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Effect of Inhibitors on Azo-Reduction of 4′-Dimethylaminoazobenzene-2-carboxylic Acid by *Clostridium* Diaphorase

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The effect of inhibitors on the azo-reduction of 4′-dimethylaminoazobenzene-2-carboxylic acid (DMABC) by *Clostridium* diaphorase together with β-nicotinamide adenine dinucleotide, reduced form (NADH) has been investigated. The reduction was inhibited by *o*-aminobenzoic acid which is a reduction product of DMABC. Lineweaver–Burk plots indicated that *o*-aminobenzoic acid was a competitive inhibitor with respect to DMABC (K_i=10.5×10^{-3} mol dm^{-3}). Various monosubstituted benzoic acid derivatives were also competitive inhibitors, among which *o*-hydroxybenzoic acid was most effective.

Bacillus diaphorase and *Clostridium* diaphorase are specific for the azo-reduction of 4′-dimethylaminoazobenzene-2-carboxylic acid (DMABC), 4′-diethylaminoazobenzene-2-carboxylic acid (DEABC) and methyl-4′-dimethylaminoazobenzene-2-carboxylate (MDMABC).[1] DMABC is reduced by these diaphorases together with β-nicotinamide adenine dinucleotide, reduced form (NADH) to form two primary arylamines, *o*-aminobenzoic acid, and *N*,*N*-dimethyl-*p*-phenylenediamine.[2] However, it was recently found that the azo-reduction of DMABC by *C.* diaphorase is inhibited by *o*-aminobenzoic acid and its analogous compounds. The effect of these inhibitors on the azo-reduction is of much interest because the reduction constitutes the initial stage in microbial degradation of azo dyes. The effect will be also exerted on the efficiency of microbial treatment of industrial wast waters containing azo dyes. In this work we examined the effect of inhibitors on *C.* diaphorase.

Experimental

Chemicals. *Clostridium* diaphorase and NADH were purchased from Oriental Yeast Co., Ltd. and Wako Pure Chemical Industries, Ltd., respectively. The enzyme and chemicals were used without purification. The enzyme was homogeneous in polyacrylamide gel electrophoresis stained with Nitro Blue Tetrazolium (R_m≈0.33). The apparent specific activity of *Clostridium* diaphorase was about 100 units/mg (25°C; with NADH and sodium 2,6-dichlorobenzenone-indophenol; pH 7.5). The prosthetic groups of the enzyme were flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). DMABC was purchased from Nacalai Chemical Ltd. and recrystallized from ethanol. The purity was verified by thin-layer chromatography using several eluent systems. *o*-Aminobenzoic acid (**1**), *p*-aminobenzoic acid (**2**), *o*-hydroxybenzoic acid (**3**), *p*-hydroxybenzoic acid (**4**), *o*-chlorobenzoic acid (**5**), *p*-chlorobenzoic acid (**6**), *o*-acetoxybenzoic acid (**7**), *o*-methoxybenzoic acid (**8**), *o*-methylbenzoic acid (**9**), and *p*-methylbenzoic acid (**10**) used as inhibitors were recrystallized from ethanol or water.

Methods. Reduction of DMABC by Diaphorase together with NADH: The azoreductase activity of diaphorase was determined as follows. A reaction mixture containing 12 to 25×10^{-6} mol dm^{-3} of DMABC (2 cm^3), 200 mg dm^{-3} of diaphorase (0.5 cm^3) and 0.6 to 12×10^{-4} mol dm^{-3} NADH (0.5 cm^3) in a final volume of 5 cm^3 of a 0.07 mol dm^{-3} potassium phosphate buffer (pH 6.1) was incubated for 5 min at 25°C. The reaction was terminated by addition of potassium hydroxide (0.5 mol dm^{-3}, 0.5 cm^3). The disappearance of DMABC was followed spectrophotometrically with a Hitachi Spectrophotometer Model 330 by observing the decrease in the absorbance of DMABC at 430 nm.

Effect of Inhibitors on the Reduction of DMABC by Diaphorase together with NADH: By adding an inhibitor of prescribed concentration in the reaction mixture mentioned above, the azoreductase activity of diaphorase was determined in a similar manner.

