An Oxygenase from Guinea-Pig Liver that Catalyses Sulphoxidation

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A mono-oxygenase catalysing the conversion of 2-ethyl-4-thioisonicotinamide (ethionamide) into its sulphoxide was purified from guinea-pig liver homogenates. The enzyme required stoichiometric amounts of oxygen and NADPH for the sulphoxidation reaction. The purified protein is homogeneous by electrophoretic, antigenic and chromatographic criteria. The enzyme has mol. wt. 85 000 and it contains 1 g-atom of iron and 1 mol of FAD per mol, but not cytochrome *P*-450. The enzyme shows maximal activity at pH 7.4 in a number of different buffer systems and the K_m values calculated for the substrate and NADPH are 6.5×10^{-5} M and 2.8×10^{-5} M respectively. The activation energy of the reaction was calculated to be 36 kJ/mol. Under optimal conditions, the molecular activity of the enzyme (mol of substrate oxidized/min per mol of enzyme) is calculated to be 2.1. The oxygenase belongs to the class of general drug-metabolizing enzymes and it may act on different compounds which can undergo sulphoxidation. The mechanism of sulphoxidation was shown to be mediated by superoxide anions.

Enzymes responsible for metabolism of drugs and foreign compounds are primarily located in the microsomal fraction of animal tissues (Brodie *et al.*, 1958; Mason *et al.*, 1965; Bloch & Hayaishi, 1966). Most of these enzymes belong to the class of microsomal oxygenases, and the well-studied pathways are ascribed to one common mechanism, namely hydroxylation (Hayaishi, 1962). A number of thioethers, used as therapeutic agents or insecticides, are metabolized in animals to sulphoxides (Clare, 1940; March *et al.*, 1955; Burns *et al.*, 1957; Walkenstein & Seifter, 1959). In addition, sulphoxidation reactions such as the conversion of chlorpromazine into its sulphoxide, have been reported (Gillette & Kamm, 1960). Nonetheless, no detailed studies have been carried out on these enzymes to determine their mechanism of action, nor has there been any attempt to purify them.

In this paper, we report the purification and properties of an enzyme from guinea-pig liver, catalysing the formation of ethionamide sulphoxide from ethionamide (2-ethyl-4-thioisonicotinamide). Ethionamide is an active tuberculostatic second-line drug used in the re-treatment of pulmonary tuberculosis resistant to one or more of the classical drugs. The activity of ethionamide *in vivo* is considerably greater than its activity *in vitro* indicating that the drug may exercise its antituberculosis action through its metabolites (Goodman & Gillman, 1970).

Several metabolites of ethionamide are excreted in the urine after administration of the drug to humans, and a few of them have been identified (Bieder *et al.*,

1961, 1963; Bieder & Mazeau, 1962, 1964; Yamamoto *et al.*, 1962, 1963). A major metabolite of ethionamide in guinea pigs is the sulphoxide. The antitubercular activity of ethionamide sulphoxide *in vitro* is 1.5 times that of the parent compound (K. Prema & K. P. Gopinathan, unpublished work).

The mechanism of sulphoxidation has been studied by us and we have presented evidence to show that sulphoxidation involves the superoxide anion (O_2^-), as in aromatic hydroxylation reactions (Prema & Gopinathan, 1974).

Materials and Methods

Materials

Ethionamide (Trescatyl) was from May and Baker Ltd., Dagenham, Essex, U.K., and was recrystallized before use. Ethionamide sulphoxide was prepared by the method of Liebermann *et al.* (1963) by treatment of ethionamide with H_2O_2 in pyridine medium and was recrystallized three times before use. Glucose 6-phosphate, barium salt (converted into the potassium salt before use), $NADP^+$, NADH, NADPH, Tris, Hepes,* Mes, DEAE-cellulose, β -alanine, dithiothreitol, 2-mercaptoethanol, iodoacetate, bovine serum albumin (fraction V), alcohol dehydrogenase, ovalbumin, β -galactosidase, peroxidase, methionine, GSH, *N*-ethylmaleimide, phenazine methosulphate and *p*-chloromercuribenzoate were purchased from Sigma Chemical Co., St. Louis,

* Abbreviations: Hepes, 2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid.

