Metabolism of xenobiotic compounds by enzymes in cell extracts of the fungus *Cunninghamella elegans*

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Cell extracts of the filamentous fungus *Cunninghamella elegans* contain epoxide hydrolase (EC 3.3.2.3), glutathione *S*-transferase (EC 2.5.1.18) and UDP-glucuronosyltransferase (EC 2.4.1.17) activities. Epoxide hydrolase activity was determined with *p*-nitrostyrene oxide as substrate and was shown to be associated with the 100000 *g* pellet obtained from disrupted mycelia. Glutathione *S*-transferase activity was demonstrated with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates. The presence of two or more glutathione *S*-transferase activities was indicated by different activity ratios for the two substrates in different extracts, and by distinct thermal denaturation curves. UDP-glucuronosyltransferase activity with 3-hydroxybenzo[*a*]pyrene as substrate was found only with the non-sedimentable fraction prepared from ruptured mycelia.

Xenobiotic chemicals may be considered to be non-nutritive, generally lipophilic, compounds that interact with organisms to display toxic and sometimes carcinogenic effects. In this context, many of the aromatic hydrocarbons that are found in the environment are considered to be xenobiotic compounds.

Mammalian enzymes involved in the metabolism of aromatic compounds have been subjected to intensive investigation. It is now known that the oxidation and subsequent elimination of aromatic hydrocarbons from animals occurs through reactions mediated by cytochrome *P*-450 (Sato & Omura, 1978), epoxide hydrolase (Oesch, 1979; Lu & Miwa, 1980), glutathione *S*-transferase (Jakoby & Keen, 1977) and UDP-glucuronosyltransferase (Nemoto & Gelboin, 1976; Dutton & Burchell, 1978; Bock & Lilienblum, 1979; Bridges, 1980) (Scheme 1).

In contrast, few published data are available pertaining to the presence or function of these enzymes in eukaryotic micro-organisms. Cytochrome *P*-450 has been detected in several fungi (Ferris *et al.*, 1976; Cerniglia & Gibson, 1978; Karenlampi *et al.*, 1980; Wiseman, 1980; Cerniglia, 1981*b*). In addition, epoxide hydrolase has been reported to be present in a protozoan (Yawetz & Agosin, 1979), and glutathione *S*-transferase has been detected in some algae, fungi and protozoa (Lau *et al.*, 1980; Yawetz & Agosin, 1980). These studies have relevance in determining the potential of micro-organisms in activating or detoxifying xeno-

biotic compounds, and Rosazza & Smith (1979) have pointed out that fungi may also serve as useful models for the study of drug metabolism.

Studies undertaken in our laboratory have demonstrated remarkable similarities between the oxidation of aromatic hydrocarbons by mammals and the filamentous fungus *Cunninghamella elegans*. For example, *C. elegans* oxidizes naphthalene (Cerniglia & Gibson, 1977), benz[*a*]anthracene (Cerniglia *et al.*, 1980; Dodge, 1981), biphenyl (Dodge *et al.*,

Scheme 1. Reactions utilized by mammals for the metabolism of aromatic hydrocarbons

1979), benzo[*a*]pyrene (Cerniglia & Gibson, 1979) and 3-methylcholanthrene (Cerniglia *et al.*, 1982) to complex mixtures of hydroxylated and conjugated products that are remarkably similar to those produced by mammalian liver. A cytochrome *P*-450 mono-oxygenase has already been implicated in the initial oxidation of naphthalene by *C. elegans* (Cerniglia & Gibson, 1978), and the formation of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene suggests that this organism also contains the enzyme epoxide hydrolase. Enzymic hydrolysis of water-soluble conjugates produced from benzo[*a*]pyrene by *C. elegans* demonstrated that UDP-glucuronosyltransferase and sulphotransferase activities may also be present (Cerniglia & Gibson, 1979). In addition, glucuronic acid and sulphate conjugates of naphthalene and biphenyl have been isolated from culture filtrates of *C. elegans* (Cerniglia, 1981*a*). The formation of glucuronide and sulphate esters from phenols may be significant steps in the fungal metabolism of aromatic hydrocarbons.