Results and Discussion

Kinetics of the Reduction of DMABC by Diaphorase together with NADH. Under conditions where non-enzymatic reduction was negligible, the amount of diaphorase used was selected so that the reaction rate was significant but small enough to remain linear for 5 min. Parallel lines were obtained in the Lineweaver–Burk plot, reciprocal of the initial reaction rate vs. that of DMABC concentration, for a fixed enzyme concentration and various NADH concentrations (Fig. 1). The reaction hence obeys the Bi Bi Uni Uni Ping Pong mechanism[3,4] where enzyme, substrates, and products in the reaction scheme are the oxidized enzyme, reduced enzyme, NADH, DMABC, NAD^+, and arylamines, respectively, as described before.[2] A straight line was obtained by plotting the ordinate intercepts in Fig. 1 against the reciprocal of NADH concentration, as shown in Fig. 2. From these data the apparent K_m(DMABC), K_m (NADH), and V_{max} values were determined to be 1.4×10^{-4} mol dm^{-3}, 4.0×10^{-4} mol dm^{-3}, and 4.3×10^{-8} mol dm^{-3} min^{-1} per mg of protein, respectively.

Effect of Inhibitors of the Reduction of DMABC by Diaphorase together with NADH. The azo-reduction of DMABC by diaphorase together with NADH was inhibited by *o*-aminobenzoic acid (**1**) which is a reduction

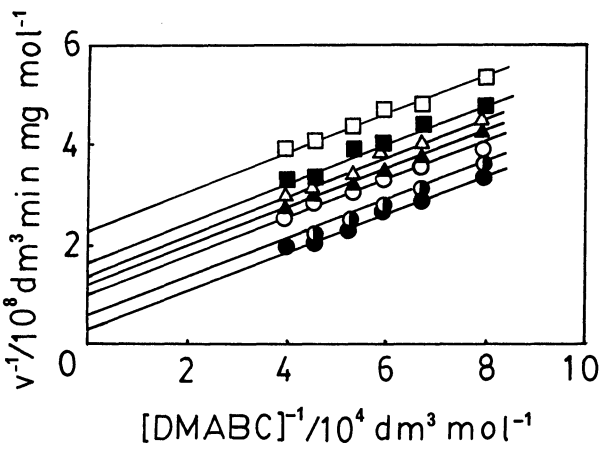

Fig. 1. Relationship between the reciprocal initial reaction rate and the reciprocal DMABC concentration (Lineweaver–Burk plots). Diaphorase concentration: 200 mg dm^{-3} NADH concentration ($\times 10^{-4}$ mol dm^{-3}): -●- 12.0, -◑- 4.9, -○- 1.5, -▲- 1.3, -△- 1.0, -■- 0.8, -□- 0.6. A reaction mixture containing DMABC, NADH, and diaphorase was incubated at 25°C, pH 6.1.

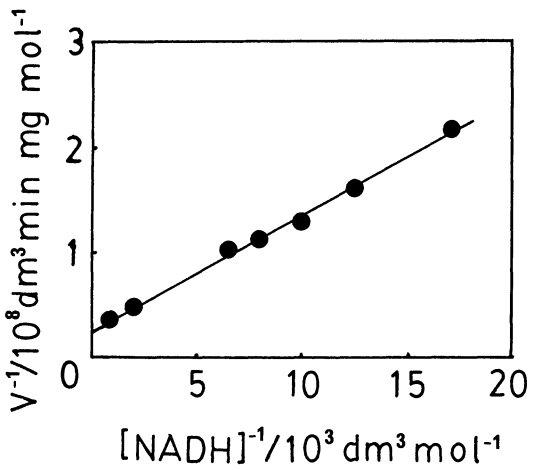

Fig. 2. Plot of the ordinate intercepts in Fig. 1 and the reciprocal NADH concentration (Lineweaver–Burk plot).

