***Agrobacterium* Ti and Ri Plasmids Specify Enzymic Lactonization of Mannopine to Agropine**

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(Received 4 February 1986; revised 22 April 1986)

A novel enzymic activity, responsible for the conversion of mannopine to agropine by lactonization, has been identified in *Agrobacterium* strains. This activity is encoded by octopine-type and agropine-type Ti or Ri plasmids, and is inducible by mannopine and agropine. In crude extracts it is stable for long periods and can be used for preparative synthesis of agropine from mannopine. The physiological role of this activity is not understood. However, it is probably involved in degradation of opines of the agropine family since it is always associated with agropine utilization in wild-type strains.

INTRODUCTION

Opines are compounds which are synthesized in crown-gall or hairy-root proliferations incited by the soil phytopathogenic bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Petit & Tempé, 1978; Tempé *et al.*, 1979; Guyon *et al.*, 1980; Petit *et al.*, 1983). These pathological overgrowths result from uncontrolled division of plant cells which have integrated in their genomes a piece of foreign DNA originally located on a large pathogenic plasmid carried by virulent *Agrobacterium* strains (Chilton *et al.*, 1977; see also Kahl & Schell, 1982). According to the opine concept (Tempé *et al.*, 1979; Guyon *et al.*, 1980; Petit *et al.*, 1983), opines are natural mediators of the relation between pathogenic agrobacteria and their hosts. Synthesis of the opines by crown-gall or hairy-root cells turns crown-gall or hairy-root proliferations into ecological niches for the pathogens, which can use opines as a nutrient source. The catabolic pathways for such degradation are also specified by the pathogenic plasmids. The opine concept also postulates that opine production in grown-gall and hairy-root proliferations and opine degradation by *Agrobacterium* strains that have incited these proliferations are essential features of the interaction between bacteria and plants in these systems. Investigations undertaken in order to test this theory have led to the discovery of several new opines, providing evidence for the validity of the theory (Guyon *et al.*, 1980; Ellis & Murphy, 1981; Petit *et al.*, 1983; Chilton *et al.*, 1984).

Among the opines so far identified are those of the agropine family (Fig. 1). These include (a) mannopinic acid [*N*2-(1'-deoxy-D-mannitol-1'-yl)-L-glutamic acid] (Tempé *et al.*, 1980; Tate *et al.*, 1982; Petit *et al.*, 1983); (b) mannopine [*N*2-(1'-deoxy-D-mannitol-1'-yl)-L-glutamine] (Tate *et al.*, 1982; Petit *et al.*, 1983); (c) agropinic acid [*N*2-(1'-deoxy-D-mannitol-1'-yl)-L-oxoproline] (Coxon *et al.*, 1980; Tate *et al.*, 1982; Petit *et al.*, 1983); and (d) agropine [*N*2-(1'-deoxy-D-mannitol-1'-yl)-L-glutamine, 1',2'-lactone] (Firmin & Fenwick, 1978; Coxon *et al.*, 1980; Guyon

Abbreviation: HVPE, high-voltage paper electrophoresis.

Fig. 1. Structural formulae of the opines of the agropine family: (a) mannopinic acid, (b) mannopine, (c) agropinic acid, (d) agropine. Galactopine and galactopinic acid are stereoisomers of mannopine and mannopinic acid, respectively, in which the polyhydroxylated sidechain has the configuration of galactitol.

et al., 1980; Tate *et al.*, 1982; Petit *et al.*, 1983). These compounds are characteristic of crown-gall tissues transformed by agropine-type, or by some of the octopine-type *A. tumefaciens* strains and of hairy-root tissues transformed by agropine- or mannopine-type *A. rhizogenes* strains (Firmin & Fenwick, 1978; Guyon *et al.*, 1980; Tepfer & Tempé, 1981; Dahl *et al.*, 1983; Petit *et al.*, 1983).

We are studying the catabolic pathways by which *Agrobacterium* strains degrade opines of the agropine family. We have found that *Agrobacterium* strains carrying octopine-type or agropine-type Ti plasmids or agropine-type Ri plasmids possess an enzyme activity responsible for the conversion of mannopine to agropine. Here we report data concerning this activity, including induction properties, substrate specificity, and localization of the genetic determinant(s). We also discuss its possible biological function. Using this activity we have developed a simple enzymic method for preparation of agropine, making this compound available in high yield and in gram quantities.

