Rat Liver DT-Diaphorase as a Nitroso-Reductase

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Reduction of several nitroso-compounds by purified DT-diaphorase from rat liver cytosol was investigated. Among nitroso-compounds tested, 1-nitroso-2-naphthol and *p*-nitrosophenol were reduced in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at rates much faster than that of nitrosobenzene. On the contrary, none of the N-nitroso-compounds tested was reduced by this enzyme. Experiments on the identification of reduction products and on the inhibition with dicoumarol and the antiserum indicated that DT-diaphorase catalyzes 4-electron reduction of C-nitroso-compounds and plays a major role in the reduction of these compounds by rat liver cytosol.

Keywords DT-diaphorase; nitroso-reductase; *p*-nitrosophenol; 1-nitroso-2-naphthol; nitrosobenzene; 4-nitroquinoline 1-oxide

Introduction

Reduction of C- and N-nitroso-compounds, some of which are carcinogenic and mutagenic, has been known to be catalyzed by several enzymes present in animal liver.[1-6] These enzymes are, for example, alcohol dehydrogenase,[3,4] aldehyde oxidase under anaerobic conditions[5] and the reconstituted system consisting of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P-450 reductase (fp_2) and cytochrome P-450 under anaerobic conditions.[6]

Rat liver cytosolic DT-diaphorase is a flavo-enzyme which catalyzes the 2-electron reduction of various quinones in the presence of NADPH or reduced nicotinamide adenine dinucleotide (NADH) as an electron donor and is strongly inhibited by dicoumarol.[7] Although the physiological role of DT-diaphorase is thought to protect animals from cytotoxicity due to the production of highly reactive semiquinone radical formed by fp_2,[8] the enzyme has also been known as a nitro- and an azo-reductase.[9,10] It is involved in the activation of a potent carcinogen, 4-nitroquinoline 1-oxide (4NQO), to its proximate form, 4-hydroxylaminoquinoline 1-oxide.[9] Recently, Horie *et al.* reported that C-nitroso reductase purified from pig heart cytosol may be DT-diaphorase with respect to its sensitivity towards dicoumarol, the cofactor requirement and the activation by addition of bovine serum albumin or Tween 20.[11]

Therefore, we examined whether DT-diaphorase purified from rat liver cytosol has reducing activity towards nitroso-compounds and, as described here, found that it catalyzes the reduction of C-nitroso-compounds but not of N-nitroso-compounds.

Experimental

Materials NADPH and NADH were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo Japan). *p*-Nitrosophenol (*p*-NSP), nitrosobenzene (NSB), *N*-nitrosodiphenylamine, *N*-nitrosodiethylamine, *N*-nitrosodimethylamine and *p*-aminophenol (*p*-AP) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 1-Nitroso-2-naphthol (NSN) and dicoumarol were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). 2,4-Dichlorophenolindophenol (DCPIP) and pyrazole were purchased from Merck (Darmstadt, FRG) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. 4-NQO was the generous gift of Dr. S. Saeki (Faculty of Pharmaceutical Sciences, Kyushu University). 3,4,5,3′,4′-Pentachlorobiphenyl (PenCB) was synthesized by the method of Saeki *et al.*[12]

Enzyme Assays DT-Diaphorase was purified from liver cytosol of 3-methylcholanthrene-treated rats as reported elsewhere.[13] The activity was assayed by the method of Ernster *et al.*[7] The incubation mixture contained 50—100 μM substrate, 0.15 mM NADPH or NADH, 50 mM Tris–HCl buffer (pH 7.5) and 10 μg of purified enzyme in a final volume of 3 ml. In the study of substrate specificity, the activity was determined by measuring the decreased absorbance of NADPH at 340 nm at 37 °C. When DCPIP was used as a substrate, the decrease of absorbance at 600 nm was measured at 25 °C. For determination of the reduction products of nitroso-compounds, the incubation mixture as described above was allowed to react for 10 min at 37 °C. After incubation, the mixture was extracted once with 6 ml of ethyl acetate and the extract was evaporated to dryness under vacuum. The residue was dissolved in ethyl acetate and then applied to a silica gel 60G plate (0.25 mm thick, Merck, Darmstadt, FRG), and developed with chloroform–methanol (9 : 1, v/v). *p*-AP, a reduction product of *p*-NSP, was quantified according to the method by Otsuka.[14] In the inhibition study using the antiserum against DT-diaphorase, the reaction was initiated by addition of NADH after the purified enzyme or the liver cytosol was incubated with the antiserum for 5 min at 37 °C.

Results and Discussion

Table I shows substrate specificity of the purified DT-diaphorase towards six nitroso-compounds together with 4NQO, methyl red and DCPIP. Among the six nitroso-compounds, NSN was the best substrate, followed by *p*-NSP. NSB was reduced at a much slower rate than NSN and *p*-NSP. The reduction was completely inhibited by addition of dicoumarol and activated about 1.3-fold by addition of 0.07% bovine serum albumin or 0.2% Tween 20, the activators of DT-diaphorase (data not shown). In contrast, none of the N-nitroso-compounds was active as a substrate of DT-diaphorase. In addition, NADH was only slightly less effective in the reduction of *p*-NSP than NADPH was (data not shown). These results suggest that

TABLE I. Substrate Specificity of Purified DT-Diaphorase from Rat Liver Cytosol

Substrate	Concentration (μM)	Activity (μmol/min/mg protein)
1-Nitroso-2-naphthol	100	240.2
p-Nitrosophenol	100	90.1
Nitrosobenzene	100	2.6
N-Nitrosodiphenylamine	100	ND
N-Nitrosodiethylamine	100	ND
N-Nitrosodimethylamine	100	ND
4-Nitroquinoline 1-oxide	100	129.1
Methyl red	50	3.97
DCPIP	80	960.5

The activity was determined by measuring the decreased absorbance of NADPH at 340 nm at 37 °C, except that, when DCPIP was used as a substrate, the activity was determined by the decreased absorbance at 600 nm at 25 °C. ND, not detected.