Table 1. Inhibitor Constants (K_i) of a Series of Monosubstituted Benzoic Acids for the Reduction of DMABC by *C.* Diaphorase

No.	Inhibitor	$K_i/10^{-3}$ mol dm^{-3}
1	*o*-Aminobenzoic acid	10.5
2	*p*-Aminobenzoic acid	16.2
3	*o*-Hydroxybenzoic acid	4.4
4	*p*-Hydroxybenzoic acid	12.6
5	*o*-Chlorobenzoic acid	7.8
6	*p*-Chlorobenzoic acid	8.1
7	*o*-Acetoxybenzoic acid	8.9
8	*o*-Methoxybenzoic acid	10.5
9	*o*-Methylbenzoic acid	6.0
10	*p*-Methylbenzoic acid	9.8

product of DMABC. The Lineweaver–Burk plots in Fig. 3 shows that **1** is a competitive inhibitor with respect to DMABC. This in turn indicates that the inhibitor **1** forms a complex with reduced diaphorase. The inhibitor constant (K_i=10.5×10^{-3} mol dm^{-3}) was derived from Dixon plots. The azo-reduction was also inhibited by various monosubstituted benzoic acid derivatives which are analogous of a reductive product **1**. These inhibitor constants of these compounds were evaluated from Dixon plots and are summarized in Table 1. *o*-Hydroxybenzoic acid (**3**) was the most effective inhibitor.

The electronic, steric, and hydrophobic effects of substituents on the inhibitor potency were examined. A correlation was noted between the hydrophobic parameter π and the K_i value of inhibitors as seen in Fig. 4. The hydrophobic parameter π is an operationally defined measure of the hydrophobic effect of a substituent: $\pi(x)=\log P(x)-\log P(H)$, where $P(x)$ is the 1-octanol/water partition coefficient of the derivative and $P(H)$ is that of the parent compound [*e.g.*, $\pi(NH_2)=\log P\text{-}(H_2NC_6H_4COOH)-\log P(C_6H_5COOH)$].[5] Hydrophobic and hydrophilic substituents exhibit positive and negative π values, respectively. The data in Fig. 4 therefore suggest that a hydrophobic compound is an effective inhibitor for the azo-reduction.

The specific activity (SA) of diaphorase was examined for the reduction of DMABC, DEABC, and MDMABC whose reduction potentials are close to each other (−0.37, −0.35, and −0.40 V vs. SCE, respectively, at pH 6.1). A distinct difference was noted in their SA values (3.3, 5.4, and 7.8×10^{-9} mol dm^{-3} min^{-1} mg^{-1}, respectively). The logarithm of SA was plotted against the ratio of organic to inorganic characters (O/I) or against the logarithm of the 1-octanol/water partition coefficient (log *P*) of the dyes. O/I and log *P* have been conveniently used as operational definition of hydrophobicity. Both the log SA vs. O/I and log SA vs. log *P* plots gave a straight line of positive slope.[1] This suggests that *C.* diaphorase has high specific activity toward hydrophobic dyes as substrate. These observations indicate that the hydrophobicity of the substrate or inhibitor is a crucial factor in their incorporation into the active site of *C.* diaphorase during azo-reduction.

To gain further insight into the mechanism of inhibition by monosubstituted benzoic acid derivatives, diaphorase was reduced by NADH under anaerobic conditions either in the presence or absence of *p*-chlorobenzoic acid (**6**). On addition of NADH to diaphorase, the absorbance peak of NADH at 340 nm was diminished (Fig. 5-B), in line with the formation of NAD^+ and reduced enzyme. Even when **6**, with no absorbance peaks beyond 250 nm, was added to the enzyme, a similar spectral change was observed. It hence appears that diaphorase reduction by NADH is

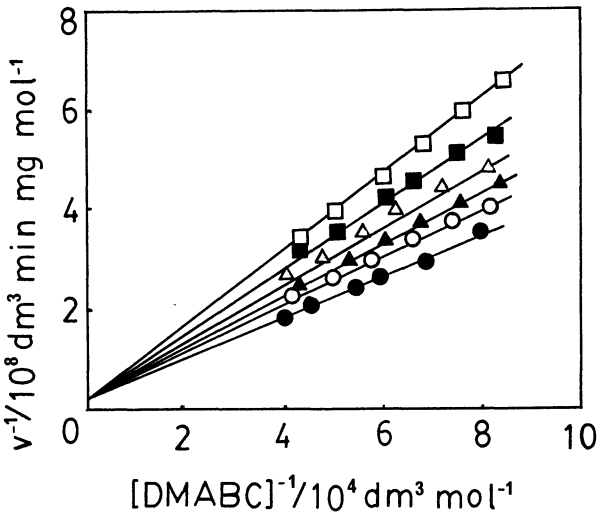

Fig. 3. Relationship between the reciprocal initial reaction rate and the reciprocal DMABC concentration (Lineweaver–Burk plots) at 25°C, pH 6.1. Diaphorase concentration: 200 mg dm^{-3}. NADH concentration: 4.9×10^{-4} mol dm^{-3}. Inhibitor concentration(*o*-aminobenzoic acid, $\times10^{-3}$ mol dm^{-3}): —●— 0.0, —○— 2.0, —▲— 3.0, —△— 4.8, —■— 6.0, —□— 9.8.