METHODS

Bacterial strains. These are listed in Table 1.

Culture media. AT mineral salts (Petit & Tempé, 1978) was used for preparing defined media. All ingredients were autoclaved except for solutions of mannityl opines and their analogues, which were filter sterilized (0.45 µm Millipore membranes). Noble agar (Difco) was used for preparing solidified media (20 g l⁻¹). Growth in liquid medium was followed by measuring OD₆₈₀. Growth on solid medium was assessed by visual inspection of Petri plates at daily to weekly intervals.

Chemicals. Mannityl opines and their analogues were synthesized as described by Petit *et al.* (1983). Stock solutions were neutralized with KOH and stored frozen at -20 °C. All other chemicals were obtained from commercial sources.

Plasmid isolation and transformation. Plasmid DNA was isolated from 1.5 ml cultures by a miniprep procedure (Farrand *et al.*, 1985). *A. tumefaciens* strain C58C1RS(pRi8196) was transformed with pJS4159K6 by the freeze-thaw technique described by Holsters *et al.* (1978).

High-voltage paper electrophoresis (HVPE). This was done as previously described (Petit *et al.*, 1983).

Paper chromatography. Descending paper chromatography was done on Whatman 3 MM paper in a 410 × 425 × 225 mm Shandon tank saturated with elution buffer (1-butanol/glacial acetic acid/water, 3:1:1, by vol.). Chromatograms were developed for about 15 h at room temperature, after which they were dried, first in a current of warm air, and then in an oven at 100 °C for 20 min.

Chemical detection of opines. Electrophoretograms and chromatograms were stained with silver nitrate reagent (Trevelyan *et al.*, 1950) and fixed as previously described (Petit *et al.*, 1983).

Estimation of protein concentration. Proteins in crude extracts were quantified by the method of Bradford (1976), using a kit from Bio-Rad.

In vivo assay of mannopine-lactonizing activity. Mannopine-lactonizing activity and conversion of agropine to mannopine were assayed in resting cells as follows. Strain ACH5 was grown to mid-exponential phase at 30 °C on an orbital shaker (Infors, 180 r.p.m.), in 125 ml AT medium supplemented with mannopine (10 mM) and ammonium sulphate (1 g l⁻¹). The bacteria were harvested by centrifugation (11000 g), washed twice with 20 ml 0.9% NaCl, and resuspended in 10 ml AT mineral medium. The resulting cell suspension was incubated at 30 °C on an orbital shaker (as above) for 3 h. Samples (5 ml) of this suspension were made 10 mM with respect to agropine or mannopine (by adding 555 µl 100 mM-agropine or mannopine solutions), and further incubated under

Table 1. *Agrobacterium* strains

Strain	Resident plasmid	Agropine utilization	Comments	Source or reference
HRI	pRiHRI	+	<i>Wild-type A. rhizogenes</i>	Petit <i>et al.</i> (1983)
8196	pRi8196	—	Wild-type agropine strain utilizing only agropine	Our collection
ACH5	pTiACH5	+	Wild-type mannopine strain	J. Schell (Max Planck Institut, Köln, FRG)
ACH5C3	—	—	Wild-type octopine strain	J. Schell
R10	pTiR10	+	Cured derivative of ACH5	Our collection
E20.8	pTiB6s3::Tn7	—	Wild-type octopine strain A Tn7 insertion mutant of pTi B6s3, unable to grow on agropine	De Greve <i>et al.</i> (1981)
Bo542	pTiBo542*	+	Wild-type agropine strain	Our collection
C58	pTiC58†	—	Wild-type nopaline strain	J. Schell
C58CIRS	—	—	Mutant obtained from C58C1, a cured derivative of pTiC58; resistant to rifampicin (100 µg ml ⁻¹) and streptomycin (500 µg ml ⁻¹)	J. Schell
C58CIRS(pTiACH5)	pTiACH5	+	<i>Transconjugants obtained into C58CIRS</i>	Our collection
C58CIRS(pArA4a)	pArA4a	—	Transconjugant containing pTiACH5 Transconjugant containing pArA4a, the avirulent plasmid of <i>A. rhizogenes</i> strain A4; utilizes mannopine and mannopinic and agropinic acids	Petit <i>et al.</i> (1983)
C58CIRS(pArA4ab)	pArA4b (= pRiA4)	+	Transconjugant containing pArA4b, the virulent plasmid from strain A4; pArA4b confers ability to utilize agropine	Petit <i>et al.</i> (1983)
C58CIRS(pA4abc)	pArA4a pArA4b pArA4c	+	Transconjugant utilizing all opines from the agropine family; pArA4c is the cointegrate of pArA4a and pArA4b	Petit <i>et al.</i> (1983)
NT1(pJS4159K1)	—	—	<i>Transformants obtained into NT1‡</i>	S. K Farrand
NT1(pJS4159K2a)	—	—	NT1 harbouring cloned <i>KpnI</i> fragments of the octopine Ti plasmid from strain 15955; K1 to K10 refer to the cloned <i>KpnI</i> fragments (nomenclature system of De Vos <i>et al.</i> , 1981)	
NT1(pJS4159K2b)	—	—		
NT1(pJS4159K3)	—	—		
NT1(pJS4159K4)	—	—		
NT1(pJS4159K5)	—	—		
NT1(pJS4159K6)	—	—		
NT1(pJS4159K7)	—	—		
NT1(pJS4159K8)	—	—		
NT1(pJS4159K9)	—	—		
NT1(pJS4159K10)	—	—		
C58CIRS(pRi8196)(pJS4159K6)	—	—	<i>Transformant and transconjugant obtained in C58CIRS</i> pJS4159K6 was introduced by transformation into C58CIRS(pRi8196)	This work

