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AZOREDUCTASE ACTIVITY OF LIVER ALDEHYDE OXIDASE

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The present study provides the first evidence that rabbit liver aldehyde oxidase supplemented with its electron donors functions as an azoreductase towards azo compounds such as methyl red, amaranth, methyl orange and *p*-dimethylaminoazobenzene.

KEYWORDS — azo compound; azo reduction; azoreductase; aldehyde oxidase; rabbit liver; methyl red; amaranth; methyl orange; *p*-dimethylaminoazobenzene

A main metabolic pathway of azo compounds is reductive cleavage of the azo linkage, which is thought to be a detoxification reaction for carcinogenic azo compounds.^{1,2)} However, the activation of a recently proposed antineoplastic drug depends on the azo reduction by a reductase so as to produce a short-lived, potent alkylating agent.³⁻⁵⁾ In addition, for azo compounds, the reductive metabolism may result in products that show carcinogenic and mutagenic effects.⁶⁻¹⁰⁾ Studies concerning azoreductase activity in mammalian livers have shown that several different enzymes, depending on substrates used, are responsible for the cleavage of the azo linkage. The reduction of *p*-dimethylaminoazobenzene could be mainly catalyzed by a liver microsomal flavoprotein, NADPH-cytochrome *c* reductase,^{11,12)} while that of amaranth was completely dependent on cytochrome P-450.^{13,14)} In addition, neopron-tosil, was reduced through three microsomal pathways corresponding to reduction by 1) NADPH-cytochrome *c* reductase, 2) cytochrome P-450 and 3) 3-methylcholanthrene-inducible, carbon monoxide-insensitive enzyme.¹⁵⁾ On the other hand, the azo reduction of methyl red could be exclusively catalyzed by a liver cytosolic enzyme, DT-diaphorase.^{16,17)} The present study provides the first example of rabbit liver aldehyde oxidase functioning as an azoreductase towards some azo compounds.

As shown in Table I, rabbit liver cytosol exhibited a significant azoreductase activity towards methyl red and methyl orange in the presence of acetaldehyde, 2-hydroxypyrimidine or N¹-methylnicotinamide under anaerobic conditions. In this case, methyl red was more readily reduced than methyl orange. The aldehyde and N-heterocyclic compounds are electron donors of aldehyde oxidase. Therefore, the result strongly suggests the involvement of rabbit liver aldehyde oxidase in the reduction of azo compounds. On the other hand, NADPH or NADH was effective in the reduction of methyl red but not methyl orange, under both anaerobic and aerobic conditions. The reduced pyridine nucleotide-linked activity appears to be due to DT-diaphorase, because the purified enzyme from rat livers functions as an oxygen-insensitive azoreductase towards methyl red in the presence of NADPH or NADH.^{16,17)} Xanthine, an electron donor of xanthine oxidase, was virtually ineffective in the

TABLE I. Azoreductase Activity of Rabbit Liver Cytosol

Addition	Azoreductase activity (nmol/min/g liver)			
	Methyl red		Methyl orange	
	Anaerobic	Aerobic	Anaerobic	Aerobic
None	17	7	7	4
Acetaldehyde	88	16	43	6
2-Hydroxypyrimidine	103	14	39	5
N ¹ -Methylnicotinamide	110	12	39	3
NADPH	206	204	10	4
NADH	93	90	7	5
Xanthine	20	6	6	5

Each value represents mean of four experiments.

TABLE II. Azoreductase Activity of Rabbit Liver Aldehyde Oxidase

Addition	Methyl red reductase activity* (nmol/min/mg protein)
None	0
Acetaldehyde	4.8
2-Hydroxypyrimidine	8.7
N ¹ -Methylnicotinamide	7.5
NADPH	0
NADH	0
Xanthine	0

Each value represents mean of four experiments.

*Assay was performed under anaerobic conditions.

reduction of both methyl red and methyl orange. As was expected, purified rabbit liver aldehyde oxidase exhibited azoreductase activity in the presence of its electron donors under anaerobic conditions (Table II).

