The proximal pathway of metabolism of the chlorinated signal molecule differentiation-inducing factor-1 (DIF-1) in the cellular slime mould *Dictyostelium*

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Stalk cell differentiation during development of the slime mould *Dictyostelium* is induced by a chlorinated alkyl phenone called differentiation-inducing factor-1 (DIF-1). Inactivation of DIF-1 is likely to be a key element in the DIF-1 signalling system, and we have shown previously that this is accomplished by a dedicated metabolic pathway involving up to 12 unidentified metabolites. We report here the structure of the first four metabolites produced from DIF-1, as deduced by m.s., n.m.r. and chemical synthesis. The structures of these compounds show that the first step in metabolism is a dechlorination of the phenolic ring, producing DIF metabolite 1 (DM1). DM1 is identical with the previously known minor DIF activity, DIF-3. DIF-3 is then metabolized by three successive oxidations of its aliphatic side chain: a hydroxylation at ω -2 to produce DM2, oxidation of the hydroxy group to a ketone group to produce DM3 and a further hydroxylation at ω -1 to produce DM4, a hydroxyketone of DIF-3. We have investigated the enzymology of DIF-1 metabolism. It is already known that the first step, to produce DIF-3, is catalysed by a novel dechlorinase. The enzyme activity

responsible for the first side-chain oxidation (DIF-3 hydroxylase) was detected by incubating [^3H]DIF-3 with cell-free extracts and resolving the reaction products by t.l.c. DIF-3 hydroxylase has many of the properties of a cytochrome *P*-450. It is membrane-bound and uses NADPH as co-substrate. It is also inhibited by CO, the classic cytochrome *P*-450 inhibitor, and by several other cytochrome *P*-450 inhibitors, as well as by diphenyliodonium chloride, an inhibitor of cytochrome *P*-450 reductase. DIF-3 hydroxylase is highly specific for DIF-3: other closely related compounds do not compete for the activity at 100-fold molar excess, with the exception of the DIF-3 analogue lacking the chlorine atom. The K_m for DIF-3 of 47 nM is consistent with this enzyme being responsible for DIF-3 metabolism *in vivo*. The two further oxidations necessary to produce DM4 are also performed *in vitro* by similar enzyme activities. One of the inhibitors of DIF-3 hydroxylase, ancymidol (IC_{50} 67 nM) is likely to be particularly suitable for probing the function of DIF metabolism during development.

INTRODUCTION

The fate of developing amoebae of the cellular slime mould *Dictyostelium*, whether they are to differentiate into stalk cells or into spores, appears to be governed by the differentiation-factor-1 (DIF-1) signalling system. DIF-1 was discovered as a dialysable factor, released during development, which could induce isolated amoebae to differentiate into stalk cells [1]. On this basis, DIF-1 was purified [2] and identified as the chlorinated alkyl phenone 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-hexan-1-one ([3]; for the structure of DIF-1 and its metabolites, see Scheme 2 below).

The biological context within which DIF-1 operates is fairly well understood. Prestalk cells first arise during development after the *Dictyostelium* amoebae have aggregated into mounds of cells (by chemotaxis towards cyclic AMP), and they arise at scattered positions within the mound [4,5]. They then sort out to form a coherent prestalk tissue, though some prestalk cells remain scattered within the prespore region [6]. Further differen-

tiation is held in abeyance through the migrating slug stage, until culmination is induced by favourable environmental factors [7]. At this point, prestalk cells vacuolate sequentially and lay down a cellulose stalk tube as they enter the top of the growing stalk. The ratio of prestalk to prespore cells and of stalk to spore cells is carefully regulated across a wide size range of aggregate and can be restored to normal after surgical removal of either precursor-cell type [8].

DIF-1 has clear-cut effects on cells. At nanomolar concentrations, it induces morphological stalk-cell differentiation and inhibits spore-cell differentiation [9]; similarly, expression of prestalk mRNAs is induced and that of prespore mRNAs inhibited [4,10,11]. Other effects of DIF-1, such as causing shape changes in cells early in development [12] and compaction of the aggregate [13], are not obviously related to the later differentiation events.

An understanding of the role of DIF-1 in development will require a molecular understanding of the DIF-1 signalling system. Though great progress in identification of the key genes involved

Abbreviations used: DIF, differentiation-inducing factor; DM, DIF metabolite; DTT, dithiothreitol; IC_{50} , inhibitor concentration producing 50% inhibition; des(Cl)-DIF-3, deschloro-DIF-3; 45K, 45000 rev./min (etc.).

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in DIF signalling is promised by the newly developed technique of insertional mutagenesis [14], this approach will be much more fruitful if there is also a suitable biochemical framework. Like hormonal and neural signals, the DIF-1 signal must be synthesized, detected by receptors, have its effects transmitted into the cell and, finally, the signal must be inactivated. Little is yet known of these processes. The pathway of DIF-1 synthesis is unknown, although a binding activity with the hallmarks of a receptor has been detected [15]. DIF-1 inactivation is accomplished by a metabolic pathway which converts it sequentially into four more-polar metabolites (DMs 1–4) and then more slowly into another four major metabolites (DMs 5–8), with a number of minor metabolites also produced [16]. Inactivation, at least as far as inducing stalk-cell differentiation is concerned, is largely accomplished by the first two steps of this pathway.

