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SULPHOXIDE REDUCTION BY RAT INTESTINAL FLORA AND BY *ESCHERICHIA COLI IN VITRO*

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Abstract—The caecal microflora from female rats show a greater ability to reduce the sulphoxide group of sulindac than either the liver or kidneys. Studies on sulphoxide reduction by *Escherichia coli* showed that NADH, NADPH and dithiothreitol (DTT), but not acetaldehyde could act as cofactors. The cytosolic fraction was responsible for about 90%, 80% and 60% of the total reducing activity with sulindac, diphenyl sulphoxide and sulphinpyrazone, respectively. The main NADPH linked activity in the *E. coli* cytosol was dependent on thioredoxin, since the activity was essentially abolished by passing through a G50 column or by the addition of anti-thioredoxin anti-serum. Partial purification and separation of sulphoxide reducing activity by DEAE-cellulose chromatography separated two main protein bands, each of which possessed sulindac reducing activity. The importance of thioredoxin for much of the NADPH dependent activity was confirmed but the eluate fractions also showed the presence of other activities with NADH, NADPH and DTT that were independent of thioredoxin. Incubation of the DEAE-cellulose eluate fractions with flosequinan and sulphinpyrazone showed that the reducing activity in the two main protein peaks showed different substrate specificities and that there were multiple sulphoxide reductase systems present in *E. coli* cytosol.

Key words: sulphoxide reduction; intestinal bacteria; *Escherichia coli* reductases; sulindac; sulphinpyrazone; flosequinan

Tissue enzymes able to reduce sulindac to the active sulphide metabolite include aldehyde oxidase from guinea pig and rabbit liver [1, 2] as well as a thioredoxin-dependent cytosolic reductase from rat liver [3] and kidney [4]. Fukazawa *et al.* [5] also reported the purification of two sulphoxide reductases from rat kidney which predominantly reduced methyl sulphoxides. In a previous paper [6] we demonstrated that the enzymes responsible for the reduction of sulindac are widely distributed in rat tissues, with the liver and kidney showing the highest activities. The activity was increased by the addition of DTT† in all the tissues investigated. Very low activities were observed with sulphinpyrazone or DPSO (Fig. 1) as substrates.

Despite the established role of the tissues in the reduction of sulphoxides both *in vivo* and *in vitro*, the intestinal microflora are also an important site for the reduction of some sulphoxide-containing drugs. Pharmacokinetic studies in rats [7], rabbits [8] and humans [9] have shown that the microflora of the gastrointestinal tract are the principal and possibly the only site of reduction of sulphinpyrazone. Similar studies on the metabolism of sulindac in normal subjects and patients with surgical ileostomies suggested that about 50% of the total sulphide was formed by the gut bacteria [10]. Incubation of these two drugs with over 200 strains of bacteria isolated from human faeces *in vitro* showed extensive reduction by both aerobes and anaerobes [11].

Several reports have described the microbial, enzymatic reduction of dimethyl sulphoxide [12, 13] and sulphoxide compounds which are normal cell constituents [14–17]. However, only a limited amount of information is available regarding the bacterial enzyme systems catalysing the reduction of sulphoxide-containing drugs. In this study, we investigated the localization and properties of the sulphoxide xenobiotic reducing enzyme systems in *Escherichia coli*.

MATERIALS AND METHODS

Materials. Sulindac and its sulphide and sulphone metabolites were supplied by Merck, Sharp and Dohme (Hoddesdon, U.K.). Sulphinpyrazone and its sulphide and sulphone metabolites were gifts from Ciba-Geigy Pharmaceuticals (Horsham, U.K.). Fenbufen was kindly supplied by Lederle Laboratories (Gosport, U.K.). DPSO and sulphide were purchased from Sigma (Poole, U.K.). Nitrendipine was a gift from Bayer (U.K.). Flosequinan and its sulphone (BTS 453554) and sulphide (BTS 53523) metabolites and the internal standard (BTS 49037) were supplied by Boots Company (Nottingham, U.K.).

Sephadex G25, Sephadex G50, PD 10 gel filtration columns and DEAE-Sephacel were all purchased from Pharmacia (Milton Keynes, U.K.). Fatty acid free bovine albumin, Freund's complete and

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† Abbreviations: DPSO, diphenyl sulphoxide; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

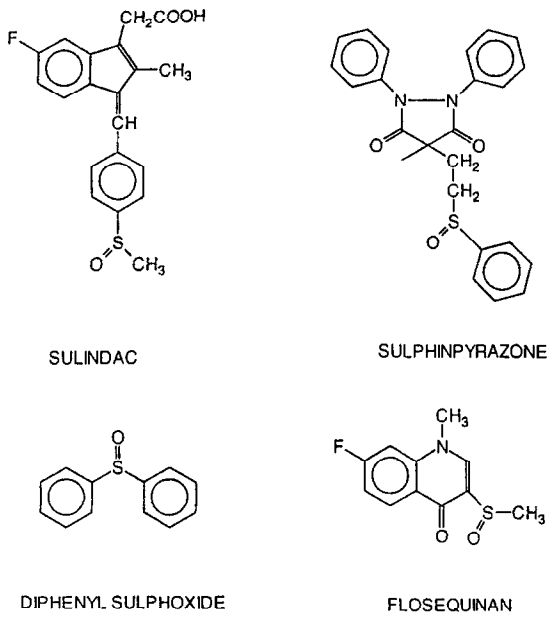

Fig. 1. Molecular structure of the substrates studied.

incomplete adjuvants and glutaraldehyde were from Sigma (Poole, U.K.). Recombinant *E. coli* thioredoxin crystals were purchased from Novabiochem (Nottingham, U.K.). Powder for Todd-Hewitt broth was obtained from Oxoid (Basingstoke, U.K.). All other chemicals and solvents were of Analar or HPLC grade from BDH (Poole, U.K.) or Sigma (Poole, U.K.).

