Kinetic analysis and substrate specificity of *Escherichia coli* dimethyl sulfoxide reductase

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We have characterized the substrate specificity of dimethyl sulfoxide reductase (DmsABC) of *Escherichia coli* by determining K_m and k_{cat} values for 22 different substrates. The enzyme has a very broad substrate specificity. The K_m values varied 470-fold, while k_{cat} values varied only 20-fold, implicating K_m as the major determinant of k_{cat}/K_m values. Sulfoxides and pyridine *N*-oxide exhibited the lowest K_m values, followed by aliphatic *N*-oxides. The k_{cat} values for these compounds also followed the same pattern. Substitution at the 2 or 3 position of the pyridine *N*-oxide ring had little effect on K_m , while substitution at the 4 position had a greater effect, and increased K_m . Negatively charged substrates were poorly accepted. A few compounds that are not *S*- or *N*-oxides were also reduced by the enzyme. Most compounds reduced by DmsABC were not toxic to *E. coli* under anaerobic growth conditions, and *E. coli* was able to use many of these compounds anaerobically as terminal electron acceptors in the presence of glycerol. Anaerobic growth on sulfoxides is solely due to DmsABC expression. However, there appears to be another as yet unidentified terminal reductase capable of using pyridine *N*-oxides as terminal electron acceptors.

Keywords: trimethylamine *N*-oxide, molybdoenzyme, anaerobic reductase, pyridine *N*-oxide, *S*- or *N*-oxide

INTRODUCTION

Dimethyl sulfoxide (DMSO) reductase (DmsABC) of *Escherichia coli* is a complex iron–sulfur molybdoenzyme capable of conserving the energy of menaquinol:DMSO oxidoreduction as a proton-motive force across the bacterial membrane (Bilous *et al.*, 1988; Bilous & Weiner, 1985b; Cammack & Weiner, 1990; Rothery *et al.*, 1995; Weiner *et al.*, 1992). This constitutive membrane-bound enzyme is composed of three subunits: a catalytic subunit containing molybdenum molybdopterin guanine dinucleotide cofactor (DmsA), an electron transfer subunit containing four [4Fe–4S] clusters (DmsB) and a membrane anchor subunit (DmsC) (Bilous *et al.*, 1988; Cammack & Weiner, 1990; Rothery & Weiner, 1991). Characterization of the molybdenum cofactor and [Fe–S] centres has proceeded using EPR (Cammack & Weiner, 1990; Rothery & Weiner, 1991, 1993) and fluorescence spectroscopy (Rothery *et al.*, 1995), and an understanding of the electron transfer pathway from menaquinol to the

molybdenum cofactor is currently being developed (Trieber *et al.*, 1994).

E. coli is capable of anaerobic growth by glycolysis, or by respiration using a variety of terminal electron acceptors. Respiratory growth of *E. coli* on several *S*- and *N*-oxides has been established, and these studies have demonstrated that the terminal reductases DmsABC and trimethylamine *N*-oxide (TMAO) reductase (TorA) play important roles in this process (Sambasivarao & Weiner, 1991a). While only DmsABC is responsible for anaerobic growth on DMSO, both DmsABC and TorA are responsible for anaerobic growth on TMAO (Sambasivarao & Weiner, 1991a). The molybdoenzyme TorA (Mejean *et al.*, 1994; Yamamoto *et al.*, 1986) is inducible (Pascal *et al.*, 1984; Silvestro *et al.*, 1988) and periplasmic (Silvestro *et al.*, 1989). The widespread occurrence in nature of DMSO and TMAO suggests that these compounds may serve as true physiological substrates for the reductases (Barrett & Kwan, 1985; Kelly & Baker, 1990; Kiene & Bates, 1990). The amino acid sequences of DmsABC and TorA are highly homologous, suggesting that the two enzymes might have similar substrate profiles (Mejean *et al.*, 1994). We have found that TorA is restricted in its substrate

Abbreviations: BV, benzyl viologen; BVH^{•+}, reduced benzyl viologen; TMAO, trimethylamine *N*-oxide.

spectrum (Iobbi-Nivol *et al.*, 1996), while DmsABC has a broad substrate specificity (Weiner *et al.*, 1988). However, these experiments examined only a few substrates of DmsABC. Therefore, it has not been possible to make conclusions regarding substrate binding, active site structure and catalysis from our knowledge of substrate specificity.

In this study, we have analysed a variety of *S*- and *N*-oxides and miscellaneous compounds in order to gain an understanding of the parameters which affect substrate binding and turnover of compounds reduced by DmsABC. The results obtained are contrasted with the substrate analysis of TorA (Iobbi-Nivol *et al.*, 1996). We have determined the toxicity of a range of DmsABC substrates, and we have monitored the ability of these substrates to serve as anaerobic terminal electron acceptors in wild-type cells, as well as in cells lacking DmsABC and/or TorA. We show that *E. coli* expresses at least one additional energy-conserving *N*-oxide reductase which is unable to use TMAO or DMSO but utilizes a subset of the compounds tested.