* This strain also contains pAtBo542, a large cryptic plasmid, and pAgBo542, a plasmid encoding production of agropine.

† All C58 derivatives also contain the large cryptic plasmid pAtC58.

‡ NT1 is C58C1.

the same conditions. Samples (1 ml) were removed at 0, 0.5, 15, 30, and 240 min, centrifuged at 11 000 *g* for 2 min, washed twice with 0.9% NaCl (1 ml) and resuspended in 10 μ l water. The resulting suspension was spotted on Whatman 3 MM paper and submitted to electrophoresis in formic acid (0.795 M)/acetic acid (1.05 M) (pH 1.9) buffer as described by Petit *et al.* (1983).

In vitro assay of mannopine-lactonizing activity and of conversion of agropine to mannopine. Samples (100 ml) of AT medium supplemented with ammonium sulphate (1 g l⁻¹) and various carbon sources were inoculated with the strain to be studied, and incubated at 30 °C on an orbital shaker until the OD₆₈₀ had reached 0.6–0.8. Bacteria were collected by centrifugation (15 min, 11 000 *g*, 4 °C), resuspended in 20 ml 0.9% NaCl and centrifuged as before. Pellets were resuspended in 1 ml ice-cold extraction buffer which, as described below, varied according to the enzyme assay. The cell suspensions were sonicated at 0 °C, twice for 4 min with a 1 min interval between pulses (Annemasse 150 TS sonicator, 20 kHz, 100 W). The resulting suspensions were centrifuged (15 min, 22 000 *g*, 4 °C) and the supernates were collected. These constituted the crude extracts.

For studying the conversion of mannopine to agropine the extraction buffer contained: potassium phosphate pH 7.5, 100 mM; dithiothreitol, 1 mM; ethylene glycol, 900 mM. The assays were performed as follows. Equal volumes (100 μ l) of bacterial crude extract (6–8 mg protein ml⁻¹), and of a 13 mM-mannopine solution (adjusted to pH 7.5) were mixed and incubated at 30 °C. Samples were generally taken from the incubation mixture at 0, 15, 30, 60 and 120 min or 150 min, immediately treated with 2 M-acetic acid (final concentration 0.4 M) and centrifuged (11 000 *g*, 4 min). Samples (4 μ l) of each supernate were spotted on Whatman 3 MM paper and analysed by HVPE.

For studying the conversion of agropine to mannopine the bacteria were sonicated in a buffer containing: glycine/NaOH pH 10.0, 100 mM; dithiothreitol, 1 mM; ethylene glycol, 900 mM. Assays were performed as follows. Extract (100 μ l, 6–8 mg protein ml⁻¹) was mixed with 100 μ l of a 13 mM-agropine solution and the mixture was incubated at 30 °C. Samples were removed at 0, 15, 30, 60 and 120 or 150 min and treated and analysed by HVPE.

