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Reduction of 2-Carboxy-4′-dimethylaminoazobenzene by *Bacillus* Diaphorase

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2-Carboxy-4′-dimethylaminoazobenzene (CDMAB) was reductively cleaved into its two component primary arylamines by *Bacillus* diaphorase together with NADH (pH 6.1, 25 °C). The reductive cleavage was found to proceed stoichiometrically by a spectrofluorometric assay of fluorescent *o*-aminobenzoic acid and a fluorescent product formed between *N,N*-dimethyl-*p*-phenylenediamine and fluorescamine. Parallel lines were obtained in Lineweaver-Burk plots of initial rate vs. CDMAB concentration for the reduction. This experimentally observed kinetic behavior is consistent with a reaction scheme in favor of the Ping Pong mechanism in which CDMAB is reduced by a reduced form of diaphorase. From a kinetic formula, the apparent K_m(CDMAB), K_m(NADH), and V_{max} values are 2.14×10^{-5} mol dm^{-3}, 7.35×10^{-5} mol dm^{-3}, and 1.8×10^{-7} mol dm^{-3} min^{-1} per mg of protein, respectively.

Most azo compounds are reduced into their parent primary arylamines by azoreductase in rat liver microsomes[1] and liver cytosol preparations.[2] Microbial reduction of water-soluble sulfonated azo compounds has been widely investigated, and it has been reported that microbial azoreductase was much more effective than liver microsomal azoreductase for a reduction of azo compounds.[3] However, there are few reports about the microbial reduction of slightly water-soluble and water-insoluble azo compounds.[4] As a part of studies on the microbiological treatment of wastewater containing various azo compounds, it is desirable to investigate on the microbial reduction (both in vivo and in vitro) of slightly water-soluble and water-insoluble azo compounds.

In this paper, the stoichiometry and kinetics of the enzymatic reduction of 2-carboxy-4′-dimethylaminoazobenzene by *Bacillus* diaphorase was followed.

Experimental

Chemicals. *Bacillus* diaphorase and NAD(P)H were purchased from Boehringer Mannheim GmbH and Wako Pure Chemical Industries, Ltd., respectively. These enzyme and chemicals were used without purification. The apparent specific activity of *Bacillus* diaphorase was about 15 units/mg (25 °C; with NADH and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; pH 8.8). The prosthetic group of the enzyme was FMN. 2-Carboxy-4′-dimethylaminoazobenzene (CDMAB) was purchased from Nakarai Chemicals Ltd. and recrystallized from ethyl alcohol. The purity was verified by thin-layer chromatography using several systems.

Methods. Reduction of CDMAB by Diaphorase together with NADH: Suitable conditions for the reduction of CDMAB by diaphorase were examined as follows. A reaction mixture containing 0.5 cm^3 of 0.2 mmol dm^{-3} CDMAB aqueous solution, 0.02 cm^3 of 1 mg cm^{-3} diaphorase solution, and 0.5 cm^3 of 0.5 mmol dm^{-3} NADH solution in a final volume of 2 cm^3 of 0.07 mol dm^{-3} potassium phosphate buffer was incubated under an atmosphere of nitrogen in an ampule at a prescribed temperature (10—37 °C) and pH (5.5—7.5). The amount of protein was determeined by the method of Layne.[5] The reaction was terminated by addition of potassium hydroxide (0.1 mol dm^{-3}, 1 cm^3) and the residual CDMAB in the mixture was extracted with butyl alcohol. The disappearance of CDMAB was determined using a spectrophotometric assay based on the decrease in the absorbance of CDMAB at 435 nm (λ_{max} 435 nm in butyl alcohol saturated with water).