Mo., U.S.A. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. The reagents for gel electrophoresis were from Eastman Kodak Co., Rochester, N.Y., U.S.A. Amido Schwartz 10B and silica gel (for t.l.c.) were from E. Merck, Darmstadt, Germany. Coomassie Brilliant Blue was from Colab Laboratories, Glenwood, Ill., U.S.A. Chlorpromazine was from May and Baker (India) Ltd., Bombay, India. Mellaryl (thioridazine) was from Sandoz (India) Ltd., Bombay, India, and was recrystallized three times from acetone before use. Noble agar was from Difco Laboratories, Detroit, Mich., U.S.A. Catalase (ox liver), cytochrome *c* (ox heart) and biotin were from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Thioisonicotinamide was from May and Baker (India) Ltd. $\alpha\alpha'$ -Bipyridyl, *o*-phenanthroline, diethyldithiocarbamate and Methylene Blue were from BDH, Poole, Dorset, U.K. EDTA, urea, 8-hydroxyquinoline, NaCN, NaN₃, thiourea, thiosemicarbazide and sodium dodecyl sulphate were from BDH, Bombay, India. Pepsin was from Calbiochem A.G., Lucerne, Switzerland. Calcium phosphate gel was prepared by the method of Colowick & Kaplan (1955).

General methods

The enzyme superoxide dismutase was prepared from sheep erythrocytes by the method of McCord & Fridovich (1969) and purified up to the DEAE-cellulose stage. A unit is defined as that amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% at 28°C.

The u.v. and visible spectra were recorded on a Unicam SP700A spectrophotometer. The i.r. spectra were recorded on a Carl Zeiss UR-10 spectrophotometer by using Nujol mulls. The fluorescence spectra were recorded on a Perkin-Elmer fluorescence spectrophotometer. Ultracentrifugations were carried out in a Beckman model L2-50 ultracentrifuge. Analytical ultracentrifugations were done in a Beckman model E analytical ultracentrifuge.

Preparation of tissue samples

Tissue samples were prepared at 0–3°C. Adult male guinea pigs were anaesthetized and bled to death, and the livers removed after perfusion and homogenization in a Waring blender with 2vol. of 1.15% (w/v) KCl. The homogenates were subjected to differential centrifugation as described by Fouts & Brodie (1957).

The enzymic activity was assayed by measuring the amount of ethionamide that disappeared, as well as the ethionamide sulphoxide formed. A typical reaction mixture contained, in a final volume of 1.5ml, 0.2 μ mol of NADPH [or an NADPH-generating system consisting of 0.13 μ mol of NADP⁺, 6.5 μ mol of glucose 6-phosphate and 0.69 unit (μ mol/min) of glucose 6-phosphate dehydrogenase], 0.36 μ mol of

substrate, 0.02M-potassium phosphate buffer, pH 7.4, and various amounts of tissue extracts. Incubations were carried out at 37°C for 90min under aerobic conditions. Ethionamide and ethionamide sulphoxide were determined by a slight modification of the method described by Bieder *et al.* (1963, 1966). The compounds were extracted into chloroform from the assay mixture after saturation with (NH₄)₂SO₄ and re-extracted into dilute HCl. The extinction of the samples at 315nm (for ethionamide) and at 395nm (for ethionamide sulphoxide) was recorded. A standard curve was prepared by using a mixture of authentic samples of the compounds. The NADPH concentrations were determined in the aqueous layer, after chloroform extraction, by recording the *E*₃₄₀ (Horecker & Kornberg, 1966).

A unit of activity is the amount of enzyme which catalyses the formation of 1nmol of ethionamide sulphoxide per min at 37°C. Specific activity is expressed as units/mg of protein.

Protein determination

The protein content was determined by the biuret method (Gornall *et al.*, 1949) in crude extracts or by the method of Lowry *et al.* (1951) in purified preparations. Bovine serum albumin fraction V (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) was used in preparing a standard curve of the protein.

Purification of the enzyme

All operations were done below 5°C and centrifugations were carried out in a Sorvall RC-2 refrigerated centrifuge.

Step I: preparation of tissue homogenates. Liver homogenates [homogenized in a Waring blender with 2vol. of 1.15% (w/v) KCl] were centrifuged at 9000g for 20min. The supernatant contained all the enzyme activity.

Step II: first ammonium sulphate fractionation. Solid (NH₄)₂SO₄ was added with gentle stirring to 125ml of the 9000g supernatant to 40% saturation. After being left for 20min, the precipitate was removed by centrifugation and discarded. (NH₄)₂SO₄ was added to the supernatant to 70% saturation, and the precipitate was collected by centrifugation after 20min. The precipitate was dissolved in a minimal volume of 0.02M-potassium phosphate buffer, pH 7.4, and desalted by dialysis against 2mM-potassium phosphate buffer, pH 7.4, overnight.

Step III: calcium phosphate gel adsorption. The enzyme from the previous step [precipitated between 40 and 70% saturation of (NH₄)₂SO₄] was adsorbed on calcium phosphate gel (20mg/ml). The adsorption was done at pH 7.4 (protein/gel ratio of 10:3) and the supernatant was removed by centrifugation. The adsorbed proteins were eluted with 0.1M-potassium phosphate buffer, pH 6.5. The eluates were pooled (40ml).