DIF-1 metabolism is also interesting for a quite different reason. Chlorinated aromatic compounds of human origin are often long-lived in the environment because they are resistant to biodegradation, efficient metabolizing enzymes presumably not having evolved [17]. Since DIF-1 is an endogenous compound and appears to have a dedicated metabolizing pathway, the study of DIF-1 metabolism presents a unique opportunity to discover how such chlorinated compounds might be efficiently metabolized.

The first step in DIF-1 metabolism is a dechlorination, catalysed by DIF-1 dechlorinase [18], to produce DM1, otherwise known as DIF-3 (the present study; [19]). As shown here, the next steps are a succession of oxidations of the aliphatic side chain of DIF-3, with the first one at least catalysed by an enzyme with the properties of a cytochrome *P*-450.

MATERIALS AND METHODS

General

Hexanophenone, hexan-3-ol, pentan-3-ol, pentan-3-one, hexan-3-one and hexanal were from Aldrich, and ketoconazole was from Janssen. NADPH, NADH, NADP⁺ and NAD⁺ were from Boehringer or Sigma. Other chemicals were of AnalaR grade from Sigma or BDH. Ancymidol was specially pure for cell culture (Sigma); [³H]methoxy-DIF-1 (79 Ci/mmol) was a custom synthesis by Amersham. [³H]Methoxy-DIF-3 was enzymically synthesized by monodechlorination of [³H]DIF-1 [18]. Briefly, DIF-1 dechlorinase activity was induced by disaggregating *Dictyostelium* cells at the mound stage of development, and the cytosolic fraction incubated with 5 mM GSH and 400 nM [³H]methoxy-DIF-1. Substrate and products were solvent extracted [20], concentrated and purified by h.p.l.c. on a Highchrom S50DS2 column with a 40–70% acetonitrile/water

linear gradient (containing 2% acetic acid). Excess solvent was evaporated under reduced pressure.

Silica-gel-precoated glass sheets (Merck 60F₂₅₄ 0.25 mm) were used for t.l.c. to monitor the chemical syntheses, and the compounds were detected by dipping the plates into aq. (NH₄)₂SO₄ and then charring or by iodine staining, or by quenching of u.v.fluorescence.

High-resolution n.m.r. spectra were recorded on a Bruker AM 400 high-resolution spectrometer with tetramethylsilane as the internal standard. *J* values are given in Hz.

M.s. was carried out on a Finnigan 4500 quadrupole instrument in the electron-impact mode. Samples were desorbed from a rhenium filament at 10 mA/s. Metabolite DM1 (DIF-3) was also analysed by g.c.–electron-impact m.s., in which samples were chromatographed on a DB5 capillary column and heated at 20 °C/min from 100 to 320 °C.

Purification of DIF-1 metabolites

About 2.2×10^{11} cells of strain Ax2 were allowed to develop on non-nutrient agar to the tipped-mound stage. They were then harvested, resuspended in NS (20 mM KCl/20 mM NaCl/1 mM CaCl₂) at $(1-2) \times 10^7$ cells/ml and shaken in flasks with 0.25–0.4 mM DIF-1 plus 0.75 mCi/l of [³H]DIF-1 for 90 min. The cells were then spun down and resuspended with more DIF-1, so that a total of 1.3 mmol of DIF-1 was used. DIF-1 and its metabolites were recovered from the supernatants by binding to and elution from XAD2 resin [2] and purified through at least two h.p.l.c. steps using ODS columns and methanol/water or acetonitrile/water gradients (both containing 2% acetic acid), essentially as described previously [16,21].

Chemical syntheses

DIF-1 and certain related compounds were synthesized as described in [22]; carboxy-DIF-3 [1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)-6-carboxyhexan-1-one], was given by S. Tudhope.

DM3 was synthesized as follows (Scheme 1).

4-Nitrohexanitrile. Sodium (23 g) in methanol (500 ml) was added dropwise to a solution of 1-nitropropane (89 g) and acrylonitrile (53 g) in methanol (250 ml) and stirred for 12 h. The mixture was acidified with acetic acid, the methanol evaporated off and the remaining liquid partitioned between dichloromethane and water and the dichloromethane layer evaporated. Chromatography on silica gel (300 g) using ethyl acetate/toluene (1:9, v/v) as eluant gave 4-nitrohexanitrile (50 g; 35%). I.r. (neat): $\tilde{\nu}$ = 1560 (NO₂), 2240 (CN) cm⁻¹.

Scheme 1 Synthesis of DM3

1-(2,6-Dihydroxy-4-methoxyphenyl)-4-nitro-hexan-1-one (compound 1, Scheme 1). Dry 5-methoxyresorcinol (5 g; 36 mmol) and 4-nitrohexanitrile (10 g; 70 mmol), dissolved in sodium-dried diethyl ether (25 ml), were placed in a round-bottomed flask containing dry ZnCl_2 (10 g; 74 mmol) and fitted with a CaCl_2 guard tube and gas-inlet tube. The mixture was kept at 0 °C using an ice/salt bath, stirred and gassed with dry HCl for 2 h. The flask was stoppered and the mixture stirred for 24 h at 5 °C. Then the mixture was poured on to ice/water and boiled for 1 h, cooled and extracted with diethyl ether (3 × 100 ml). The organic fractions were combined, washed once with water, dried (over anhydrous MgSO_4) and evaporated. Chromatography on silica gel (200 g) using ethyl acetate/toluene (1:19, v/v) as eluant gave compound 1 (0.60 g; 6%) and *(4,6-dihydroxy-2-methoxyphenyl)-4-nitro-hexan-1-one* (3.74 g; 37%).