METHODS

Materials. Potential substrates (Fig. 1) of DmsABC were purchased from Sigma or Aldrich, except for biotin sulfoxide, which was synthesized from biotin (Melville, 1954), and DMSO, which was obtained from Fisher Scientific. For substituted pyridine *N*-oxides, all compounds with substituents in the 2, 3 and 4 position were obtained whenever possible.

Strains and plasmids. *E. coli* strains used in this study were: HB101 [*supE44 hsdS20 2(r_Bm_B) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (Boyer & Roulland-Dussoix, 1969); MC4100 [*F⁻ ara D139 (lacI POZYA-argF)U169 rpsL150 relA1 fli-5301 deoCl PtsF25 rbsR*] (Casadaban, 1976); LCB128 [as MC4100; *torA::Mud1 (Ap^r-lac)*] (M. Chippaux, Marseilles, France); DSS401 (as MC4100; Δ *dms* Km^r) (Sambasivarao & Weiner, 1991a); and DSS501 (as LCB128; Δ *dms* Km^r) (Sambasivarao & Weiner, 1991a). For overexpression of DmsABC, bacteria were transformed with pDMS160 (Rothery & Weiner, 1991). All manipulations of plasmids and strains were carried out essentially as described by Sambrook *et al.* (1989).

Media and growth conditions. Bacteria were grown anaerobically on minimal media as indicated previously by Bilous & Weiner (1985a), with the following exceptions. Peptone (0.16%) was substituted for Casamino acids, and streptomycin sulfate was not included in the growth medium. Arginine was included at a final concentration of 0.005% as necessary. When appropriate, 50–100 µg ampicillin ml⁻¹ and 40 µg kanamycin ml⁻¹ were included in the growth medium. The ability of *S*- and *N*-oxides to sustain growth on glycerol minimal media was tested by growing cells in sealed screw-capped tubes containing a small air bubble to allow mixing using a continuously rotating wheel. Quantitative growth experiments were performed in Klett flasks (Sambasivarao & Weiner, 1991a).

Assessment of the toxicity of DmsABC substrates by inhibition of anaerobic growth of *E. coli*. HB101 grown overnight in LB was used as a 1% inoculum for 3 ml of unsolidified LB top agar (0.7%). This sample was mixed and poured onto LB agar plates. Sterilized filters were dipped into filter-sterilized substrate to the point of saturation and placed on LB agar plates overlaid with LB top agar containing bacteria. All potential DmsABC substrates were used at 300 mM, except

3-amidopyridine *N*-oxide (100 mM). Platinum catalyst and plates were placed in anaerobic jars that were subsequently repeatedly evacuated and filled with H₂/CO₂ (10:90, v/v). After 72 h at 37 °C, the diameter of the zone of clearing (absence of growth) around the substrate-soaked filters was measured. Fumarate and nitrate were used as non-toxic controls. K₂CrO₄ and ampicillin were used as positive toxic controls.

Purification of DmsABC. HB101(pDMS160) was grown in 19 l carboys and harvested using a Pellicon membranes system as indicated previously (Weiner *et al.*, 1988). The cell paste was resuspended in buffer (Rothery & Weiner, 1991), and everted envelopes were prepared as indicated by Bilous & Weiner (1985a). The membrane fraction was extracted with Triton X-100 as indicated by Cammack & Weiner (1990). DmsABC was purified from solubilized membrane proteins on a DEAE-cellulose (Whatman DE-52) column as indicated previously (Weiner *et al.*, 1988), except that a 0–0.2 M KCl gradient was used, equivalent to ten column volumes, or on a DEAE-Sepharose CL-6B column with a 50–300 mM NaCl gradient equivalent to two column volumes.

Kinetic analysis. Substrate-dependent oxidation of the electron donor, reduced benzyl viologen (BVH^{•+}), was monitored at 570 nm using a Gilford 250 spectrophotometer. MOPS (50 mM; pH 7.0) was used as the buffer for all assays, and electron acceptor (substrate) was varied as necessary. For open-cuvette (non-stoppered) assays, using degassed MOPS buffer, benzyl viologen (BV) was used at a final concentration of 0.2 mM (ϵ of BVH^{•+} = 7.8×10^3 l mol⁻¹ cm⁻¹) and sodium dithionite at a concentration of 0.33 mM. The final assay volume was kept constant, with the ordered addition of BV, substrate, dithionite and enzyme. Closed-cuvette assays (stoppered) were performed using degassed buffer that was subsequently bubbled with nitrogen. Buffer was transferred to a cuvette, which was then stoppered. Hamilton syringes were used for ordered addition as above. A stable baseline was confirmed prior to addition of enzyme. For closed-cuvette assays, BV was used at a final concentration of 0.2 mM, and dithionite was added to approximately 0.1 mM, to give an absorbance of about 1.0. Closed-cuvette assays were used unless indicated in the text. All assays were performed at 30 °C using equilibrated buffer and a jacketed cuvette holder, except for the comparison of open- and closed-cuvette assays and membrane-bound, solubilized and purified enzyme, which were performed at 25 °C. One unit of enzyme activity corresponds to 1 µmol BVH^{•+} oxidized min⁻¹, or 0.5 µmol *S*- or *N*-oxide reduced min⁻¹. Between three and nine assays were performed at each substrate concentration, and five to eight different substrate concentrations were examined to determine each K_m . The values used were means. In all cases, the standard deviation of the mean was less than 10%. Whenever possible, a range of substrate concentrations was chosen that varied from at least two times less than the K_m to five times greater than the K_m . This range of substrate concentration could not be achieved in some instances, due to limitations resulting from the minimum concentration of substrate that can be used in the assay, or substrate inhibition. We ensured that only a small fraction of the substrate was utilized during the course of the experiment. K_m and V_{max} values were determined from Eadie-Hofstee plots (Fersht, 1977). The K_m values obtained are apparent K_m values using a saturating concentration of BV. k_{cat} values were determined by estimating the enzyme purity from PAGE analysis, as indicated below. Assays were performed on freshly diluted enzyme that was previously beaded into liquid nitrogen and stored at -70 °C. The diluted sample was stored on ice and used during the day to obtain reproducible results. Compounds found not to be substrates were tested for their ability to inhibit the transfer of electrons from reduced DmsABC