The present paper describes the localization and properties of glutathione *S*-transferase, epoxide hydrolase and UDP-glucuronosyltransferase in *C. elegans*. To our knowledge, this represents the first report of a UDP-glucuronosyltransferase activity that is entirely soluble and the first demonstration of its presence in a micro-organism.

Materials and methods

Organism and growth conditions

The isolation of *Cunninghamella elegans* has been described (Cerniglia & Perry, 1973). The organism was subcultured as described previously (Cerniglia & Gibson, 1977). Experimental cultures were grown by blending the mycelial growth from an agar plate in 50 ml of sterile saline (0.9% NaCl). A 10 ml portion was used to inoculate 100 ml of Sabouraud dextrose broth (Difco Laboratories). The seed culture was grown for 24 h at 30°C on a rotary shaker operating at 180 rev./min and then used to inoculate a 1-litre flask containing 300 ml of Sabouraud dextrose broth. In experiments designed to determine glutathione *S*-transferase and UDP-glucuronosyltransferase activities, the secondary culture was grown for 24–48 h under the same conditions as for the seed culture. Epoxide hydrolase activity was determined after secondary cultures were grown for 67 h. In the experiments described, extracts for the determination of UDP-glucuronosyltransferase activity were obtained from cells that were treated with 25 mg of benz[*a*]anthracene in 250 µl of *NN*-dimethylformamide for 9 h before being harvested. Subsequent work indicated that UDP-glucuronosyltransferase activity is constitutively produced in fungi grown in Sabouraud dextrose broth alone. In fact, none of the enzyme activities

examined appears to be induced by xenobiotic compounds. Naphthalene or 1-chloro-2,4-dinitrobenzene failed to induce higher glutathione *S*-transferase activities, and treatment with benzo[*a*]pyrene did not increase epoxide hydrolase activity.

Preparation of subcellular fractions

Mycelia were collected by filtration and broken into pea-sized fragments (10 g portions) before being blended in liquid N₂. After a blending for 3.0 min (UDP-glucuronosyltransferase and epoxide hydrolase) or 5.0 min (glutathione *S*-transferase), 50 ml of cold buffer was added to each preparation, and blending was continued for a further 2.0 min. Buffers used were: buffer A (glutathione *S*-transferase), 0.1 M-potassium phosphate buffer, pH 7.5, containing EDTA (1 mM), dithiothreitol (1 mM) and glycerol (20%, v/v); buffer B (UDP-glucuronosyltransferase), 0.1 M-potassium phosphate buffer, pH 7.5, containing dithiothreitol (0.5 mM) and reduced glutathione (0.5 mM); buffer C (epoxide hydrolase), 25 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid/KOH buffer, pH 7.2, containing EDTA (1.5 mM), dithiothreitol (1 mM) and glycerol (10%, v/v).

Broken mycelia were centrifuged at 12000 *g* for 15 min to remove cell debris. The supernatant solution was filtered through glass-wool to remove floating lipid and then centrifuged at 100000 *g* for 1 h. The microsomal pellet was washed with the appropriate buffer, and enzyme activity in the pellet and supernatant fractions was determined as described below.

Enzyme assays

Glutathione *S*-transferase activity was assayed as described previously (Habig *et al.*, 1974). The 1 ml reaction mixtures typically contained 300–600 µg of protein. In all experiments non-enzymic rates were determined and subtracted from the rates obtained with cell extracts.

A high-pressure liquid-chromatographic assay (Westkaemper & Hanzlik, 1980) was modified to determine epoxide hydrolase activity in *C. elegans*. A DuPont Zorbax ODS column was utilized, and compounds were eluted isocratically with a solvent composed of 45% (v/v) methanol in water at a flow rate of 1.3 ml/min. Product formation was determined quantitatively by use of a calibration curve prepared with synthetic *p*-nitrostyrenediol.

