DL- α -Difluoromethyl[3, 4- 3 H]arginine metabolism in tobacco and mammalian cells

Inhibition of ornithine decarboxylase activity after arginase-mediated hydrolysis of DL- α -difluoromethylarginine to DL- α -difluoromethylornithine

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DL- α -Difluoromethylarginine (DFMA) is an enzyme-activated irreversible inhibitor of arginine decarboxylase (ADC) *in vitro*. DFMA has also been shown to inhibit ADC activities in a variety of plants and bacteria *in vivo*. However, we questioned the specificity of this inhibitor for ADC in tobacco ovary tissues, since ornithine decarboxylase (ODC) activity was strongly inhibited as well. We now show that [3, 4- 3 H]DFMA is metabolized to DL- α -difluoromethyl[3, 4- 3 H]ornithine ([3, 4- 3 H]DFMO), the analogous mechanism-based inhibitor of ODC, by tobacco tissues *in vivo*. Both tobacco and mammalian (mouse, bovine) arginases (EC 3.5.3.1) hydrolyse DFMA to DFMO *in vitro*, suggesting a role for this enzyme in mediating the indirect inhibition of ODC by DFMA in tobacco. These results suggest that DFMA may have other effects, in addition to the inhibition of ADC, in tissues containing high arginase activities. The recent development of potent agmatine-based ADC inhibitors should permit selective inhibition of ADC, rather than ODC, in such tissues, since agmatine is not a substrate for arginase.

INTRODUCTION

Arginine decarboxylase (ADC) catalyses the first step in a pathway leading to putrescine biosynthesis in plants and bacteria (Slocum *et al.*, 1984; Tabor & Tabor, 1985), although these organisms can also synthesize putrescine directly via ornithine decarboxylase (ODC). In many plant tissues ADC activity appears to be the rate-limiting factor in overall polyamine biosynthesis; consequently the ADC enzyme represents an important target for the inhibition of polyamine biosynthesis in these tissues (Slocum & Galston, 1987).

In addition to simple competitive inhibitors, such as D-arginine and α -methylarginine (Kallio *et al.*, 1981), several mechanism-based irreversible inhibitors of ADC have been described (see Bitonti *et al.*, 1987). One such compound, DL- α -difluoromethylarginine (DFMA), has been widely employed as a specific inhibitor of ADC in a variety of plants and bacteria since its initial description and characterization by Kallio *et al.* (1981). Although there is a large body of literature reporting the inactivation of ADC by DFMA both in tissue extracts and *in situ*, the specificity of this drug has been less thoroughly investigated than that of its counterpart DL- α -difluoromethylornithine (DFMO) (Pritchard *et al.*, 1981; Seeley *et al.*, 1982; Pegg, 1987). The presumed specificity of DFMA for ADC is based not only on the enzyme-dependent mechanism of inhibition, but also on the assumption that DFMA is not metabolized in tissues. However, we observed that DFMA significantly inhibited ODC activity in tobacco ovary tissues *in vivo*, suggesting that this drug was being metabolized to DFMO or some other ODC inhibitor (Slocum & Galston, 1985a). We

postulated that a simple arginase-mediated hydrolysis of DFMA could produce DFMO and demonstrated that both bovine and tobacco arginases hydrolysed DFMA *in vitro*, on the basis of the production of urea (Slocum & Galston, 1985b). Similarly, Mussell *et al.* (1987) used this assay to show hydrolysis of DFMA by *Verticillium* extracts, which were shown to have high arginase activities. The more direct demonstration of DFMO production from DFMA by arginase proved to be more difficult until radiolabelled DFMA recently became available.

In this report, we provide evidence for an arginase-mediated hydrolysis of [3, 4- 3 H]DFMA in tobacco ovary tissues, resulting in the accumulation of [3, 4- 3 H]DFMO and significant inhibition of ODC activity in these tissues. Our findings suggest that, in cells containing high arginase activities, the effects of DFMA, and possibly other arginine-analogue inhibitors, on polyamine metabolism may not be entirely due to the specific inhibition of ADC.

