STUDIES ON BACTERIAL NITROREDUCTASES. ENZYMES INVOLVED IN REDUCTION OF AROMATIC NITRO COMPOUNDS IN *ESCHERICHIA COLI*

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Enzymes involved in reduction of methyl *p*-nitrobenzoate in *Escherichia coli* B/r were oxygen-insensitive and precipitated between 30 and 60% ammonium sulfate saturation from cell-free extracts of the strain. The reductases were resolved by DEAE-cellulose column chromatography into three enzymes, NADH-linked, NAD(P)H-linked and NADPH-linked ones. These enzymes were flavoprotein which could be inactivated by dialysis against 1 M potassium bromide and could be reactivated by FMN. The NADH-linked and NAD(P)H-linked reductases were sensitive to dicumarol and exhibited menadione reductase activities. Aromatic nitro compounds with electron-withdrawing *p*-substituents were easily reduced by the NAD(P)H-linked reductase.

Keywords —aromatic nitro compound; methyl *p*-nitrobenzoate; bacterial nitro-reductase; menadione reductase activity; flavoenzyme; *Escherichia coli* B/r; DEAE-cellulose column chromatography

Aromatic nitro compounds are widely used as clinical medicines, agricultural chemicals, industrial materials and so on. In addition, recent studies showed that some polycyclic aromatic nitrohydrocarbons are present in various environmental samples such as airborne particulates, diesel emission exhaust and carbon black.^{1–10)} Although many of them are regarded as direct-acting mutagens in bacterial test systems^{11–13)} because of occurrence of their mutagenic activities without S9-Mix, these nitro compounds seem to undergo bioactivation by nitroreductases present in bacteria, which are used as tester strains.

In regard to such bacterial nitroreductases, however, little information is available now, besides an Asnis' brief report that a flavoprotein capable of mediating the reduction of *p*-nitrobenzoic acid was precipitable with ammonium sulfate from cell-free extracts of *Escherichia coli*.¹⁴⁾

The present study was carried out by using methyl *p*-nitrobenzoate as a substrate in order to

explore enzymes involved in reduction of aromatic nitro compounds in *E.coli* B/r.

EXPERIMENTAL

Chemicals—NADPH, NADH, FAD, FMN and dicumarol were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) was purchased from Whatman Ltd. Riboflavin and *p*-nitrobenzoic acid were obtained from Yoneyama Chemical Industries, Ltd. Menadione and *p*-nitroacetophenone were purchased from Nakarai Chemicals, Ltd. *p*-Nitroaniline, *p*-nitrobenzaldehyde and *p*-dinitrobenzene were obtained from Wako Pure Chemical Industries, Ltd. Methyl *p*-nitrobenzoate, *p*-chloronitrobenzene, *p*-nitrophenol, *p*-nitroanisole, *p*-nitrobenzenesulfonic acid, *p*-bromonitrobenzene, *p*-nitrobenzenesulfonamide and *p*-nitrobenzophenone were purchased from Tokyo Kasei Kogyo Co., Ltd.

Bacteria—*E.coli* B/r was used in this study. Nitrofurazone low- and high-resistant mutants were prepared from the parent strain by the two

step mutational method as described previously.¹⁵⁾

Preparation of Cell-free Extracts—Late-log-phase cells were harvested from 2 l of the culture, resuspended in 67 mM phosphate buffer (pH 7.2) and sonicated for 10 periods for 2 min each at full power in a Tomy ultrasonic disruptor (UR-200P). After centrifugation at $13000 \times g$ for 15 min, the supernatant (cell-free extract) was subjected to ammonium sulfate fractionation. In the case of the 30–60% ammonium sulfate fraction, the precipitated protein was collected, redissolved in 15 ml of 10 mM phosphate buffer (pH 7.2) containing 50 mM NaCl and dialyzed against 2 l of the same buffer for 12 h. The dialyzed solution was concentrated to about 6 ml by ultrafiltration through a collodion membrane and centrifuged at $22000 \times g$ for 20 min to remove any insoluble materials. The clear supernatant was subjected to DEAE-cellulose (DE-52) column chromatography.