1-(2,6-Dihydroxy-4-methoxyphenyl)hexan-1,4-dione (compound 2). TiCl_3 (6.0 g; 39 mmol) in water (16 ml) was added under N_2 to a solution of the nitro derivative, compound 1 (1.00 g; 3.5 mmol) in tetrahydrofuran (20 ml) and stirred for 12 h. Chromatography on silica gel (100 g) using ethyl acetate/toluene (1:9, v/v) as eluant gave compound 2 (0.32 g; 36%) δ_{H} (CDCl_3) 1.12 (3H, t, J 7.4), 2.60 (2H, q, J 7.4), 2.90 (2H, t, J 6.4), 3.33 (2H, t, J 6.4), 3.77 (3H, s, OCH_3), 5.91 (2H, s, 3-H 5-H), 10.35 (2H, s, 2 OH); fast-atom-bombardment m.s.: M^{+} 253.

1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1,4-dione (compound 3, DM3). Aliquots of freshly prepared chlorine water were added to a solution of the dione (compound 2) (0.10 g; 0.4 mmol) in chloroform. The reaction was monitored by t.l.c. (dichloromethane/diethyl ether, 19:1, v/v) deactivated by lightly spraying the plates with water. The addition of aliquots of chlorine water was stopped when no more of the dione was detectable by t.l.c. The mixture was partitioned between chloroform and water, the chloroform layer being washed with water and evaporated to a low volume of chloroform. Chromatography on silica gel (100 g) using chloroform/methanol/ammonia (95:5:1, by vol.) as eluant gave compound 3 (0.08 g; 70%): m.p. 182–184 °C [from acetone/light petroleum (b.p. 40–60 °C)]; δ_{H} (400 MHz; $[\text{^2H}]$ chloroform) 1.11 (3H, t, J 7.4), 2.57 (2H, q, J 7.4), 2.80 (2H, t, J 6.0), 3.42 (2H, t, J 6.0), 3.70 (3H, s, OCH_3), 6.11 (2H, s, OH), 7.28 (1H, s, 5-H) and *(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1,4-dione* (0.02 g; 16%). δ_{H} (400 MHz; $[\text{^2H}]$ chloroform) 1.11 (3H, t, J 7.3), 2.55 (2H, q, J 7.3), 2.83 (2H, t, J 6.0), 3.43 (2H, t, J 6.0), 3.96 (3H, s, OCH_3), 10.33 (2H, s, 2OH).

Growth and development of cells

Strain V12M2 was grown in association with *Klebsiella aerogenes* on SM agar [23]. Residual bacteria were removed by several washes with KK2 (20 mM potassium phosphate, pH 6.2) and a final wash with 10% NS (100% NS is 20 mM KCl/20 mM NaCl/1 mM CaCl_2). Cells were plated on 1.7% agar containing 10% NS or KK2+2 mM MgSO_4 at a density of $(4\text{--}8) \times 10^6$ cells/cm² and developed at 13 °C overnight and at 22 °C for a few more hours until the desired stage was reached.

Cell lysis and fractionation

Cells, harvested at the appropriate developmental time were pelleted, frozen at –70 °C and assayed within a week.

(a) Small-scale lysis (< 10⁸ cells)

Lysis buffer [50 mM sodium phosphate/100 mM NaCl/10%

(v/v) glycerol, pH 7.8] was added to the frozen pellet and vigorously pipetted up and down to break big lumps; this procedure yielded lysates of activity comparable with filter lysis. Cell debris was removed by a short centrifugation (2 min at 10000 g at 4 °C) and the supernatant assayed directly.

(b) Large scale lysis

Cells collected from several plates were pelleted, frozen in solid CO_2 , taken up in lysis buffer at a density of $(1\text{--}3) \times 10^8$ cells/ml and subjected to another cycle of freeze–thawing. The cell suspension was filtered through Millipore AP10 prefilters and then forced through a 3 mm-pore-size Nuclepore membrane. When DIF-1 dechlorinase was to be assayed in the same sample, 1 mM DTT was included in the lysis buffer; DTT had no effect on DIF-3 hydroxylase activity.

In a standard microsomal preparation, the lysate was centrifuged for 5 min at 1300 g to remove nuclei and unbroken cells, at 10000 g (12000 rev./min; Sorvall SS-34 rotor) and at 349000 g for 30 min (95000 rev./min in Beckman TL-100; rotor TLA100.3).

DIF-3 hydroxylase is induced by disaggregation of cell masses in a similar manner to DIF-1 dechlorinase (P. Morandini and R. R. Kay, unpublished work). High-activity lysates were prepared as follows. Cells were taken at mound/tipped-mound stage and shaken for 1 h at 22 °C at a density of 2×10^7 /ml in KK2; microsomal fractions were prepared as described above.