Step IV: adsorption and elution from DEAE-cellulose. The enzyme [40ml of eluate from the $\text{Ca}_3(\text{PO}_4)_2$ gel] from the previous step was treated with DEAE-cellulose (1g of DEAE-cellulose to 5ml of the enzyme) which was activated by the procedure of Peterson & Sober (1962). After being stirred for 20min, the slurry was centrifuged; the supernatant did not show any enzyme activity. The residue was treated with 0.1M-potassium phosphate buffer, pH 7.4 (15ml). The enzyme activity was eluted completely in three extractions (5ml for each extraction). Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the DEAE-eluate as in Step II to 40% saturation. The protein precipitating between 40 and 70% saturation of $(\text{NH}_4)_2\text{SO}_4$ was collected and dissolved in 10ml of potassium phosphate buffer (pH below 6).

Step V: second ammonium sulphate fractionation. To the enzyme (10ml of the concentrated eluate) from the previous step, a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ (pH adjusted to 7.5) was added to a saturation of 30%. The precipitated protein, containing all the enzyme activity, was collected and dissolved in 0.02M-potassium phosphate buffer, pH 6.5 (8ml).

Step VI: gel filtration on Sephadex G-200. The enzyme (8ml) was passed through a column (2.2cm $\times$ 43cm) of Sephadex G-200. The column had been previously equilibrated with 0.02M-potassium phosphate buffer, pH 6.5. Individual fractions were assayed for enzyme activity. The active fractions (two fractions equal to 6ml) were pooled and rechromatographed on a column (2.2cm $\times$ 43cm) of Sephadex G-200 under similar conditions.

Disc electrophoresis

Polyacrylamide-gel electrophoresis was done by the method of Ornstein & Davis (1962) by using Tris-glycine buffer, pH 8.3, or β -alanine-acetic acid buffer, pH 4.3, on 7.5% (w/v) polyacrylamide gels. The gels were stained for 30min with Amido Schwartz or Coomassie Brilliant Blue and destained with 7% (v/v) acetic acid by diffusion.

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969).

Determination of the molecular weight of the enzyme

The approximate molecular weight of the enzyme was determined by the gel-filtration method (Andrews, 1965), sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) and the sucrose-gradient-centrifugation method (Martin & Ames, 1961). The molecular weight was also calculated by the determination of sedimentation velocity in the analytical ultracentrifuge.

Determination of iron

The iron content of the enzyme was measured by the bathophenanthroline method, after wet ashing,

as described by Van de Bogart & Beinert (1967).

A sample of enzyme (1.08mg of protein in 0.6ml of deionized water) was ashed completely with 0.1 ml of 60% (w/v) HClO_4 , 0.1 ml of concn. HNO_3 and 0.1 ml of concn. H_2SO_4 . The residue was taken up in 0.5ml of deionized water and the colour reaction was carried out. The amount of iron present was calculated from a calibration curve obtained by subjecting known concentrations of a standard (fresh solution of FeCl_3) to a similar procedure to that described above.

Preparation of antiserum

Antiserum against purified enzyme was prepared from rabbits. Subcutaneous injections of purified enzyme (2.5mg of protein) in Freund's complete adjuvant were given; the injections were repeated on the 7th and 14th day, and the rabbits' serum was tested by the Ouchterlony (1967) double-diffusion method. Immunoelectrophoresis at pH 8.6 was carried out in agar gel (1.5%, w/v, Ionagar 2 in 25mm-sodium diethyl barbiturate, pH 8.6, containing 0.1% NaN_3) with 50mm-sodium diethyl barbiturate, pH 8.6, in the reservoir (Feinstein, 1968).

Oxygen-uptake studies

O_2 -uptake studies were carried out in Warburg flasks at 37°C by conventional Warburg techniques (Umbreit *et al.*, 1957), to measure the gas consumed during the course of the enzyme reaction. The reaction was started by the addition of substrate from the side arm.

Results

Intracellular localization of enzyme activity

The oxidation of ethionamide was catalysed by an enzyme system in the 9000g supernatant fraction of the liver homogenate (Table 1). The specific activity was slightly greater in the 9000g supernatant fraction than in the whole homogenate. Most of the activity was sedimented at 105000g for 1h suggesting that the enzyme is associated with the microsomal fraction.

Ethionamide was metabolized by microsomal preparations supplied with NADPH directly or with an NADPH-generating system consisting of NADP^+ , glucose 6-phosphate and glucose 6-phosphate dehydrogenase. No oxidation was observed when the 9000g supernatant was supplemented with NADP^+ alone (in the absence of glucose 6-phosphate and glucose 6-phosphate dehydrogenase) or with NADH. These results indicate that ethionamide is oxidized by a microsomal enzyme that requires NADPH.

Purification of the enzyme

The results of purification of the enzyme are summarized in Table 2, which shows that about 160-

Table 1. *Intracellular distribution of enzyme activity*

The assay system was as described in the Materials and Methods section. The protein concentrations used were 20–25 mg per total assay mixture.