Assay for Reductase Activity towards Aromatic Nitro Compounds—A typical incubation mixture consisted of 0.15 μ mol of methyl *p*-nitrobenzoate, 0.15 μ mol of NADPH or NADH, and an enzyme solution in a final volume of 3 ml of 67 mM phosphate buffer (pH 7.2). Aerobic incubation was carried out at 37°C in an open cell and anaerobic incubation in a Thunberg cell, respectively, by measuring the decrease in absorbance at 340 nm (the absorption maximum of NADPH or NADH) in a Hitachi 320 ultraviolet spectrophotometer. An incubation mixture from which the substrate was omitted was used as a reference. A preliminary experiment showed that when the reaction proceeded, the decrease of NADPH or NADH in the incubation mixture was parallel to that of the substrate to form methyl *p*-hydroxylaminobenzoate and methyl *p*-aminobenzoate as reaction products under both aerobic and anaerobic conditions.

Assay for Menadione Reductase Activity—An incubation mixture consisted of 0.15 μ mol of menadione, 0.15 μ mol of NADPH or NADH, and an enzyme solution in a final volume of 3 ml of 67 mM phosphate buffer (pH 7.2). Incubation

was carried out in an open cell, by measuring the decrease in absorbance at 340 nm as described above. An incubation mixture from which the substrate was omitted was used as a reference.

Determination of Protein—Protein concentration was determined by the method of Lowry *et al.* with bovine serum albumin as a standard.¹⁶⁾

RESULTS AND DISCUSSION

As shown in Table I, the cell-free extracts from *E. coli* B/r exhibited a significant methyl *p*-nitrobenzoate reductase activity under both aerobic and anaerobic conditions to a similar extent. This fact suggests that the reductases in *E. coli* B/r are oxygen-insensitive. When the cell-free extracts were subjected to ammonium sulfate fractionation, the reductases were mainly precipitated between 30 and 60% ammonium sulfate saturation. In these cases, NADPH and NADH were equally effective as electron donors.

The reductase activities in the parent strain of *E. coli* B/r could be fractionated into three well-defined peaks of NADH-linked, and both NADPH- and NADH-linked (NAD(P)H-linked), and NADPH-linked activities on a DEAE-cellulose column (Fig. 1a).

E. coli B/r acquires resistance to a nitrofurant derivative such as nitrofurazone in two mutational steps, associating with stepwise loss of oxygen-insensitive nitrofurant reductase activities.¹⁵⁾ Therefore, we examined whether similar alteration on the methyl *p*-nitrobenzoate reductase was observed in the mutational steps or not. As shown in Fig. 1b and 1c, the NADPH-linked reductase activity was lost in the first mutational step and then the NAD(P)H-linked reductase activity was lost in the second mutational step. The NADH-linked reductase activity was irrelevant to such mutation of *E. coli* B/r.

As shown in Table II, the NADH-linked and NAD(P)H-linked reductases could be almost completely inactivated by dialysis against 1 M potassium bromide and could be reactivated by FMN, but not by FAD or riboflavin. On the

other hand, the inactivation of the NADPH-linked reductase was to a lesser extent by this dialysis procedure, but the enzyme could also be reactivated by FMN. Thus, these methyl *p*-nitrobenzoate reductases proved to be flavoenzymes.

Furthermore, dicumarol, a menadione reductase inhibitor, inhibited the NAD(P)H-linked reductase markedly, while it inhibited the NADH-linked one to a lesser extent. However, the inhibitor exhibited little effect on the NADPH-linked reductase (Table III).

In such dialysis and inhibition experiments concerning the NAD(P)H-linked reductase, no significant difference was observed between NADPH and NADH, suggesting that the NADPH- and NADH-linked activities of the reductase are due to a single enzyme protein.

The fact that the NADH-linked and NAD(P)H-linked reductases were sensitive to dicumarol led us to a comparative study on methyl *p*-nitrobenzoate reductases and menadione reductases in *E.coli* B/r. When the cell-free extracts from the strain were chromatographed on a DEAE-cellulose column, the menadione reductase activities were fractionated into three peaks of NADH-linked, NAD(P)H-linked

and NADPH-linked activities (Fig. 2). Among them, the NADH-linked and NAD(P)H-linked activities were eluted at the same positions as the NADH-linked and NAD(P)H-linked methyl *p*-nitrobenzoate reductase activities, respectively. On the other hand, the peak position of the NADPH-linked activity was different from that of the NADPH-linked methyl *p*-nitrobenzoate reductase activity.