Enzyme assays

Protein concentration was measured by dye binding (Bio-Rad) using lysozyme as standard, membrane fractions having first been solubilized with 0.5 M NaOH. The DIF-1 dechlorinase assay was as described in [18]. DIF-3 hydroxylase activity was assayed by incubating cell extracts with [^3H]DIF-3 (79 Ci/mmol). The reaction was carried out in 1.5 ml Eppendorf tubes for up to 30 min at 23 °C in 100 ml of lysis buffer supplemented with $1 \times$ proteinase inhibitors (1000 $\times$ mix in ethanol: leupeptin, 5 mg/ml; pepstatin A, 2.5 mg/ml; benzamidine, 0.15 g/ml) and containing 10 nM DIF-3 (3.6×10^4 c.p.m.) and 1 mM NADPH, unless otherwise stated. The reaction was terminated by adding 0.5 ml of ethyl acetate/hexane (9:1, v/v) containing 200 mg/ml butylated hydroxytoluene. The organic phase, containing substrate and metabolites, was removed, dried down, redissolved in 40 ml of methanol/chloroform (1:1, v/v) containing 1 mM 2-mercaptoethanol and chromatographed on t.l.c. plates (Whatman LK6D silica) in ethyl acetate/hexane/acetic acid (40:10:1, by vol.). T.l.c. plates were sprayed with En³Hance (du Pont/NEN) and exposed at –70 °C to preflashed X-OMAT film (Kodak). To quantify enzyme activity, labelled bands were scraped off the t.l.c. plates into 0.5 ml of 95% ethanol, scintillation fluid added and the radioactivity determined by scintillation counting; this allowed the proportion of substrate metabolized to be determined.

Reaction was linear with protein concentrations up to 1 mg of microsomal protein/ml, providing that less than 85% of the substrate was consumed. BSA at 2 mg/ml in the assay was later found to improve enzyme activity, especially in microsomal preparations.

For the CO-inhibition experiments, gas mixtures were formed in gas traps by measuring volumes of displaced water and forced into tapering Pasteur pipettes dipped into the samples.

A unit of DIF-3 hydroxylase activity is defined as the amount of enzyme able to metabolize 1 pmol of DIF-3/h to products (DM2, DM3 and DM4 combined).

RESULTS

Structure of the proximal DIF-1 metabolites

[³H]DIF-1 is sequentially converted by *Dictyostelium* cells, at the appropriate stage of development, into four more polar metabolites, DMs 1–4, and eventually to at least eight further metabolites [16]. Metabolites DMs 1–4 were recovered from the medium of cells incubated with 250–400 nM [³H]DIF-1 by binding to the non-polar resin XAD-2 and purified through at least two reverse-phase h.p.l.c. steps. We obtained about 0.1 mmol of pure DM4, sufficient for both m.s. and n.m.r., smaller amounts of DM2 and DM3, sufficient for m.s. but not n.m.r., and even smaller amounts of DM1, sufficient only for use as a radiochemical tracer. However, when DIF-1 was incubated with a *Dictyostelium* lysate supplemented with GSH [18], larger amounts of a metabolite were produced whose structure is determined below and which was indistinguishable from DM1 by h.p.l.c. and t.l.c. (results not shown); prolonged incubation produced a second metabolite not normally detectable in living cells. Co-elution of this second metabolite on h.p.l.c. and t.l.c. with authentic material (synthesized as in [22]) showed that it is doubly dechlorinated DIF-1 [i.e. deschloro-DIF-3 [des(Cl)-DIF-3]; Scheme 2; results not shown].

The structures of the other metabolites (Scheme 2) were established as follows.

DM1

Metabolite DM1 co-eluted on both t.l.c., h.p.l.c. and g.c. with the previously identified stalk-cell-inducing compound, DIF-3 [1-(3-chloro-4-methoxy-2,6-dihydroxyphenyl)-hexan-1-one] [19]. The electron-impact mass spectrum of DM1 was identical with that of DIF-3 with a series of 3:1 doublet ions (consistent with the presence of a single chlorine) at m/z 272/274 (M^+), 254/256 ($-H_2O$), 229/231 ($-C_3H_7$), 216/218 ($-C_4H_8$) and 201/203 (base peak, $-C_5H_{11}$) (Scheme 2, cf. [19]). These data show that DM1 is DIF-3 [des(Cl)-DIF-1], confirming our preliminary conclusion [18]. Dechlorination is therefore the first step in the metabolism of DIF-1.

DM2 and DM3

Mass spectra were generated from DM2 and DM3 (Figure 1); each contained a 3:1 doublet at m/z 201/203 as the base peak, indicating that each metabolite contained the 3-chloro-4-methoxy-2,6-dihydroxyphenylcarbonyl (DIF-3) nucleus, and that metabolism had occurred on the side chain. The mass spectrum of DM2 shows that DM2 arises through a single hydroxylation of DIF-3, with the doublet ion at m/z 288/290 (M^+) and loss of water (m/z 270/272). The spectrum is consistent with hydroxylation at ω -2 with α -cleavage leading to doublets at m/z 259/261 ($-C_2H_5$) and 229/231 ($C_{10} \cdot Cl \cdot O_4 \cdot H_8^+$); the latter ion, which is also present in DM3, is stabilized through the cyclization with the ring hydroxy group to form a six-membered protonated ion ($-CO-CH_2-CH_2-OH^+-aryl$). The ion pair at m/z 227/229 may arise through dehydration (to m/z 270/272), double-bond migration to generate the enone and cyclization to form the analogous cyclic dehydro ion ($-CO-CH=CH-OH^+-aryl$). The intensity ratio of m/z 227/229 varied during acquisition of the spectrum; this appears to be associated with the varying degree of thermally induced dehydration, although there may also be some contribution to m/z 229/231 from small amounts of DM3 arising through partial aerial oxidation. The mass spectrum of DM3 shows that oxidation of the hydroxy group in DM2 has occurred to form the corresponding ketone (M^+ : m/z 286/288).