Addition	Specific activity (nmol of ethionamide sulphoxide formed/min per mg of protein)
Whole homogenate	0.10
Post-nuclear (300g supernatant)	0.12
Nuclear fraction (300g sediment)	—
Post-mitochondrial (9000g supernatant)	0.13
Mitochondrial (9000g sediment)	—
Post-microsomal (100000g supernatant)	0.02
Microsomal (100000g sediment)	0.09
Microsomal+soluble fraction (mixed)	0.12

Table 2. *Purification of enzyme*

For details see the text. A unit of activity is the amount of enzyme that catalyses the formation of 1 mol of ethionamide sulphoxide/min at 37°C. Specific activity is expressed as units/mg of protein.

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity	Specific activity	Percentage recovery
9000g supernatant	125	108	13 500	10.8	1350	0.10	100
40–70% satd. (NH ₄) ₂ SO ₄	30	110	3300	39.0	1180	0.36	88
Ca ₃ (PO ₄) ₂ gel eluate	40	41	1640	24.6	984	0.60	72
DEAE-cellulose eluates	10	12.8	128	62.7	627	4.90	46
Alkaline (NH ₄) ₂ SO ₄ (0–30% satd.)	8	6.8	54	73.0	583	10.80	43
Sephadex G-200	4	7.4	29.6	118.0	474	16.00	35

fold purification has been achieved with a recovery of 35%. At the stage of Sephadex G-200 filtration, the enzyme preparation showed the presence of at least three distinct protein peaks (Fig. 1).

The enzymically active fractions, when rechromatographed on a Sephadex G-200 column, showed one peak of protein (with a slight shoulder, see the inset of Fig. 1) and enzyme activity. The active fractions (between 161 and 164 ml of effluent volume) from this step were used as the purified enzyme.

Product of enzyme activity. At the end of the reaction, the product of the enzyme activity, along with the excess of unchanged substrate, was extracted into chloroform and was re-extracted into acid medium. The acid layer was evaporated and t.l.c. and paper chromatography were carried out. The chromatograms were developed by the ascending technique in the solvent system, butan-1-ol-acetic acid-water (4:1:5, by vol.). Spots were yellow and could also be located by spraying with Dragendorff's reagent (Prema & Gopinathan, 1974). The *R_F* values of the enzymic product and the authentic samples were identical.

The spots from t.l.c. were eluted with dilute HCl and the u.v., visible and i.r. spectra were recorded

and compared with those of authentic ethionamide and ethionamide sulphoxide.

From these criteria, the product of enzyme reaction has been identified as ethionamide sulphoxide.

Homogeneity of the purified enzyme. The purified enzyme on passage through another Sephadex G-200 column (for molecular-weight determinations) showed a single coincident peak of protein and enzyme activity. The enzyme showed only one band on polyacrylamide-gel electrophoresis at pH 8.3 or at 4.5.

Immunoelectrophoresis of the enzyme with rabbit antiserum as well as Ouchterlony double-immuno-diffusion analysis also revealed a single protein component.

Determination of molecular weight

The molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column, by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis and by sucrose-gradient centrifugation. For sodium dodecyl sulphate-polyacrylamide-gel electrophoresis, protein samples were incubated at 37°C for 2 h in 0.01 M-sodium phosphate buffer,

Fig. 1. Gel filtration of the enzyme on Sephadex G-200

The column (2.2cm×43cm) was equilibrated with 0.02M-phosphate buffer, pH6.5. The flow rate was 15ml/h. The inset shows rechromatography on the same column under identical conditions. ○—○, Protein; ○---○, enzyme activity (units).

Fig. 2. pH-activity profiles of sulfoxide-forming enzyme in different buffer systems

For details see the text. ○, Tris-HCl; ■, HEPES; △, Tris-maleate; ●, Mes.

Fig. 3. Effect of temperature on enzyme activity

Experimental details are described in the text. The inset shows the Arrhenius plots for the calculation of activation energy.

pH 7.0, containing 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol before loading in the gel; 10% (w/v) polyacrylamide gels were used. The distribution of the enzyme activity in sucrose gradients was also studied. The enzyme was treated with sodium dodecyl sulphate and loaded on the gradient. The enzyme activity was distributed in the same fractions as the untreated enzyme, though most of the activity was lost on sodium dodecyl sulphate treatment, indicating that the enzyme contains only one subunit. Thus the molecular weight calculated by these three methods was 85000.

The sedimentation pattern of the enzyme in the analytical ultracentrifuge gives a single symmetrical boundary.

Properties

(a) *pH optima*. The relationship between pH and the enzymic activity was studied and the results are presented in Fig. 2. The pH optimum was 7.4, in several buffer systems (Tris-HCl, Tris-maleate, Mes and Hepes buffers). Although the maximal activity observed in potassium phosphate buffer was also at pH 7.4, the peak was less sharp.