The azoreductase activity of diaphorase was determined as follows. A solution containing 19.8 to 72.8 nmol of CDMAB and 20 μg of diaphorase in 1.7 cm^3 of 0.07 mol dm^{-3} potassium phosphate buffer (pH 6.1) was placed in a spectrophotometric Thunberg cuvette. A solution containing 0.24 to 2.4 μmol of NADH in 0.3 cm^3 of 0.07 mol dm^{-3} potassium phosphate buffer was placed in the stopper of the Thunberg cuvette. Two solutions were bubbled independently with nitrogen gas for about 10 min. The reaction was initiated by mixing the two solutions. The mixture was incubated at 25 °C. The activity of diaphorase was determined in a spectrophotometer by recording the decrease in the absorbance of CDMAB at 430 nm (λ_{max} 430 nm in potassium phosphate buffer solution; pH 6.1) for 0—2 min and using the molar absorptivity of CDMAB (21.8 dm^3 mmol^{-1} cm^{-1}).

Assay of Reduction Products of CDMAB by Diaphorase: The products, *o*-aminobenzoic acid (*o*-ABA) and *N,N*-dimethyl-*p*-phenylenediamine (DMPA), were determined using a gas chromatographic-mass spectrometric (GC-MS) and a spectrofluorometric assay.

The reaction mixture was similar to that described above. The reaction (3 cm^3, pH 11) was terminated by addition of potassium hydroxide and the mixture was charged into a column of Extrelut 3 (purchased from Merck Co., Inc.) in an atmosphere of nitrogen and in the dark. Hexane (15 cm^3) was passed through the column after 10—15 min. Only one of the products, DMPA, was extracted into the hexane under these conditions with a recovery of about 90%. Then, 25 cm^3 of ether containing 0.1 cm^3 of acetic acid was passed through the column. Unreduced CDMAB was first extracted into the ether. Subsequently, the other product, *o*-ABA, was extracted into the ether with a recovery of about 100%.

The hexane of the extract containing DMPA was slowly removed by aspiration. To the residue (1), ethyl acetate and trifluoroacetic anhydride (TFAA) were added and the mixture was allowed to stand for 5 d at room temperature or for 6

h at 50 °C. Then 0.001 cm^3 was injected onto the GC-MS. On the other hand, the hexane extract containing DMPA was back extracted into 2 cm^3 of sodium acetate buffer (pH 4.0) and the upper hexane layer was carefully removed by aspiration in the dark. Fluorescamine solution (0.2 mg of fluorescamine in 1 cm^3 of acetone, 1 cm^3) was added to the aqueous phase with vigorous mixing. After 30 min, the fluorescence of the product formed between DMPA and fluorescamine was measured at 25 °C in a spectrofluorometer using excitation and emission wavelengths of 400 and 488 nm, respectively. A standard curve of DMPA was prepared by runs of the entire procedure with known amounts of authentic sample passed through the column of Extrelut 3.

The ether extract containing *o*-ABA was dehydrated by adding a few drops of 2,2-dimethoxypropane (DMP), and then the ether was evaporated under reduced pressure. To the residue (2), ethyl acetate and TFAA were added and 0.001 cm^3 was injected onto the GC-MS.[6] On the other hand, to the residue (2), 2 cm^3 of 0.07 mol dm^{-3} potassium phosphate buffer (pH 6.1) was added, and then the fluorescence of *o*-ABA was measured at 25 °C in a spectrofluorometer using excitation and emission wavelengths of 355 and 429 nm, respectively. A standard curve of *o*-ABA was prepared from the authentic sample subjected to the entire procedure.

Apparatus and Conditions: Visible spectra: a Hitachi Spectrophotometer Model 330 was used. Fluorescent spectra: a Hitachi Spectrofluorometer Model 650-10 was used. GC-MS: a Hitachi Mass Spectrometer Model M-52 was used; a 1 m×3 mm glass column was packed with Silicone OV-17 (2%) on Chromosorb WAW DMCS (80—100 mesh); samples were 0.001 cm^3. Operational conditions: column temperature 110—140 °C; carrier gas He; chamber voltage 20 eV.

Results and Discussion

Conditions for the Reduction of CDMAB by Diaphorase. The azoreductase activity of diaphorase was investigated using either NADH or NADPH as electron donor. Through the investigation it has been proved that the activity is supported favorably by NADH. Suitable conditions for the reduction of CDMAB by diaphorase together with NADH are as follows: (1) the optimum pH is 6.1; (2) the optimum temperature is 25 °C. The representative result is shown in Fig. 1.

