists of aglycone, aklavinone and trisaccaride moiety, L-cinerulose, 2-deoxy-L-fucose and L-rhodosamine (1,2). Aclacinomycin A shows a potent antitumor activity against L1210 and P388 leukemia and some solid tumors, and shows low cardiac toxicity (1,3).

In the course of the studies of metabolism of aclacinomycin A, we found the hepatic reductase responsible for reductive cleavage at C-7 position of anthracyclines and anthracyclinones to be identical with microsomal NADPH-cytochrome P-450 reductase (4).

Through further studies on the metabolism of aclacinomycin A, we found that the compound MA144 N1 was produced by the reduction of the keto group of L-cinerulose of aclacinomycin A to L-rhodinose

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by rat liver homogenate in the presence of NADH (Fig. 1).

In this paper, we describe the presence of cinerulose reductase in rat liver microsomal membrane and some of its catalytic properties.

Materials and Methods

Chemicals: Aclacinomycin A and MA144 N1 were obtained from the culture of St. galilaeus MA144-M1 in our labolatory (1,2). NADH was purchased from Böehringer Mannheim GmbH and Oriental Yeast Co., LTD. Pre-coated TLC silica gel $60F_{254}$ was obtained from Merck Co.

Enzyme preparation: Adult male rats of Wistar strain were killed by decaptation, and the liver was wuickly exised, rinsed with ice-cold 0.15 M KCl, and used immediately or stored frozen at -25°C. Subcellular fractionation was carried out by the modification of De Duve et al. (5) described by Sedgwick and Hübscher (6). The minced livers were suspended in 9 volumes of 0.3 M sucrose solution containing 2.0 mM EDTA, pH 7.0 and homogenized by a Potter homogenizer with 6 times up and down movements. The homogenate was centrifuged at 1,000 x g for 10 minutes to remove nuclei and debris. The supernatant was subsequently spun at 7,300 x g for 10 minutes to sediment the mitochondrial fraction. The supernatant was further centrifuged at 11,700 x g for 20 minutes to remove the lysosomal fraction. The resulting supernatant was spun at 100,000 x g for 60 minutes to obtain the microsomal fraction. Each fraction was suspended in the same sucrose solution as described above and 100,000 x g supernatant was refered to as the soluble fraction. Protein was determined by the method of Lowry et al. (7) using bovine serum albumin as the standard.

Enzyme activity: The stock solution of aclacinomycin A was prepared by dissolving in 5.0 mM acetic acid at a concentration of 2.0 mM. The reaction mixture, in a total volume of 1.0 ml, consisted of NADH 0.2 mM, aclacinomycin A 0.1 mM, potassium phosphate buffer 0.1 M, pH 7.0 and suitable amount of enzyme. The reaction was started by the addition of NADH solution, carried out with standing at 37°C for 30 minutes, and then stopped by the addition of 2.5 ml of chloroform-methanol (1:1 v/v). The chloroform layer obtained by centrifugation at 3,000 rpm for 5 minutes was evaporated to dryness. The residue was dissolved in 100 µl of chloroform-methanol (1:1 v/v) and 50 µl of sample was spotted on silica gel TLC, then developed with a solvent system of chloroform-methanol (10:1 v/v). The product was identified by the direct comparison with MA144 N1 obtained from microbial culture (2). The amount of the product was measured with a Shimadzu dual-wave length TLC scanner, Model CS-900 at 430 nm. The unit of the activity was defined as µmoles of the product formed under above condition, and specific activity was defined as units per mg of protein. Km value was calculated from Lineweaver-Burk's plot (8).

Results and Discussion

The result of subcellular fractionation of NADH-dependent

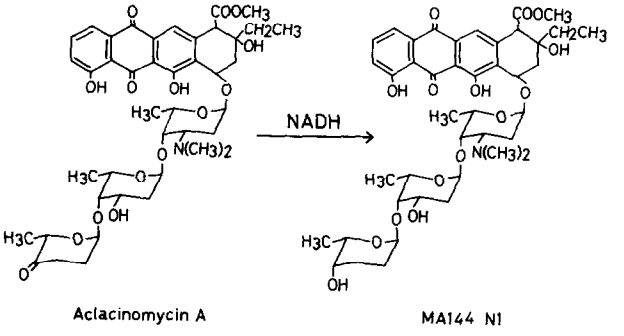

Fig. 1. Reduction of aclacinomycin A to MA144 N1 by rat liver homogenate.

cinerulose reductase is shown in Table 1. The highest activity was found in the microsomal fraction and relatively high activity was observed in the nuclei and debris fractions. The activity in the microsomal fraction was not released by washing with 0.15 M KCl containing 10 mM Tris-HCl and 0.25 mM EDTA, pH 7.4 or with 0.1 M potassium phosphate buffer, pH 7.4.

The reaction was strictly dependent on NADH, and NADPH and reduced glutathione were inactive, and addition of NADPH to NADH showed no effect (Table 2). These results indicate that NADH-dependent cinerulose reductase is present in liver microsomes.

The reaction catalyzed by microsomes proceeded linearly at least for 60 minutes and proportionally to the microsomal protein concentration (Fig. 2). Heating at 100°C for 5 minutes completely abolished the reductase activity. As shown in Fig. 3, the optimum pH for NADH-dependent cinerulose reductase was around pH 7.0 in potassium phosphate buffer and a similar result was obtained with Tris-HCl buffer.