(b) *Effect of temperature*. The effect of temperature on enzymic activity was studied and the results are presented in Fig. 3 which shows that maximum activity was observed at about 39°C. Further increase in the temperature resulted in rapid loss of activity. There was total inactivation of the enzyme between 55° and 60°C within 1 min. By using Arrhenius plots

Fig. 4. U.v.-absorption spectrum of the purified enzyme
The concentration of protein was 500 μ g/ml.

Fig. 5. Visible absorption spectrum of the purified enzyme
Concentration of protein was 6.3 mg/ml. —, Enzyme; ----, FAD.

(of $\log v$ versus $1/T$), the energy of activation was calculated to be 36 kJ/mol.

(c) *Spectral properties*. The purified enzyme was yellow and showed fluorescence. It had an absorption maximum at 278 nm in the u.v. spectrum (Fig. 4) and a typical iron flavoprotein spectrum, with a characteristic maximum at 425 nm. A shoulder between 450 and 460 nm was observed (Fig. 5). The fluorescence spectrum of the enzyme is given in Fig. 6 which shows that the enzyme has a characteristic flavin fluorescence spectrum, with the excitation maxima at 375 and 460 nm and an emission maximum at 520 nm. The fluorescence peaks disappear completely on addition of dithionite.

(d) *Nature of flavin in the enzyme*. The enzyme activity was stimulated by the addition of FAD, or to a lesser extent by FMN, to the standard assay system. To characterize the nature of the flavin co-enzyme, the procedure described by Siegel *et al.* (1973) was adopted. The purified enzyme (3.6 mg of protein/ml) was kept for 3 min in a boiling-water bath and denatured protein was removed. Suitable dilutions of the supernatant were made and u.v., visible and fluorescence spectra were then recorded.

Heat-treated supernatants were also analysed chromatographically on paper in three different solvent systems. The R_F values obtained were compared

Fig. 6. Fluorescence-excitation and -emission spectra of the purified enzyme

Protein concentration was 300 $\mu\text{g/ml}$ in potassium phosphate buffer, pH 6.5 (0.02M). (a) The excitation wavelength was varied and emission was recorded at 520nm. (b) The emission wavelength was varied at a fixed excitation wavelength of 460nm. The pattern is identical if the excitation is at 370nm. ●-----●, Effect of sodium dithionite.

Table 3. Paper chromatography of the flavin component of the enzyme

For details see the text.

Sample	Solvent system	...	R_F value		
			t-Butyl alcohol-water (3:1, v/v)	Lutidine saturated with water	5% (w/v) Na_2HPO_4
Riboflavin			0.47	0.48	0.27
FMN			0.37	0.84	0.47
FAD			0.23	0.61	0.39
Enzyme flavin			0.24	0.60	0.38

with those of authentic FAD, FMN and riboflavin. The results are presented in Table 3. By comparing the fluorescence-emission spectra of the enzyme with those of authentic FAD subjected to similar treatment, the amount of flavin in the enzyme was calculated.

From the data presented, it is clear that the flavin present is FAD; the content of FAD in the enzyme has been estimated to be 1 mol/mol of enzyme.

Effect of substrate concentration

The effect of varying the substrate concentration on the enzyme reaction was studied (Fig. 7, inset). The K_m value for ethionamide was calculated to be $5.9 \times 10^{-5} \text{M}$ (Fig. 7). The K_m value for ethionamide, calculated on the basis of oxygen-uptake studies (Warburg), was $7.0 \times 10^{-5} \text{M}$. The K_m value for NADPH was calculated to be $2.8 \times 10^{-5} \text{M}$.

Requirement for oxygen

The oxidation of ethionamide required O_2 . Bubbling O_2 through the assay mixture resulted in stimulation of the enzyme activity (the specific activity increased from 0.19 to 0.22) whereas bubbling N_2 through the assay mixture led to complete absence of enzyme activity. Under completely anaerobic conditions (under vacuum) also no activity was observed.

The O_2 -uptake studies carried out in Warburg flasks showed that stoichiometric amounts of O_2 were consumed as the oxidation proceeded. The stoichiometry of the reaction at different time-intervals (at a fixed high concentration of substrate) as well as at different concentrations of substrate (at a fixed time-interval) was established. The results are presented in Tables 4 and 5, which show that 1 mol of ethionamide sulphoxide was formed for every 2 atoms of oxygen

Fig. 7. Determination of K_m value

The inset shows the effect of varying the substrate concentration on the rate of reaction. For details see the text.