EXPERIMENTAL

Studies *in vivo* of [3 H]DFMA metabolism

Flowers from tobacco plants (*Nicotiana tabacum* cv. 'Wisconsin 38') maintained under standard greenhouse conditions were excised at the 'Day 7–8' stage (Slocum & Galston, 1985a), approx. 2 days after fertilization. The cut end of the pedicel of each excised flower was immersed in 1.0 ml of 1 mM-potassium phosphate buffer, pH 5.8, containing 0.4 μ M-[3, 4- 3 H]DFMA (12.8 μ Ci; New England Nuclear, Boston, MA, U.S.A.; sp. radio-

Abbreviations used: ADC, arginine decarboxylase (EC 4.1.1.19); DFMA, DL- α -difluoromethylarginine; DFMO, DL- α -difluoromethylornithine; ODC, ornithine decarboxylase (EC 4.1.1.17); MGBG, methylglyoxal bis(guanylhydrazone).

activity 31.4 Ci/mmol) in a glass vial. The mouth of the vial was then sealed with Parafilm wrapped around the protruding flower. Replicate flowers were allowed to take up the label for 18 h at 22 °C under high-intensity mixed tungsten/fluorescent lighting. After labelling, the flowers were dissected and various tissues were homogenized directly in 20% (v/v) HClO₄ (100 mg fresh wt. of tissue/ml of acid). The homogenates were then hydrolysed in 6 M-HCl at 110 °C for 16 h to ensure that any label covalently bound to proteins would be released for subsequent analysis. To evaluate the stability of [³H]DFMA during the acid-hydrolysis step, a [³H]-DFMA/buffer control was processed in a manner identical with that for the experimental samples. The acid hydrolysates were evaporated to dryness at 80 °C, then redissolved in 20% HClO₄. The labelled products in these samples were identified and quantified by using an amino acid analyser.

Analysis of [³H]DFMA metabolites

Samples in 20% HClO₄ were adjusted to 100 µl in 0.2 M-lithium citrate sample dilution buffer, pH 2.2, applied to a 6 mm × 20 cm cation-exchange column (type W-3P spherical resin; Beckman) on a Beckman 119CL automatic amino acid analyser. Samples were eluted with 1.0 M-lithium citrate buffer, pH 3.75, and the column was regenerated between samples with 0.3 M-LiOH. The column temperature was held constant at 65 °C. Under these conditions, acidic and neutral amino acids passed directly through the column, but the basic amino acids and their analogues were separated. The column was calibrated with radioactively labelled standards: DL-α-[3, 4-³H]DFMO, DL-α-[3, 4-³H]DFMA, L-[1-¹⁴C]-ornithine (all from New England Nuclear) and DL-[1-¹⁴C]arginine (Research Products International). Peak retention times for the standards were determined by monitoring radioactivity with a Flo-One flow-through radioactivity detector attached to the column (Radiomatic Instruments Co., Tampa, FL, U.S.A.). Owing to the inherent insensitivity of the detector for ³H in this configuration, 1 min fractions were collected, and radioactivity in these fractions was quantified by scintillation counting.

Assays for arginase-mediated hydrolysis of DFMA *in vitro*

Two types of experiments were performed to examine the hydrolysis of DFMA by arginase *in vitro*. In the first type, [³H]DFMA (2 µM) was incubated in the presence of 50 units (1 unit = 1 µmol of urea/min at 37 °C, pH 9.7) of highly purified bovine arginase, or approx. 0.002 unit of arginase activity in a tobacco ovary tissue homogenate, for 30 min, at 37 °C. The reaction was stopped by the addition of 20% HClO₄ on ice, and the labelled products in the reaction mixture were identified with an amino acid analyser, as described above.

In the second type of experiment, kinetic analyses of the bovine and tobacco arginases, with unlabelled DL-DFMA (Merrell Dow) or L-arginine as substrates, were carried out by using colorimetric assays for either urea (Schimke, 1970) or L-ornithine (Chinard, 1952) production. Bovine arginase (2 units) or tobacco arginase (0.002 unit) activity was assayed in the presence of various concentrations of substrate (5, 10, 25, 50 and 100 mM; pH 9.5) for 10 min at 37 °C.

Before each assay, the arginases were preincubated in

3 × Assay Buffer (150 mM-3-cyclohexylaminopropane-1-sulphonic acid buffer, 15 mM-MnCl₂, 1.5% bovine serum albumin, pH 9.5) for 20 min at 25 °C. Then 200 µl of the substrate (300 mM; pH 9.5) was combined with 100 µl of preincubated arginase on ice. The reaction was started by placing the tubes in the 37 °C water bath and stopped by the addition of 100 µl of 20% HClO₄. The specificity of arginase for other guanidino compounds which are structurally related to arginine (an arginine homologue (canavanine), an intermediate in polyamine biosynthesis (agmatine), or inhibitors of polyamine biosynthesis [arcaine, methylglyoxal bis(guanylhydrazine) (MGBG)]) was also examined.