Incubation with rat liver, kidneys and caecum contents. Adult female rats were killed by cervical dislocation and the liver, kidneys and contents of the caecum were immediately removed and prepared as 33% (w/v) homogenates in 0.1 M sodium phosphate buffer, pH 7.4 using a Potter glass–Teflon homogenizer. The homogenates (0.5 mL) were incubated with sulindac (200 μg in 1.5 mL 0.1 M phosphate buffer, pH 7.4 to give a final concentration of 0.28 mM) for 1 hr at 37° with shaking under an atmosphere of oxygen-free nitrogen. The reaction was stopped by the addition of HCl (2 M; 2 mL) and then internal standard (50 μg of sulphinpyrazone sulphide) was added. The incubates were immediately extracted with organic solvent and analysed by HPLC as described below.

Escherichia coli *BM*1023 *cultures and incubation with sub-cellular fractions. Escherichia coli* BM 1023 and other purified bacteria strains isolated from human faeces were provided by Dr M. Hill and co-workers of the Bacterial Metabolism Research Laboratories (Salisbury, U.K., as described in Ref. 11). All *E. coli* BM 1023 cultures were grown in freshly prepared, autoclaved Todd-Hewitt broth containing 0.03% sodium formaldehyde sulphoxylate. The stock *E. coli* suspensions were checked regularly for any contamination by culturing a sample on agar plates which were then incubated at 30° for 24 hr. No contamination was detected throughout the period during which these experiments were performed. The *E. coli* cells were harvested by centrifugation at 10,000 *g* for 15 min. They were then washed with 20 mM Tris-HCl buffer containing 20 mM KCl (pH 7.4; buffer A) and re-suspended in a small volume of the same buffer.

The suspension of washed *E. coli* cells was transferred to a 10 mL glass pot placed in a salted ice bath and the cells were sonicated for four cycles (15 sec on/1 min off) at a maximum power using a Soniprep 150 sonicator (MSE, U.K.). Any undisrupted cells in the suspension were removed by centrifugation at 10,000 *g* for 15 min. The supernatant was centrifuged again at 100,000 *g* for 60 min to obtain the cytosol. The 100,000 *g* pellets were homogenized in buffer A and centrifuged at 100,000 *g* for 60 min to give the washed membrane fractions. All centrifugations were performed at 4°.

A typical incubation mixture contained cytosol or membrane fraction (1.5–2.0 mg total protein), substrate (5–15 μg) and cofactors in 0.1 M sodium phosphate buffer, pH 7.4 to a final volume of 2.0 mL. The mixture was incubated at 37° for 3 hr with shaking under an atmosphere of oxygen-free nitrogen. The internal standard was added and the reaction was immediately stopped either by adding 2 M HCl (2 mL; for sulindac and sulphinpyrazone incubations) or immediately freezing the tube at −20° (for DPSO and flosequinan).

The effect of dithiothreitol during preparation of cytosol. Escherichia coli cells suspended in buffer A containing DTT (1 mM) were sonicated as described above. The suspension was then centrifuged at 10,000 *g* for 15 min at 4°. The resulting supernatant was centrifuged at 100,000 *g* for 60 min at 4° to obtain the cytosolic fraction. The DTT in this fraction was removed by passing through a Pharmacia PD10 gel filtration column (1 × 8 cm; containing Sephadex G25) previously equilibrated with buffer A. The column was eluted with the same buffer and fractions (1.5 mL) were collected. Fractions 2–4, which contained the main protein peak but not dithiothreitol, were pooled together and used for incubation. The activity was compared to that of cytosol prepared with buffer A not containing DTT.

Removal of thioredoxin from E. coli *cytosol.* The method was based on that of Larson and Larsson [18]. Thioredoxin is eluted in the void volume of a Sephadex G25 column but is retained by a Sephadex G50 column [18]. *Escherichia coli* 100,000 *g* supernatant (1.5 mL) was applied to a Sephadex G-25 gel filtration column (1.0 × 10 cm) previously equilibrated with 20 mM potassium phosphate buffer, pH 7.4 containing 0.1 mM EDTA. Proteins were eluted with the same buffer at a flow rate of 1 mL/min. The void volume (6 mL) containing the main protein peak was collected.

Half of the void volume (3.0 mL) collected from the Sephadex G-25 column was applied to a Sephadex G-50 column (1.5 × 60 cm) previously equilibrated with the same buffer. Proteins were eluted with the same buffer at a flow rate of 0.17 mL/min. The void volume (15 mL) was collected. All gel filtration chromatography was performed at 4°.

The original cytosol, the Sephadex G-25 void volume and the Sephadex G-50 void volume were incubated with sulindac in the presence of either

NADH (0.5 mM), NADPH (0.5 mM) or dithiothreitol (5 mM). Pure *E. coli* thioredoxin (manufactured by recombinant DNA technology) was also added to the incubation containing the Sephadex G-50 void volume.

Preparation of anti-sera against E. coli *thioredoxin.* Antisera were prepared using a method similar to that described by Lunn *et al.* [19]. A nonapeptide corresponding to the active site sequence of *E. coli* thioredoxin was synthesized chemically by Dr J.W. Conlan (Microbiology, University of Southampton, U.K.). The sequence of the peptide was Trp–Ala–Glu–Trp–Cys–Gly–Pro–Cys–Lys. The peptide (1.3 mg) was added to bovine serum albumin (2.6 mg; fatty acid free) dissolved in 0.1 M sodium phosphate buffer, pH 7.5 (0.2 mL). Glutaraldehyde (0.1 mL of a 0.2 mM solution in 0.1 M sodium phosphate buffer) was added dropwise to the solution with stirring. The reaction was incubated at room temperature for 30 min. The peptide–BSA conjugate was obtained by passing the solution through a Sephadex G25 column (0.9 × 10 cm) previously equilibrated with 0.1 M sodium phosphate buffer, pH 7.5. The void volume containing the main protein peak was collected by monitoring the absorbance of the eluate at 280 nm.