Table 4. *Stoichiometry of the enzymic conversion of ethionamide at different time-intervals*

The assays were carried out in Warburg flasks at 37°C. The reactions were carried out in a final volume of 3.0 ml, and all the components of the standard assay (final volume 1.5 ml) were doubled. The amount of enzyme protein used was 200 μ g.

Time (min)	Product formed (μ mol)	Oxygen consumed (μ mol)	NADPH oxidized (μ mol)
5	0.01	0.01	0.01
10	0.02	0.02	0.02
20	0.04	0.04	0.05
40	0.09	0.10	0.11
60	0.11	0.11	0.14
90	0.15	0.16	0.17
120	0.16	0.18	0.18
150	0.17	0.18	0.17
180	0.16	0.19	0.18

consumed; concomitantly 1 mol of NADPH was oxidized.

Effect of thiol inhibitors, metal-chelating agents and other reagents

Thiol inhibitors such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetate and HgCl_2 strongly inhibited sulphoxidation (Table 6). The inhibition caused by *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetate could at least partially be re-

Table 5. *Stoichiometry of the enzymic conversion at different concentrations of substrate*

The assay system was as described in Table 4, except that the substrate concentration was varied as indicated. The reactions were terminated at 90 min.

Substrate added (μ mol)	Product formed (μ mol)	Oxygen consumed (μ mol)	NADPH oxidized (μ mol)
0.05	0.04	0.05	0.05
0.10	0.08	0.09	0.10
0.20	0.10	0.10	0.11
0.30	0.13	0.15	0.15
0.40	0.16	0.17	0.17
0.50	0.16	0.20	0.20
0.75	0.17	0.26	0.18
1.00	0.17	0.21	0.18
1.50	0.16	0.20	0.18

versed by the addition of GSH, 2-mercaptoethanol and dithiothreitol. These results indicate that a thiol group is involved in the enzymic activity.

Metal-chelating agents such as EDTA, $\alpha\alpha'$ -bipyridyl, *o*-phenanthroline and diethyldithiocarbamate caused very little inhibition of the enzyme. 8-Hydroxyquinoline caused more than 40% inhibition at 0.1 mM.

Reducing agents such as GSH, 2-mercaptoethanol and dithiothreitol did not have any effect on the enzyme activity. Methylene Blue (NADPH-oxidizing agent) inhibited the enzyme completely even at 0.01 M. CO inhibited the enzyme by 50%, whereas compound SKF 525A, a known inhibitor of the microsomal oxidases, did not cause any inhibition. Inhibitors such as cyanide, azide, fluoride, arsenite and arsenate were also studied. Of the compounds tested, only cyanide inhibited enzyme activity, by 60% at 1 mM. In these studies, the enzyme and the inhibitor were treated for 10 min at 37°C before the addition of substrate. By increasing the time of treatment of the enzyme with cyanide, complete inhibition of activity was observed. The cyanide-treated enzyme did not show any activity after dialysis; however, part of the activity (10%) was recovered on addition of 0.1 mM- Fe^{3+} to the dialysed preparation.

Measurement of iron content

In addition to the sensitivity to inhibition by cyanide and the iron-chelating agents, the enzyme showed the characteristic absorption at 450 nm indicating the presence of iron. The CO differential absorption spectrum, however, did not reveal the presence of cytochrome *P*-450 or other cytochromes; hence all the iron may be present as non-haem iron. The iron content was estimated to be 1 g-atom/mol of enzyme.

Table 6. *Effect of chelating agents, thiol inhibitors and reversal of inhibition*

The composition of the reaction mixture was the same as given in the Materials and Methods section, except that the reaction mixture also contained the inhibitors as indicated. The enzyme was incubated with the inhibitor for 10 min before the addition of substrate at 37°C. In the case where the reversal of inhibition was studied, the reagents were added (after 10 min incubation of the enzyme and inhibitor) and further incubated for 10 min at 37°C. The concentration of reversing agents used was 0.1 mM at all three concentrations of inhibitors. The enzymic reaction was started by addition of the substrate. The amount of enzyme used was 100 µg of protein.

Addition	Concn. of inhibitor	...	1 mM	Inhibition (%)	
				0.1 mM	0.01 mM
<i>p</i> -Chloromercuribenzoate			100	83	62
<i>p</i> -Chloromercuribenzoate + GSH			78	52	0
<i>p</i> -Chloromercuribenzoate + 2-mercaptoethanol			62	39	0
<i>p</i> -Chloromercuribenzoate + dithiothreitol			73	50	0
<i>N</i> -Ethylmaleimide			74	62	35
<i>N</i> -Ethylmaleimide + GSH			39	23	0
<i>N</i> -Ethylmaleimide + 2-mercaptoethanol			34	15	0
<i>N</i> -Ethylmaleimide + dithiothreitol			52	34	0
Iodoacetate			72	47	42
Iodoacetate + GSH			37	29	0
Iodoacetate + 2-mercaptoethanol			31	19	0
Iodoacetate + dithiothreitol			51	19	0
EDTA			10	4	—
αα'-Bipyridyl			14	10	—
<i>o</i> -Phenanthroline			15	0	—
8-Hydroxyquinoline			60	41	—
Diethyldithiocarbamate			20	20	—

Table 7. *Effect of metal ions*

The assay system was as described in Table 6. The enzyme (100 µg of protein) was treated with the metal ion for 10 min before the addition of substrate.