UDP-glucuronosyltransferase activity, with 3-hydroxybenzo[*a*]pyrene as the substrate, was determined by a sensitive fluorometric assay (Singh & Wiebel, 1979). Product formation was determined quantitatively by use of a standard curve constructed with synthetic 3-hydroxybenzo[*a*]pyrene. The fluorescence coefficient of the phenol and the

glucuronide product are equivalent at the respective wavelength maxima. Initial assays were conducted as described at a reaction temperature of 35°C.

Thermal denaturation studies

Thermal denaturation experiments were performed in a water bath equilibrated at 48°C. Samples (5 ml) in test tubes were placed in the bath, and removed after 1, 2, 4, 5, 8, 10 and 15 min and placed on ice. After centrifugation at 12000g, 100 μ l portions of the supernatant solution were used to determine glutathione *S*-transferase activities with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates.

Analytical methods

Spectrophotometric assays and u.v.-absorption spectra were obtained with a Beckman 25 spectrophotometer. For thermal denaturation studies on glutathione *S*-transferase, reproducible low activities were obtained with an Aminco DW-2 spectrophotometer.

Epoxide hydrolase assays were performed with a Waters model 440 high-pressure liquid chromatograph fitted with a DuPont Zorbax ODS column.

UDP-glucuronosyltransferase assays were performed with an Aminco-Bowman SPF-500 spectrofluorometer. In localization experiments an SPX Fluorolog spectrofluorometer was used.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chemicals

The following chemicals were purchased from Eastman Chemicals, Rochester, NY, U.S.A.: 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride, 2-bromo-4'-nitroacetophenone and 1,2-dichloro-4-nitrobenzene. Ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, reduced glutathione and UDP-glucuronic acid (ammonium salt) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 1,2-Epoxy-3,3,3-trichloropropane, *p*-nitrobenzyl alcohol, *trans*-4-phenylbut-3-en-2-one and 4-nitropyridine *N*-oxide were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 3-Hydroxybenzo[*a*]pyrene was provided by Dr. David Longfellow of the National Cancer Institute Carcinogenesis Research Program. *p*-Nitrostyrene oxide and *p*-nitrostyrenediol were synthesized as described by Westkaemper & Hanzlik (1980). All other materials were of the highest available purity.

Results

Glutathione S-transferase

Cell extracts, prepared by blending *C. elegans* mycelia in liquid N₂, were found to contain

glutathione *S*-transferase activity. This activity was located almost exclusively in the cytosol fraction. However, a small amount of enzyme (1–2%) was always detected in the membrane fraction that was obtained by centrifugation at 100000g. This activity was not removed by repeated washing, and it is not known at this time whether the microsomal fraction contains a distinct glutathione *S*-transferase or whether the activity is due to soluble protein that is trapped in membrane vesicles.

Glutathione *S*-transferase activity was observed with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates. Specific activities in different preparations ranged from 7 to 28 nmol/min per mg of protein and from 20 to 100 nmol/min per mg of protein for 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl alcohol respectively. No activity was observed with 1,2-dichloro-4-nitrobenzene, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, 4-nitropyridine *N*-oxide, *trans*-4-phenylbut-3-en-2-one and ethacrynic acid. The rate of the enzymic reaction with 1-chloro-2,4-dinitrobenzene was linear with protein concentration up to at least 1.48 mg of protein/ml of reaction mixture. The reaction was also linear with respect to time for at least 5 min. Dithiothreitol and glycerol were both necessary for the maintenance of enzyme activity. When cell extracts were dialysed against buffer A in the absence of these reagents, enzyme activity was lost and could not be restored by the addition of dithiothreitol or glycerol either singly or in combination. When 1-chloro-2,4-dinitrobenzene was used as a substrate the pH optimum of the enzyme-catalysed reaction was 7.6. An apparent K_m of 1.4 mM for reduced glutathione was obtained by Lineweaver-Burk analysis of initial-velocity data when 1-chloro-2,4-dinitrobenzene (1.0 mM) was used as the second substrate.