Preparation of crude extracts for preparative conversion of mannopine to agropine. A flask containing 2 l AT medium supplemented with ammonium sulphate (1 g l⁻¹), mannitol (0.5 g l⁻¹, as a non-inducing, low-cost growth substrate) and mannopine (150 mg l⁻¹, as the inducer of the activity) was inoculated with strain ACH5 and the culture was grown at 30 °C on an orbital shaker to an OD₆₈₀ of 0.6–0.8. Bacteria were collected by centrifugation (15 min, 11 000 *g*, 4 °C), resuspended in 400 ml 0.9% NaCl and further centrifuged. The pellet was resuspended in 20 ml ice-cold extraction buffer (potassium phosphate pH 7.5, 50 mM; dithiothreitol, 1 mM; ethylene glycol, 180 mM). The suspension was sonicated as described above, the resulting homogenate was centrifuged (15 min, 22 000 *g*, 4 °C) and the supernate was collected. This constituted the crude extract.

Enzymic preparation of agropine. Lactonization of mannopine to agropine by crude bacterial extracts was performed in a final volume of 60 ml as follows. At the beginning of the incubation the reaction mixture had the following composition: bacterial extract from a 2 l culture (final protein content 2.7–3.3 mg ml⁻¹); potassium phosphate pH 7.5, 50 mM; dithiothreitol, 1 mM; ethylene glycol, 180 mM; mannopine, 65 mM. The reaction mixture was incubated at room temperature with magnetic stirring (200 r.p.m.). After an initial period of 15 min, a solution of mannopine (65 mM) in extraction buffer (see above) was continuously fed into the reactor as the reaction mixture was removed at the same rate through three immersible ultrafiltration units (Millipore immersible CX30), connected to a vacuum flask. In our experimental device, a residual pressure of 5330 Pa (40 mmHg) provided a flow rate of about 20 ml h⁻¹. After the addition of the mannopine-containing solution, fresh buffer without mannopine was supplied for 6 h to the flask before the reaction was finally terminated. During the incubation, the composition of the reaction mixture was monitored by subjecting samples to analysis by HVPE at pH 1.9.

Extraction and purification of agropine from the reaction mixture. Agropine and mannopine were separated from other components of the reaction mixture by ion-exchange chromatography. The mixture (950–1000 ml) was percolated over a column of sulphonated polystyrene resin (250 ml, Bio-Rad AG 50W-X8, 20–50 mesh, H⁺ form) to retain the cations. Neutral substances and anions, including agropinic acid formed by spontaneous rearrangement of mannopine and agropine during the incubation, were not retained and the column was rinsed with 300 ml water. At the beginning of the process the pH of the column eluate was 3.5; it dropped to 2.6–2.7 during the fixation step and rose to 3.2 during the rinse. Elution was performed with 2 M-aq. NH₃. During elution, the pH first decreased to 2.55, and then rose. Agropine and mannopine were eluted together from the column between pH 2.85 and 11.0. Agropine- and mannopine-containing fractions were pooled. These fractions (about 600 ml) were dried at 40 °C in a rotary evaporator under vacuum.

This mixture was dissolved in 80 ml water and the entire sample was percolated over a 20 ml column of anion-exchange resin (Bio-Rad AG1-X8, 25–50 mesh, OH⁻ form). Agropine was detected in the effluent after 20 ml had been eluted, and the column was washed with 10 mM-aq. NH₃ (to avoid desorption of mannopine) until no more of the lactone was detected in the eluate. After rinsing with 50 ml of water, mannopine and retained agropine were recovered from the column by elution with 1 M-acetic acid (175 ml).

Chemical transformation of agropine to agropinic acid. Agropine (1 mg) was dissolved in 100 μ l 10 mM-NaOH and heated in a sealed tube for 1 h at 100 °C. After cooling, 1 μ l of the mixture was analysed by HVPE at pH 1.9.

Fig. 2. Opine conversions by whole cells. *A. tumefaciens* strain ACH5 was grown in AT medium supplemented with mannopine. The cells were harvested, washed and resuspended in AT medium containing: (a) mannopine for 1 min; (b) agropine for 240 min. After these incubations the cells were harvested, washed and assayed for opine content by HVPE as described in Methods. Abbreviations: M, mannopine; A, agropine; NS, neutral sugars.