Scheme 2 Structures of DIF-1 and its proximal metabolites

The structures of DIF-1 and DIF-3 were determined previously, principally by m.s. and chemical synthesis [3,22]; determination of the structures of the other compounds is reported in the present paper. Metabolism inactivates DIF-1: in the stalk-cell-differentiation bioassay, DIF-3 has 4% of its bioactivity and the other metabolites less than 2% [15,16]. Des(Cl)-DIF-3 can be produced from DIF-1 by cell lysates, but is not normally detectable in intact cells.

Apart from the base peak at m/z 201/203, the only other intense doublet in the spectrum is at m/z 229/231 ($C_{10} \cdot Cl \cdot O_4 \cdot H_{10}^+$) which occurs through cleavage α to a carbonyl at ω -2 and cyclization with the ring hydroxy group (cf. DM2). These data are consistent with oxidation occurring at ω -2 to give the hexan-1-one-4-ol (DM2) and the hexan-1,4-dione (DM3) metabolites respectively. Confirmation of the structure of DM3 was obtained by synthesis. 1-(3-Chloro-4-methoxy-2,6-dihydroxyphenyl)-hexan-1,4-dione (4-oxo-DIF-3) was prepared by the Hoesch reaction of 4-nitrohexanitrile with 5-methoxyresorcinol, reduced with $TiCl_2$ and chlorinated (Scheme 1). The synthetic material was co-eluted on t.l.c. and h.p.l.c. with DM3 and generated a similar mass spectrum with intense doublet ions at m/z 286/284 (M^+), 229/231 and 201/203. As DM3 arises from DM2, the structure of DM2 is confirmed as the hexan-1-one-4-ol derivative of DIF-3. The stereochemistry of DM2 has not been determined.

DM4

DM3 is further metabolized to DM4. The mass spectrum of

Figure 1 Desorption electron-impact mass spectra of DIF-1 metabolites DM1–DM4

DM1 was identified as DIF-3 {des(Cl)-DIF-1; cf. [19]}. The mass spectra of DM2–4 gave the doublet ions at m/z 201/203 as base peak, confirming that each metabolite contained the DIF-3 nucleus. Metabolism has occurred on the alkyl chain to give the hexan-1-one-4-ol (DM2, m/z 288), hexan-1,4-dione (DM3, m/z 286) and hexan-1,4-dione-5-ol (DM4, m/z 302) derivatives.

DM4 showed that it contained the DIF-3 nucleus (m/z 201/203) and that it had a mass 16 units higher than DM3 (M^+ : m/z 302/304), which would correspond to further hydroxylation (i.e. hydroxy-4-oxo-DIF-3; Figure 1). The mass spectrum of DM4 is consistent with further hydroxylation at ω -1: the doublet at m/z 229/231 ($C_{10} \cdot Cl \cdot O_4 \cdot H_{10}^+$) is also present in the hexan-1,4-dione side chain of DM3 and the intense doublet at m/z 257/259 arises through loss of CH_3CHOH through α cleavage. Thus metabolite DM4 is probably the hexan-1,4-dione-5-ol derivative of DIF-3. The possibility that DM4 is a carboxylic acid was discounted by examination of the mass spectrum of ω -carboxy-DIF-3 (a gift from Dr. S. Tudhope, Miles Research, Slough, Berks., U.K.; the acid exhibited a markedly different spectrum from DM4 with ions at m/z 302/304, 266/268, 255/257, 236/238, 229/231, 216/218 and 201/203).

The identity of DM4 was confirmed from the n.m.r. spectrum (Figure 2). DM4 in $[^2H]$ chloroform showed a single aromatic singlet (δ 6.13, 1H) and a single aromatic methoxy singlet (δ 3.92, 3H). The spectrum after exchange with 2H_2O showed a methyl doublet (δ 1.50, 3H) and a low-field methine 1:3:3:1 quartet (δ 4.41, 1H), suggesting the presence of a $-CH(OH)CH_3$ grouping. This was further supported by the observation of an exchangeable

doublet at δ 3.47, which was shown by a decoupling experiment to be coupled to the methine signal; presumably the extremely low concentration of solute was one reason why the exchange rate of this hydroxyl signal was low enough to allow its coupling to the methine proton to remain resolved. The two lines immediately to lowfield of the hydroxy doublet (δ 3.49 and δ 3.50), despite their appearance, do not originate from DM4; their origin remains uncertain, but the higher-field line (which, unlike the lower-field line, is only slightly diminished on 2H_2O exchange) may well be due to the methyl signal of a trace of methanol. The remaining four protons of DM4 form an AA'BB' spin system that gives rise to the two complex multiplets centred at δ 3.52 and δ 2.83. The fact that no detectable inter-proton couplings exist between this AA'BB' spin system and the resonances of the $CH(OH)CH_3$ grouping very strongly suggests that the new carbonyl group of DM4 is located at C_4 . Consistent with this, the multiplet assigned to the C_3 methylene group has the higher-field shift, since it is adjacent to an isolated ketone functionality rather than an aromatic ketone functionality as in the case of the C_2 methylene group, and, furthermore, the C_3 methylene multiplet has the greater diastereotopicity (A–B shift difference), since it is the closer to the chiral centre (C-5).

Figure 2 Proton n.m.r. spectra of DM4 (a) in $[^2\text{H}]$ chloroform alone and (b) in $[^2\text{H}]$ chloroform after shaking with $^2\text{H}_2\text{O}$

The spectra were acquired from about 100 nmol of DM4; only the region of the spectrum containing the signals from the side-chain protons is shown.