Fig. 1. Structures of the *S*- and *N*-oxides used in this study.

to TMAO, by inclusion in the cuvette at a concentration equal to that of TMAO.

Analytical techniques. Protein concentrations were determined by the method of Markwell *et al.* (1978). SDS-PAGE was carried out according to Laemmli (1970), and gels were stained (Lemire *et al.*, 1982) and scanned with a Joyce-Loebel Chromo-scan 3 densitometer to determine the purity of DmsABC. DmsABC bands were confirmed by Western blotting (Sambasivarao & Weiner, 1991b).

RESULTS

Dithionite inhibits DmsABC

Several assays have been developed to monitor the activity of terminal reductases, including DmsABC. The most widely used is a spectrophotometric assay which measures the substrate-dependent oxidation of BVH⁺⁺. Both open-cuvette (Oren & Truper, 1990; Sambasivarao & Weiner, 1991b; Yamamoto *et al.*, 1989) and closed-cuvette

(Cecchini *et al.*, 1986; Neumann & Simon, 1984) versions of this assay have been developed (see below). We examined the kinetic parameters of DmsABC with TMAO using both assays. With membrane-bound DmsABC, the K_m values determined using either assay were similar (open cuvette, 32.3 mM; closed cuvette, 25.4 mM). However, when assays were carried out with purified DmsABC, we noted at least a fourfold higher apparent K_m value for TMAO under open-cuvette conditions (open cuvette, 71.6 mM; closed cuvette, 18.0 mM). Similar results were obtained for all substrates tested (data not shown). The closed cuvette K_m for purified DmsABC was close to the membrane-bound value. The k_{cat} values were depressed by about 15% under open-cuvette conditions. When the assays were performed with freshly solubilized DmsABC, an intermediate K_m (42 mM) for TMAO was obtained utilizing the open-cuvette assay, potentially resulting from enzyme with mixed stability.

Fig. 2. K_m for TMAO varies with dithionite concentration. DmsABC was assayed in the closed-cuvette assay as indicated in Methods, with increasing concentrations of dithionite added to the cuvette.

Further investigation of assay conditions indicated that the apparent K_m differences resulted from the dithionite used to produce BVH^{+} . The open-cuvette assay requires an excess of dithionite to BV, whereas the closed-cuvette assay excludes oxygen (using degassed and nitrogen-bubbled buffer in a stoppered cuvette), and uses only enough dithionite to reduce a portion of the BV. To demonstrate that dithionite is responsible for the increase in apparent K_m observed when using the open-cuvette assay, the closed-cuvette assay was performed but altered to use increasing concentrations of dithionite, up to the value normally used in the open-cuvette assay. Fig. 2 shows that as the dithionite concentration increases, the apparent K_m increases. However, the apparent K_m reaches a plateau at lower dithionite concentrations, and at a value approximately equivalent to that determined by closed-cuvette assay conditions. This plateau in apparent K_m suggests that the closed-cuvette assay is more appropriate for accurate kinetic analysis. Dithionite appears to approximate a competitive inhibitor, as excess substrate can reverse the effect (data not shown). It was not possible to quantitate this inhibition because of assay constraints. We found that the product of dithionite oxidation, sodium bisulfite, was also an inhibitor of DmsABC, but the inhibition observed under our assay conditions was not due to bisulfite, as half-maximal inhibition for bisulfite occurred at 25 mM, and a maximal level of only 0.66 mM was present in the cuvette.

The interaction of dithionite with the enzyme explains the order of addition effects previously observed (J. H. Weiner & D. MacIssac, unpublished results). S- or N-oxide substrates must be present before the enzyme comes into contact with dithionite to prevent depressed k_{cat} values, suggesting that the oxide substrate protects the enzyme from dithionite inactivation.

Utilization of N-oxides by DmsABC

A wide variety of N-oxides (Fig. 1) were examined for their ability to act as electron acceptors. The apparent K_m and k_{cat} values obtained under closed-cuvette conditions are listed in Table 1.

Examination of a group of substituted pyridine N-oxides led to some interesting findings about substrate specificity. Unsubstituted pyridine N-oxide has a K_m of 0.094 mM. Substitution at the 2 position did not appear to alter K_m or k_{cat} significantly. Hydrophobic substituents, CH_3 , CH_2OH and amido groups are preferred (low K_m), whereas hydroxy and carboxy groups are not accepted, resulting in lower k_{cat} and very high K_m values. Substitution in the 4 position generally increased the K_m (0.25–0.5 mM), but had little effect on k_{cat} , resulting in intermediate k_{cat}/K_m values.

