A COMPARATIVE STUDY ON 1-NITROPYRENE AND NITROFURAZONE REDUCTASES IN *ESCHERICHIA COLI*

NORIKO NARAI, SHIGEYUKI KITAMURA, AND KIYOSHI TATSUMI

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima, 734, Japan

(Received December 22, 1983)

1-Nitropyrene and nitrofurazone reductases in *Escherichia coli* B/r were studied comparatively. Nitrofurazone reductase activity was oxygen-insensitive, whereas 1-nitropyrene reductase activity was markedly inhibited by oxygen in both intact cells and cell-free preparations. The former activity depended upon reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate, whereas the latter activity upon flavin-adenine dinucleotide (FAD) as well as the reduced pyridine nucleotide. *E. coli* B/r acquired resistance to nitrofurazone in two mutational steps, associated with stepwise loss of the oxygen-insensitive nitrofurazone reductase activity. However, 1-nitropyrene reductases were not affected at all by the mutation. These facts indicated that the major enzymes responsible for the reduction of 1-nitropyrene and nitrofurazone in *E. coli* B/r were different from each other.

1-Nitropyrene reductases were resolved by diethylaminoethyl-cellulose column chromatography into four enzymes all of which seem to reduce FAD, too. Among them, three enzymes appear to be able also to catalyze the reduction of nitrofurazone under anaerobic conditions.

Keywords—nitropolycyclic aromatic hydrocarbon; 1-nitropyrene; nitrofurazone derivative; nitrofurazone; bacterial nitroreductase; flavin-adenine dinucleotide reductase activity; *Escherichia coli* B/r; 1-nitropyrene reductase; nitrofurazone reductase

Nitropolycyclic aromatic hydrocarbons (nitro-PAHs) which have been found in airborne particulates^{1,2)} and diesel engine emission,^{3,4)} and also in xerographic toners⁵⁾ and copies,⁶⁾ have been noted as a new type of environmental pollutants. These nitro compounds are among the most potent mutagens⁶⁻⁸⁾ and some of them have proved to be carcinogenic.^{9,10)} Generally, the metabolic nitroreduction appears to be essential for such biological activities of aromatic nitro compounds. Indeed, the mutagenicity of nitro-PAHs in *Salmonella typhimurium* depends upon nitroreduction of these compounds by nitroreductases of the tester strain.^{7,11)}

Our recent study¹²⁾ showed that nitroreduction of nitro-PAHs such as 1-nitropyrene and 2-nitrofluorene in rabbit liver is mainly due to aldehyde oxidase and cytochrome P-450. On the other hand, in regard to bacterial nitro-PAH

reductases, Messier *et al.*¹³⁾ and Lu *et al.*¹⁴⁾ showed that 1-nitropyrene reductase is present in *S. typhimurium*. Rosenkranz *et al.*¹¹⁾ indicated the existence in *S. typhimurium* of a multiplicity of nitroreductase activities of varying specificities. More recently, Kinouchi *et al.*¹⁵⁾ examined 1-nitropyrene reductase activity of aerobic and anaerobic bacteria, and found that the activity of anaerobic bacteria was much higher than that of aerobic bacteria. In addition, Kinouchi and Ohnishi¹⁶⁾ isolated four kinds of 1-nitropyrene reductases, one of which was purified about 416-fold, from anaerobic bacteria, *Bacteroides fragilis*.

Previously, we reported enzymes involved in the reduction of nitrofurazone and *p*-substituted nitrobenzene derivatives in *Escherichia coli* B/r.¹⁷⁻¹⁹⁾ We wish to describe here a comparative study on 1-nitropyrene and nitrofurazone reductases in *E. coli* B/r.

EXPERIMENTAL

Materials — 1-Nitropyrene was synthesized by the method of Ristagno and Shine.²⁰⁾ Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) was donated by Ueno Fine Chemicals Industry, Ltd. 1-Aminopyrene was purchased from Tokyo Kasei Kogyo Co., Ltd. and diethylaminoethyl (DEAE)-cellulose (DE-52) from Whatman Ltd., respectively. Nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), flavin-adenine dinucleotide (FAD), glucose-6-phosphate, bakers yeast glucose-6-phosphate dehydrogenase and horse liver alcohol dehydrogenase were obtained from Sigma Chemical Co.