RESULTS

In vivo studies

In order to understand how mannityl opines are catabolized by *A. tumefaciens*, we first determined whether metabolic intermediates could be detected in cells grown with various members of this opine family. Strain ACH5 was grown with mannopine as sole source of carbon. The cells were then harvested, washed, incubated with the same opine or with agropine, and analysed for intracellular products by HVPE as described in Methods. As shown in Fig. 2, such cells rapidly converted the added mannopine to a compound co-migrating with agropine. Cells incubated with agropine accumulated this lactone opine; however, after prolonged incubation, small amounts of a compound co-migrating with mannopine could be electrophoresed from the cells.

In vitro analysis

We examined cell-free extracts of strain ACH5 for an activity which would convert mannopine to agropine. Extracts prepared as described in Methods were incubated with mannopine or with agropine. *In vitro* assays were first improved with regard to buffer, pH and substrate concentration. The lactonizing activity was best demonstrated in 50 mM-potassium phosphate buffer, pH 7.5, with an optimal concentration of 2–6.5 mM-mannopine. The reverse activity, conversion of agropine to mannopine, was highest in 50 mM-glycine/NaOH buffer, pH 10.0, with an optimal substrate concentration of 5–10 mM (data not shown). As shown in Fig. 3, the extracts carried out a time-dependent conversion of mannopine to agropine (identified as indicated below). When agropine was added to such extracts, a compound electrophoresing with the same mobility as mannopine was detected. However, the rate of appearance of this compound was far less than seen for agropine when mannopine was added as substrate (compare Figs 4a and 4b).

Fig. 3. Mannopine-lactonizing activity in cell-free extracts. Extracts from various *Agrobacterium* strains were prepared and incubated with mannopine as described in Methods. Samples were removed from the incubations at the times (in min) indicated, treated with acetic acid and spotted on Whatman 3MM paper for analysis by HVPE in formic acid/acetic acid buffer, pH 1.9. (a) Extract from the Ti plasmidless strain C58C1RS pregrown in AT supplemented with mannose. Similar results were obtained with strain ACH5C3 or when either of these strains was grown in AT supplemented with both mannose and mannopine. (b) Extract from the octopine strain ACH5 pregrown in AT supplemented with mannose. Similar results were obtained with C58C1RS(pTiACH5). (c) Extract from strain ACH5 pregrown in AT supplemented with both mannose and mannopine. Similar results were obtained with C58C1RS(pTiACH5). The degree of conversion at the 60 min points was judged as 0 (a), + (b) and +++ (c) on the scale described in Table 2. Abbreviations are as described in Fig. 2 and: AA, agropinic acid; S, standard mix containing agropine, mannopine and agropinic acid.

Identification of the product as agropine

In order to characterize the compound produced from mannopine, the *in vitro* assay was scaled up to a preparative level (see below). The product from this reaction, purified by ion-exchange chromatography (see below and Methods), was crystallized from methanol (Tate *et al.*, 1982) and identified as agropine by the following criteria.

The crystalline material melted at 181 °C (reported melting point for agropine, 177/178 °C; Tate *et al.*, 1982), and its IR and NMR spectra (data not shown) were identical to those of agropine (Tate *et al.*, 1982).

The crystalline product co-chromatographed with agropine in 1-butanol/acetic acid/water (3:1:1, by vol.) and co-electrophoresed with agropine in ammonium carbonate (5 g l⁻¹, pH 9.8) and in acetic acid/formic acid (pH 1.9) buffer systems. Heating the product under alkaline conditions yielded agropinic acid (Coxon *et al.*, 1980; Tate *et al.*, 1982).

Finally, the product was tested for its biological activity. In growth experiments, the utilization pattern of the product by various *A. tumefaciens* and *A. rhizogenes* strains was identical to that of agropine. Strains C58C1RS, ACH5C3, 8196 and C58C1RS(pArA4a) did not grow on medium containing the product as sole carbon source, whereas strains R10, ACH5, HRI and C58C1RS(pRiA4) grew well on this medium.

It is clear from these results that the product is indeed agropine and that strain ACH5 contains an activity capable of lactonizing mannopine to agropine.

