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ESCHERICHIA COLI PYRIDINE 1-OXIDE REDUCTASE

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SUMMARY

Pyridine 1-oxide reductase activity was studied in cell-free extracts of *Escherichia coli*, Strain 9723 and Strain B (ATCC 11303). Reduced pyridine nucleotides serve as a physiological electron donor for the reduction process; reduced methyl viologen serves as a very effective artificial electron donor. Reaction conditions for the assay of enzymatic pyridine 1-oxide reduction are given, and evidences are presented which indicate that the pyridine 1-oxide reductase activity is distinct from nitroaryl reductase, nitrate reductase, nitrite reductase and other known reductase activities.

INTRODUCTION

In a previous study, it was found that whole cells of *Escherichia coli* 9723 catalyze the reduction of certain pyridine 1-oxides to the corresponding pyridines[1]. It was later found upon incubation of selected pyridine 1-oxides and quinoline 1-oxides with growing cells of both *E. coli* 9723 and *E. coli* 11303, employing paper chromatographic analysis, that most of the pyridine 1-oxides served as substrates for the reduction process; however, the quinoline 1-oxides tested were not reduced. Since the reduction process apparently exhibited a preference for the *N*-oxide grouping of the pyridine ring, it was anticipated that the activity might be due to the presence of a pyridine ring-specific enzyme(s). Little information of the reduction of pyridine 1-oxides by bacterial enzyme systems is to be found in the literature, and the studies reported are neither exhaustive nor definitive[2,3]. Studies have been conducted on pyridine 1-oxide reductions catalyzed by mammalian liver preparations; in the one reported case of partial purification of the liver reductase activity, questions still remained concerning the reaction components necessary for activity and the physiological significance of the reduction of pyridine 1-oxides[4].

The study reported herein is concerned with the reduction of pyridine 1-oxides by cell-free extracts of both *E. coli* 9723 and *E. coli* 11303. Cofactor and reaction condition requirements have been determined, and evidence is given supporting the conclusion that the pyridine 1-oxide reductase activity is distinct from activities involved in the reduction of other types of nitrogen–oxygen bonds.

MATERIALS AND METHODS

Materials

The materials were purchased as follows: pyridine nucleotides, flavin nucleotides, glucose 6-phosphate dehydrogenase (Type XI), and methyl viologen, from Sigma Chemical Company; glucose 6-phosphate (disodium), from General Biochemicals; and alcohol dehydrogenase (yeast), from Worthington Biochemical Corporation. The pyridine 1-oxides were kindly provided by Dr. P. T. Sullivan*.

Culture methods

The *E. coli* strains were cultured on ANDERSON's[5] glucose salts medium supplemented with 0.5% yeast extract. The incubations were conducted at 37° for 10–12 h in 16-l carboys under aeration. The cells were harvested with a Sharples Super Centrifuge. *E. coli* B (ATCC 11303) frozen cell paste (General Biochemicals, late log, Kornberg medium) was also used as an enzyme source.

Cell-free extracts

All procedures involving cell-free extracts were carried out at 0–5°. Protein concentration was measured by the spectrophotometric method of MURPHY AND KIES[6].

30 g (wet weight) of *E. coli* cells were suspended in 50 ml of a medium containing 0.01 M potassium phosphate, pH 7.5, and 0.01 M 2-mercaptoethanol (Medium A). The suspension was then subjected to mechanical disruption in a Braun Model MSK Mechanical Cell Homogenizer with glass beads (0.10–0.11 mm diameter, 1-ml beads per 5 ml cell suspension) for 4 min. Unbroken cells and debris were removed by centrifugation at 28 000 × *g* for 20 min. A Parr Cell Disruption Bomb was also used to prepare cell-free extracts. Cells (60 g per 100 ml Medium A) were exposed to 1500 lb/inch^2 of nitrogen for two 30-min pressurizations. After centrifugation to remove undisrupted cells and debris, the cell-free extracts were stored at −40°.