Bacteria — *E.coli* B/r was used in this study. Nitrofurazone low- and high-resistant mutants were isolated from the parent strain by the two step mutational method as described previously.¹⁷⁾

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 3 min each at full power in a Tomy ultrasonic disruptor (UR-200P). The suspension was centrifuged at $12000 \times g$ for 10 min and the supernatant (cell-free extract) was subjected to ammonium sulfate fractionation. The protein precipitated between 0 and 60% ammonium sulfate saturation was redissolved in 10 ml of 10 mM phosphate buffer (pH 7.2) containing 50 mM NaCl and dialyzed against 3 l of the same buffer for 12 h. The dialyzed solution was centrifuged at $22000 \times g$ for 20 min to remove any insoluble materials. The clear supernatant was chromatographed on a DEAE-cellulose (DE-52) column.

Incubation — Aerobic incubation was performed at 37°C in an open vessel and anaerobic incubation in a Thunberg tube or a Thunberg cell, respectively. In the latter case, the tube or cell was gassed for 3 min with N₂ and then evacuated with an aspirator for 5 min.

Assay of 1-Nitropyrene Reductase Activity — After incubation, the mixture was

adjusted to pH 10 with 1N NaOH and then extracted twice with 5 ml each of *n*-heptane. The extract, after removal of the solvent, was subjected to gas chromatography (GLC) to determine 1-aminopyrene produced. The GLC was carried out using a Shimadzu GC-4CM gas chromatograph equipped with a flame ionization detector and a 2 m × 3 mm i.d. glass column packed with 3% Dexsil 400 GC on Chromosorb W. Operation conditions: Injection port and detector temperature, 310°C; column temperature, 260°C; an internal standard, chlorpromazine. Retention times were 13.5 min for 1-aminopyrene and 16 min for 1-nitropyrene, respectively.

Assay of Nitrofurazone Reductase Activity — After incubation, the amount of nitrofurazone reduced was calculated from the decrease in absorbance at 370 nm, which was determined in a Hitachi 320 spectrophotometer.

Assay of FAD Reductase Activity — After incubation, the amount of FAD reduced was calculated from the decrease in absorbance at 450 nm.

Nonenzymatic Reduction of 1-Nitropyrene by FADH₂ — The anaerobic experiment was carried out in a Thunberg tube. The cell-free extract from *E.coli* B/r (0.25 mg of protein) was incubated for 1 h at 37°C with FAD and a NADH-generating system. At the end of incubation, the yellow color due to FAD faded almost completely. After heated for 5 min in a boiling water bath to inactivate enzymes, the mixture was cooled to room temperature, added to 1-nitropyrene and then further incubated for 30 min at 37°C. The amount of 1-aminopyrene produced was extracted with *n*-heptane and determined by GLC as described above.

Determination of Protein — Protein was determined by the method of Lowry *et al.*²¹⁾ with bovine serum albumin as a standard or by measuring absorbance at 280 nm.

RESULTS AND DISCUSSION

Table I shows nitroreductase activities towards 1-nitropyrene and nitrofurazone of the intact cells

of *E.coli* B/r. The nitrofurazone reductase activity was oxygen-insensitive, whereas the 1-nitropyrene reductase activity was markedly inhibited by oxygen, suggesting that distinct enzymes are responsible for the reduction of 1-nitropyrene and nitrofurazone in *E.coli* B/r. It is known that nitrofurans reductases in *E.coli* can be classified into two groups based on their sensitivity to oxygen: Oxygen-insensitive nitrofurans reductases and oxygen-sensitive nitrofurans reductases.^{22,23)} The present results described above indicated that the former enzymes play a prominent role in the reduction of nitrofurazone in *E.coli*, whereas the latter enzymes function only as minor nitrofurans reductases.

As shown in Table II, the cell-free extract from the strain also exhibited oxygen-insensitive nitrofurazone reductase activity when supplemented with NADPH or NADH. The NADPH-linked activity was much stronger than the NADH-linked one. However, no stimulating effect of FAD was observed at all. On the other hand, the cell-free extract exhibited a significant 1-nitropyrene reductase activity in the presence of both the reduced pyridine nucleotide and FAD under anaerobic conditions. In this case, NADH was much more effective than NADPH. FAD could be replaced with flavin mononucleotide (FMN), riboflavin or methyl viologen (data not shown). These results indicated that an electron carrier such as FAD is required as an essential fac-

tor in the reduction of 1-nitropyrene by the bacterial enzymes. However, no significant activity above the control experiment was found when the cell-free extract was supplemented with FAD alone (data not shown).