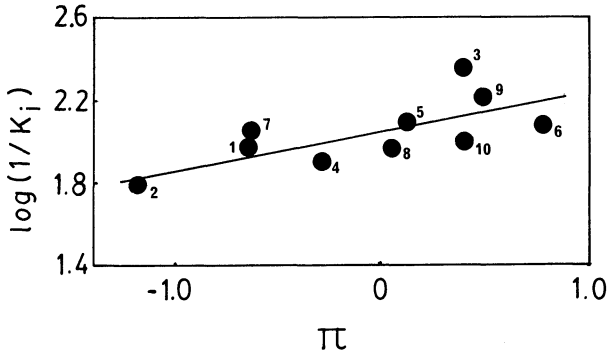

Fig. 4. Relationship between the hydrophobic bonding substituent constants (π) and the logarithums of the reciprocal inhibitor constant. See Table 1 for the compound numbering. $\log(1/K_i)=0.26\pi+2.06$ (n=10, r=0.70, s=0.12).

not inhibited by **6** under these conditions.

The azo-reduction of DMABC by diaphorase was also weakly inhibited by 1.5×10^{-3} mol dm^{-3} of *N*,*N*-dimethyl-*p*-phenylenediamine which is another reduction product of DMABC.

When *o*-aminobenzoic acid or *N*,*N*-dimethyl-*p*-phenylenediamine was added to the medium, the reduction of DMABC by diaphorase was inhibited. The effect of these inhibitors on the azo-reduction is worth further examination in the microbial treatment of industrial wast waters containing azo dyes and other organic compounds.

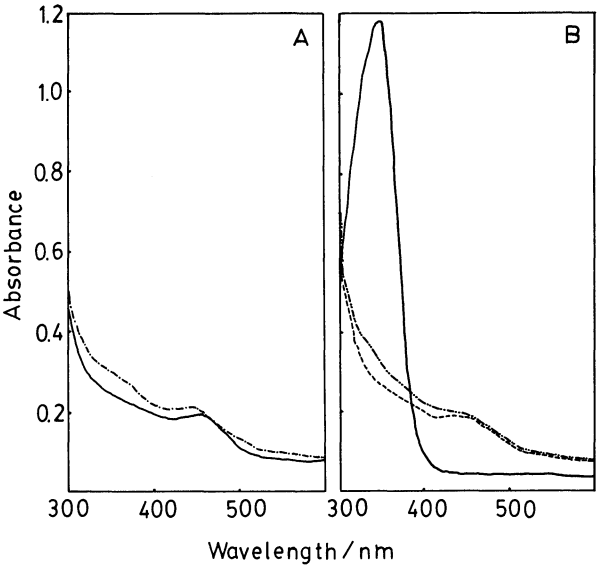

Fig. 5. Effect of *p*-chlorobenzoic acid on the anaerobic reduction of *C.* diaphorase. A. Diaphorase solution (—) and diaphorase plus *p*-chlorobenzoic acid solution (—•—) in an anaerobic cell deoxygenated by 10 min bubbling with nitrogen. B. NADH solution (—) added successively with diaphorase (----) and *p*-chlorobenzoic acid (—••—) in an anaerobic cell. A: —; 1.33 g dm^{-3} diaphorase, —•—; 1.33 g dm^{-3} diaphorase and 3×10^{-3} mol dm^{-3} *p*-chlorobenzoic acid. B: —; 0.2×10^{-3} mol dm^{-3} NADH, – – –; 1.33 g dm^{-3} diaphorase and 0.2×10^{-3} mol dm^{-3} NADH, —••—; 1.33 g dm^{-3} diaphorase, 0.2×10^{-3} mol dm^{-3} NADH, and 3×10^{-3} mol dm^{-3} *p*-chlorobenzoic acid.

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