Stoichiometry of the Reduction of CDMAB by Diaphorase together with NADH under Anaerobic Conditions. The stoichiometry of the reduction of CDMAB by diaphorase together with NADH was determined using a spectral assay based on the decrease in concentration of CDMAB and a spectrofluorometric assay based on the increase in amount of reduction products.

The magnitude of spectral change at 430 nm was initially proportional to the amount of NADH added. After addition of 250, 500, or 750 nmol of NADH to a solution containing 250 nmol of CDMAB, the spectral change (ΔA_{430}) was 0.17, 0.34, or 0.35, respectively. These results indicate that the reduction of 1 mol of CDMAB requires 2 mol of NADH under anaerobic conditions as reported by Huang et al.[2]

Reduction products were detected by GC-MS. The products were found having the same *m/z* (rel intensity) 215 (M^+; 26) and 146(100) with the TFA derivative of authentic *o*-ABA and the same *m/z* (rel intensity) 328 (M^+; 47), 231(45), and 162(100) with the TFA derivative of authentic DMPA. It has been confirmed from these results that CDMAB is reduced by diaphorase together with NADH to form two primary arylamines, *o*-ABA and DMPA.

The products, *o*-ABA and DMPA, in the reaction mixture could be simultaneously determined by spectrofluorometry since a satisfactory separation of the

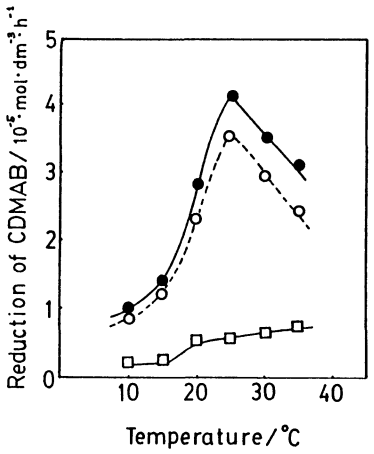


Fig. 1. Effect of temperature on reduction of CDMAB (5×10^{-5} mol dm^{-3}) by NADH (1.25×10^{-4} mol dm^{-3}) in the presence of diaphorase (10 $\mu g\,cm^{-3}$).
The reaction mixture containing CDMAB, NADH, and diaphorase was incubated under anaerobic conditions in an ampule at pH 6.1.
—●— total reduction, --○-- enzymatic reduction, —□— non-enzymatic reduction.

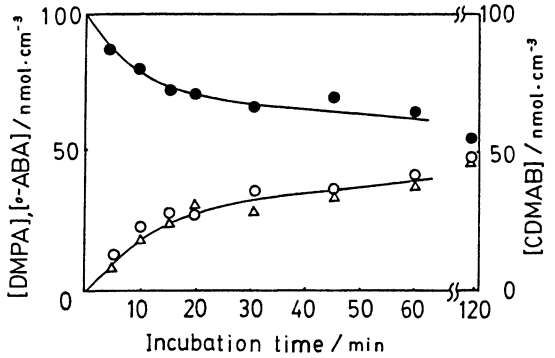
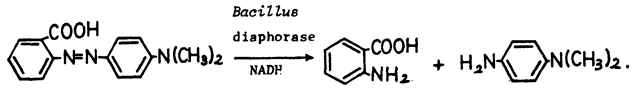


Fig. 2. Relationship between concentration of products (DMPA and *o*-ABA) and CDMAB on reduction of CDMAB (1.0×10^{-4} mol dm^{-3}) by NADH (1.0×10^{-4} mol dm^{-3}) in presence of diapholase (10 $\mu g\,cm^{-3}$).
The reaction mixture containing CDMAB, NADH, and diaphorase was incubated under anaerobic conditions in a Thunberg tube at 25 °C, pH 6.1.
—●— 2-carboxy-4′-dimethylaminoazobenzene (CDMAB), —○— *N,N*-dimethyl-*p*-phenylenediamine (DMPA), —△— *o*-aminobenzoic acid (*o*-ABA).