As shown in Table III, *E.coli* B/r acquires resistance to nitrofurazone in two mutational steps to form low-resistant and then high-resistant mutants, associating with stepwise loss of the oxygen-insensitive nitrofurans reductase activity. However, such stepwise loss of the activity was not observable with 1-nitropyrene reductases.

From these results, we concluded that the major enzymes responsible for the reduction of 1-nitropyrene and nitrofurazone in *E.coli* B/r are different from each other.

In addition, methyl *p*-nitrobenzoate reductases in *E.coli* B/r are oxygen-insensitive and exhibit their activities without FAD, similar to nitrofurazone reductases.¹⁹⁾ Also, 1-nitropyrene reductases from *Bacteroides fragilis* are much resistant to oxygen and able to reduce 1-nitropyrene without FAD.¹⁶⁾ These facts suggest that 1-nitropyrene reductases differ from not only major nitrofurazone reductases but also methyl *p*-nitrobenzoate reductases in *E.coli* B/r and that 1-nitropyrene reductases in *E.coli* B/r also differ from those in anaerobic bacteria such as *Bacteroides fragilis* in their properties.

The cell-free extract from nitrofurazone high-resistant mutants was chromatographed on a

TABLE I. 1-Nitropyrene and Nitrofurazone Reductase Activities in Intact Cells of *Escherichia coli* B/r

Condition	1-Nitropyrene reductase activity (nmol/h/10 ¹⁰ cells)	Nitrofurazone reductase activity (μmol/15 min/10 ¹⁰ cells)
Aerobic	0.5	50.9
Anaerobic	22.5	52.3

With 1-nitropyrene, the incubation mixture consisted of 0.2 μmol of the substrate and 4.5×10^{10} cells in a final volume of 2.5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 1 h under aerobic or anaerobic conditions as described in Experimental. With nitrofurazone, the incubation mixture consisted of 0.25 μmol of the substrate and 0.18×10^{10} cells in a final volume of 5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 15 min under aerobic or anaerobic conditions. Each value represents mean of three experiments.

DEAE-cellulose column, followed by measuring reductase activities of each fraction towards 1-nitropyrene, FAD and nitrofurazone. As shown in Fig. 1a, 1-nitropyrene reductase activities were eluted as four well-defined peaks (I, II, III and IV) of NADH-linked, and both NADH- and NADPH-linked activities. This fact indicated that at least four kinds of enzymes function as 1-nitropyrene reductases in *E.coli* B/r. Next, FAD

reductase activities were also fractionated into four peaks (I, II, III and IV) of NADH-linked, and both NADH- and NADPH-linked activities (Fig. 1b). These peak positions were completely identical with those of 1-nitropyrene reductase activities described above. On the other hand, five elution peaks (II, III, IV, V and VI) of NADH-linked, NADPH-linked, and both NADH- and NADPH-linked activities were observed when

TABLE II. *1-Nitropyrene and Nitrofurazone Reductase Activities in Cell-Free Extract from Escherichia coli B/r*

Addition	1-Nitropyrene reductase activity (nmol/h/mg protein)		Nitrofurazone reductase activity (μmol/5 min/mg protein)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
None (control)	0.8	3.1	0	0
NADPH	1.2	8.6	616	600
NADPH, FAD	1.5	21.6	615	619
NADH	1.2	7.9	117	130
NADH, FAD	3.0	130.5	110	130

With 1-nitropyrene, the incubation mixture consisted of 0.2 μmol of the substrate, the cell-free extract (0.3 mg of protein), 0.25 μmol of FAD and a NADPH-generating system (1 μmol of NADP, 10 μmol of glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase) or a NADH-generating system (1 μmol of NAD, 10 units of alcohol dehydrogenase and 50 μl of ethanol) in a final volume of 2.5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 1 h under aerobic or anaerobic conditions as described in Experimental. With nitrofurazone, the incubation mixture consisted of 0.15 μmol of the substrate, the cell-free extract (0.12 mg of protein), 0.25 μmol of FAD and a NADPH- or NADH-generating system in a final volume of 2.5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 5 min under aerobic or anaerobic conditions. Each value represents mean of three experiments.