Characterization of the mannopine-lactonizing activity

Assayed under the conditions described in Methods, the lactonizing activity was stable when samples were preincubated for 5 min periods at temperatures up to 55 °C (Fig. 4 a). In addition, cell-free extracts could be kept for as long as 50 h at room temperature without appreciable loss of activity (see below). However, the activity was completely destroyed by heating the extracts at 65 °C for 5 min.

The specificity of the activity was determined by examining the lactonization of various

Fig. 4. Heat sensitivity of mannopine-lactonizing and agropine-converting activities. Cell-free extracts from strain ACH5 grown on mannopine-containing medium (10 mM) were treated for 5 min at various temperatures, cooled on ice, and incubated with mannopine (a) or agropine (b), at 30 °C, as described in Methods. Abbreviations are as described in Figs 2 and 3. The spots close to the origin are ethylene glycol.

mannityl opine analogues. Galactopine [*N*2-(1'-deoxygalactitol-1'-yl)-L-glutamine: Chilton & Chilton., 1984; P. Guyon & J. Tempé, unpublished] was converted to a compound which co-electrophoresed with the corresponding chemically prepared lactone (data not shown), albeit with less efficiency as compared with mannopine. The activity had no detectable effect on mannopinic acid, or on galactopinic acid [*N*2-(1'-deoxygalactitol-1'-yl)-L-glutamic acid: Tempé *et al.*, 1980; Chilton & Chilton, 1984), as judged by HVPE.

Mannopine-lactonizing activity associated with Ti and Ri plasmids

Cell-free extracts from various agrobacteria were examined for the ability to lactonize mannopine. The activity could be detected only in extracts from strains harbouring octopine Ti or agropine Ti or Ri plasmids (Table 2). Strain C58C1RS, which has no Ti plasmid, lacked the activity. However, when pTiACH5, an octopine-type Ti plasmid, was introduced into this strain, it acquired the activity. Similar results were obtained when ACH5 and its Ti-plasmid-cured derivative, ACH5C3, were examined. The activity was present in strain HRI, an *A. rhizogenes* strain able to catabolize agropine, but was undetectable in *A. rhizogenes* 8196, which

Table 2. *In vitro* assays of mannopine-lactonizing activity in various *Agrobacterium* strains

Strain	Mannopine-lactonizing activity*		
	Growth medium† :		
	Man	Man + Mop	Man + Agr
Wild-type <i>A. rhizogenes</i>			
HRI	+	+	+++
8196	0	0	0
Wild-type <i>A. tumefaciens</i> and their derivatives			
ACH5	+	+++	+++
ACH5C3	0	0	0
R10	+	+++	+++
E20.8	0	0	0
Bo542	+	+++	+++
C58	ND	0	ND
C58C1RS	0	0	0
Transconjugants obtained into C58C1RS			
C58C1RS(pTiACH5)	+	+++	+++
C58C1RS(pArA4a)	0	0	0
C58C1RS(pArA4b)	+	+	+++
C58C1RS(pA4abc)	+	+++	+++
Transformants obtained into NT1			
NT1(pJS4159K1) to NT1(pJS4159K5)	0	0	0
NT1(pJS4159K6)	+	‡	+
NT1(pJS4159K7) to NT1(pJS4159K10)	0	0	0
Transformant and transconjugant obtained into C58C1RS			
C58C1RS(pRi8196)(pJS4159K6)	+	+++	ND

* Mannopine-lactonizing activity in bacterial extracts was assayed as described in Methods. The activity was estimated after 1 h incubation by visual inspection of electrophoretograms as follows (see also Fig. 2): 0, no agropine detected (no visible spot of agropine); +, 20–50% of the silver-nitrate positive material appeared as agropine; +++, more than 90% of the silver-nitrate-positive material was agropine; ND, not determined.

† Cells were grown in AT minimal medium supplemented with $(\text{NH}_4)_2\text{SO}_4$ (0.2%) and with the carbon source indicated. Abbreviations and final concentrations: Man, mannose (0.2%); Mop, mannopine (2 mM); Agr, agropine (2 mM).