Tobacco arginase was prepared by homogenizing ovary tissues in 5 vol. of 100 mM-Hepes/50 mM-MnCl₂/5 mM-dithiothreitol, pH 7.5, on ice, followed by centrifugation at 12000 g for 30 min at 4 °C. The supernatant was dialysed against 10 mM-Hepes/1 mM-dithiothreitol, pH 7.5, and used in arginase assays. Freeze-dried bovine arginase (Sigma) was dissolved at 470 units/ml in the same dialysis buffer.

[³H]DFMA hydrolysis by mouse liver homogenates

Mouse livers were homogenized in 10 vol. of 100 mM-Hepes/50 mM-MnCl₂/5 mM-dithiothreitol, pH 7.5, and centrifuged at 12000 g for 30 min at 4 °C. The supernatant was dialysed overnight against 10 mM-Hepes/1 mM-dithiothreitol, pH 7.5, and used directly in arginase assays, as described above. The enzyme (0.1 ml) was incubated with 1 µCi of [³H]DFMA for 3 h, and then the reaction was stopped by addition of 0.2 ml of 0.4 M-HClO₄. Labelled products in the supernatant were analysed by h.p.l.c. using a Whatman Partisil-SCX 10 column coupled to a flow-through radioactivity detector, as described by Bitonti *et al.* (1986).

Protein determination

The soluble protein contents of homogenates were measured by the protein dye-binding assay of Bradford (1976), with α-globulin (Cohn Fraction II; Sigma) as the standard.

RESULTS

We have previously demonstrated the specificity of DFMA as an inhibitor of ADC activity in tobacco homogenates (Slocum & Galston, 1985a). In these extracts, arginase-mediated hydrolysis of DFMA to DFMO, and the resultant inhibition of ODC activity, are not seen. However, ADC is extracted from the tissue and assayed under conditions (pH 7.5, chelated Mn²⁺ co-factor) which would not permit arginase activity. This is not the case for DFMA supplied to the tissue *in vivo*, where ODC inhibition is approx. 40% of that seen with DFMO alone.

As shown in Table 1, DFMA is indeed a substrate for both the tobacco arginase and bovine arginase. The bovine enzyme exhibits an apparent *K_m* of 83.3 mM for DFMA, compared with 27.8 mM for the tobacco arginase. In contrast, the bovine arginase shows a *K_m* of 2.6 mM for its normal L-arginine substrate, whereas the tobacco enzyme has a *K_m* of 20.8 mM, which is not significantly lower than that for the DFMA substrate. These kinetic data were obtained by assaying for urea production (Schimke, 1970), but similar results were independently obtained by assaying L-ornithine pro-

Table 1. Specific activity and substrate K_m values for bovine and tobacco arginases

	Specific activity (μ mol of urea/h per mg of protein)		Apparent K_m	
	Bovine	Tobacco	Bovine	Tobacco
L-Arginine	460.3	1.2	2.6 mM	20.8 mM
DFMA	26.4	0.6	83.3 mM	27.8 mM

duction (Chinard, 1952) from the L-arginine substrate. For the bovine arginase, a K_m of 3.1 mM for L-arginine was estimated by this method. The production of DFMO from the DFMA substrate could not be monitored by this colorimetric method for two reasons: (1) under standard assay conditions, the ninhydrin reagent reacted with DFMO to give measurable A_{515} readings only at concentrations of 2 mM or greater, although absorbance was linear between 2 and 10 mM-DFMO, and (2) DFMA standards exhibited A_{515} readings almost identical with those for DFMO, even though L-arginine does not react with the ninhydrin reagent under these assay conditions (Chinard, 1952). The reason for the positive DFMA ninhydrin reaction is unclear, since t.l.c. analysis of the DFMA standard indicated that the sample did not contain detectable amounts of DFMO or ornithine contaminants (results not shown).

The K_m values for DFMA, which is a mixture of both the D- and L-isomers, are considerably higher than might be expected for L-DFMA alone. D-Arginine was shown to be a competitive inhibitor for both enzymes and exhibited a K_i of 3.9 mM for L-arginine and 0.8 mM for DFMA with the bovine arginase (results not shown).