The K_m and inhibition characteristics of 2-hydroxymethylpyridine N-oxide were not determined due to the ability to accept electrons directly from BVH^{+} at high substrate concentrations.

Only a few aliphatic N-oxides were available. 4-Methylmorpholine N-oxide and TMAO have relatively low k_{cat}/K_m values. They have substantially higher turnover numbers compared to the other compounds tested, and substantially higher K_m values. The detergent dimethyldodecylamine N-oxide was a reasonable substrate, although this compound forms micelles at a concentration of 2.1 mM (Neugebauer, 1990). The K_m was lower than the critical micelle concentration, but there was no break in the Eadie–Hofstee plot at the critical micelle concentration, suggesting that DmsABC is capable of reducing substrate that is part of a micelle. The K_m for dimethyldodecylamine N-oxide was substantially lower than for the other aliphatic N-oxides, possibly due to multiple interactions between the dodecyl sidechain and the enzyme, or possibly due to partitioning of substrate into the Triton X-100/protein micelles.

Utilization of S-oxides

In general, the sulfoxides had similar K_m values to that of pyridine N-oxide and those substituted in the 2 and 3 position, and k_{cat} values near 100 (Table 2). Because the sulfoxides have lower k_{cat} values than pyridine N-oxide compounds, some of the substituted pyridine N-oxides are the best substrates on the basis of k_{cat}/K_m values.

The substrates DL-methyl phenyl sulfoxide and DL-methionine sulfoxide present the enzyme with different enantiomers. At this time, it is unknown which enantiomers are utilized by DmsABC. This question is being examined by polarimetric analysis.

Utilization of miscellaneous substrates

Bromate and chlorate exhibited measurable k_{cat} values of 401 and 7.3 s⁻¹, respectively. The K_m for bromate was 31.3 mM and the k_{cat}/K_m was 12.8 s⁻¹ mM⁻¹. It was not possible to calculate a K_m value for chlorate, due to the

Table 1. Kinetic parameters for *N*-oxide substrates of DmsABC, and their ability to support or inhibit anaerobic growth of *E. coli* in the presence of glycerol

All assays were performed using the closed-cuvette assay. K_m values were calculated for those substrates with a reasonable k_{cat} . For those compounds where K_m values were not determined, k_{cat} values were estimated using excess substrate. Anaerobic growth on these compounds (30 mM) in the presence of glycerol was assessed visually as no growth (—), very poor growth (+/—), growth (+) or good growth (++) . Bacteria grown in minimal media with no terminal electron acceptor were used as the negative control, and with fumarate as the positive control. ND, Not determined.

Substrate	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	Growth	Growth inhibition
2-Chloropyridine <i>N</i> -oxide	7130	0.043	307	++	No
3-Amidopyridine <i>N</i> -oxide	5280	0.045	237	++	No
3-Methylpyridine <i>N</i> -oxide	2600	0.089	231	++	No
2-Methylpyridine <i>N</i> -oxide	2690	0.092	247	++	No
3-Hydroxymethylpyridine <i>N</i> -oxide	2280	0.094	214	++	No
Pyridine <i>N</i> -oxide	2580	0.094	243	++	No
3 α -Hydroxybenzylpyridine <i>N</i> -oxide	1450	0.158	229	—	No
4-Phenylpyridine <i>N</i> -oxide	1205	0.246	297	—	No
4-Hydroxymethylpyridine <i>N</i> -oxide	607	0.372	226	++	No
4-Methylpyridine <i>N</i> -oxide	592	0.452	268	++	No
4-Chloropyridine <i>N</i> -oxide	413	0.513	212	+/—	No
Dimethyldodecylamine <i>N</i> -oxide	287	0.83	239	—	Yes
3-Hydroxypyridine <i>N</i> -oxide	135	3.17	429	++	No
3-Carboxypyridine <i>N</i> -oxide	34	4.94	168	++	No
4-Methylmorpholine <i>N</i> -oxide	52	11.1	573	++	No
TMAO	59	20.2	1203	++	No
2-Hydroxymethylpyridine <i>N</i> -oxide	ND	ND	89.3	+	ND
4-Carboxypyridine <i>N</i> -oxide	ND	ND	30.3	++	No
2-Mercaptopyridine <i>N</i> -oxide	ND	ND	8.0	—	No
2-Carboxypyridine <i>N</i> -oxide	ND	ND	8.0	+/—	No

Table 2. Kinetic parameters for *S*-oxide substrates of DmsABC, and their ability to support or inhibit anaerobic growth of *E. coli* in the presence of glycerol

Measurements are as described in the legend to Table 1. All assays were performed using closed cuvettes.