‡ A low induction of the mannopine-lactonizing activity can be observed in this condition.

cannot utilize this opine. Furthermore, as judged with the C58C1RS transconjugants (Table 2), the activity appeared to be associated with the Ri plasmid of strain A4, a strain which contains a cointegrate plasmid system (White & Nester, 1980). Finally, we were able to locate the determinant for the lactonizing activity to *Kpn*I fragment 6 (nomenclature system according to De Vos *et al.*, 1981) of the octopine-type Ti plasmid, pTi15955 (Table 2). Consistent with this, strain E20.8, an agropine non-catabolizing Tn7 insertion mutant of strain B6 (De Greve *et al.*, 1981) lacks the activity. In this mutant, the transposon maps to a position within *Kpn*I fragment 6 of pTiB6 (De Greve *et al.*, 1981).

Inducibility of the lactonizing activity

The mannopine-lactonizing activity was low in cells grown in media lacking agropine or mannopine (Table 2). Nor could high activities be demonstrated in cells pregrown with mannopinic acid or agropinic acid (data not shown). However, when the agropine-utilizing strains were grown in the presence of either mannopine or agropine the activity was easily detected. Interestingly, the activity in extracts from strain HRI, or from strain C58C1RS(pArA4b), was not increased when the cells were grown in the presence of mannopine. In addition, strain NT1(pJS4159K6) showed low activities under all growth conditions tested.

The results presented above suggest that the mannopine-lactonizing activity is inducible during growth on both mannopine and agropine. However, strain NT1(pJS4159K6) failed to show high levels of activity when incubated with either opine. Nor could this strain utilize either agropine or mannopine as sole carbon source. We introduced pJS4159K6 into NT1 containing pRi8196, which encodes a mannopine transport system, but lacks mannopine lactonase activity. When this construct was grown in the presence of mannopine, the lactonizing activity was increased to a level similar to that seen in the inducible wild-type strain. However, this strain remained unable to grow on agropine as sole carbon source.

Preparative enzymic synthesis of agropine

Of the mannityl opines, agropine is the most difficult to synthesize chemically and the product yield is low (about 10% of the input mannopine precursor). Because the mannopine-lactonizing activity appeared to be stable *in vitro*, and because high levels could be found in cells induced by preincubation with mannopine, we sought to determine if the system could be used for the enzymic synthesis of agropine on a preparative scale.

Cells from 2 l of culture were harvested and washed, and an extract was prepared as described in Methods. When mannopine was added to the extract at a concentration of 65 mM, about 85% was converted to agropine in 20 min. Given this rapid conversion, a system was set up whereby mannopine at a concentration of 65 mM was continuously added to the reaction mixture at a rate of 20 ml h⁻¹; reaction products were removed from the vessel at an equal rate (see Methods). Under these conditions, the composition of the reaction mixture remained stable for at least 50 h. However, during such prolonged incubations, flow rates generally decreased due to coating of the ultrafiltration units with a sticky material. When flow rates became unacceptable, the units could be disassembled, washed with distilled water and reassembled without major reduction in the efficiency of the system.

The reaction constituents contained in the ultrafiltration collection flask were preparatively fractionated by ion-exchange chromatography on sulphonated polystyrene resin. Agropine-containing fractions eluted from this column were identified by HVPE, pooled and concentrated *in vacuo*, to yield 15.2 g of a white powder. Analysis by HVPE showed this to be a mixture of about 80% agropine and 20% mannopine. Contaminating amounts of mannopine were removed by chromatography on an anion-exchange column (see Methods). After rotary evaporation the agropine fraction weighed 11 g (63% yield). This fraction was estimated by HVPE to be 98% pure agropine. The acidic eluate from the anion-exchange column yielded about 4.2 g product, judged by HVPE to be approximately 70% mannopine and 30% agropine.

DISCUSSION

Little is known about the pathways involved in the catabolism of the mannityl opines by *Agrobacterium*. In this work we describe an activity apparently associated with the catabolism of agropine, the lactonized derivative of mannopine. The activity was first identified in whole cells by its ability to cause the lactonization of mannopine (Fig. 2). This lactonizing activity was easily demonstrated in cell-free extracts from cells previously grown with either mannopine or agropine. Physical and chemical analysis established that the lactone product formed by this activity is agropine. The fact that only agropine-catabolizing *Agrobacterium* strains are able to utilize the product and that the pattern of utilization is identical to that of authentic agropine (Table 1) is strong biological proof for its identity. Two lines of evidence indicate that the lactonization of mannopine is due to a real enzyme activity. First, the activity is heat-sensitive (Fig. 4a); second, the activity, barely detectable in cells grown with mannose, is greatly elevated in cells grown with mannopine or agropine. This induction effect shows that the activity is not a nonspecific consequence of adding mannopine to crude, cell-free extracts.