products was achieved using the column of Extrelut. The result is shown in Fig. 2. The expected stoichiometry for the reduction of the azo linkage of CDMAB by diaphorase together with NADH has been demonstrated. The reaction was completed within 120 min under the conditions adopted. Consequently these results indicate that *Bacillus* diaphorase consumes 2 mol of NADH as electron donor when 1 mol of CDMAB is reduced to *o*-ABA and DMPA under the present experimental conditions. The reduction of CDMAB by diaphorase is summarized as follows:



CDMAB *o*-ABA DMPA

Hydrazo intermediates are presumably formed in the reduction of azo compounds.[1,7] While CDMAB can be reduced to the hydrazo intermediate with sodium sulfite, at pH 5—8 CDMAB is known to be reduced to the amines via a four-electron reaction by electrode reaction.[8] The hydrazo intermediate was not detected in the present experiment.

Kinetic Studies. Under the conditions minimizing the non-enzymatic reduction (the non-enzymatic reaction was made not to occur during 2 min), the amounts of diaphorase used were selected so that the reaction rate was significant but small enough to remain linear for at least 2 to 3 min. Initial slopes of recorder tracings were taken as initial rates. Parallel lines were obtained in the Lineweaver-Burk plot of initial rate vs. CDMAB concentration at fixed enzyme and various NADH concentrations, as shown in Fig. 3. The result can be explained by the Ping Pong mechanism; Alberty,[9] suggested two kinds of mechanisms, Sequential and Ping Pong, as a result of inspection of double reciprocal plots of initial rate vs. substrate concentration (Lineweaver -Burk plot). In theory, lines in Lineweaver-Burk plots for the Ping Pong mechanism are parallel in contrast with the Sequential mechanism which exhibits converging lines. The feature of the Ping Pong mechanism is that the enzyme reacts with one of the substrates to form a complex, which in turn breaks down to yield a modified enzyme with dissociation of the product before the second substrate binds the enzyme. On the basis of the stoichiometry of reduction of CDMAB previously described, the result can therefore be summarized as the following reaction scheme, where E is the enzyme, diaphorase, and Eox and Ered are the oxidized and reduced forms of the enzyme, respectively:

$$\mathrm{Eox}+2\mathrm{NADH}+2\mathrm{H}^+ \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{Eox}\text{-}(\mathrm{NADH})_2\text{-}(\mathrm{H}^+)_2,$$

$$\mathrm{Eox}\text{-}(\mathrm{NADH})_2\text{-}(\mathrm{H}^+)_2 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{Ered}+2\mathrm{NAD}^+,$$

$$\mathrm{Ered}+\mathrm{CDMAB} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{Ered}\text{-}\mathrm{CDMAB},$$

$$\mathrm{Ered}\text{-}\mathrm{CDMAB} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} \mathrm{Eox}+\mathrm{DMPA}+o\text{-}\mathrm{ABA},$$

Eox: E-$(FMN)_2$, Ered: E-$(FMNH_2)_2$.

This mechanism leads to the following expression as for the Bi Bi Uni Uni Ping Pong mechanism using Cleland's nomenclature,[10] in which the initial reaction rate in a steady-state treatment depends on the square of the concentration, $[NADH]^2$, in the form[11,12]