Furthermore, relationship between acquisition of resistance to nitrofurazone and alteration of these menadione reductase activities in *E.coli* B/r was also examined by DEAE-cellulose column chromatography (data not shown). In the first mutational step, the NADPH-linked methyl *p*-nitrobenzoate reductase activity was lost as described above, whereas three menadione reductase activities shown in Fig. 2 remained without any alteration. In the second mutational step, the NAD(P)H-linked menadione reductase activity was lost completely together with the NAD(P)H-linked methyl *p*-nitrobenzoate reductase activity. After mutation, the NADH-linked menadione and methyl *p*-nitrobenzoate reductase activities still remained at the same position. These facts suggest that the NADH-linked and NAD(P)H-linked, but not the

TABLE I. *Methyl p-Nitrobenzoate Reductase Activities of Cell-free Extract and Ammonium Sulfate Fraction from Escherichia coli B/r*

Preparation	Methyl <i>p</i> -nitrobenzoate reductase activity			
	NADPH		NADH	
	Aerobic	Anaerobic	Aerobic	Anaerobic
	nmol/min/mg protein			
Cell-free extract	28.8	33.6	29.0	33.6
Ammonium sulfate fraction				
0—30%	1.5	—	0.3	—
30—60%	70.8	71.4	68.1	73.3
>60%	5.4	—	3.6	—

The assay was carried out by using 0.02 ml (0.4 mg protein) of the cell-free extract, 0.2 ml (3.3 mg protein) of the 0—30% ammonium sulfate fraction, 0.01 ml (0.2 mg protein) of the 30—60% ammonium sulfate fraction or 0.2 ml (0.7 mg protein) of the supernatant after 60% ammonium sulfate saturation as an enzyme solution. Each value represents mean of three experiments.

NADPH-linked, methyl *p*-nitrobenzoate reductases are a kind of menadione reductase in *E. coli* B/r.

Table IV shows reduction of various kinds of *p*-substituted aromatic nitro compounds by the

NAD(P)H-linked methyl *p*-nitrobenzoate reductase. The nitro compounds with electron-withdrawing substituents (COCH_3 , NO_2 , COOCH_3 etc.) were much more easily reduced than those with electron-repelling substituents

FIG. 1. Chromatography of Methyl *p*-Nitrobenzoate Reductases from *Escherichia coli* B/r on a DEAE-cellulose Column

The chromatography was carried out on a column (1.5×12 cm) of DE-52 equilibrated with 10 mM phosphate buffer (pH 7.2) containing 50 mM NaCl. After adsorption of the enzyme solution on the column, elution was carried out with 50 ml of the same phosphate buffer, followed by a gradient of increasing NaCl concentration. The mixing chamber held 50 ml of the phosphate buffer (pH 7.2) containing 50 mM NaCl and the reservoir held 50 ml of the phosphate buffer containing 250 mM NaCl. Fractions (2 ml) were collected at a flow rate of 15 ml/h. Methyl *p*-nitrobenzoate reductase activity of each fraction was assayed with 0.01 ml of the sample as described in Experimental. a: Parent strains of *E. coli* B/r. b: Nitrofurazone low-resistant mutants isolated in the first mutational step. c: Nitrofurazone high-resistant mutants isolated in the second mutational step. The amount of protein applied to a column was 120 mg in all cases (a, b and c).

(NH₂, OCH₃, OH *etc.*) by the reductase.

Previously, Asnis ¹⁴⁾ suggested that enzymes involved in reduction of aromatic nitro compounds were different from those catalyzing reduction of nitrofuran derivatives in *E.coli*. How-

ever, our preliminary study showed that when the cell-free extracts from *E.coli* B/r were again fractionated by DEAE-cellulose column chromatography, three peaks of NADH-linked, NAD(P)H-linked and NADPH-linked nitrofu-

TABLE II. *Effects of Dialysis against 1 M Potassium Bromide on Methyl p-Nitrobenzoate Reductases from Escherichia coli B/r, and Effect of Flavins on the Dialyzed Enzymes*