Assay procedures

Pyridine 1-oxide reductase. The reaction mixture contained 2.0 mM 4-hydroxymethylpyridine 1-oxide; 100 mM potassium phosphate buffer, pH 8.0; 0.3 mM NADP; 8.0 mM glucose 6-phosphate (disodium); glucose 6-phosphate dehydrogenase, 1.0 I.U.; 0.05 ml enzyme preparation and deionized water to give a total volume of 0.5 ml. When methyl viologen was the electron donor, the assay mixture contained 2.0 mM 4-hydroxymethylpyridine 1-oxide; 100 mM potassium phosphate buffer, pH 8.0; 0.1 mM methyl viologen; 4.0 mM sodium dithionite in 9.5 mM sodium bicarbonate; 0.05 ml enzyme preparation and deionized water to give a total volume of 0.5 ml.

The reaction was started by the addition of enzyme, allowed to proceed for 20 min at 37°, and terminated by the addition of 0.5 ml of 0.2 M HCl. The amount of pyridine derivative produced was determined by the colorimetric procedure of GORSKAYA AND YARYM-AGAEVA[7]. The absorption maximum for 4-hydroxymethylpyridine, employing this procedure, was found to be 614 nm.

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Nitroaryl reductase. The reaction mixture was the same as that for pyridine 1-oxide reductase (described above) with the exception that 1.5 mM *m*-dinitrobenzene was substituted for 4-hydroxymethylpyridine 1-oxide. The incubation time was 10 min at 37°, and the reaction was terminated by the addition of 0.5 ml of 3.0 M HCl. The precipitated protein was removed by centrifugation and the *m*-nitroaniline present in the supernatant was determined by the colorimetric method of BRATTON AND MARSHALL[8].

Nitrate reductase. The reaction mixture consisted of 10 mM KNO_3; 100 mM potassium phosphate buffer, pH 7.5; 0.1 mM methyl viologen; 8.0 mM sodium dithionite in 9.5 mM sodium bicarbonate; 0.05 ml enzyme preparation and distilled water to give a total volume of 0.5 ml. The assay was started by the addition of enzyme, was incubated 10 min at 37°, and was terminated by the addition of 0.1 ml of 1.0 M barium acetate. The nitrite formation was determined by the method of HEREDIA AND MEDINA[9].

Fractionation of crude enzyme extracts

Streptomycin sulfate and protamine sulfate precipitations. 2 ml of a 1% protamine sulfate solution were added to 100 ml of crude enzyme preparation, followed immediately by the addition of 6.67 ml of a 10% streptomycin sulfate solution. The resulting suspension was stirred for 15–30 min and then was centrifuged for 15 min at 28 000 × *g*. The pellet was discarded.

Ammonium sulfate precipitation. Cold, saturated ammonium sulfate solution, pH 7.0, was employed. The amount of saturated solution used was determined from standard ammonium sulfate concentration conversion tables to obtain 40% and 55% saturated fractions. The 55% saturated fraction was resuspended in 20% of the original volume of 0.005 M potassium phosphate buffer containing 0.01 M 2-mercaptoethanol (Medium B). The solution was dialyzed 2 h against Medium B.

DEAE-cellulose chromatography. Washed DEAE-cellulose was equilibrated with Medium B and packed in a 2.5 cm × 45 cm column. Approx. 200 mg of the dialyzed ammonium sulfate fraction was applied to the column. Reductase activity was eluted with a phosphate buffer gradient (0.005 M to 0.5 M) containing 0.01 M 2-mercaptoethanol.

RESULTS AND DISCUSSION

The reduction of various pyridine 1-oxides to the corresponding pyridine derivatives by whole cells of *E. coli* 9723 and *E. coli* 11303 is given in Table I. Paper chromatography was utilized to detect the reduction products. Most of the pyridine 1-oxides tested were reduced; however, neither quinoline 1-oxide nor 8-hydroxyquinoline 1-oxide were reduction substrates. As can be seen in the table, the only pyridine 1-oxide (of those tested) that is not reduced is the highly substituted 5-methoxy-4-chloro-2-malonylpyridine 1-oxide.