Antisera were prepared by immunization of female albino New Zealand rabbits (approx. 3 kg). Before immunization, a 20 mL blood sample was taken from the rabbit to obtain the pre-immune serum. The peptide–bovine serum albumin conjugate (500 μg; equivalent to 90 μL of the above solution) was mixed with phosphate buffer saline (pH 7.4; 0.21 mL) containing 2 mM DDT, and left to stand at room temperature for 10 min. A further 0.7 mL phosphate buffer saline was added and the resulting solution was emulsified in 1.0 mL of Freund's complete adjuvant. This was injected intramuscularly into the thigh muscle of each side (0.5 mL each) of the rabbit. The booster doses were injected one month and 3 months after the initial dose. Booster doses were prepared using half the amount of peptide–BSA conjugate emulsified in Freund's incomplete adjuvant. Blood was withdrawn from the rabbits 4 weeks after each injection and allowed to clot before separation of the antiserum by centrifugation. The terminal bleed of the rabbit was performed 4 weeks after the second booster dose under thiopentone anaesthesia.

Characterization of antisera. The titre of antisera against thioredoxin was determined by inhibition of the thioredoxin catalysed reduction of insulin disulphide [20, 21]. A typical assay mixture contained bovine insulin (0.13 mM), EDTA (2.0 mM), DTT (1.0 mM), *E. coli* thioredoxin (1.0 μM) and pre-immune serum or anti-serum (2–4 μL) in 0.1 M potassium phosphate buffer pH 7.4 to a final volume of 0.6 mL. The serum was pre-incubated with thioredoxin in the assay mixture at room temperature for 3 min prior to the addition of insulin. The reaction was initiated by adding the bovine insulin and the resulting precipitation of the insulin free chains monitored by measuring the absorbance at 650 nm every minute for 60 min at a temperature of 27 ± 2° using a Beckman DU-50 spectrophotometer. Thioredoxin activity was measured by two parameters: (a) the time for the free chains to start precipitation, defined as the time required to give an increase of 0.01 absorbance unit at 650 nm over a stable base-line recording; (b) the rate of precipitation, defined as the maximum increase in absorbance per minute at 650 nm. Inhibition of thioredoxin activity was calculated from the differences between the pre-immune and anti-serum using these two parameters. Control tubes (which contained insulin and dithiothreitol but no thioredoxin) were also incubated with pre-immune serum and anti-serum as described above to investigate the effect of the presence of serum on the reaction.

Partial purification of sulindac reductases from E. coli *cytosol.* Sulindac reductases in *E. coli* cytosol were partially purified by the following procedures. A typical example of the result of purification of the reductase activity is shown in Table 1.

Table 1. Partial purification and separation of *E. coli* cytosolic sulindac reductases

Fraction	Volume (mL)	Total protein (mg)	Cofactor	Total activity (U)*	Specific activity (U/mg protein)	Yield (%)
Cytosol	3.0	191	NADH	34.4	0.18	100
			NADPH	39.7	0.21	100
			DTT	78.9	0.41	100
Ammonium sulphate	5.9	110	NADH	12.1	0.10	35
			NADPH	29.3	0.26	74
			DTT	70.8	0.64	90
DEAE-cellulose						
Peak 1†	8.0	31.1	NADH	11.1	0.36	32
			NADPH‡	26.1	0.84	66
			DTT	19.2	0.62	24
Peak 2§	4.0	31.3	NADH	3.9	0.13	11
			NADPH‡	3.9	0.12	10
			DTT	8.2	0.26	10

* 1 unit of activity is defined as the amount of enzyme that catalysed the reduction of 1 nmol of sulindac/hr under the assay conditions described in the Methods section.

† Corresponds to fraction 3–6 (Fig. 4).

‡ Values represent activities with the addition of thioredoxin (5 μg).

§ Corresponds to fraction 15–16 (Fig. 4).

- *Step 1—Preparation of 100,000 g supernatant*: The cytosolic fraction of approximately 5 g (wet weight) of *E. coli* cells was obtained by sonication and centrifugation as described above.
- *Step 2—Ammonium sulphate precipitation*: Powdered ammonium sulphate (1.16 g) was added slowly to 3.0 mL of the 100,000 *g* supernatant to give 60% saturation. The mixture was stirred gently for 60 min at room temperature, and then centrifuged at 3000 *g* for 20 min. The precipitate was redissolved in 20 mM Tris-HCl buffer (pH 8.5), containing 20 mM KCl (1.3 mL). This solution was desalted by passing through a short column (1 × 6 cm) of Sephadex G25 previously equilibrated with the same buffer.
- *Step 3—DEAE-cellulose chromatography*: The ammonium sulphate precipitated fraction (0–60%) was applied to a DEAE-cellulose column (Sephacel, 1.0 × 10 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.5), containing 20 mM KCl and 0.1 mM EDTA. Proteins were initially eluted with 18 mL of the same buffer and then with 24 mL of 10 mM potassium phosphate buffer (pH 7.4) containing 500 mM KCl and 0.1 mM EDTA at a flow rate of 0.26 mL/min. Eluate fractions of 2.0 mL were collected. Each fraction was incubated with the four substrates (sulindac, sulphinpyrazone, DPSO and flosequinan) in the presence of cofactors (NADH, 0.5 mM; NADPH, 0.5 mM or DTT, 5 mM) at 37° under anaerobic conditions for 3 hr. In order to assess the importance of thioredoxin, each fraction was also incubated with the substrates and cofactors plus the addition of *E. coli* thioredoxin (5 μg). Samples were analysed by HPLC.