TABLE III. *1-Nitropyrene and Nitrofurazone Reductase Activities in Cell-Free Extracts from Escherichia coli B/r and Its Nitrofurant-Resistant Mutants*

Strain	1-Nitropyrene reductase activity (nmol/h/mg protein)		Nitrofurazone reductase activity (μmol/5 min/mg protein)	
	NADH	NADPH	NADH	NADPH
Parent	130.5	21.6	130	600
Low-resistant	166.5	28.9	88	78
High-resistant	157.2	34.9	35	0

The incubation was carried out as described in the legend of Table II. In the assay of nitrofurazone reductase activity FAD was omitted from the incubation mixture. Each value represents mean of three experiments.

FIG. 1. Elution Patterns of 1-Nitropyrene, Nitrofurazone and FAD Reductase Activities in DEAE-cellulose Column Chromatography of the Cell-Free Extract from *Escherichia coli* B/r

The chromatography was carried out on a column (1.5×20 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 up to 350 mM. Fractions (2.2 ml) were collected at a flow rate of 22 ml/h. With 1-nitropyrene, the incubation mixture consisted of 0.2 μ mol of the substrate, 1 ml of each fraction (an enzyme source), 0.25 μ mol of FAD and a NADPH- or NADH-generating system (See the legend of Table II) in a final volume of 2.5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 1 h under anaerobic conditions. With nitrofurazone, the incubation mixture consisted of 0.15 μ mol of the substrate, 0.5–1 ml of each fraction and a NADPH- or NADH-generating system in a final volume of 2.5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 1.5 h under anaerobic conditions. With FAD, the incubation mixture consisted of 0.25 μ mol of the substrate, 0.2–0.5 ml of each fraction and a NADPH- or NADH-generating system in a final volume of 2.5 ml of 67 mM phosphate buffer (pH 7.2). The incubation was carried out for 15 min under anaerobic conditions.

oxygen-sensitive nitrofurazone reductase activities were assayed (Fig. 1c). Among them, peaks II, III and IV were eluted at the same positions as those of 1-nitropyrene or FAD reductase activities described above. These results strongly suggest that enzymes capable of reducing FAD in *E. coli* B/r are responsible for the reduction of 1-nitropyrene in the presence of its electron donor and FAD under anaerobic conditions, and that some of them also function as oxygen-sensitive nitrofurazone reductases without FAD.

Furthermore, the results suggest that FADH_2 , which is formed by enzymatic reduction of FAD, reduces 1-nitropyrene to the corresponding amine. To examine this possibility, FADH_2 was reacted with 1-nitropyrene under anaerobic conditions as described in Experimental. As a result, FADH_2 was gradually reoxidized, accompanying the increase in absorbance at 450 nm, and the GLC analysis of the incubation mixture showed that 1-aminopyrene had been produced non-enzymatically. As shown in Fig. 2, the reduction ratio of 1-nitropyrene depended upon the amount of FAD which was added to the incubation mixture. These facts led us to conclusion that the reduction of 1-nitropyrene to the correspond-

ing amine by 1-nitropyrene reductases in *E. coli* is mediated by an electron carrier such as FAD.