The reduction process was studied in cell-free extracts of both *E. coli* 9723 and *E. coli* 11303 employing 4-hydroxymethylpyridine 1-oxide as substrate. The specific activities of and the requirements for the pyridine 1-oxide reductase activities from the two bacterial strains were found to be virtually identical. In the early studies both NADPH and NADH were found to be effective electron donors for the

TABLE I

STUDIES OF THE REDUCTION OF VARIOUS PYRIDINE AND QUINOLINE 1-OXIDES BY *Escherichia coli*

Bacterial cells were cultured in a salts–glucose medium. After approx. 6 h growth, sterile solutions of the various pyridine and quinoline 1-oxides were added (final concentration, 0.6 mg/ml), and incubation was continued for an additional 12 h. Reduction products were identified by paper chromatography of the growth medium as previously described[1].

N-Oxide	*E. coli 9723*	*E. coli 11303*
4-Hydroxymethylpyridine 1-oxide	Completely reduced	Partially reduced
3-Hydroxymethylpyridine 1-oxide	Partially reduced	Detectable reduction
2-Hydroxymethylpyridine 1-oxide	Completely reduced	Partially reduced
β-(4-Pyridyl 1-oxide)-DL-alanine	Completely reduced	Completely reduced
β-(3-Pyridyl 1-oxide)-DL-alanine	Completely reduced	Completely reduced
β-(2-Pyridyl 1-oxide)-DL-alanine	Completely reduced	Completely reduced
5-Methoxy-4-chloro-2-chloromethylpyridine 1-oxide	Not reduced	Not reduced
Quinoline 1-oxide	Not reduced	Not reduced
8-Hydroxyquinoline 1-oxide	Not reduced	Not reduced

reduction process, the former reductant being somewhat more effective (see Table II). Both FMN and FAD were found to stimulate the enzyme activity somewhat; however, there appears to be no absolute requirement for these flavins, in that neither restore any of the activity lost upon dialysis. Very significant loss of reductase activity was encountered upon dialysis for periods longer than 2 h (25–30% at 2 h). The addition of a variety of cations, such as Fe^{2+}, Fe^{3+}, Mo^{6+}, Mg^{2+}, Mn^{2+}, Co^{2+}, K^{+}, *etc.*, failed to restore activity to dialyzed preparations. 2-Mercaptoethanol at a concentration of 10 mM was routinely added to all crude enzyme preparations and to all dialysis media; however, little stimulation of enzyme activity and only a small increase in stability of the enzyme was effected by this addition. As also seen in Table II, reduced methyl viologen is an effective electron donor *in lieu* of reduced pyridine nucleotides.

TABLE II

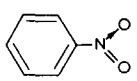
ELECTRON DONOR REQUIREMENTS FOR PYRIDINE 1-OXIDE REDUCTION IN CELL-FREE EXTRACTS OF *E. coli* 11303

4-Hydroxymethylpyridine 1-oxide was employed as substrate. The reaction mixture composition and experimental conditions are described in MATERIALS AND METHODS. Specific activity is in terms of μmoles of product produced per min per mg protein. The NADH generating system consisted of 0.3 mM NAD, 0.1 M 95% ethanol and 1.0 I.U. alcohol dehydrogenase. The NADPH generating system consisted of 0.3 mM NADP, 80 mM glucose 6-phosphate and 1.0 I.U. glucose 6-phosphate dehydrogenase. When FMN or FAD were added to the reaction mixture, the concentrations were 0.02 mM. The method of TANIGUCHI AND ITAGAKI[10] was employed when reduced methyl viologen was used as an electron donor.