In addition, the comparative ability of some chemicals to inhibit the azoreductase and aldehyde oxidase activities of the purified enzyme was examined. As shown in Table III, both activities were similarly susceptible to inhibition by all of these chemicals. In this case, it is interesting that SKF 525-A, an inhibitor of microsomal mixed function oxidase, shows an inhibitory effect on rabbit liver aldehyde oxidase.

TABLE III. Effect of Various Chemicals on Azoreductase Activity of Rabbit Liver Aldehyde Oxidase

Addition	Concentration (M)	Methyl red reductase activity*	Aldehyde oxidase activity
		Control (%)	
None	-	100	100
Menadione	5×10^{-6}	4	0
Chlorpromazine	1×10^{-4}	18	15
Dicumarol	1×10^{-4}	11	15
Disulfiram	1×10^{-4}	29	20
SKF 525-A	2×10^{-4}	14	2

Each value represents mean of four experiments.

*Assay was performed in the presence of 2-hydroxypyrimidine under anaerobic conditions.

TABLE IV. Azoreductase Activity of Rabbit Liver Aldehyde Oxidase towards Some Azo Compounds

Compound	Azoreductase activity* (nmol/min/mg protein)
Methyl red	10.0
Amaranth	5.3
Methyl orange	4.0
<u>p</u> -Dimethylaminoazobenzene	2.7

Each value represents mean of four experiments.

*Assay was performed in the presence of 2-hydroxypyrimidine under anaerobic conditions.

Table IV shows that the purified enzyme supplemented with 2-hydroxypyrimidine can also catalyze the reduction of amaranth, methyl orange and p-dimethylaminoazobenzene as well as methyl red in varying degrees. By thin-layer chromatography, dimethyl-p-phenylenediamine was detected as a reduction product from methyl red, methyl orange and p-dimethylaminoazobenzene; and 1-naphthylamine-4-sulfonic acid as a reduction product from amaranth.

From these results, we concluded that rabbit liver aldehyde oxidase is involved as an azoreductase in the cleavage of the azo linkage.

EXPERIMENTAL

Male albino rabbits (2.0-2.5 kg) were used for enzyme preparation. Liver aldehyde oxidase was purified by the method of Rajagopalan *et al.*¹⁸⁾ Liver cytosol was prepared by homogenizing the liver in 4 volumes of 1.15% KCl, followed by centrifuging the homogenate for 20 min at $9,000 \times g$ and then centrifuging the supernatant for 60 min at $105,000 \times g$. In the assay of azoreductase activity, a typical incubation mixture consisted of 0.1 μ mol of an azo compound,

1 μ mol of an electron donor and an enzyme source in a final volume of 2.5 ml of 0.1 M K₂Na-phosphate buffer (pH 7.4). Anaerobic incubation was performed at 37°C using a Thunberg tube. The tube was gassed for 3 min with oxygen-free nitrogen, evacuated with an aspirator for 5 min and again gassed with nitrogen. In the aerobic experiments, the incubation was performed in an open vessel. The amount of an azo compound which was reduced during incubation was calculated from the decrease in absorbance at 427 nm for methyl red, 520 nm for amaranth, 438 nm for *p*-dimethylaminoazobenzene and 462 nm for methyl orange. The assay of aldehyde oxidase was performed by the method of Felsted *et al.*,¹⁹⁾ measuring the increase in optical density at 300 nm which accompanies the oxidation of N¹-methylnicotinamide to the 2- and 4-pyridones. Protein was determined by the method of Lowry *et al.*²⁰⁾ with bovine serum albumin as a standard. The reduction products from the azo compounds were examined by thin-layer chromatography using a silica gel plate (Kieselgel 60F-254, Merck). When developed with CHCl₃-MeOH-ammonia water (14:6:1) and visualized by spraying the plate with an Ehrlich reagent, the R_f values of dimethyl-*p*-phenylenediamine and 1-naphthylamine-4-sulfonic acid were 0.80 and 0.19, respectively.

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