Preliminary observations suggested that there may be more than one glutathione *S*-transferase in the soluble fraction. Thus storage of cell extracts at 4°C resulted in a steady decline in activity with 1-chloro-2,4-dinitrobenzene as the substrate. After the loss of approx. 60% of the activity the rate of enzyme inactivation decreased significantly. Also, the dialysis of freshly prepared extracts against buffer A for 24 h resulted in a fractional loss of activity, whereas older extracts maintained activity on overnight dialysis. These observations are shown in Table 1 with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates. Over a 96 h period at 4°C the activity with 1-chloro-2,4-dinitrobenzene decreased by approx. 40%. In contrast, only 2% of the activity with *p*-nitrobenzyl chloride was lost during the same time period. When freshly prepared cell extracts were heated at 48°C for different time periods, the activity with 1-chloro-2,4-dinitrobenzene rapidly declined. After 6 min more than 80% of the enzyme activity was

Table 1. *Glutathione S-transferase activity in freshly prepared and aged cell extracts from Cunninghamella elegans*
For full experimental details see the Materials and methods section. Values for two separate enzyme preparations are given.

Cell extract	Substrate	Specific activity (nmol of product formed/min per mg of protein)	
		1-Chloro-2,4-dinitrobenzene	<i>p</i> -Nitrobenzyl chloride
Fresh		12.4; 10.8	22.9; 51.1
Aged		7.1; 6.9	22.8; 49.9
	Decline (%)	42.7; 36.1	0.4; 2.3

Fig. 1. Effect of heat treatment on glutathione *S*-transferase activity in freshly prepared *Cunninghamella elegans* cell extracts (a) and in cell extracts that had been stored at 4°C for 72 h (b).

For full experimental details see the Materials and methods section. ●, Activity with *p*-nitrobenzyl chloride; ○, activity with 1-chloro-2,4-dinitrobenzene.

lost, compared with a 10% loss of activity with *p*-nitrobenzyl chloride (Fig. 1a). When the same experiment was conducted with an extract that had been stored at 4°C for 3 days, no discernable differences were observed in the loss of activity with both substrates (Fig. 1b).

Epoxide hydrolase

Fractions prepared by differential centrifugation were tested for epoxide hydrolase activity with

Fig. 2. High-pressure liquid-chromatographic resolution of a methanol extract obtained from a reaction mixture containing *p*-nitrostyrene oxide and *Cunninghamella elegans* microsomal fraction.

For full experimental details, including assays of epoxide hydrolase activity, see the Materials and methods section. Key to peaks: I, unretained material; II, *p*-nitrostyrene diol (6.4 min); III, *p*-nitrobenzyl alcohol (internal standard, 9.7 min); IV, *p*-nitrostyrene oxide (24 min).

p-nitrostyrene oxide as the substrate. When this substrate was incubated with membrane preparations (100 000 *g* pellet) a polar product was detected by high-pressure liquid chromatography (Fig. 2). The reaction product had a retention time (6.4 min) and absorption spectrum identical with those given by synthetic *p*-nitrostyrenediol. The specific activities of three different microsomal preparations were in the range 1.2–1.5 nmol/min per mg of protein. These activities can be considered minimum values, since a 15 min reaction was required for reproducible quantification of product formation. When fungal microsomal preparations were incubated with

1.0mM-1,2-epoxy-3,3,3-trichloropropane for 3 min before the addition of *p*-nitrostyrene oxide, product formation was completely abolished.