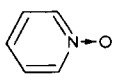
Electron donor added to reaction mixture	*Pyridine 1-oxide reductase specific activity (× 10³)*
None	2.0
NADH (1.0 mM)	3.2
NADPH (1.0 mM)	6.8
NADH generating system	5.3
NADPH generating system	10.3
NADPH generating system *plus* FMN	10.5
NADPH generating system *plus* FAD	10.0
Methyl viologen, reduced	26.0

Although the reduction of pyridine 1-oxides has been reported to occur in certain crude microbial extracts[2,3,11], no detailed studies of the nature and substrate specificity of the enzyme(s) involved in the reduction process have appeared in the literature. On the other hand, many reports have appeared delineating the enzymatic reactions in microbial systems involved in the reduction of the nitrogen–oxygen bond in a variety of groups and ions; *e.g.* nitroaryl-, nitrate-, nitrite- and hydroxylamine reductase activities[3,9,12–19]. All of these reductase activities have been shown to be pyridine nucleotide-dependent; at least in crude preparations. It was considered possible that the reduction of pyridine 1-oxides by extracts of *E. coli* could be attributable to one of the above nitrogen–oxygen reductase types; accordingly, studies were made to determine the identity, or lack thereof, of *E. coli* pyridine 1-oxide reductase activity with other nitrogen–oxygen bond reductases found in the same organism.

Nitroaryl reductase activity has been studied in *E. coli* and other organisms[17–19]. The reduction of pyridine 1-oxides by nitroaryl reductase activity appears reasonable in light of the obvious structural and electronic similarities of these compounds.



Nitrobenzene



Pyridine 1-oxide

Table III summarizes studies of the effects of various additives and/or reaction conditions on the reductions of *m*-dinitrobenzene and 4-hydroxymethylpyridine 1-oxide by cell-free extracts of *E. coli* 11303. It should be noted that in addition to the different responses of the enzymatic reductions of *m*-dinitrobenzene and 4-hydroxymethylpyridine 1-oxide, elicited by various treatments and additions, the ratio of specific activities for the reductions of these compounds in crude extracts varied considerably from preparation to preparation.

Further evidence which indicates that nitroaryl reductase is not responsible for the reduction of pyridine 1-oxides is provided by attempts to purify the latter activity. As can be seen in Table IV, the activities for the reduction of pyridine 1-oxides and for nitroaryls do not fractionate together; moreover, it is obvious that the pyridine 1-oxide reductase activity is lost in the course of the purification attempts. This loss of activity was always found when a variety of other fractionation methods were employed after the $(NH_4)_2SO_4$ fractionation step, *e.g.* adsorption on calcium phosphate gel, Sephadex gel chromatography, carboxymethyl Sephadex chromatography, and disc gel electrophoresis. On the other hand, nitroaryl reductase activity could readily be purified by employment of a number of the above fractionation methods.

The facile loss of the pyridine 1-oxide reductase activity indicated the possibility that one or more other enzymes and/or cofactors necessary for the activity were being removed by the fractionation processes. Indirect evidence for the participation of other required factors, even in crude enzyme preparations, is shown in Fig. 1. A sigmoidal response results when enzyme activity is plotted against increasing enzyme preparation concentration. Such a plot is indicative of the presence of limiting amounts of a dissociable cofactor(s) or of another enzyme(s) which becomes limiting at high dilution[20].

TABLE III

EFFECTS OF VARIOUS ADDITIVES AND TREATMENTS ON THE REDUCTION OF *m*-DINITROBENZENE AND OF 4-HYDROXYMETHYLPYRIDINE 1-OXIDE BY CELL-FREE EXTRACTS OF *E. coli* 11303

The normal reaction mixture compositions and experimental methods are given in the MATERIALS AND METHODS section.

