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Properties of Nitrofuran Reductases from *Escherichia coli* B/r

TAKAYOSHI DOI,[a,1)] HIDETOSHI YOSHIMURA,[a] and KIYOSHI TATSUMI*,[b]

Faculty of Pharmaceutical Sciences, Kyushu University,[a] 3-1-1, Maidashi, Higashi-ku, Fukuoka 812, Japan and Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[b] 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

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The properties of oxygen-insensitive nitrofuran reductase from *Escherichia coli* B/r were investigated by using cell-free preparations. Both the reduced nicotinamide-adenine dinucleotide phosphate (NAD(P)H)-linked and the NADPH-linked nitrofuran reductases are flavoenzymes containing riboflavin 5′-phosphate as a prosthetic group. The former enzyme appears to be a kind of NAD(P)H-linked menadione reductase.

Keywords——*Escherichia coli* B/r; nitrofuran reductase; menadione reductase; NAD(P)H-linked enzyme; NADPH-linked enzyme; flavoenzyme; DEAE-cellulose column chromatography

Bacterial nitrofuran reductases seem to be involved in the bioactivation of nitrofuran derivatives.[2,3)] Our previous paper[4)] showed that oxygen-insensitive nitrofuran reductases from *Escherichia coli* B/r could be resolved by diethylaminoethyl (DEAE)-cellulose column chromatography into reduced nicotinamide-adenine dinucleotide phosphate (NAD(P)H)-linked and NADPH-linked enzymes. The present study was carried out in order to explore the properties of these nitrofuran reductases from *E.coli* B/r.

Experimental

Materials——Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) was donated by Ueno Fine Chemical Industry, Ltd. Menadione and *o*-iodosylbenzoic acid were purchased from Nakarai Chemicals, Ltd., dicumarol from Wako Pure Chemical Industries, Ltd., riboflavin from Yoneyama Chemical Industries, Ltd. and DEAE-cellulose (DE-52) from Whatman Ltd. NADPH, NADP, NADH, NAD, flavin-adenine dinucleotide (FAD), riboflavin 5′-phosphate (FMN), *p*-chloromercuribenzoic acid and glucose-6-phosphate were obtained from Sigma Chemical Co. Baker's yeast glucose-6-phosphate dehydrogenase and horse liver alcohol dehydrogenase were also purchased from Sigma Chemical Co.

Preparation of Cell-free Extracts——*E. coli* B/r was used throughout. The cell-free extracts of the strain were prepared as described previously.[4)]

Enzyme Assay——Incubation was carried out at 37°C in an open vessel. For the assay of nitrofuran reductase activity, the incubation mixture consisted of 0.15 μmol of nitrofurazone, an NADPH-generating system (10 μmol of glucose-6-phosphate, 0.15 μmol of NADP and 1 unit of glucose-6-phosphate dehydrogenase) or an NADH-generating system (50 μl of ethanol, 0.15 μmol of NAD and 10 units of alcohol dehydrogenase) and enzyme solution in a final volume of 3 ml of 67 mM phosphate buffer (pH 7.2). The nitrofuran reductase activity was calculated from the observed decrease in the absorbance at 370 nm (the absorption maximum of nitrofurazone) in a Hitachi 124 ultraviolet (UV) spectrophotometer. For the assay of menadione reductase activity, the incubation mixture consisted of 0.15 μmol of menadione, 0.15 μmol of NADPH or NADH, and enzyme solution in a final volume of 3 ml of 67 mM phosphate buffer (pH 7.2). The menadione reductase activity was also calculated from the observed decrease in the absorbance at 340 nm (the absorption maximum of NADPH or NADH).

Preparation of Deflavoenzymes——The preparation was performed by a slight modification of the method originally described for the preparation of D-amino acid oxidase apoenzyme.[5)]

Determination of Protein Concentration——Protein concentration was determined by the method of Lowry *et al.*[6)] with bovine serum albumin as a standard (Table I and II) or by measuring absorbance at 280 nm (Fig. 1).

Results and Discussion

Oxygen-insensitive nitrofuran reductase and menadione reductase activities in *E.coli* B/r were comparatively studied by DEAE-cellulose column chromatography. The nitrofuran reductase activities were fractionated into both NADPH- and NADH-linked (NAD(P)H-linked), and NADPH-linked activities as described previously.[4] On the other hand, the menadione reductase activities were fractionated into three peaks of NADH-linked, NAD(P)H-linked and NADPH-linked activities. Among them, the NAD(P)H-linked menadione reductase activity was eluted at the same position as the NAD(P)H-linked nitrofuran reductase activity, whereas the peak position of NADPH-linked menadione reductase activity was different from that of the NADPH-linked nitrofuran reductase activity (Fig. 1). This finding suggests that the NAD(P)H-linked nitrofuran reductase in *E. coli* B/r is a kind of NAD(P)H-linked menadione reductase