Substrate	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	Growth	Inhibition
Tetramethylene sulfoxide	2080	0.06	119	++	No
Methionine sulfoxide	663	0.09	61.1	++	No
DL-Methyl phenyl sulfoxide	483	0.21	99.6	+	No
DMSO	455	0.18	79.9	++	No
Dithane 1-oxide	ND	ND	28.4	ND	No
Dibutyl sulfoxide	ND	ND	<4	—	No
Biotin sulfoxide	ND	ND	<4	+/—	No
DL-Methyl(phenylsulfinyl)acetate	ND	ND	<4	—	No
Diphenyl sulfoxide	ND	ND	<4	—	Yes

low k_{cat} . Interestingly, hydroxylamine (K_m 47.5 mM, k_{cat} 431 s⁻¹) and diethylhydroxylamine (k_{cat} > 389 s⁻¹) were substrates, although high concentrations of hydroxylamine inhibited enzyme activity. The K_m for diethylhydroxylamine could not be determined, since it accepts electrons directly from BVH⁺ at high substrate concen-

trations. Diethylhydroxylamine was capable of supporting anaerobic growth. Adenosine *N*-oxide was not used by DmsABC when MOPS was used as the buffer. However, when 50 mM potassium acetate buffer (pH 5.5) was used, low activity was observed (k_{cat} 10 s⁻¹). Growth was also observed on this substrate. Dimethyl sulfone and

Fig. 3. Growth of wild-type *E. coli* and selected mutants on *S*- and *N*-oxide substrates. Cells were grown in 160 ml sidearm Klett flasks at 30 °C on a stirred water bath. (a) MC4100 wild-type; (b) DSS401 $\Delta dmsABC$; (c) LCB128*torA*; (d) DSS501 $\Delta dmsABC$ *torA*. Terminal electron acceptors were used as follows: 70 mM TMAO ($\square$); 30 mM DMSO ($\diamond$); 70 mM 3-carboxypyridine *N*-oxide ($\circ$); 25 mM 4-methylpyridine *N*-oxide ($\triangle$). Growth readings were taken as necessary with a Klett-Summerson spectrophotometer equipped with a no. 66 filter, using minimal medium as a blank.

triethanolamine borate are substrates with k_{cat} values of 12.8 and 4.2 s⁻¹, respectively. A number of additional compounds were examined, but triethylamine *N*-oxide picrate, K₂CrO₄, NaSeO₃, 5-chlorofurazan 3-oxide, 4-nitropyridine *N*-oxide and 4-nitroquinoline *N*-oxide accept electrons directly from BVH⁺⁺, and thus could not be tested as substrates of DmsABC.

Inhibition of DmsABC activity

Compounds that were found to be poor substrates were tested for their ability to act as inhibitors of DmsABC activity with TMAO as substrate. Because TMAO has a high K_m , non-substrates would not need to have particularly low K_i values to inhibit enzyme activity. Dibutyl sulfoxide, DL-methyl(phenylsulfinyl)acetate, diphenyl sulfoxide, 2-mercaptopyridine *N*-oxide, triethanolamine borate, chlorate, and 2-carboxypyridine *N*-oxide were not able to inhibit DmsABC activity. Biotin sulfoxide (33.5% inhibition) and dimethyl sulfone (12.4% inhibition) were found to be weak inhibitors of DmsABC activity. 3-Carboxypyridine, the product of 3-carboxypyridine *N*-oxide reduction, was a poor inhibitor (27.3%).

Growth inhibition by *S*- and *N*-oxides

It is possible that DmsABC may detoxify various *S*- and *N*-oxides encountered in the environment, providing a selective advantage for the organism. We determined the

toxicity of our collection of substrates to anaerobic growth of *E. coli*. Under the conditions described in Methods, most DmsABC substrates were not toxic to anaerobically growing *E. coli* (Tables 1 and 2). However, diphenyl sulfoxide, hydroxylamine – a protein modifier – and dimethyldodecylamine *N*-oxide – a detergent – did cause zones of clearing around the substrate-soaked filters.

Ability of *S*- and *N*-oxides to act as terminal electron acceptors

Many of the substrates were able to support anaerobic growth of *E. coli* with glycerol as carbon and energy source (Tables 1 and 2). Most of the *N*-oxides tested could be used by *E. coli* as a terminal electron acceptor for anaerobic growth. Exceptions were 2-mercaptopyridine, 4-phenylpyridine, 4-nitropyridine and 3 α -hydroxylbenzylpyridine *N*-oxides. Dimethyldodecylamine *N*-oxide inhibited anaerobic growth, presumably due to its detergent properties. The *S*-oxide compounds reduced by DmsABC were able to support anaerobic growth, whereas those which were not reduced by DmsABC did not support growth.

Fig. 3(a) shows anaerobic growth curves of *E. coli* MC4100 grown on selected *S*- and *N*-oxides. TMAO, 3-carboxypyridine *N*-oxide and 4-methylpyridine *N*-oxide supported essentially identical growth rates, and these were comparable to growth rates with fumarate and

pyridine *N*-oxide as terminal acceptor (data not shown). MC4100 grew well on DMSO, but a longer lag preceded exponential growth. Stationary phase was reached in about 20 h. Tetramethylene sulfoxide supported very limited growth (data not shown).