HPLC analysis. Sulindac, sulphinpyrazone, DPSO and their metabolites were analysed by HPLC as described previously [6]. Flosequinan and its metabolites were analysed using 7-chloro-1-methyl-3-methylsulphinyl-4-quinolone (BTS 49037, 10 μg) as the internal standard. The drugs were extracted with chlorobutane/1,2-dichloroethane (4:1, v/v; 4 mL) by shaking for 15 min and the upper organic layer was separated by centrifugation and pipetted into another tube. The solvent was evaporated to dryness by heating the tubes at 70°, assisted by passing a stream of oxygen-free nitrogen into the tubes. The drugs were re-dissolved in 800 μL of mobile phase (double distilled water/methanol/acetonitrile; 73:20:7 by volume). An aliquot (100 μL) of the solution was injected into a HPLC system consisting of a Waters Associates WISP 712 injector, a model M6000A pump, a Hypersil 3ODS column (15 cm; 4.6 mm, i.d.), a model 481 detector set at 254 nm and a model 745 integrator. The sulphide metabolite was also detected by monitoring the eluate with a Waters 420-AC fluorescence detector (254 nm excitation; 425 nm emission wavelength) connected to a Phillips PM8252A recorder. The solvent flow rate was 2.0 mL/min. The peak heights were measured and the ratios of flosequinan or its metabolites to that of the internal standard were calculated. The intra-assay coefficient of variation averaged 5% in the relevant concentration range studied (1–15 μg per incubate).

Table 2. Reduction of sulindac and sulphinpyrazone by rat tissue homogenates and caecum contents

Substrate	Sulphide formed (μmol/g tissue/hr)		
	Liver	Kidney	Caecum contents
Sulindac (N = 6)	0.11 ± 0.04	0.08 ± 0.01	1.15 ± 0.45
Sulphinpyrazone (N = 5)	<0.01	ND	0.32 ± 0.09

Results represent the mean ± SD. All incubations were performed under anaerobic conditions. ND, not detectable

RESULTS

Reduction of sulindac and sulphinpyrazone by rat liver, kidney and caecum contents

The reducing activity of these three sites are compared in Table 2. Considerable reduction of sulindac occurred with the liver and kidney homogenates; however, the reducing activity of the

Table 3. Effects of DTT and pH on the reduction of sulindac by *E. coli* 10,000 *g* supernatant

DTT concentration (mM)		Sulphide formed (nmol/mg protein/hr)		
Buffer A	Incubation mixture	pH 6.5	pH 7.5	pH 8.5
1*	0	ND	ND	ND
1*	1	0.18 ± 0.02	0.35 ± 0.02	0.28 ± 0.03
0†	0	0.01 ± 0.01	ND	0.01 ± 0.01
0†	1	0.14 ± 0.01	0.33 ± 0.05	0.30 ± 0.04

Results are mean ± for three experiments. *Escherichia coli* cells were sonicated in buffer A with or without DTT and the 10,000 *g* supernatant was incubated with sulindac under anaerobic conditions. ND, not detected.

* DTT was present in buffer A for re-suspending and sonicating the cells. After sonication, DTT was removed by passing the 10,000 *g* supernatant through a Pharmacia PD 10 column (1 × 8 cm).

† Buffer A for re-suspending and sonicating the cells contained no DTT.

Table 4. Localization of the sulphoxide reductase activities in *E. coli*

Substrate	Cofactor	Cytosol		Washed membrane	
		Activity*	% Total activity†	Activity*	% Total activity†
Sulindac	NADH	1.49 ± 0.65	87.6 ± 9.2	0.17 ± 0.12	12.4 ± 9.2
	NADPH	0.50 ± 0.13	100	ND	0
	Acetaldehyde	ND	—	ND	—
	DTT	0.48 ± 0.05	91.2 ± 1.1	0.04 ± 0.01	8.8 ± 1.1
Sulphinpyrazone	NADH	0.07 ± 0.02	100	ND	0
	NADPH	0.07 ± 0.01	60.6 ± 3.3	0.07 ± 0.01	39.4 ± 3.3
	Acetaldehyde	ND	—	ND	—
	DTT	0.02 ± 0.01	56.7 ± 8.2	0.03 ± 0.01	43.3 ± 8.2
DPSO	NADH	1.48 ± 0.44	79.8 ± 4.4	0.55 ± 0.08	20.2 ± 4.4
	NADPH	ND	—	ND	—
	Acetaldehyde	ND	—	ND	—
	DTT	ND	0	0.32 ± 0.02	100

Results are mean ± SD for three experiments performed under anaerobic conditions. NADH and NADPH were added at 0.5 mM and acetaldehyde and DTT at 5 mM. ND, not detected.
* Activity is expressed as sulphide formed (nmol/mg protein/hr).
† Total activity was calculated as described in Methods.

caecum contents was about ten times higher than that of the liver. For sulphinpyrazone, the caecum contents also showed high reducing activity, but the drug was not reduced by the liver or the kidney. The lower activity detected with sulphinpyrazone compared with sulindac suggests that this compound is a poor substrate for the bacterial enzymes.

Role of dithiothreitol in enzyme preparation

During the initial stages of the present study, considerable reduction of sulindac was detected with the cultured *E. coli* whole cells but not in the initial 10,000 *g* supernatant obtained after sonication of the cells as described above. It was suspected that the sonication process might have inactivated the enzymes or destroyed part of the electron transfer chain. Other disintegration methods, including osmotic shock and freeze–thaw–lysozyme treatments, were tried with limited success. However, when DTT (1 mM) was added to the buffer for suspending the cells before sonication, reducing activity was then detected in the subsequent 10,000 *g* supernatant. The results in Table 3 show that DTT was acting simply as a cofactor for the reduction of sulindac rather than stabilizing the enzyme during the sonication process, since addition of DTT to the incubation mixture produced comparable activities even when DTT was not present in the original sonication suspension. The highest activity was detected at pH 7.5 and decreasing the pH also decreased the activity considerably.