Thus the structure of DM4 is confirmed as the hexan-1,4-dione-5-ol derivative of DIF-3; again the stereochemistry has not been determined.

These results therefore show that DIF-1 is successively modified during metabolism by a monodechlorination, followed by a hydroxylation at C-4 (ω -2) of the hexyl side chain, an oxidation of this hydroxy group to a ketone and then a further hydroxylation, this time at C-5 (ω -1).

DIF-1 metabolism in cell-free lysates

The first enzymic activity involved in DIF-1 breakdown is a cytosolic GSH-dependent dechlorinase which converts DIF-1 into DIF-3 in cell extracts [18]. Preliminary experiments showed that subsequent metabolites could be produced from DIF-3 when cellular membranes are present in the lysate and the reaction is supplemented with NADPH [18]. This fact and the chemistry of these reactions suggest that metabolism of DIF-3 is carried out by a cytochrome *P*-450 enzyme. Cytochrome *P*-450s are a group of ubiquitous enzymes which are involved in the metabolism of a wide variety of endogenous and exogenous chemicals [24–26]. They require NADPH as a co-substrate as well as molecular oxygen, and the most common reaction catalysed by *P*-450s is mono-oxygenation [27,28].

DIF-3 metabolism in cell-free lysates

To investigate further the activities responsible for the second and subsequent steps in DIF metabolism, $[^3\text{H}]\text{DIF-3}$ was prepared enzymically from $[^3\text{H}]\text{DIF-1}$ with DIF-1 dechlorinase, as described in the Materials and methods section. Lysates and microsomal fractions, prepared from cells at the tipped-mound stage of development (where, *in vivo*, DIF-1 metabolism is maximal [16]), readily convert $[^3\text{H}]\text{DIF-3}$ into more polar metabolites in the presence of NADPH (Figure 3). Three metabolites are detectable, one of which is only present at low

Figure 3 Time course of $[^3\text{H}]\text{DIF-3}$ metabolism by cell lysates

Microsomal membranes were prepared from cells of strain V12M2 at the tipped-mound stage and aliquots taken from the reaction at the indicated times and the reaction stopped by extraction with ethyl acetate. Substrate and products were separated by t.l.c. and revealed by autoradiography as described in the Materials and methods section.

levels. These metabolites accumulate simultaneously, but their ratios can vary according to enzyme activity and assay conditions such as temperature and cofactor (not shown). Chromatography on silica t.l.c. plates in two different solvent systems (ethyl acetate/hexane/acetic acid, 30:20:1, by vol.; chloroform/methanol/acetic acid/water, 30:7:1:1, by vol.) using the authentic compounds as standards [16], shows that these metabolites correspond to DM2, 3 and 4 (results not shown). The additional, less-polar band appearing above the DIF-3 on t.l.c. is dechlorinated DIF-3, which is present as a contaminant in our $[^3\text{H}]\text{DIF-3}$ preparation. Since DIF-3 is converted into DM2 by the monohydroxylation of the alkyl side chain (Scheme 2), the enzyme carrying out this reaction will be referred to as 'DIF-3 hydroxylase'.

Localization of DIF-3 hydroxylase

When the lysate was fractionated by differential centrifugation (1000 *g* for 5 min; 12000 *g* for 15 min; 78000 *g* for 120 min; and 349 000 *g* for 30 min), the activity was highly enriched in the 78000 *g* pellet [Figure 4, '45K' (45000 rev./min); note the different protein concentrations], though it is necessary to reach 349000 *g* ('95K') to sediment the residual 20% of activity. This result is consistent with a microsomal location for the enzyme. It is also apparent from Figure 4 that at least some, if not all, of the activity converting DM2 into DM3 and DM4 is also in the microsomal fraction.

General and kinetic properties of DIF-3 hydroxylase

The pH optimum of enzyme activity, determined in phosphate buffer, was at about pH 7.8, with activity decreasing rapidly at higher and lower pH (results not shown). Activity in Tricine was lower, but also fell off above pH 7.8.

Assay mixtures containing microsomes and $[^3\text{H}]\text{DIF-3}$ were supplemented with various pyridine cofactors: both NADPH and NADH at 1 mM supported enzyme activity, although NADH was somewhat less efficient (results not shown). An equimolar mixture of both cofactors was as effective as NADPH alone, while the corresponding oxidized cofactors did not sustain substantial activity. The K_m for NADPH is probably below its physiological level (10–100 μM), because decreasing the

Figure 4 DIF-3-metabolizing activities are predominantly in the microsomal fraction

A lysate was prepared from V12M2 cells at the tipped-mound stage of development by forced filtration and fractionated by differential centrifugation. Two experiments are shown. In the first experiment (lanes 1–9) a cell lysate was centrifuged at 1000 g for 5 min (lanes 2 and 3; '3K'), the supernatant was centrifuged again at 12000 g for 15 min (lanes 4 and 5; '12K') and this supernatant centrifuged at 78000 g for 120 min (lanes 6–9; '45K'). In the second experiment, the lysate was prepared in the presence of 1 mM DTT and centrifuged at 349000 g for 30 min ('95K') after being cleared at 12000 g for 15 min. The following amounts of protein were assayed: lanes 1–5, 200 mg; lane 6, 100 mg; lane 7, 20 mg; lane 8, 400 mg; lane 9, 100 mg; lane 11, 100 mg; lane 12, 10 mg; lane 13, 500 mg and lane 14, 125 mg. The des(Cl)-DIF-3 in lane 13 is produced by dechlorination of DIF-3 by DIF-1 dechlorinase present in the cytosol, using endogenous GSH as cofactor. Lane 15 contains a [3 H]DIF-1 standard.