As reported previously (Sambasivarao & Weiner, 1991a), *E. coli* DSS401, with the *dmsABC* operon deleted, grew well on TMAO, whereas no growth was observed on DMSO. This strain exhibited slower growth on 3-carboxypyridine *N*-oxide and 4-methylpyridine *N*-oxide (Fig. 3b). *E. coli* LCB128, lacking the *torA* gene, grew well on all the substrates tested (Fig. 3c). *E. coli* lacking both *torA* and *dmsABC* (DSS501) was unable to grow on sulfoxides and TMAO, but, surprisingly, maintained the ability to grow on 3-carboxypyridine *N*-oxide (Fig. 3d), pyridine *N*-oxide and 3-hydroxypyridine *N*-oxide (data not shown). These results indicate that an alternative terminal reductase which conserves the redox energy of these pyridine *N*-oxides must be present in *E. coli*. This alternative terminal reductase does not appear to be a secondary activity of DmsABC or TorA, as this enzyme possesses a different substrate specificity.

DISCUSSION

We conclude that: (i) DmsABC has a very broad substrate specificity, and analysis of the data gives us a better understanding of the parameters which affect substrate binding and turnover; (ii) K_m values vary 470-fold, while k_{cat} values vary only 20-fold, implicating K_m as the major determinant of k_{cat}/K_m values; (iii) DmsABC plays an important role in the utilization of *S*- and *N*-oxides; (iv) we have determined that there is another, as yet unidentified, terminal reductase that is capable of utilizing a subset of these compounds.

Two classes of *S*- and *N*-oxide reductases have been identified (Iobbi-Nivol *et al.*, 1996). One class has broad substrate specificity, and includes the periplasmic DMSO/TMAO reductases of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (McEwan *et al.*, 1987; Satoh & Kurihar, 1987). The second class has much more limited substrate specificity, and includes the TMAO reductases of *Shewanella* and *E. coli* (Clarke & Ward, 1988; Iobbi-Nivol *et al.*, 1996). DmsABC appears to be most similar to the *Rhodobacter* enzymes. Although the substrate specificity of DmsABC overlaps with TorA, the poorest DmsABC substrates are the best TorA substrates, and vice versa. This may allow these proteins to play complementary roles in the cell.

If we compare all the substrates solely on the basis of k_{cat} , we can identify three compounds (TMAO, 4-methylmorpholine *N*-oxide and 3-hydroxypyridine *N*-oxide) with substantially higher k_{cat} values than all the other compounds tested. These compounds also have the highest K_m values, and therefore relatively poor k_{cat}/K_m values, suggesting that they are poor substrates. This is obviously not the case, as cells grow well using these compounds as terminal electron acceptors. Most substituted pyridine *N*-oxides have k_{cat} values between 200

and 300 s⁻¹, and sulfoxides have k_{cat} values between 60 and 120 s⁻¹. A very large range of K_m values was observed for these compounds, indicating that substrate affinity has a primary role in determining enzyme specificity.

Redox potential would be expected to affect k_{cat} much more than K_m . Pyridine *N*-oxides have lower redox potentials and are more difficult to reduce than aliphatic *N*-oxides, because the electrons from the oxygen are in resonance with the aromatic ring, stabilizing the unstable N–O bond (Ochaiai, 1953, 1967). It is possible that the lower redox potentials of pyridine *N*-oxides could be an influencing factor in the lower k_{cat} values observed for these compounds.

Reduction of *N*-oxides has been suggested to occur after protonation (Kubota & Miyazaki, 1962; Ochaiai, 1967). As aliphatic *N*-oxides are more easily protonated than pyridine *N*-oxides (Kubota & Miyazaki, 1962), it is possible that this factor may also contribute to the higher k_{cat} values observed for aliphatic *N*-oxides.

A pattern is observed when the K_m values are examined. The K_m values are approximately equivalent for pyridine *N*-oxide and pyridine *N*-oxides substituted in the 2 and 3 position. Pyridine *N*-oxides substituted in the 4 position have higher K_m values. Carboxy-substituted pyridine *N*-oxides do not follow the rule above, and are not good substrates. This is presumably due to their negative charge.

The K_m values for the pyridine *N*-oxides and sulfoxides are observed to be lower than for the aliphatic *N*-oxides. This may be for steric reasons, suggesting that the enzyme may be willing to accept compounds with two substituents more easily than those with three substituents. The only aliphatic *N*-oxide that does not follow this rule is dimethyldodecylamine *N*-oxide, and it is possible that the lower K_m value observed could be due to additional contacts between the dodecyl sidechain and the enzyme, or possibly due to partitioning of substrate into Triton X-100/protein micelles.

Compact sulfoxides (DMSO and tetramethylene sulfoxide) or those with one extended substituent are accommodated by the enzyme (DL-methionine sulfoxide and DL-methyl phenyl sulfoxide). However, those that are bulky and those with two extended substituents are not [dibutyl sulfoxide, DL-methyl(phenylsulfinyl)acetate and diphenyl sulfoxide]. This agrees with X-ray structures of molybdopterin- and tungstopterin-containing aldehyde oxidoreductases, and molybdopterin-containing DMSO reductase from *R. sphaeroides*, all of which show that the active site is accessible only through a narrow tunnel (Chan *et al.*, 1995; Romao *et al.*, 1995; Schindelin *et al.*, 1996).