Localization of sulindac reductase in E. coli

The ability of NADPH, NADH, acetaldehyde and DTT to function as cofactors was investigated. Acetaldehyde had no effect on the reducing activity towards sulindac of any of the *E. coli* fractions studied (Table 4). The sulindac reductases using NADPH as electron donor were only present in the cytosol. Enzymes able to utilize NADH or DTT as cofactors were also found to be predominantly localized in the cytosol.

Effects of thioredoxin on the reduction of sulindac

After passing through the Sephadex G50 column, the reducing activity of the *E. coli* cytosol in the presence of NADH was higher when compared to that of the Sephadex G25 void volume, whereas that with DTT as cofactor was reduced to 39% (Table 5). However, no reducing activity was detected when the Sephadex G50 void volume was incubated with NADPH. When pure *E. coli* thioredoxin (2 μg) was

Table 5. Reduction of sulindac by *E. coli* cytosol—effect of removal of thioredoxin from the cytosol

Fraction	% Total activity of G-25 void volume*		
	+NADH (0.5 mM)	+NADPH (0.5 mM)	+DTT (5 mM)
Sephadex G-25 void volume (total)	100	100	100
Sephadex G-50 void volume (total)	243	0	39
Sephadex G-50 void volume (total)			
+2 μg thioredoxin	238	44	32
+4 μg thioredoxin	127	41	39
+8 μg thioredoxin	171	41	30

The results represent the mean of two experiments performed under anaerobic conditions. The data are presented as a % of the total activity present in the G-25 void volume.
* The total reducing activity of the Sephadex G-25 void volume was 2.03 (nmol sulindac reduced/hr) with NADH (0.5 mM), 4.62 with NADPH (0.5 mM) and 6.65 with DTT (5 mM). Half of the void volume from the G-25 column was then passed through a G-50 column and the void volume collected.

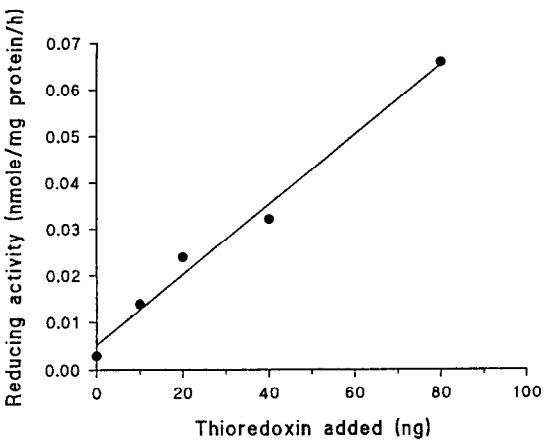

Fig. 2. Thioredoxin dependence of sulindac reduction by *E. coli* Sephadex G-50 void volume in the presence of NADPH (0.5 mM). Values represent the mean of two experiments performed under anaerobic conditions ($r = 0.993$).

added to the Sephadex G50 void volume, the NADPH-dependent reducing activity was restored to more than 40% indicating the presence of an NADPH/thioredoxin dependent system; but increased amounts of thioredoxin did not give any further increase in activity. In contrast, addition of thioredoxin did not alter the NADH or DTT dependent activity. This suggests that the decrease in activity of the Sephadex G50 void volume with DTT as a cofactor compared to the Sephadex G25 void volume was not due to a depletion of thioredoxin. The involvement of thioredoxin in the NADPH-dependent activity was confirmed when the Sephadex G50 void volume was incubated with low concentrations of pure *E. coli* thioredoxin (0–80 ng) in the presence of NADPH (Fig. 2). The rate

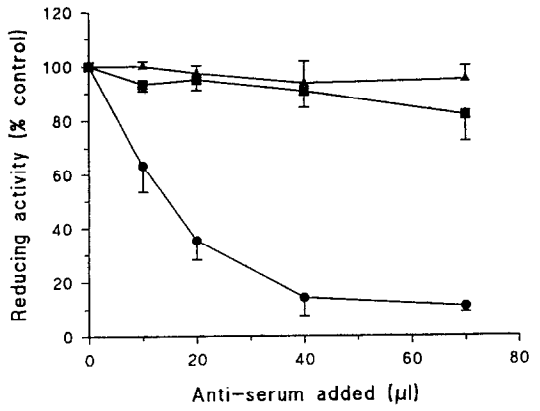

Fig. 3. Effects of anti-thioredoxin anti-serum (μL added per incubation) on the reduction of sulindac by *E. coli* cytosol with NADPH (0.5 mM; circles), NADH (0.5 mM; triangles) and dithiothreitol (5 mM; squares). Percentage activity was calculated by comparing the reducing activity in the presence of anti-serum to that of the same amount of pre-immune serum. Results are mean ± SD for four experiments performed under anaerobic conditions. *$P < 0.05$ compared to the corresponding amount of pre-immune serum using Student's *t*-test for paired data.

of sulindac reduction was directly proportional to the amounts of thioredoxin added to the incubation mixture within this range ($r = 0.993$).

Effects of anti-thioredoxin anti-serum

The reducing activity of the cytosol with NADPH as cofactor was increasingly inhibited with increasing amounts of the anti-serum (Fig. 3). A maximum of about 90% inhibition was obtained with 40 μL or 70 μL of the anti-serum. The same amounts of anti-serum did not give any significant inhibition of reduction in the presence of NADH or DTT. These results, together with those described above indicate the possibility of two sulindac reductase systems, one involving thioredoxin which is coupled to NADPH and one not involving thioredoxin and utilizing NADH.