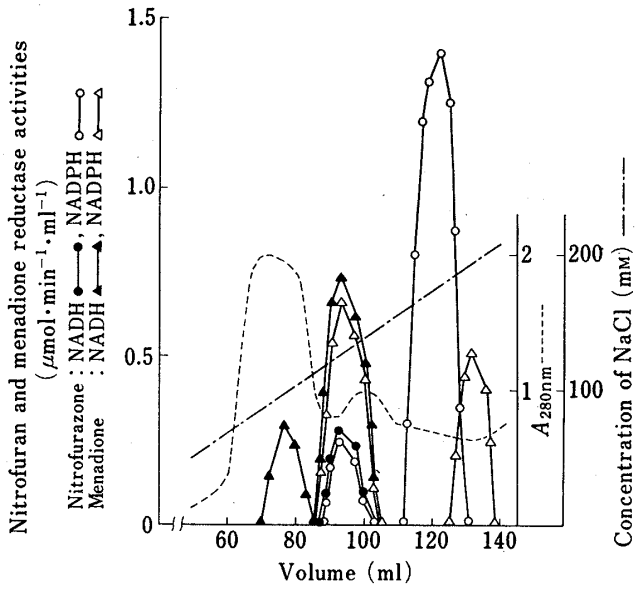

Fig. 1. Chromatography of Oxygen-insensitive Nitrofuran Reductase and Menadione Reductase from *Escherichia coli* B/r on a DEAE-cellulose Column

The chromatography was carried out by using 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 enzyme solution (120 mg protein) 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 10 mM phosphate buffer (pH 7.2) containing 50 mM NaCl and the reservoir held 50 ml of the phosphate buffer containing 250 mM NaCl. Fractions (1 ml each) were collected at a flow rate of 5 ml/h. The nitrofuran and menadione reductase activities of each fraction were assayed as described in "Experimental."

The following study demonstrated that the oxygen-insensitive nitrofuran reductases in *E. coli* B/r are flavoenzymes. As shown in Table I, the NAD(P)H-linked nitrofuran reductase could be inactivated completely and the NADPH-linked nitrofuran reductase could be inactivated partially by dialysis against 1 M potassium bromide. Both inactivated enzymes could be effectively reactivated by addition of FMN. Similarly, the NAD(P)H-linked

Table I. Effect of Dialysis against 1 M Potassium Bromide on the Activities of Nitrofuran Reductase and Menadione Reductase from *Escherichia coli* B/r

Preparation	NAD(P)H-linked nitrofuran reductase[a]	NADPH-linked nitrofuran reductase	NAD(P)H-linked menadione reductase[b]
	μmol·min⁻¹·mg⁻¹ protein⁻¹ (% of control)		
Enzyme before dialysis	0.161(100)	0.628(100)	0.448(100)
Enzyme after dialysis	0 (0)	0.223(35.0)	0.022(4.9)
+FMN (10^{-4} M)	0.129(80.1)	0.523(83.3)	0.371(82.8)
+FAD (10^{-4} M)	0.020(12.4)	0.340(54.1)	0.075(16.7)
+Riboflavin (10^{-4} M)	0.013(8.1)	0.130(20.7)	0.058(12.9)

The peak fractions of the NAD(P)H-linked and NADPH-linked enzymes were used as enzyme sources. Each value represents the mean of three experiments.
a) In the presence of an NADPH-generating system.
b) In the presence of NADH.

menadione reductase was inactivated significantly by the dialysis and reactivated by addition of FMN (Table I).

As shown in Table II, furthermore, both the NAD(P)H-linked nitrofuran reductase and the NAD(P)H-linked menadione reductase were inhibited to similar degrees by dicumarol, *p*-chloromercuribenzoic acid and *o*-iodosylbenzoic acid.

These results support the view that the NAD(P)H-linked nitrofuran reductase in *E. coli* B/r is a kind of menadione reductase as described above.

TABLE II. Effect of Various Chemicals on the Activities of Nitrofuran Reductase and Menadione Reductase from *Escherichia coli* B/r

Addition	NAD(P)H-linked nitrofuran reductase[a]	NAD(P)H-linked menadione reductase[b]
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (% of control)	
None	0.156(100)	0.392(100)
Dicumarol (10^{-5} M)	0 (0)	0 (0)
p-Chloromercuribenzoic acid (10^{-3} M)	0.034(21.8)	0.109(27.8)
o-Iodosylbenzoic acid (10^{-3} M)	0.071(45.5)	0.188(48.0)

The peak fraction of the NAD(P)H-linked enzyme was used as an enzyme source. Each value represents the mean of three experiments.
a) In the presence of an NADPH-generating system.
b) In the presence of NADH.

References and Notes

1) Present address: *Central Research Division, Takeda Chemical Industries, Ltd., Jusohonmachi, Yodogawa-ku, Osaka 532, Japan.*
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