The effects of the substrate concentration are shown in Fig. 4A and 4B. Km value for aclacinomycin A was calculated as 2.1 x 10^{-5} M. The saturation curve of the enzyme with NADH is sigmoidal. The reciprocal of the velocity plotted against the reciprocal

Table 1

Subcellular Distribution of NADH-Dependent Cinerulose Reductase

Fraction	Protein	Total Activity	Specific Activity
	(mg)	(unit)	(unit/mg)
Nuclei+Debris	711	10.9	0.015
Mitochondria	381	0.7	0.002
Lysosomes	169	2.0	0.012
Microsomes	269	29.3	0.109
Soluble	704	2.8	0.004

The rat liver, 13.5 g, was fractionated and assayed as described in Materials and Methods.

Table 2

Cofactor Requirement of Microsomal Cinerulose Reductase

Cofactor	MA144 N1 Production
	(nmoles/tube)
NADH	22.8
NADPH	0.9
NADH + NADPH	21.9
Reduced glutathione	0.4

Microsomal protein 0.23 mg was used in the assay and cofactors added were at the concentration of 0.2 mM.

of the square concentration of NADH gave a straight line (Fig. 4B). The concentration of NADH to exhibit half maximal activity was

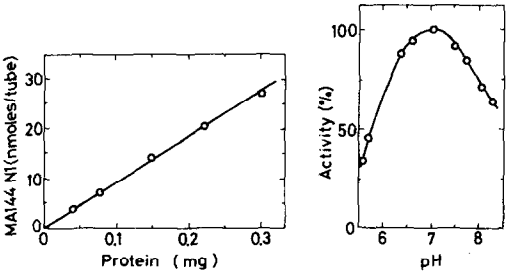

Fig. 2. (left) Effect of the concentration of microsomal cinerulose reductase. Assay conditions described in Materials and Methods were used with various amount of microsomal protein.

Fig. 3. (right) Effect of pH on microsomal cinerulose reductase. Microsomal protein 0.23 mg was used in the assay with various pH values of 0.1 M potassium phosphate buffer.

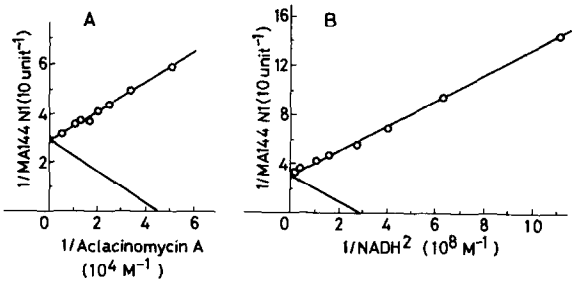

Fig. 4. Effect of the substrate concentration on microsomal cinerulose reductase. Microsomal protein 0.23 mg was used in the assay with various amount of aclacinomycin A or NADH. A: Effect of aclacinomycin A concentration. B: Effect of NADH concentration.

calculated as 6.2×10^{-5} M.

The enzyme activity was highly sensitive to various detergents, such as Triton X-100, sodium deoxycholate and sodium dodecyl sulfate (Table 3), over 90 % of the activity was inhibited by these detergents at the concentration of 0.05 %, while Tween 60, Tween 40 and Brij 35 showed a milder effect than the above three detergents at the same concentration. A higher concentration of Triton X-100 and sodium deoxycholate, tested up to 1.0 %, inhibited the reaction more strongly.

There are several NADH-dependent reactions in liver micro-

Table 3

Effect of Detergent on Microsomal Cinerulose Reductase

Detergent	Concentration	Activity
	(%)*	(%)
Control	----	100
Triton X-100	0.05	9
Sodium deoxycholate	0.05	3
Tween 60	0.05	56
Tween 40	0.05	44
Brij 35	0.05	26
Sodium dodecyl sulfate	0.05	3

Microsomal protein 0.23 mg was used in the assay and detergent was added to reaction mixture as the indicated concentration.
*The percentage was expressed as v/v for Triton X-100, Tween 60 and Tween 40, and w/v for sodium deoxycholate, Brij 35 and sodium dodecyl sulfate.

somal membrane; desaturation of fatty acid (9), hydroxylamine reduction (10), reduction of N-oxide (11), hydroxylation of benzo(a)pyrene and aniline (12,13), O-deethylation of p-nitrophenetol and 7-ethoxycoumarin (14,15), N-demethylation of benzphetamine (16), decomposition of hydroperoxide (17), cyclization of squalene (18), epoxidation of vitamin K_3 (19), DT diaphorase (20), testosterone 17β-dehydrogenase (21), 3α- and 11β-hydroxysteroid dehydrogenase (22) and 20β-hydroxysteroid dehydrogenase (23). Considering the substrate specificity and other properties of these enzymes, the microsomal cinerulose reductase described above might be a new enzyme.

Although aclacinomycin A is not a common molecule in mammals, this enzyme seems to have a naturally occuring substrate and play

an important role in vivo, because the enzyme showed a relatively low Km value for aclacinomycin A, 2.1×10^{-5} M.

More precise properties of this enzyme will be reported elsewhere.

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