Partial purification of sulindac reductases and substrate specificity

Each eluate fraction collected from the DEAE-cellulose column was incubated with the four substrates in the presence of various cofactors. Two main peaks of enzyme activity for sulindac reduction were separated (Fig. 4). The first peak appeared in the void volume of the initial washing (fractions 3–6) and the second peak corresponded to fractions 15–17. In the first peak eluted, significant reducing activity was detected with NADH or DTT as cofactors, but very low activity was detected in the presence of NADPH. However, much higher activity was observed with NADPH following the addition

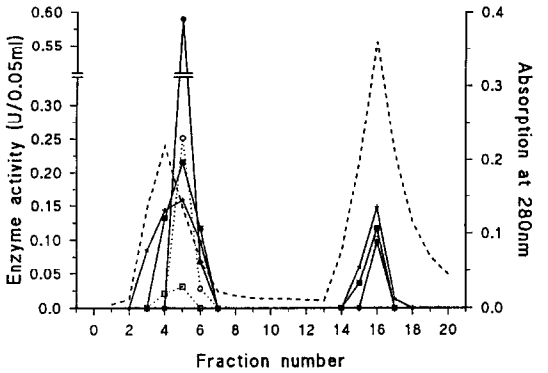

Fig. 4. DEAE-cellulose chromatography of sulindac reducing activity in *E. coli* cytosol. The ammonium sulphate fraction was applied to a DEAE-cellulose column (Sephacel, 1.0 × 10 cm) equilibrated with 20 mM Tris–HCl buffer, pH 8.5, containing 20 mM KCl and 0.1 mM EDTA. Proteins were first eluted with 18 ml of the same buffer and then with 24 mL of 10 mM potassium phosphate buffer, pH 7.4, containing 500 mM KCl and 0.1 mM EDTA. The flow rate was 0.26 mL/min. Eluate fractions of 2 mL were collected. Protein was estimated by the absorption of the eluate at 280 nm (shown as a dashed line). Each fraction (0.05 mL aliquot) was incubated in the presence of NADH (0.5 mM; circles), NADPH (0.5 mM; squares) or DTT (5 mM; stars). Incubations containing NADH or NADPH were studied in both the absence (open symbols and dotted lines) and presence (solid symbols and lines) of *E. coli* thioredoxin (5 μg). The enzyme activity is expressed as nmols sulphide formed/mg protein/hr.

of thioredoxin, demonstrating again the dependence of the NADPH linked reducing activity on thioredoxin. The high activity with NADH alone was also increased by more than two-fold in the presence of thioredoxin.

Comparatively lower enzyme activities were detected in the second peak, although the amount of protein present was considerably higher than in the early peak. Activity with NADPH as cofactor was observed only when thioredoxin was added to the incubations. However, contrary to the results from the early peak, adding thioredoxin to fraction number 16 with NADH as cofactor had no obvious effect on the activity.

The enzyme activity of the different fractions towards flosequinan (Fig. 5) was quite different from that observed with sulindac. Significant reducing activity was detected only in proteins eluted with the initial buffer. The activity associated with the second main protein peak (fractions 15–17) was generally extremely low. The highest activities with NADH and DTT were found in fraction 4 but the highest activity with NADPH was in fraction 5, as in the case of sulindac. A significant main difference with sulindac was that adding thioredoxin did not have any effect on the reducing activity of any fraction or cofactors. The presence of NADPH alone without addition of thioredoxin was sufficient to give an activity comparable to that detected with NADH or DTT. These results suggest that different enzyme systems may be responsible for the reduction of sulindac and flosequinan.

The reducing activity towards sulphinpyrazone was detected only with DTT as a cofactor in fractions 2–5 (Fig. 6). The activity was comparatively much lower than that observed with sulindac and flosequinan (note the scales of enzyme activity). None of the fractions showed any reducing activity towards DPSO under the same incubation conditions.

Localization of the reductases for sulphinpyrazone and DPSO in E. coli

Unlike the sulindac reductases which are mainly found in the cytosol of *E. coli* (Table 4), the reductases for sulphinpyrazone and DPSO were found in both the cytosolic and membrane fractions, depending on the cofactor used (Table 4). It is interesting to note that NADPH did not act as an electron donor in the reduction of DPSO. These results suggest that the enzyme systems responsible for reducing sulphinpyrazone and DPSO may be even more complicated than those demonstrated for sulindac.

DISCUSSION

Despite the established role of the intestinal microflora in the reduction of sulphoxide-containing drugs, there are few data on the enzymic mechanisms responsible. In contrast to the findings in mammalian tissues, acetaldehyde had no effect on the reduction of the substrates by the *E. coli* strain studied. The presence of aldehyde oxidase which receives electrons directly from acetaldehyde has not been reported in

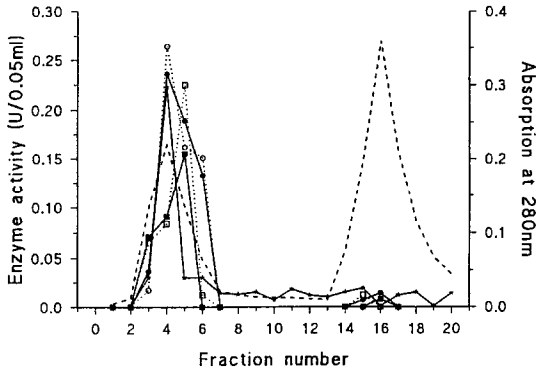

Fig. 5. DEAE-cellulose chromatography of flosequinan reducing activity in *E. coli* cytosol. The ammonium sulphate fraction was applied to a DEAE-cellulose column (Sephacel, 1.0 × 10 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.5, containing 20 mM KCl and 0.1 mM EDTA. Proteins were first eluted with 18 mL of the same buffer and then with 24 mL of 10 mM potassium phosphate buffer, pH 7.4, containing 500 mM KCl and 0.1 mM EDTA. The flow rate was 0.26 mL/min. Eluate fractions of 2 mL were collected. Protein was estimated by the absorption of the eluate at 280 nm (shown as a dashed line). Each fraction (0.05 mL aliquot) was incubated in the presence of NADH (0.5 mM; circles), NADPH (0.5 mM; squares) or DTT (5 mM; stars). Incubations containing NADH or NADPH were studied in both the absence (open symbols and dotted lines) and presence (solid symbols and lines) of *E. coli* thioredoxin (5 μg). The enzyme activity is expressed as nmols sulphide formed/mg protein/hr.