Addition	Concn. of inhibitor	...	Inhibition (%)		
			1 mM	0.1 mM	10 µM
AgNO ₃			100	95	75
CuSO ₄			100	96	73
HgCl ₂			100	100	85
Zinc acetate			96	85	73
CdSO ₄			100	82	79
MgSO ₄			0	2	—
MnSO ₄			0	0	0
FeSO ₄			-8*	0	0
Fe ₂ (SO ₄) ₃			-23*	-4*	—

* There was stimulation of enzyme activity.

Effect of metal ions

Incubation of the enzyme with Ag⁺, Hg²⁺, Cu²⁺, Cd²⁺ and Pb²⁺ led to complete inhibition of enzyme activity (Table 7) whereas Mg²⁺ and Mn²⁺ did not have any effect. Fe²⁺ and Fe³⁺ activated the reaction by 8 and 25% respectively.

Effect of urea

The purified enzyme, on treatment with urea, loses activity. After dialysis the enzyme activity is at least partially regained (Table 8).

Table 8. *Effect of urea on enzyme activity (effect on sulph-oxidation)*

The enzyme was incubated with various concentrations of urea for 15 and 30 min. The urea-treated enzyme (treated for 30 min) was dialysed against water (18 h, three changes) and the activity was measured. The enzyme reactions were started by addition of substrate.

Concn. of urea (M)	Time of treatment	...	Activity remaining (%)		
			Before dialysis		After dialysis*
			15 min	30 min	
2			50	34	52
4			40	26	48
6			34	20	50
8			14	4	60

* Enzyme treated for 30 min.

Effect of sulphur-containing compounds

The effect of some endogenous substrates such as methionine and biotin which undergo sulphoxidation was tested. The other compounds tested include thiourea, thiosemicarbazide and thioisonicotinamide (all of which contain the -CSNH₂ group) as well as drugs such as chlorpromazine and thioridazine which are known to undergo sulphoxidation. The effects of these compounds were analysed at lower (half the

Table 9. *Effect of sulphur-containing compounds*

The assays were carried out as described in the Materials and Methods section. The inhibitors were used at concentrations, half of, equal to, twice and ten times that of the substrate. The amount of enzyme used was 100 μ g of protein.

Inhibitor	Concn. of inhibitor (M)	Inhibition (%)			
		0.5	1.0	2.0	10.0
Methionine		0	0	2	0
Biotin		0	0	2	39
Thiosemicarbazide		54	58	75	98
Thiourea		59	100	100	100
Thioisonicotinamide		55	100	100	100
Thiouracil		0	0	0	0
Cystine		0	0	0	0
Chlorpromazine		52	100	100	100
Mellaryl (thioridazine)		45	62	100	100

molarity of the substrate), equal and higher (twice and ten times the molarity of the substrate) concentrations (Table 9).

Methionine, cystine and thiouracil had no effect on sulphoxidation; biotin caused 39% inhibition when used in 10-fold excess over substrate. Thiourea, thiosemicarbazide, thioisonicotinamide, chlorpromazine and thioridazine inhibited the enzyme by about 50% at half the concentration of the substrate; most of these compounds inhibited the enzyme completely at equimolar and higher concentrations. Fensulfthion (*O'*-diethyl-*O*-4-methylsulphinyl phenyl phosphorothioate), a sulphur-containing insecticide also inhibited the enzyme completely.

Effect of superoxide dismutase

On treatment of the enzyme with superoxide dismutase (prepared from sheep erythrocytes), there was complete inhibition of sulphoxide formation. The pattern of inhibition was linear with increasing concentrations of superoxide dismutase added. In the standard assay system containing 100 μ g of the enzyme, when 150 units of superoxide dismutase was added, there was inhibition, and on doubling the concentration of dismutase there was complete inhibition. The results show the involvement of superoxide anions in the sulphoxidation reaction.

Molecular activity

The molecular activity of the enzyme (defined as the number of mol of substrate oxidized/min per mol of enzyme, under optimal conditions) was calculated to be 2.1.