We have demonstrated that *E. coli* has the ability to use a wide variety of *S*- and *N*-oxides as terminal electron acceptors, resulting in anaerobic growth in the presence of glycerol. TMAO has widespread natural occurrence in nature as an osmostabilizer in salt water fish, and DMSO is found in sea water as part of the global sulfur cycle (Barrett & Kwan, 1985; Kelly & Baker, 1990; Kiene &

Bates, 1990). Many of these compounds are also man-made. DMSO is used as a solvent and is found as an effluent from wood pulp mills (Wood, 1981). Substituted pyridine *N*-oxides are found as soil contaminants, in crude petroleum and in coal tar, and are used as organic solvents and drugs (Shukla, 1984). DmsABC is a key enzyme in utilization of these compounds, as shown by the work reported herein. The presence of DmsABC in *E. coli* may result in a selective advantage for the organism to enable it to use a wide variety of *S*- and *N*-oxides as they are encountered in the environment, including utilizing *S*-oxide drugs found in the intestine (Lee & Renwick, 1995).

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REFERENCES

- Barrett, E. L. & Kwan, H. S. (1985). Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol* **39**, 131–149.
- Bilous, P. T. & Weiner, J. H. (1985a). Dimethyl sulfoxide reductase activity by anaerobically grown *Escherichia coli*. *J Bacteriol* **162**, 1151–1155.
- Bilous, P. T. & Weiner, J. H. (1985b). Proton translocation coupled to dimethyl sulfoxide reduction in anaerobically grown *Escherichia coli* HB101. *J Bacteriol* **163**, 369–375.
- Bilous, P. T., Cole, S. T., Anderson, W. F. & Weiner, J. H. (1988). Nucleotide sequence of the *dmsABC* operon encoding the anaerobic dimethylsulphoxide reductase of *Escherichia coli*. *Mol Microbiol* **2**, 785–795.
- Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementary analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459.
- Cammack, R. & Weiner, J. H. (1990). Electron paramagnetic resonance spectroscopic characterization of dimethyl sulfoxide reductase of *Escherichia coli*. *Biochemistry* **29**, 8410–8416.
- Casadaban, M. J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *Mol Biol* **104**, S41–S55.
- Cecchini, G., Ackrell, B. A. C., Deshler, J. O. & Gunsalus, R. P. (1986). Reconstitution of quinone reduction and characterization of *Escherichia coli* fumarate reductase activity. *J Biol Chem* **261**, 1808–1814.
- Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W. W. & Rees, D. C. (1995). Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science* **267**, 1463–1469.
- Clarke, G. & Ward, B. (1988). Purification and properties of trimethylamine *N*-oxide reductase from *Shewanella* sp. NCMB 400. *J Gen Microbiol* **134**, 379–386.
- Fersht, A. (1977). The basic equations of enzyme kinetics; and enzyme-substrate complementarity and theories of enzyme catalysis. In *Enzyme Structure and Mechanism*, pp. 84–102, 244–271. San Francisco: W. H. Freeman.
- Iobbi-Nivol, C., Pommier, J., Simala-Grant, J., Mejean, V. & Giordano, G. (1996). High substrate specificity and induction characteristics of trimethylamine *N*-oxide reductase of *Escherichia coli*. *Biochim Biophys Acta* **1294**, 77–82.
- Kelly, D. P. & Baker, S. C. (1990). The organosulphur cycle: aerobic and anaerobic processes leading to turnover of C_1 sulphur compounds. *FEMS Microbiol Rev* **87**, 241–246.
- Kiene, R. P. & Bates, T. S. (1990). Biological removal of dimethylsulphide from sea water. *Nature* **345**, 702–705.
- Kubota, T. & Miyazaki, H. (1962). Polarography of pyridine *N*-oxide and its alkyl derivatives. *Bull Chem Soc Jpn* **35**, 1549–1551.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lee, S. C. & Renwick, A. G. (1995). Sulfoxide reduction by rat intestinal flora and by *Escherichia coli*. *Biochem Pharmacol* **49**, 1567–1576.
- Lemire, B. D., Robinson, J. J. & Weiner, J. H. (1982). Identification of membrane anchor polypeptides of *Escherichia coli* fumarate reductase. *J Bacteriol* **152**, 1126–1131.
- McEwan, A. G., Wetzstein, H. G., Meyer, O., Jackson, J. B. & Ferguson, S. J. (1987). The periplasmic nitrate reductase of *Rhodobacter capsulatus*; purification, characterisation and distinction from a single reductase for trimethylamine-*N*-oxide, dimethylsulphoxide and chlorate. *Arch Microbiol* **147**, 340–345.
- Markwell, M. A. D., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**, 206–210.
- Mejean, V., Iobbi-Nivol, C., Lepelletier, M., Giordano, G., Chippaux, M. & Pascal, M.-C. (1994). Trimethylamine *N*-oxide anaerobic respiration in *Escherichia coli*: involvement of the *tor* operon. *Mol Microbiol* **11**, 1169–1179.
- Melville, D. B. (1954). Biotin sulfoxide. *J Biol Chem* **208**, 495–501.
- Neugebauer, J. M. (1990). Detergents: an overview. *Methods Enzymol* **182**, 239–253.
- Neumann, S. & Simon, H. (1984). On a non-pyridine nucleotide-dependent 2-oxo-acid reductase of broad substrate specificity from two *Proteus* species. *FEBS Lett* **167**, 29–32.
- Ochiai, E. (1953). Recent Japanese work on the chemistry of pyridine 1-oxide and related compounds. *J Org Chem* **18**, 534–551.
- Ochiai, E. (1967). *Aromatic Amine Oxides*, pp. 6–17, 91–97. Amsterdam: Elsevier.
- Oren, A. & Truper, H. G. (1990). Anaerobic growth of halophilic archaeobacteria by reduction of dimethyl sulfoxide and trimethylamine *N*-oxide. *FEMS Microbiol Lett* **70**, 33–36.
- Pascal, M.-C., Burini, J.-F. & Chippaux, M. (1984). Regulation of the trimethylamine *N*-oxide (TMAO) reductase in *Escherichia coli*: analysis of *tor*:Mud1 operon fusion. *Mol Gen Genet* **195**, 351–355.
- Romao, M. J., Archer, M., Moura, I., Moura, J. J. G., LeGall, J., Engh, R., Schneider, M., Hof, O. & Huber, R. (1995). Crystal structure of the xanthine oxidase-related aldehyde oxido-reductase from *D. gigas*. *Science* **270**, 1170–1176.
- Rothery, R. A. & Weiner, J. H. (1991). Alteration of the iron-sulfur cluster composition of *Escherichia coli* dimethyl sulfoxide reductase by site-specific mutagenesis. *Biochemistry* **30**, 8296–8305.
- Rothery, R. A. & Weiner, J. H. (1993). Topological characterization of *Escherichia coli* DMSO reductase by electron paramagnetic