REFERENCES

- 1) J. N. Pitts, Jr., K. A. van Cauwenberghe, D. Grosjean, J. P. Schmid, D. R. Fitz, W. L. Belser, Jr., G. B. Knudson and P. M. Hynds: Atmospheric reactions of polycyclic aromatic hydrocarbons: Facile formation of mutagenic nitro derivatives, *Science*, **202**, 515–519 (1978).
- 2) H. Tokiwa, S. Kitamori, K. Takahashi and Y. Ohnishi: Mutagenic and chemical assay of extracts of airborne particulates, *Mutat. Res.*, **77**, 99–108 (1980).
- 3) T. C. Pederson and J. S. Siak: The role of nitroaromatic compounds in the direct-acting mutagenicity of diesel particle extracts, *J. Appl. Toxicol.*, **1**, 54–60 (1981).
- 4) X. B. Xu, J. P. Nachtman, S. M. Rappaport, E. T. Wei, S. Lewis and A. L. Burlingame: Identification of 2-nitrofluorene in diesel exhaust particulates, *J. Appl. Toxicol.*, **1**, 196–198 (1981).
- 5) H. S. Rosenkranz, E. C. McCoy, D. R. Sanders, M. Butler, D. K. Kiriakides and R. Mermelstein: Nitropyrenes: Isolation, identification, and reduction of mutagenic impurities in carbon black and toners, *Science*, **209**, 1039–1043 (1980).
- 6) G. Löfroth, E. Hefner, I. Alfheim and M. Möller: Mutagenic activity in photocopies, *Science*, **209**, 1037–1039 (1980).
- 7) R. Mermelstein, D. K. Kiriakides, M. Butler, E. C. McCoy and H. S. Rosenkranz: The extraordinary mutagenicity of nitropyrenes in bacteria, *Mutat. Res.*, **89**, 187–196 (1981).
- 8) H. Tokiwa, R. Nakagawa and Y. Ohnishi: Mutagenic assay of aromatic nitro compounds with *Salmonella typhimurium*, *Mutat. Res.*, **91**, 321–325 (1981).
- 9) H. Ohgaki, N. Matsukura, K. Morino, T. Kawachi, T. Sugimura, K. Morita, H. Tokiwa and T. Hirota: Carcinogenicity in rats of the mutagenic compounds 1-nitropyrene and 3-nitrofluoranthene, *Cancer Lett.*, **15**, 1–7 (1982).
- 10) K. El-Bayoumy, S. S. Hecht and D. Hoffmann: Comparative tumor initiating activity on mouse skin of 6-nitrobenzo[a]pyrene, 6-nitrochrysene, 3-nitroperylene, 1-nitropyrene and their parent hydrocarbons, *Cancer Lett.*, **16**, 333–337 (1982).
- 11) E. J. Rosenkranz, E. C. McCoy, R. Mermelstein and H. S. Rosenkranz: Evidence for the existence of distinct nitroreductases in *Salmonella typhimurium*: roles in mutagenesis, *Carcinogenesis*, **3**, 121–123 (1982).
- 12) S. Kitamura, N. Narai, M. Hayashi and K. Tatsumi: Rabbit liver enzymes responsible for reduction of nitropolycyclic aromatic hydrocarbons, *Chem. Pharm. Bull.*, **31**, 776–779 (1983).
- 13) F. Messier, C. Lu, P. Andrews, B. E. McCarry, M. A. Quilliam and D. R. McCalla: Metabolism of 1-

FIG. 2. Nonenzymatic Reduction of 1-Nitropyrene by FADH_2

- nitropyrene and formation of DNA adducts in *Salmonella typhimurium*, *Carcinogenesis*, **10**, 1007–1011 (1981).
- 14) C. Lu, D. R. McCalla and B. E. McCarry: The 1-nitropyrene reductases of *Salmonella typhimurium*, *Chem.-Biol. Interact.*, **43**, 67–71 (1983).
- 15) T. Kinouchi, Y. Manabe, K. Wakisaka and Y. Ohnishi: Biotransformation of 1-nitropyrene in intestinal anaerobic bacteria, *Microbiol. Immunol.*, **26**, 993–1005 (1982).
- 16) T. Kinouchi and Y. Ohnishi: Purification and characterization of 1-nitropyrene nitroreductases from *Bacteroides fragilis*, *Appl. Environ. Microbiol.*, **46**, 596–604 (1983).
- 17) K. Tatsumi, T. Doi, N. Koga, H. Yoshimura, H. Koga and T. Horiuchi: Studies on oxygen-insensitive nitrofurantoin reductase in *Escherichia coli* B/r, *J. Biochem. (Tokyo)*, **89**, 855–859 (1981).
- 18) T. Doi, H. Yoshimura and K. Tatsumi: Properties of nitrofurantoin reductases from *Escherichia coli* B/r, *Chem. Pharm. Bull.*, **31**, 1105–1107 (1983).
- 19) S. Kitamura, N. Narai and K. Tatsumi: Studies on bacterial nitroreductases. Enzymes involved in reduction of aromatic nitro compounds in *Escherichia coli*, *J. Pharm. Dyn.*, **6**, 18–24 (1983).
- 20) C. V. Ristagno and H. J. Shine: Ion radicals. XX. Aromatic nitration. A new preparation of 3-nitroperylene and 1-nitropyrene, *J. Am. Chem. Soc.*, **93**, 1811–1812 (1971).
- 21) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275 (1951).
- 22) R. E. Asnis, F. B. Cohen and J. S. Gots: Studies on bacterial resistance to furacin, *Antibiot. Chemother.*, **2**, 123–129 (1952).
- 23) R. E. Asnis: The reduction of furacin by cell-free extracts of furacin-resistant and parent-susceptible strains on *Escherichia coli*, *Arch. Biochem. Biophys.*, **66**, 208–216 (1957).