Expt. No.	*Additions or treatments*	*Nitroaryl reductase activity (I.U. × 10³)*	*Pyridine 1-oxide reductase activity (I.U. × 10³)*	*Nitroaryl reductase/ pyridine 1-oxide reductase activity ratio*
1	Guanidine·HCl in reaction mixture			
	None	10.6	7.9	1.3
	0.1 M	11.8	7.1	1.7
	0.2 M	12.4	4.6	2.7
	0.5 M	11.3	2.1	5.4
2	Urea in reaction mixture			
	None	7.9	4.2	1.9
	6 M	7.5	0.8	9.3
3	Preheat treatment of extract*			
	Not preheated	12.6	5.3	2.4
	Preheated	11.0	2.8	3.9
4	Pretreatment of extract to pH 4.0**			
	Untreated	8.7	4.4	2.0
	pH treated	8.4	2.4	3.5
5	Dialysis of extract			
	Undialyzed	17.1	5.1	3.4
	Dialyzed 4 h	17.1	1.3	13.2

* A portion of the cell-free extract was heated at 55° for 5 min; the assay temperature was 37°.

** A portion of the cell-free extract was taken to pH 4.0, allowed to stand for a short time, and then the pH was adjusted to 7.0 for use in the assay.

TABLE IV

FRACTIONATION DATA FOR PYRIDINE 1-OXIDE REDUCTASE AND NITROARYL REDUCTASE ACTIVITIES

The normal reaction mixture compositions and experimental methods are given in MATERIALS AND METHODS. The substrate for pyridine 1-oxide reductase was 4-hydroxymethylpyridine 1-oxide and was *m*-dinitrobenzene for nitroaryl reductase. Specific procedures are described in MATERIALS AND METHODS.

Treatment	*Specific activity (I.U. × 10³)*		*Yield (%)*		*Purification (-fold)*	
	Pyridine 1-oxide reductase	*Nitroaryl reductase*	*Pyridine 1-oxide reductase*	*Nitroaryl reductase*	*Pyridine 1-oxide reductase*	*Nitroaryl reductase*
Crude	3	15	—	—	—	—
Protamine sulfate, streptomycin sulfate	2	28	60	100	1.0	1.9
Ammonium sulfate (40–55%)	9	28	39	65	4.5	1.9
DEAE-cellulose	0.05	330	none	60	—	22.0

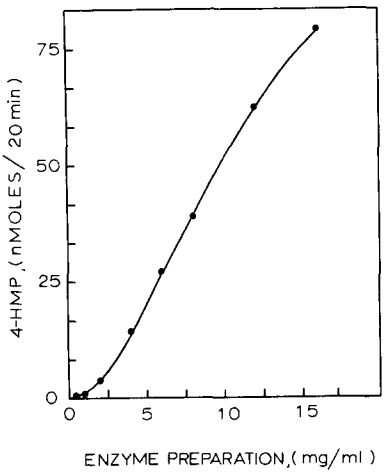


Fig. 1. Pyridine 1-oxide reductase activity with increasing concentrations of enzyme preparation. The substrate employed was 4-hydroxymethylpyridine 1-oxide; the usual procedures and incubation mixtures were employed as described in MATERIALS AND METHODS. 4-HMP, 4-hydroxymethylpyridine.

Escherichia coli has been shown to contain two types of nitrate reductases, an assimilatory type and a respiratory type[10,13,21,22]. The respiratory nitrate reductase, which functions under anaerobic conditions and is principally particulate, has an apparent requirement for cytochrome b_1 as an intermediate electron carrier. It was found that preparations of cytochrome-rich fractions from *E. coli* reproducibly stimulated pyridine 1-oxide reductions when NADPH was employed as electron donor. The results of one such experiment are summarized in Table V.

TABLE V

PYRIDINE 1-OXIDE REDUCTION IN THE PRESENCE OF CYTOCHROME-RICH PREPARATIONS

The complete reaction mixtures are described in MATERIALS AND METHODS. The method of FUJITA AND SATO[23] was used through the ammonium sulfate fractionation step. 0.1 ml of the preparation was added to the reaction mixture.