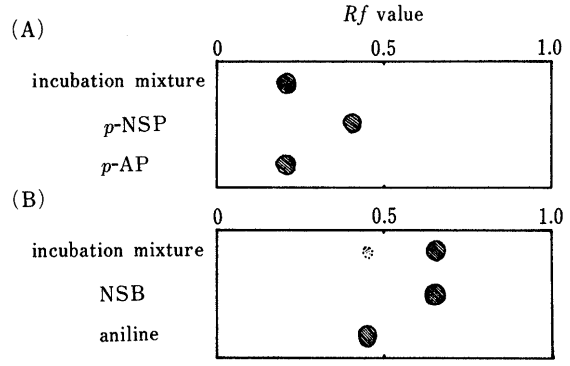

Fig. 1. Thin-Layer Chromatography of Reduction Product of *p*-NSP (A) and NSB (B)

The reduction product was extracted with ethyl acetate and applied to a silica gel plate. The developing solvent used was chloroform–methanol (9 : 1, v/v).

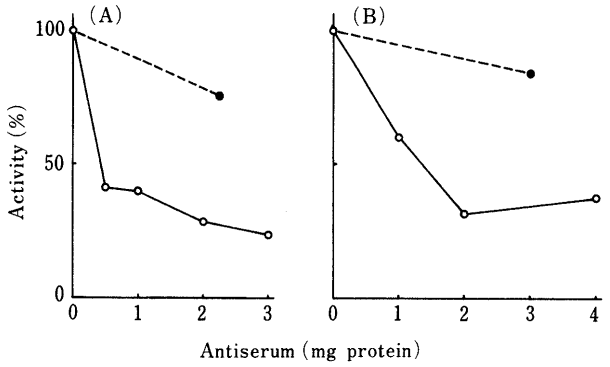

Fig. 2. Effects of Antiserum against Purified DT-Diaphorase on Reduction of *p*-NSP by Purified Enzyme (A) and Liver Cytosol from 3,4,5,3′,4′-Pentachlorobiphenyl-Treated Rats (B)

After the purified enzyme and liver cytosol were preincubated with control serum (●---●) or antiserum (○—○) for 5 min at 37 °C, the reaction was initiated by addition of NADH. The activity was determined by measuring the decreased absorbance of NADH at 340 nm at 37 °C.

DT-diaphorase participates in the reduction of C-nitroso-compounds as well as various quinones, 4-NQO and methyl red.

The ethyl actate extracts of reaction mixtures of *p*-NSP and NSB by DT-diaphorase were submitted to thin-layer chromatography for identification. As shown in Fig. 1, only one spot was detected in each extract. The products were identified as *p*-AP and aniline, respectively, by comparison of the *Rf* values and colorations with those of the corresponding authentic samples. The yellow coloration of both products with Ehrlich reagent also supported that they possess a primary amino group. These results indicated that DT-diaphorase catalyzes the 4-electron reduction of *p*-NSP and NSB, similar to nitro-compounds. We could not establish the structure of the reduction product(s) of NSN because of its very low stability.

Figure 2 shows the effect of antiserum against rat liver cytosolic DT-diaphorase on the reduction of *p*-NSP catalyzed by the purified enzyme and the liver cytosol from PenCB-treated rats. The antiserum inhibited 70—75% of the reducing activity of *p*-NSP by both enzyme sources. This result further confirmed that DT-diaphorase can function as a nitroso-reductase.

TABLE II. Inhibitory Effect of Dicoumarol and Pyrazole on Reduction of *p*-Nitrosophenol by Liver Cytosol from Untreated and 3,4,5,3′,4′-Pentachlorobiphenyl-Treated Rats

Inhibitor	*p*-Aminophenol formed (nmol/min/mg protein)	
	Untreated	PenCB-treated
None	13.79 (100)	165.57 (100)
Dicoumarol (10^{-4} M)	3.03 (22)	9.93 (6)
Pyrazole (10^{-4} M)	11.72 (85)	160.60 (97)

Values are the means of three determinations and figures in parentheses are the relative ratios to the controls. PenCB dissolved in corn oil was injected i.p. to male Wistar rats at a single dose of 5 mg/kg and rats were killed 5 d thereafter.

Further, the degree of the contribution of DT-diaphorase to the formation of *p*-AP from *p*-NSP by the liver cytosol from untreated and PenCB-treated rats was determined using some inhibitors. As shown in Table II, in the untreated cytosol, dicoumarol inhibited 78% of the total activity of *p*-AP formation. Pyrazole, a specific inhibitor of alcohol dehydrogenase,[15] showed 15% inhibition of *p*-NSP reduction. These results indicate that DT-diaphorase plays a major role in the reduction of *p*-NSP by rat liver cytosol under aerobic conditions. PenCB has been known to be the most toxic polychlorinated biphenyl and to markedly induce DT-diaphorase.[16] The reducing activity towards *p*-NSP of PenCB-treated cytosol was elevated about 12 times that of the untreated cytosol, and 94% of the activity was inhibited by dicoumarol, supporting the strong participation of DT-diaphorase in this reduction.

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