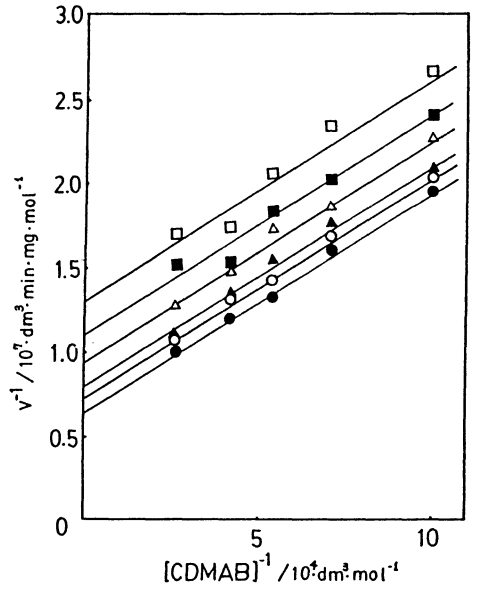


Fig. 3. Relationship between reciprocal intial reaction rate and reciprocal concentration of CDMAB (Lineweaver-Burk plots).
Concentration of diaphorase; 10 $\mu g\,cm^{-3}$. Concentration of NADH ($\times 10^{-5}$ mol dm^{-3}). —●— 120, —○— 60, —▲— 30, —△— 24, —■— 18, —□— 12.
The reaction mixture containing CDMAB, NADH and diaphorase was incubated under anaerobic conditions in a spectrophotometric Thunberg cuvette at 25°C, pH 6.1.

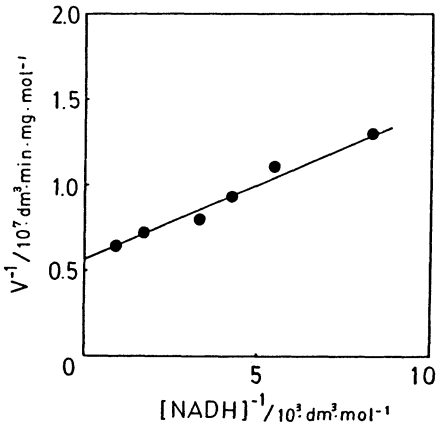


Fig. 4. Relationship between intercepts on the ordinate of Fig. 3 and reciprocal of concentration of NADH (Lineweaver-Burk plots).

$$\frac{V}{v} = 1 + \frac{K_m(\text{coen.})\, K'_m(\text{coen.})}{[\text{coen.}]^2} + \frac{2K_m(\text{coen.})}{[\text{coen.}]} + \frac{K_m(\text{dye})}{[\text{dye}]} \quad (1)$$

where

coen.: NADH, dye: CDMAB,

$$V = \frac{k'_{+2}\, k_{+2}\, k_{+4}}{k'_{+2}\, k_{+2} + k'_{+2}\, k_{+4} + k_{+2}\, k_{+4}} E_0 ,$$

$$K_m(\text{dye}) = \frac{k'_{+2}\, k_{+2}(k_{-3} + k_{+4})}{k_{+3}(k'_{+2}\, k_{+2} + k'_{+2}\, k_{+4} + k_{+2}\, k_{+4})} ,$$

$$K_m(\text{coen.}) = \frac{k_{+2}\, k_{+4}(k_{-1} + k'_{+2})}{k_{+1}(k'_{+2}\, k_{+2} + k'_{+2}\, k_{+4} + k_{+2}\, k_{+4})} ,$$

$$K'_m(\text{coen.}) = \frac{k'_{-1}}{k'_{+1}} .$$

The square term in Eq. 1 becomes negligible, provided [NADH] $\gg K_m$(NADH) or K'_m(NADH). Equation 1 may be rearranged to

$$\frac{V}{v} = 1 + \frac{2K_m(\text{coen.})}{[\text{coen.}]} + \frac{K_m(\text{dye})}{[\text{dye}]} . \quad (2)$$

Equation 2 predicts that a plot of v^{-1} vs. [NADH]$^{-1}$ will be linear. [NADH] under the present experimental conditions was varied in the range $12—120\times10^{-5}$ mol dm^{-3}. These conditions are higher than the tentative K_m(NADH) value, 7×10^{-5} mol dm^{-3}. In Fig. 4 data are presented to show that a secondary plot of the intercepts of the Lineweaver-Burk plots of Fig. 3 vs. NADH concentration gives a straight line. Equations 1 and 2 are in good agreement with the data in Figs. 3 and 4. Thus the experimental data suggest the correctness of the above reaction scheme that in the sequence of substrate binding and product release, CDMAB is reduced by a reduced form of the enzyme.

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