Components of reaction mixture	*Pyridine 1-oxide reductase activity (I.U. × 10³)*
Cytochrome-rich preparation	0.005
Crude enzyme preparation alone	2.5
Crude enzyme preparation *plus* cytochrome-rich preparation	4.1
DEAE-cellulose fraction alone	0.05
DEAE-cellulose fraction *plus* cytochrome-rich preparation	0.11

As with the respiratory nitrate reductase of *E. coli*, reduced methyl viologen serves as an excellent electron donor for the enzymatic reduction of pyridine 1-oxides (see Table II). Several experiments were conducted to determine whether the activity

responsible for the anaerobic reduction of pyridine 1-oxides is distinct from that involved in the anaerobic reduction of nitrate in *E. coli*.

It has been reported that the respiratory nitrate reductase of *E. coli* is induced when the cells are grown in the presence of nitrate under anaerobic conditions[10,13]. Table VI summarizes experiments conducted with whole cells of *E. coli* 11303 which were cultured anaerobically with or without either KNO_3 or 3-hydroxymethylpyridine 1-oxide in the growth media. The assay conditions were both aerobic and anaerobic; the electron donor was methyl viologen. The respiratory nitrate reductase activity was induced approx. 2-fold when nitrate was added to the growth media; a contrary effect is exerted on pyridine 1-oxide reductase in that its activity is repressed over 3-fold by the addition of nitrate. It is also seen from the table that the pyridine 1-oxide reductase activity is not significantly affected by the presence of a pyridine 1-oxide in the growth medium. The reason why nitrate, but not pyridine 1-oxides, in the growth media should affect pyridine 1-oxide reductase activity is not apparent.

TABLE VI

EFFECT OF GROWTH AND ASSAY CONDITIONS ON NITRATE REDUCTASE AND PYRIDINE 1-OXIDE REDUCTASE ACTIVITIES

Cells were grown as described in MATERIALS AND METHODS with the exception that the flasks were maintained under a nitrogen atmosphere. All reaction mixtures and the aerobic experimental procedures are described in MATERIALS AND METHODS. The anaerobic assay tubes were flushed with nitrogen and stoppered during incubation. The colorimetric assay procedures are described in MATERIALS AND METHODS.

Cell growth conditions	*(I.U. × 10^4) : assay conditions and substrate*			
	Aerobic KNO_3	*Anaerobic KNO_3*	*Aerobic 4-hydroxymethyl-pyridine 1-oxide*	*Anaerobic 4-hydroxymethyl-pyridine 1-oxide*
Anaerobic (no additions)	9.7	17	15	52
Anaerobic (KNO_3)	28	34	19	17
Anaerobic (3-hydroxymethylpyridine 1-oxide)	15	19	19	60

Another series of studies which indicated that the respiratory nitrate reductase and pyridine 1-oxide reductase are distinct activities involved competition studies. Fig. 2 summarizes the results of these studies. Plot A shows that nitrate, at a concentration approx. 10-fold greater than the K_m value for nitrate reductase, has no effect on the enzymatic reduction of pyridine 1-oxide. Plot B shows the lack of effect of 4-hydroxymethylpyridine 1-oxide on the reduction of nitrate. A concentration of the pyridine 1-oxide 10-fold greater than the K_m value for pyridine 1-oxide reductase was employed. The K_m values for KNO_3 and 4-hydroxymethylpyridine 1-oxide were found to be 0.9 mM and 2.0 mM respectively when the electron donor is reduced methyl viologen. The general reaction conditions followed for the K_m determinations are also given under Fig. 2.