Preparation	Methyl <i>p</i> -nitrobenzoate reductase activity of			
	NADH-linked enzyme	NAD(P)H-linked enzyme		NADPH-linked enzyme
		NADPH	NADH	
nmol/min/mg protein (% of control)				
Enzyme before dialysis	48 (100)	268 (100)	326 (100)	216 (100)
Enzyme after dialysis	2 (4.2)	16 (6.0)	26 (8.0)	60 (27.8)
+ FMN (10 ⁻⁵ M)	46 (95.8)	246 (91.8)	316 (97.0)	181 (83.8)
+ FAD (10 ⁻⁵ M)	9 (18.8)	27 (10.1)	82 (25.2)	56 (25.9)
+ Riboflavin (10 ⁻⁵ M)	2 (4.2)	27 (10.1)	49 (15.0)	71 (32.9)

The peak fractions of the NADH-linked (0.1 ml; 0.1 mg protein), NAD(P)H-linked (0.1 ml; 0.03 mg protein) and NADPH-linked (0.1 ml; 0.1 mg protein) enzymes (Fig. 1) were used as enzyme sources. Deflavoenzymes of these reductases were prepared by a slight modification of the method originally described in the preparation of D-amino acid oxidase apoenzyme. ¹⁷⁾ A solution (2 ml) of each reductase was dialyzed at 4°C against a solution (300 ml) of 0.1 M sodium pyrophosphate (pH 8.5) containing 1 M KBr. The dialysate was changed four times over a 2-d period. The dialyzing medium was then changed to 67 mM phosphate buffer (pH 7.2) to remove KBr. Dialysis was continued for 1-d against 1 l of the dialyzing medium. Each value represents mean of three experiments.

TABLE III. *Inhibitory Effect of Dicumarol on Methyl p-Nitrobenzoate Reductases from Escherichia coli B/r*

Dicumarol	Methyl <i>p</i> -nitrobenzoate reductase activity of			
	NADH-linked enzyme	NAD(P)H-linked enzyme		NADPH-linked enzyme
		NADPH	NADH	
	nmol/min/mg protein (% of control)			
None	64 (100)	319 (100)	399 (100)	273 (100)
10 ⁻⁶ M	49 (76.6)	207 (64.9)	267 (66.9)	
10 ⁻⁵ M	18 (28.1)	0 (0)	8 (2.0)	235 (86.1)

The peak fractions of the NADH-linked (0.1 ml; 0.1 mg protein), NAD(P)H-linked (0.1 ml; 0.03 mg protein) and NADPH-linked (0.1 ml; 0.1 mg protein) enzymes (Fig. 1) were used as enzyme sources. Each value represents mean of three experiments.

razone reductase activities were eluted at the same positions as the corresponding methyl *p*-nitrobenzoate reductase activities, respectively.

These nitrofurazone reductase activities exhibited the same behavior as the methyl *p*-nitrobenzoate reductase activities in two mutational

FIG. 2. Chromatography of Menadione Reductases from *Escherichia coli* B/r on a DEAE-cellulose Column

The chromatography was carried out as described in the legend of Fig. 1. Menadione reductase activity of each fraction was assayed with 0.01 ml of the sample as described in Experimental.

TABLE IV. Reduction of *p*-Substituted Aromatic Nitro Compounds by NAD(P)H-linked Methyl *p*-Nitrobenzoate Reductase from *Escherichia coli* B/r

R	Reductase activity nmol/min/mg protein	R	Reductase activity nmol/min/mg protein
NH ₂	9	CHO	273
OCH ₃	13	COCH ₃	363
COOH	32	SO ₂ NH ₂	363
OH	36	CO-	419
Cl	41		
Br	62	NO ₂	560
SO ₃ H	135	COOCH ₃	560

The peak fraction (0.1 ml, 0.03 mg protein) of the NAD(P)H-linked enzyme (Fig. 1) was used as an enzyme source. Each value represents mean of three experiments.

steps described above (data not shown). In addition, the NAD(P)H-linked, but not the NADPH-linked, nitrofurazone reductase was markedly sensitive to dicumarol as described previously.¹⁵⁾ The NADH-linked nitrofurazone reductase was also sensitive to the inhibitor. These facts suggest that the NADH-linked, NAD(P)H-linked and NADPH-linked enzymes shown in Fig. 1 can mediate not only the reduction of aromatic nitro compounds but also that of nitrofuran derivatives in *E.coli* B/r.

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