The lactonizing activity is present only in *Agrobacterium* strains harbouring specific Ti or Ri plasmids. Agropine-type strains lose the activity when cured of their Ti plasmids. When a Ti or Ri plasmid from an agropine-catabolizing strain is introduced into a cured recipient, the activity is readily detectable (Table 2). Furthermore, the lactonizing activity is specific to Ti or Ri

plasmids from strains able to utilize agropine, and is not detectable in agrobacteria harbouring pTiC58 or pRi8196. Nor are these strains able to grow on agropine as sole source of carbon. It is interesting that the lactonizing activity appears to be associated with the Ri plasmid component from *A. rhizogenes* strain A4. This plasmid only encodes the catabolism of agropine. Utilization of mannopine and mannopinic acid is associated with pArA4a (Petit *et al.*, 1983).

Genetic analysis has allowed us to localize the determinant for the mannopine-lactonizing activity on the octopine-type Ti plasmid (e.g. from strains ACH5, B6, R10 or 15955). When strains harbouring recombinant clones containing various *KpnI*-generated inserts of pTi15955 were examined, only the construct containing *KpnI* fragment 6 showed activity (Table 2). This is consistent with our observation that a Tn7 insertion located in the *KpnI* fragment 6 of pTiB6 abolished the ability of this strain to grow on agropine (De Greve *et al.*, 1981) and simultaneously its mannopine-lactonizing activity (Table 2).

The function of the lactonizing activity is unclear. However, in strains harbouring wild-type plasmids the activity is associated with the catabolism of agropine. Mutants lacking the activity fail to catabolize agropine. Furthermore, the activity is inducible by exogenous agropine. However, at least *in vitro*, the activity preferentially converts mannopine to agropine. While the reverse reaction can be detected in both whole cells and cell-free extracts (Figs 2 and 4*b*), it has not been established that both are carried out by the same enzyme, even though the thermosensitivity pattern of the two activities is identical (Fig. 4*a, b*). The lactonizing activity is not sufficient for the catabolism of agropine. When pJS4159K6 was introduced into a strain harbouring pRi8196, the construct remained unable to utilize the lactone opine (Table 1). This may be due to the lack of an agropine transport system. Interestingly, the construct harbouring the cloned *KpnI* fragment 6 and pRi8196 was inducible for agropine lactonase, while the corresponding strain without pRi8196 had only a low uninducible level of the activity. One explanation is that pRi8196 only provides a mannopine transport system which allows induction of the agropine lactonase determinant located on the *KpnI* fragment 6. It could be also suggested that the activity is involved in the catabolism of mannopine by first converting it to agropine. Consistent with this is our observation that cells grown with mannopine accumulated substantial amounts of intracellular agropine. However, mutants lacking the lactonizing activity are still able to utilize mannopine.

Although the physiological role for this activity is unknown, the enzymic conversion of mannopine to agropine can be used for the preparative synthesis of this lactone. Until now, agropine has been prepared by chemical lactonization of mannopine (Tate *et al.*, 1982; Petit *et al.*, 1983). The reported yields for this reaction are low, the main product formed being agropinic acid (90%). Attempts to improve this yield by changing the reaction conditions have been unsuccessful (M. Tate, personal communication and our own results). With the enzymic reaction conditions we describe, we can routinely prepare gram quantities of agropine in yields approaching 70%. The product obtained after two chromatographic separations is of sufficient purity for use as a selective growth substrate for agropine- or octopine-type *Agrobacterium* strains. The activity is inducible, and is stable for at least 50 h at room temperature, making it an ideal system for an enzyme-based preparative synthesis.

This work was supported in part by grants from Centre National de la Recherche Scientifique, from Institut National de la Recherche Agronomique and from Ministère de l'Industrie et de la Recherche. Stephen K. Farrand was supported by grants CA19401 from the National Cancer Institute and 84-CRCR-1-1421 from the United States Department of Agriculture.

The authors thank Dr Marie-Hélène Brillanceau for determination of physical constants of agropine, and Dr Max E. Tate for helpful discussions and for assistance during preparation of this manuscript.

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