Nitrite reductase activity in *E. coli* has been reported; LAZZARINI AND ATKIN-

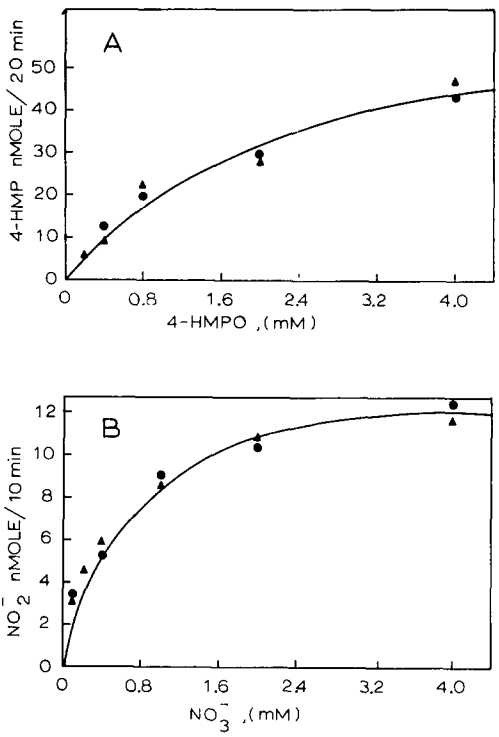


Fig. 2. The effects of NO^-_3 on pyridine 1-oxide reductase and of 4-hydroxymethylpyridine 1-oxide(4-HMPO) on nitrate reductase activities. (A) Effect of NO^-_3 on the reduction of 4-HMPO. The usual procedures and incubation mixtures were employed as described in MATERIALS AND METHODS with the exception of the addition designated below (methyl viologen was employed as electron donor). ●—●, no addition; ▲—▲, KNO_3 (10 mM) in reaction mixture. 4-HMP, 4-hydroxymethylpyridine. (B) Effect of 4-hydroxymethylpyridine 1-oxide on the reduction of NO^-_3. The usual procedures and incubation mixtures were employed with the exception of the addition designated below. ●—●, no addition; ▲---▲, 4-hydroxymethylpyridine 1-oxide (20 mM) in reaction mixture.

SON[12] found that crude extracts of *E. coli*, strain Bn, apparently contain three nitrite reductase activities. One is specific for NADPH as electron donor, a second is specific for NADH for the source of electrons, while a third activity is associated with the 144 000 × *g* particulate fraction and will utilize benzyl viologen as electron donor. The latter nitrite reductase activity, while similar to the pyridine 1-oxide reductase activity of *E. coli* 11303 with respect to utilization of artificial electron donors, differs from pyridine 1-oxide reductase with respect to sedimentability. The pyridine 1-oxide reductase activity remains in the 144 000 × *g* supernatant fraction. In other experiments the addition of nitrite to reaction mixtures did not result in competition with 4-hydroxymethylpyridine 1-oxide for a catalytic reduction site. Further, the presence of high concentrations of hydroxylamine in the reaction mixtures did not result in a diminution of the rate of pyridine 1-oxide reduction.

The identity of *E. coli* pyridine 1-oxide reductase with sulfate and sulfite reductases from this organism can be discounted. With the sulfate reductase there is an absolute requirement for ATP[24]. In numerous attempts, no stimulation of *E. coli* pyridine 1-oxide reductase could be demonstrated by the addition of ATP

to the reaction mixtures. The sulfite reductase of *E. coli* has been shown to be strongly inhibited by cyanide[25]. The pyridine 1-oxide reductase of *E. coli*, on the other hand, is not inhibited by cyanide.

The physiological role of the pyridine 1-oxide reductase activity of *E. coli* is unclear, and its constitutive presence is puzzling. The specificity, fractionation, competition, and induction-repression data indicate that the activity is distinct from other characterized activities which catalyze the reduction of the nitrogen–oxygen bond (*e.g.* nitroaryl, respiratory nitrate, nitrite or hydroxylamine reductase activities) and the reduction of the sulfur-oxygen bond (*e.g.* sulfate and sulfite reductase activities). The great lability of the pyridine 1-oxide reductase indicates a possible participation of other factors in the reduction process. The data obtained from the use of methyl viologen and of cyrochrome-rich preparations would indicate a terminal electron acceptor role of the *N*-oxide function. However, the assignment of such a role for the pyridine 1-oxide reductase activity is premature and awaits further study.

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