UDP-glucuronosyltransferase

Initial experiments revealed that UDP-glucuronosyltransferase activity in cell extracts were present only in the soluble fraction. The product formed from UDP-glucuronic acid and 3-hydroxybenzo[*a*]pyrene gave a fluorescence spectrum that was identical with that given by 3-benzo[*a*]pyrenyl-glucopyranosiduronic acid. Since UDP-glucuronosyltransferase is a microsomal enzyme in higher organisms, a mild breakage procedure was devised to determine if enzyme activity could be detected in the microsomal fractions prepared from *C. elegans*. Freshly harvested mycelia (18.4g wet wt.) were suspended in 180ml of buffer B and gently disrupted by nine strokes with a motor-driven Teflon pestle in a glass homogenizer. This procedure released only 164mg of protein, which was separated into nuclear, mitochondrial, microsomal and soluble fractions by a series of centrifugation steps. Activity in the particulate fractions was not detectable to a level of 1 pmol/min per mg of protein. The specific activity of the soluble fraction was shown to be 125 pmol/min per mg of protein.

Discussion

The observations reported in the present paper show that the filamentous fungus *Cunninghamella elegans* contains enzyme activities involved in the biotransformation of aromatic compounds that are similar to the enzymes found in mammals (Jakoby, 1980). For example, the glutathione *S*-transferase activities share several properties with the same enzymes found in mammalian liver. These include soluble localization, enzyme multiplicity and stabilization with glycerol and a thiol reagent. The fungal enzymes were active with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride, both of which are excellent substrates for liver glutathione *S*-transferases.

The physiological function of the fungal glutathione *S*-transferases is not known. Since 1,2-epoxy-3-(*p*-nitrophenoxy)propane will not serve as a substrate, the enzymes may not play a major role in the conjugation of glutathione with arene oxides (Bend *et al.*, 1976). When *C. elegans* is grown in the presence of 1-chloro-2,4-dinitrobenzene, a compound with an absorption spectrum similar to that of *S*-(2,4-dinitrophenyl)glutathione accumulates in the culture medium. In addition, the detection of a soluble NADPH-dependent glutathione reductase activity in *C. elegans* suggests that the fungus maintains an intracellular pool of reduced glutathione. These findings (L. P. Wackett & D. T. Gibson, unpublished work) show that the organism

can detoxify halogenated aromatic compounds, although it is uncertain whether or not this is the endogenous function of this group of enzymes.

Epoxide hydrolase activity in *C. elegans* is membrane-bound and strongly inhibited by 1,2-epoxy-3,3,3-trichloropropane. These are properties exhibited by liver microsomal epoxide hydrolase (Lu & Miwa, 1980). Soluble epoxide hydrolase activity with *p*-nitrostyrene oxide as the substrate was not detected in *C. elegans*. However, a soluble epoxide hydrolase that is active with *trans*- β -methylstyrene oxide, but not styrene oxide, has been reported (Ota & Hammock, 1980). A soluble epoxide hydrolase with different substrate specificity may also be present in *C. elegans*.

Most reports on mammalian UDP-glucuronosyltransferases have indicated that this enzyme is membrane-bound. However, cytosolic fraction from human liver has been shown to be active in the glucuronidation of the 16 α -hydroxy group in oestradiol (Rao *et al.*, 1970). The authors did, however, suggest that *in vivo* the enzyme may be associated with the endoplasmic reticulum. The results obtained in the present study also indicate the presence of a soluble UDP-glucuronosyltransferase activity. The method of cell breakage was extremely mild and yielded microsomal preparations that contained epoxide hydrolase activity. We have also noticed that the supernatant solution obtained from centrifugation at 12000g contains less activity than did the same supernatant fluid after centrifugation at 100000g. This diminished activity may be due to sequestration of substrate by membranes or the presence of a microsomal β -glucuronidase activity.

In summary, *C. elegans* contains enzyme activities involved in the biotransformation of xenobiotic compounds that are similar to those found in mammals. Further investigations will be required to determine if these enzymes participate in essential metabolic reactions or are present to protect the fungus from the effects of environmental chemicals.

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