We examined the extent to which other guanidino

Table 2. Specificity of bovine arginase for L-arginine and some structurally related guanidino compounds

Assays were carried out at 37 °C for 30 min. Arginase (25 units) was added to 200 mM-Hepes buffer, pH 8.0, containing 10 mM-MnCl₂ + 125 mM concentration of the compound tested. N.D., not detected.

Substrate	Arginase activity (μ mol of urea/h)
MGBG	N.D.
Arcaïne	0.08
L-Arginine	18.3
D-Arginine	N.D.
DFMA	11.6
Canavanine	N.D.
Agmatine	N.D.

compounds, structurally related to L-arginine and DFMA, might also serve as arginase substrates (Table 2 and Fig. 1). Assays of bovine arginase activity, utilizing these various substrates, were carried out at pH 8 to resemble more closely conditions *in vivo*. Only L-arginine (not D-arginine) and DFMA supported appreciable arginase activity, whereas arcaïne, a reported inhibitor of agmatine iminohydrolase (EC 3.5.3.12; Goldenberg *et al.*, 1983), was hydrolysed to a small extent. MGBG, a spermidine-analogue inhibitor of S-adenosylmethionine decarboxylase (EC 4.1.1.50; Williams-Ashman & Schenone, 1972), and agmatine, the decarboxylation product of ADC activity, were not substrates. Finally, the guanidino-oxy analogue of arginine, canavanine, which is a substrate for arginase in jack bean (Downum *et al.*, 1983), was not hydrolysed.

We examined the metabolic fate of [³H]DFMA

Fig. 1. Arginine and some structurally related guanidino compounds

Fig. 2. Identification of [^3H]DFMA metabolites from tobacco floral tissues labelled *in vivo* and from bovine and tobacco arginase assays *in vitro*.

Elution profiles for (a) radiolabelled standards [3, 4- ^3H]DFMO, [3, 4- ^3H]DFMA and L-[1- ^{14}C]ornithine (L-Orn); peaks 1 and 3 are unidentified contaminants in the L-[1- ^{14}C]arginine standard in this mixture, which is not eluted from the column during a standard 60 min run, and peak 2 is an unidentified contaminant in the L-[1- ^{14}C]ornithine standard; (b) purified bovine arginase assay mixture; (c) DFMA control for labelling studies *in vivo*; (d) hydrolysate of tobacco ovary tissue labelled *in vivo*; peaks 1 and 2 are unidentified metabolites.

incubated with purified bovine arginase *in vitro* and in tobacco tissues *in vivo*, by amino acid analysis of acid hydrolysates of these samples. As shown in Fig. 2(a), labelled standards of DFMO and DFMA were well separated from each other and from L-ornithine. L-Arginine was not eluted from the column during a standard 60 min run, but was rapidly eluted when the column was regenerated with LiOH.

The metabolism of [^3H]DFMA to [^3H]DFMO by purified bovine arginase (Fig. 2b) and by the tobacco ovary tissues (Fig. 2d) provides further support for our previous observations (Slocum & Galston, 1985b) of DFMA inhibition of ODC activity, and the presumed arginase-mediated hydrolysis of this drug to DFMO in tobacco tissues. Approx. 45% of the [^3H]DFMA was converted into [^3H]DFMO by bovine arginase during a 30 min incubation, and no other labelled compounds were seen in this reaction mixture (Fig. 2b). In contrast, two unidentified compounds (in addition to DFMO and DFMA) are seen in homogenates of labelled tobacco ovary tissues (Fig. 2d). The remaining [^3H]DFMA represents only 27% of the total radioactivity in the four peaks, again suggesting that this compound is readily metabolized in these tissues. A similar labelling pattern was seen in homogenates of petal and sepal tissues, but less [^3H]DFMA was metabolized to [^3H]DFMO, consistent with the 3-fold lower arginase activity that we measured in these tissues (results not shown). Since these samples were acid-hydrolysed, both free and protein-bound labelled metabolites were analysed. Acid hydrolysis did not produce detectable amounts of [^3H]DFMO

Fig. 3. Hydrolysis of [^3H]DFMA to [^3H]DFMO in mouse liver homogenate

□, + enzyme; ○, no enzyme.

or other compounds in the [^3H]DFMA control sample (Fig. 2c).