Discussion

By incorporating one atom of molecular oxygen into substrate, mono-oxygenases catalyse a variety of reactions namely hydroxylation, dealkylation, epoxide and *N*-oxide formation, desaturation, aromatiza-

tion of steroids and acid amide formation (Yamamoto *et al.*, 1967; Gillette *et al.*, 1957). The participation of a flavin cofactor has been reported for each type of mono-oxygenase reaction and an electron-transport system is associated with several mono-oxygenase reactions. Kimura & Suzuki (1967) have described the components of the electron-transport system in adrenal steroid hydroxylase as a flavoprotein (adrenodoxin reductase), a non-haem iron protein and possibly cytochrome *P*-450.

The enzyme described in the present paper is a mono-oxygenase which catalyses sulphoxide formation, which specifically requires NADPH; it is a flavoprotein, containing one g-atom of iron/mol of enzyme and FAD as the flavin component. In other oxygenases such as L-lysine mono-oxygenase, imidazole acetate mono-oxygenase and *p*-hydroxybenzoate hydroxylase FAD has also been identified as the flavin component; however, the latter enzymes do not contain iron or copper (Yamamoto *et al.*, 1966; Yano *et al.* 1966). There are other enzymes, such as the lactonizing enzyme for 2,3-dioxocamphene, which requires FMN (Conrad *et al.*, 1961), and the phenylalanine hydroxylase of mammalian liver, which requires a reduced pteridine derivative (Kaufman, 1964).

The sulphoxidation enzyme appears to be associated with the microsomal fraction in the liver homogenates but is easily solubilized during fractionation procedures. The enzyme contains non-haem iron and no cytochrome *P*-450 was detected by CO differential spectroscopy. Further, compound SKF 525A, a known inhibitor of the microsomal oxidation involving cytochrome *P*-450, had no effect on the enzyme. Although cyanide caused almost complete and irreversible inactivation of the enzyme, it was necessary to preincubate the enzyme with cyanide for more than 20 min to bring about the effect. Similarly, other iron-chelating agents such as $\alpha\alpha'$ -bipyridyl or *o*-phenanthroline also caused only partial inhibition of

the enzyme. The enzyme was extremely sensitive to inhibition by thiol-group reagents and metal ions such as Hg^{2+} and Ag^{+} ; the inhibition by thiol reagents could be at least partially reversed by GSH, dithiothreitol, and 2-mercaptoethanol. No attempts have been made to estimate the thiol-group content of the protein.

The purified protein is homogeneous by electrophoretic criteria (it moved in a single band on polyacrylamide gels either at pH 4.5 or 8.3) and by antigenic, immunoelectrophoretic and ultracentrifugal criteria. It has an approximate molecular weight of 85000, as determined by electrophoretic, column-chromatographic or sucrose-gradient-centrifugation methods, and presumably contains one subunit, because the molecular weight determined on sodium dodecyl sulphate-polyacrylamide gels was in agreement with the value obtained from the other methods. On sucrose-density-gradient centrifugation after treatment with denaturation agents such as sodium dodecyl sulphate, the enzyme activity was distributed similarly to the untreated enzyme, although 50% of the activity was lost as a result of treatment. This presumably indicates that the enzyme contains only one subunit. On treatment with urea, a significant amount of enzyme activity was lost; however, more than 50% of the activity was regained after the urea had been removed by dialysis.

Although a number of sulphur-containing compounds which are normal metabolites or which can undergo sulfoxidation in biological systems (e.g. methionine, biotin, cystine and thiouracil) were tested in the enzyme reaction, none of them seemed to undergo the reaction with the purified enzyme. However, the method used was indirect because they were tested as inhibitors of the ethionamide sulfoxidation reactions, at different concentrations. Nonetheless, under these conditions compounds such as thiosemicarbazide, thiourea, chlorpromazine and thioridazine acted as competitive inhibitors of ethionamide sulfoxidation. Further, chlorpromazine sulfoxide has been identified as the product of the reaction from chlorpromazine. The enzyme therefore may have a role in the sulfoxidation of drug molecules in general and may act on a variety of compounds capable of undergoing sulfoxidation.

The sulfoxidation reaction may be of special significance with certain drugs such as ethionamide because it is readily metabolized to the sulfoxide on administration to the experimental animals and man (Bieder *et al.*, 1961, 1963; Bieder & Mazeau, 1962, 1964). Ethionamide sulfoxide appears to be more potent *in vitro* than the parental compound itself, in its antituberculosis action (K. Prema & K. P. Gopinathan, unpublished work); this finding is in agreement with the observation that the drug is more potent *in vivo* than *in vitro* (Goodman & Gillman, 1970).

The mechanism of sulfoxidation was also examined by us. The enzyme superoxide dismutase, prepared from sheep erythrocytes, inhibits the sulfoxidation reaction indicating the involvement of superoxide anion in the reaction. In addition, the sulfoxidation has also been demonstrated in a non-enzymic system which can generate superoxide anions (Prema & Gopinathan, 1974). Therefore the involvement of superoxide anions in yet another class of reaction, namely sulfoxidation, also appears to be certain.

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