spectroscopy of an engineered [3Fe-4S] cluster. *Biochemistry* **32**, 5855–5861.

Rothery, R. A., Simala-Grant, J. L., Johnson, J. L., Rajagopalan, K. V. & Weiner, J. H. (1995). Association of molybdopterin guanine dinucleotide with *Escherichia coli* dimethyl sulfoxide reductase: effect of tungstate and a *mob* mutant. *J Bacteriol* **177**, 2057–2063.

Sambasivarao, D. & Weiner, J. H. (1991a). Differentiation of the multiple S- and N-oxide reducing activities of *Escherichia coli*. *Curr Microbiol* **23**, 105–110.

Sambasivarao, D. & Weiner, J. H. (1991b). Dimethyl sulfoxide reductase of *Escherichia coli*: an investigation of function and assembly by use of *in vivo* complementation. *J Bacteriol* **173**, 5935–5943.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Satoh, T. & Kurihar, F. N. (1987). Purification and properties of dimethyl sulfoxide reductase containing a molybdenum cofactor from a photodenitrifier *Rhodospseudomonas sphaeroides* f.s. *denitrificans*. *J Biochem* **102**, 191–197.

Schindelin, H., Kisker, C., Hilton, J., Rajagopalan, K. V. & Rees, D. C. (1996). Crystal structure of DMSO reductase: redox linked changes in molybdopterin coordination. *Science* **272**, 1615–1621.

Shukla, O. P. (1984). Microbial transformation of pyridine derivatives. *J Sci Ind Res* **43**, 98–116.

Silvestro, A., Pommier, J. & Giordano, G. (1988). The inducible trimethylamine N-oxide reductase of *Escherichia coli* K12: bio-

chemical and immunological studies. *Biochim Biophys Acta* **954**, 1–13.

Silvestro, A., Pommier, J., Pascal, M.-C. & Giordano, G. (1989). The inducible trimethylamine N-oxide reductase of *Escherichia coli* K12: its localization and inducers. *Biochim Biophys Acta* **999**, 208–216.

Trieber, C. A., Rothery, R. A. & Weiner, J. H. (1994). Multiple pathways of electron transfer in dimethyl sulfoxide reductase of *Escherichia coli*. *J Biol Chem* **269**, 7103–7109.

Weiner, J. H., MacIsaac, D. P., Bishop, R. E. & Bilous, P. T. (1988). Purification and properties of *Escherichia coli* dimethyl sulfoxide reductase, an iron-sulfur molybdoenzyme with broad substrate specificity. *J Bacteriol* **170**, 1505–1510.

Weiner, J. H., Rothery, R. A., Sambasivarao, D. & Trieber, C. A. (1992). Molecular analysis of dimethyl sulfoxide reductase: a complex iron-sulfur molybdoenzyme of *Escherichia coli*. *Biochim Biophys Acta* **1102**, 1–18.

Wood, P. M. (1981). The redox potential for dimethyl sulphoxide reduction to dimethyl sulphide. *FEBS Lett* **124**, 11–14.

Yamamoto, I., Okubo, M. & Ishimoto, M. (1986). Further characterization of trimethylamine N-oxide reductase from *Escherichia coli* a molybdoprotein. *J Biochem* **99**, 1773–1779.

Yamamoto, I., Hohmura, M. & Ishimoto, M. (1989). A novel gene, *torB*, for trimethylamine N-oxide reductase in *Escherichia coli*. *J Gen Appl Microbiol* **35**, 95–105.

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