Figure 5 K_m of DIF-3 hydroxylase

A high-specific-activity extract was prepared as described in the Materials and methods section. Serial dilutions from DIF-3 stocks were made in dimethyl sulphoxide and added to the reaction mixtures. Solvent never exceeded 2% of the final volume of the assay. The reaction was carried out for 20 min and processed as usual. BSA (2 mg/ml) and proteinase inhibitors were included in the buffer. (a) Hyperbolic graph fitted with a Michaelis–Menten curve. (the inset, b) Lineweaver–Burk plot using the data from (a).

NADPH concentration from 1 mM to 3 μ M resulted only in a 30% decrease in enzyme activity (results not shown).

Lysates of high specific activity were prepared as described in the Materials and methods section and used to determine the kinetic parameters of DIF-3 hydroxylase. From the Lineweaver–Burk plot shown in Figure 5, a K_m of 47 nM for DIF-3 and a V_{max} of 3.0×10^3 units/mg can be calculated. DIF-1 is estimated at 100–200 nM in the tissue of the migrating slug and DIF-3 is

Figure 6 Specificity of DIF-3 hydroxylase

Standard assay mixtures containing 100 nM [3 H]DIF-3 were supplemented with additional unlabelled DIF-3 or DIF-3 analogues. AS2 and AS3 are the two other possible isomers of DIF-3 in the position of the methoxy group: 1-(3-chloro-2,4-dihydroxy-6-methoxyphenyl)hexan-1-one and 1-(3-chloro-4,6-dihydroxy-2-methoxyphenyl)hexan-1-one; COOH-DIF-3 bears a terminal carboxylic group on the alkyl chain: 1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)-6-carboxyhexan-1-one. Results are given normalized to the proportion of [3 H]DIF-3 converted into metabolites at a final concentration of 100 nM DIF-3.

5–10-fold lower in concentration [2,29]: DIF-3 is therefore in the 10–40 nM range in slug tissue, close to the K_m of DIF-3 hydroxylase. The K_m of DIF-3 hydroxylase is therefore consistent with this enzyme carrying out DIF-3 hydroxylation *in vivo*.

DIF-3 hydroxylase is highly specific for DIF-3

A range of DIF-related substances was tested for inhibition in the enzymic assay. DIF-1 is DIF-3 with an additional chlorine atom; des(Cl)-DIF-3 is DIF-3 lacking the chlorine atom; DIF-2, a minor DIF activity produced during *Dictyostelium* development [19], is the homologue of DIF-1, having one carbon atom less in the side chain; AS2 and AS3 are the two isomers of DIF-3 in which the position of the methoxy group on the ring is varied, and COOH-DIF-3 is an analogue in which the terminal methyl group of the alkyl chain has been replaced by a carboxy group.

The picture that emerges (Figure 6) is of a very specific enzyme which does not tolerate additions to the substrate structure and requires all the substituents in their proper spatial configuration. For example, DIF-1, which differs from DIF-3 only in having one additional chlorine atom on the aromatic ring [19], does not compete with the natural substrate even at 100-fold molar excess. The only analogue competing effectively with DIF-3 is des(Cl)-DIF-3, which must fit into the enzyme pocket, since it has the same substituents as DIF-3 except the Cl atom, which is replaced by a smaller H on the aromatic ring.

Other distantly related compounds were also tested for inhibition (results not shown): hexanophenone, a DIF-3 devoid of all the ring substituents, did not show any competition at 10^4 -fold molar excess, but blocked the reaction at 10^6 -fold molar excess; a similar behaviour was shown by hexanal, while hexanol, pentanol and hexanone did not compete, even at a 10^6 -fold molar excess.

It should be noted that these experiments do not show that DIF-3 hydroxylase actually metabolizes any of the substrate analogues (though we have evidence that both [3 H]DIF-1 and [3 H]des(Cl)-DIF-3 can be metabolized by the membrane preparations), but it does follow that if any analogues are metabolized, they are much poorer substrates than DIF-3. Thus

Figure 7 Effect of CO on DIF-3 hydroxylase activity

A mixture of the indicated gases was forced for 1 min through 200 μ l of microsomal extract at 1 mg/ml contained in screw-capped Eppendorf tubes which were then immediately closed. DIF-3 and NADPH had been previously placed inside the cap and were mixed with the extracts by a short, low-speed centrifugation.

Table 1 Inhibitors of DIF-3 hydroxylase

Standard assay mixtures were supplemented with different inhibitors over a wide concentration range as described. The results were quantified and the concentration of inhibitor required for 50% inhibition presented. The value for norethisterone is only approximate.

Inhibitor	IC ₅₀
Ancymidol	67 nM
Paclobutrazol (SS)	2.1 μ M
Metyrapone	60 μ M
Ketoconazole	70 μ M
Norethisterone	$\approx$ 400 μ M
Diphenyliodonium	2 μ M

whether the enzyme metabolizes the analogues or not, the active site must be highly specific for DIF-3.