Consistent with our finding that DFMA was hydrolysed to DFMO by bovine arginase, we observed a similar metabolism of this inhibitor in mouse liver extracts, which contain high arginase activities (Fig. 3). Approx. 50% of the labelled DFMA was hydrolysed to DFMO during a 3 h incubation.

DISCUSSION

We have provided evidence for the metabolism of DFMA to DFMO in tobacco ovary tissues *in vivo* and supporting data obtained *in vitro* suggesting the involvement of arginase in this metabolism. We have found no evidence that either DFMO or α -methylornithine (Abdel-Monem *et al.*, 1974), a competitive inhibitor of ODC, is further metabolized by ornithine transcarbamoylase (EC 2.1.3.3) in the urea cycle (R. D. Slocum, unpublished work), although it is certainly possible that DFMO and/or DFMA are metabolized by enzymes other than arginase and ornithine transcarbamoylase. Peaks 1 and 2 in Fig. 2(d) may represent such metabolites.

We did not investigate whether arginase hydrolyses other arginine-analogue inhibitors, such as DL- α -methylarginine (Kallio *et al.*, 1981), DL- α -monofluoromethylarginine (Bitonti *et al.*, 1987) or (E)-DL- α -monofluoromethyldehydroarginine (Bitonti *et al.*, 1987), but it seems likely that these compounds would be converted into their ornithine analogues as well. Although this might limit the usefulness of these compounds as specific ADC inhibitors in plant tissues exhibiting high arginase activities, they undoubtedly will find application in *Escherichia coli* and other bacteria which do not contain arginase (Tabor & Tabor, 1985).

In arginase-containing tissues, a new group of potent agmatine-analogue inhibitors of ADC (Bitonti *et al.*, 1987) may prove useful, since we have shown that agmatine is not a substrate for arginase in the present study. One of these compounds, DL- α -monofluoromethylagmatine, inhibited 95% of the ADC activity in oat and barley leaf extracts at 0.1 mM concentration (Bitonti *et al.*, 1987). However, it remains to be seen whether these agmatine inhibitors are substrates for enzymes such as agmatine iminohydrolase or agmatinase, which might portend problems for their use as specific ADC inhibitors *in vivo*.

Arginase-mediated hydrolysis of DFMA to DFMO may also explain the observed DFMA inhibition of polyamine biosynthesis and mycelial growth in several fungi. Rajam & Galston (1985) reported that the growth of several isolates of phytopathogenic fungi was more strongly inhibited by DFMA than by DFMO, and this inhibition was reversible by exogenous putrescine or spermidine. This was surprising, since, with one exception (Uhlemann & Reinbothe, 1977), ADC activity has not been found in fungi, and putrescine biosynthesis occurs via the ODC pathway (Tabor & Tabor, 1985). Mussell *et al.* (1987) reported that a similar DFMA inhibition of growth in cultures of the tomato-wilt fungus *Verticillium dahliae* was reversed by putrescine. They found that extracts of this fungus contained high arginase activity and hydrolysed DFMA to DFMO, assayed by urea production, suggesting that DFMA was inhibiting polyamine biosynthesis and growth at the level of ODC, after its arginase-mediated conversion into DFMO. Boyle and co-workers (S. M. Boyle, personal communication) have found that several fungal dermatophytes are more sensitive to DFMA than to DFMO, again suggesting the metabolism of DFMA via arginase. One possible reason for the enhanced growth inhibition of DFMA, relative to that of DFMO, is that this compound is more readily taken up by the cells. This possibility requires further investigation. DFMA may also non-specifically

inhibit growth in some fungal pathogens, such as *Helminthosporium maydis*, in which DFMA effects were not reversed by exogenous putrescine (Birecka *et al.*, 1986).

As was recently reported, DFMA and related arginine-analogue inhibitors of ADC may have application in the treatment of Chagas' disease, which is caused by the New World trypanosome *Trypanosoma cruzi* (Kierszenbaum *et al.*, 1987). However, our finding that DFMA is rapidly hydrolysed to DFMO in mouse liver extracts suggests that this drug will be converted into DFMO in the body. Since DFMO is ineffective in inhibiting the growth of this trypanosome, it may be expected that high serum concentrations of DFMA would have to be maintained in order for these drugs to be chemotherapeutically effective. An attractive alternative might be the treatment of this disease with agmatine-analogue inhibitors, which would not be expected to be metabolized by arginase, since we have shown that agmatine is not a substrate for this enzyme.

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