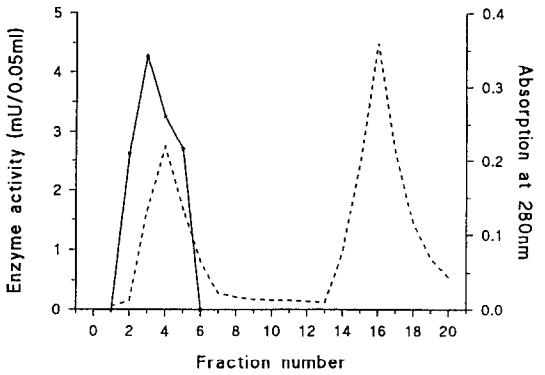

Fig. 6. DEAE-cellulose chromatography of sulphinpyrazone reducing activity in *E. coli* cytosol. The ammonium sulphate fraction was applied to a DEAE-cellulose column (Sephacel, 1.0 × 10 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.5, containing 20 mM KCl and 0.1 mM EDTA. Proteins were first eluted with 18 mL of the same buffer and then with 24 mL of 10 mM potassium phosphate buffer, pH 7.4, containing 500 mM KCl and 0.1 mM EDTA. The flow rate was 0.26 mL/min. Eluate fractions of 2 mL were collected. Protein was estimated by the absorption of the eluate at 280 nm (shown as a dashed line). Each fraction (0.05 mL aliquot) was incubated in the presence of NADH (0.5 mM), NADPH (0.5 mM) or DTT (5 mM; stars). *Escherichia coli* thioredoxin (TXR) (5 μg) was also added to incubations containing NADH or NADPH. No activity was detected with NADH, NADPH or with the addition of *E. coli* thioredoxin. The enzyme activity is expressed as nmols sulphide formed/mg protein/hr.

procaryotic cells [22]. The membrane bound molybdoenzyme in *E. coli* which reduced dimethyl sulphoxide as reported by Weiner *et al.* [23] is unlikely to be aldehyde oxidase due to the large difference in native molecular weight. Our results of lack of stimulation by acetaldehyde may simply reflect the absence of aldehyde oxidase or a functionally equivalent enzyme in *E. coli*.

DTT has been shown to support the activity of several thioredoxin dependent enzymes [18, 24]. The possible involvement of thioredoxin in the reduction of sulindac by *E. coli* was initially indicated from the results demonstrating the stimulatory effects of DTT on the reaction (Table 3). *Escherichia coli* cytosol passed through a Sephadex G50 column was inactive in reducing sulindac in the presence of NADPH, but activity was restored by adding pure *E. coli* thioredoxin (Fig. 2). Together with the inhibition obtained using the anti-thioredoxin antiserum (Fig. 3), these results show that sulindac is reduced by a NADPH–thioredoxin-dependent cytosolic enzyme system in *E. coli*. The electron transfer from NADPH to thioredoxin is catalysed by the FAD-containing enzyme thioredoxin reductase which is highly specific for NADPH and oxidised thioredoxin [25, 26]. Since neither *E. coli* thioredoxin plus NADPH alone nor DTT alone reduced sulindac, a terminal reductase is also required. This suggests