Further evidence that DIF-3 is a cytochrome *P*-450

The chemistry of the reaction catalysed by DIF-3 hydroxylase, the requirement for NADPH and its microsomal location all suggest that it is a cytochrome *P*-450 mono-oxygenase. To obtain further evidence that this is so, we tested the effect of several cytochrome *P*-450 inhibitors on enzyme activity (Figure 7 and Table 1). CO is the classic cytochrome *P*-450 inhibitor. Figure 7 shows that the formation of DM2, DM3 and DM4 was strongly inhibited by CO (1:1 mixture with air); reduction of the oxygen concentration was not the cause of the inhibition, since the control with argon showed the same activity as air alone. Likewise, diphenyliodonium chloride, an irreversible inhibitor of cytochrome *P*-450 reductase [30] inhibited DM2 and DM3 formation when present at micromolar concentrations (Table 1).

We also tested the effects of a number of specific inhibitors of plant and fungal *P*-450 enzymes [31]. The anti-fungal drug ketoconazole and metyrapone were both potent inhibitors but norethisterone, a specific suicide inhibitor of aromatase [32], was, as expected, without effect. Paclobutrazol, a compound which

inhibits the cytochrome *P*-450 hydroxylase involved in gibberellic acid biosynthesis [33] was a fairly potent inhibitor of DIF-3 hydroxylase. However, the most potent inhibitor was ancymidol, another inhibitor of the cytochrome *P*-450 involved in gibberellic acid biosynthesis [34,35]. Ancymidol inhibited DIF-3 hydroxylase with an IC₅₀ of 67 nM and reduced enzymic activity to undetectable levels at a concentration of 50 μ M. Its potency and membrane permeability make ancymidol a particularly useful inhibitor, and we have found that it can essentially abolish DIF-3 hydroxylase activity in living cells without other obvious adverse effects (results not shown).

DISCUSSION

Our identification of the first four products of DIF-1 metabolism shows that DIF-1 is initially metabolized by a reductive dechlorination of the phenolic ring [18], and then by three successive oxidations of the aliphatic side chain. Further metabolism undoubtedly occurs, as living cells convert DM4 into another group of four metabolites of which DMs 6, 7 and 8 are the major end products of DIF-1 metabolism [16,21]. These later metabolites have not been characterized in detail, though it is known that they retain their chlorine atoms [21].

All the properties of DIF-3 hydroxylase indicate that it is a cytochrome *P*-450 enzyme, though final proof will only come with purification of the protein or cloning of the gene. Thus it catalyses a mono-oxygenation, is membrane-bound, uses NADPH as its cofactor and is inhibited by CO and other cytochrome *P*-450 inhibitors [36,37]. Certain cytochrome *P*-450 enzymes are capable of multiple rounds of oxidation on the same molecule [34,38–43]; for example, a methyl group can be oxidized to an alcohol, then to an aldehyde and sometimes released as formic acid; in other cases the same enzyme can oxidize different carbon atoms. It is therefore possible that DIF-3 hydroxylase also performs multiple oxidations to form, successively, DM2, DM3 and DM4. Alternatively, additional, distinct enzymes in our microsomal preparations may perform these later oxidations.

Both DIF-1 dechlorinase and DIF-3 hydroxylase are highly specific for their respective substrates and have K_m values suitable for the metabolism of these compounds *in vivo* (the present work; [18]). Both enzymes are also developmentally regulated, appearing in cells towards the end of aggregation, when DIF-1 levels start to rise [44] and both are inducible in living cells by DIF-1 [16,45] (P. Morandini and R. R. Kay, unpublished work). There therefore seems little doubt that these enzymes, and presumably the entire DIF-1 metabolizing pathway, are dedicated to DIF metabolism and do not metabolize DIF as an adventitious consequence of some other function.

The most obvious role of this DIF-1 metabolizing pathway is to regulate DIF-1 levels in the aggregate by inactivating DIF-1 in a controlled way. Since DIF-1 dechlorinase expression is induced by DIF-1 and the enzyme is essentially restricted to prestalk cells, we have suggested that DIF-1 levels are regulated by negative feedback and that this mechanism in turn controls the proportion of prestalk cells that differentiates in the aggregate. It appears that prestalk cells destroy their own inducer and thereby set their own numbers [45,46].

Though DIF-1 has so far only been detected in the cellular slime moulds ([21,47], but see [48]), its metabolism shows some similarities to the metabolism of other lipophilic signal molecules. Both DIF-1 and the thyroid hormones [49] are metabolized by a reductive dehalogenation of a phenolic ring by enzymes using GSH as the cofactor. Both DIF-3 (in all probability) and the retinoids [50] are oxidized by cytochrome *P*-450 enzymes. There may also be a different sort of parallel. Both thyroid hormones

and the retinoids [51,52] are activated in their target tissues by metabolism; conceivably DIF-1 may also be activated (and subsequently inactivated) by metabolism, though there is no firm evidence for this to date [15,16].

The biochemical definition of the proximal pathway for DIF-1 metabolism, the identification, purification and synthesis of some of the metabolites and the characterization of DIF-3 hydroxylase provide the basis for investigating the role of DIF metabolism *in vivo*. Ancymidol will also be a useful tool for this purpose, since it is a potent inhibitor of DIF-3 hydroxylase (IC_{50} of 67 nM) and blocks DIF-1 metabolism in living cells at DIF-3, without any other obvious deleterious effects (P. Morandini and R. R. Kay, unpublished work).

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