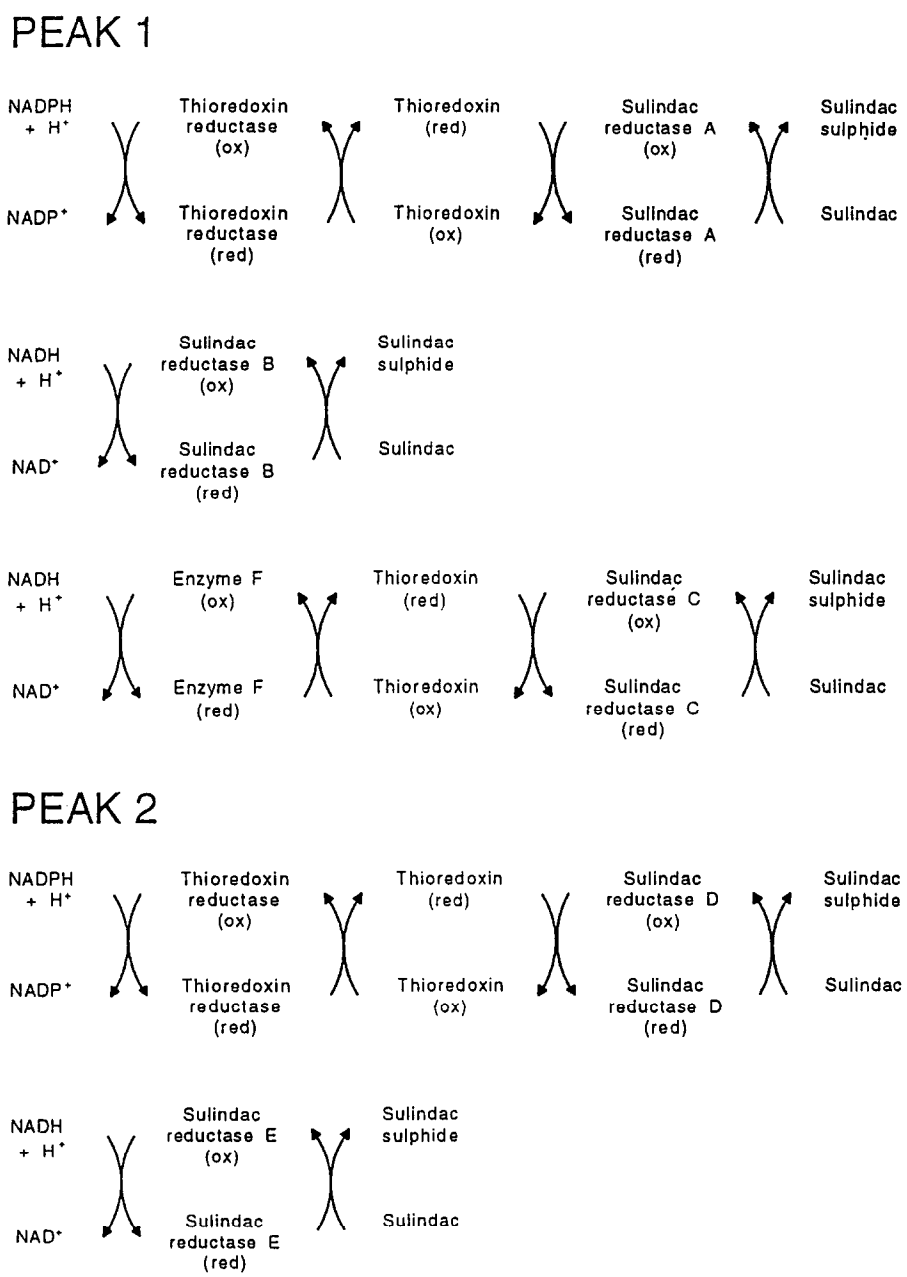

Fig. 7. Proposed sulphoxide reductase systems in *E. coli* cytosol separated by DEAE-cellulose chromatography.

the participation of the whole thioredoxin system, i.e. NADPH, thioredoxin reductase and thioredoxin in the reduction of sulindac.

A more complex profile of the sulindac reducing enzyme systems in *E. coli* emerged after the purification procedures. Detailed analysis of the results in Fig. 4 suggests that peak I (fractions 3–6) contains three enzyme systems (Fig. 7): one linked to NADPH and thioredoxin, one linked to NADH and one linked to NADH and thioredoxin. The NADPH–thioredoxin enzyme system has been discussed above. The NADH linked reductase receives electrons from NADH directly accounting for the NADH–thioredoxin-independent activity. The third enzyme involves both NADH and thioredoxin, but presumably not thioredoxin reductase since this is specific for NADPH.

According to the results in Fig. 4, two more enzymes are probably present in peak 2 (fractions 15–16) (Fig. 7). These enzymes show characteristics of the NADPH–thioredoxin enzyme and the NADH linked enzymes discussed above except that the sulindac reductases are different due to the different elution time. The systems depicted in Fig. 7 are simple representations of the possible enzyme systems present in *E. coli* cytosol according to the results of the DEAE-cellulose chromatography. The possible contribution of DTT has been deliberately omitted from the above systems but it is likely that it acts as an electron donor to the terminal reductases in those systems involving thioredoxin. However, considerable reducing activity was detected with DTT in fraction 3 which showed no activity with NADPH plus thioredoxin, suggesting that DTT may possibly be supplying electrons to an enzyme system not dependent on the thioredoxin system. Overall, these results show that the reduction of sulindac by *E. coli* is mediated by multiple enzyme systems, at least two of which are dependent on the thioredoxin system.

The diversity of the xenobiotic sulphoxide reducing enzyme systems in *E. coli* is further demonstrated from the findings using the other sulphoxides as substrates. The reduction of flosequinan occurred only with the initial peak of reductase activity and required the same cofactors as for sulindac, but the NADPH linked activity was not dependent on thioredoxin. This suggests that NADPH is also able to supply electrons to an enzyme system different from the thioredoxin system. Furthermore, the NADH and NADPH-dependent enzymes did not reduce sulphinpyrazone or DPSO, and only sulphinpyrazone was reduced slightly in the presence of DTT. However, reducing activity towards these two substrates was present as membrane bound enzymes (Table 4), although the actual activity is much lower than that shown towards sulindac. These apparently complex enzyme systems warrant further investigations.

Sulindac and flosequinan are both methyl sulphoxides while sulphinpyrazone and DPSO are aromatic sulphoxides. Our previous studies [6] have shown that the two phenyl sulphoxides are poor substrates for mammalian tissue reductases. Although the existence of specific methyl sulphoxide reductases, such as those present in rat kidney cytosol [5] has not been demonstrated in the present study due to the relatively small number of substrates used, our findings suggest such a trend may well exist in *E. coli*.

As in mammalian tissues [3, 4, 6] it is clear from the data presented above that the thioredoxin system also plays an important part in the reduction of xenobiotic sulphoxides by *E. coli*. Various other sulphoxide reductases in *E. coli* and other microorganisms have been reported by other workers mostly using endogenous substrates [12, 19, 27, 28]. In the present study, we showed that there are likely to be at least five different cytosolic enzyme systems in *E. coli* for the reduction of sulindac, plus possibly separate enzyme(s) for the reduction of flosequinan, sulphinpyrazone and DPSO. Therefore, the total number of xenobiotic sulphoxide reducing enzyme systems in *E. coli* exceeds that previously reported for endogenous substrates and that the enzyme systems appear far more diverse than those reported in mammalian tissues.

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