Genetic and biochemical characterization of an enantioselective amidase from Agrobacterium tumefaciens strain d3

Sandra Trott, Reinhard Bauer, Hans-Joachim Knackmuss and Andreas Stolz

An enantioselective amidase was purified to homogeneity from Agrobacterium tumefaciens d3. The enzyme has a molecular mass of about 490000 Da and is composed of identical subunits with a molecular mass of about 63000 Da. The purified enzyme converted racemic 2-phenylpropionamide to the corresponding S-acid with an enantiomeric excess (ee) value > 95% at almost 50% conversion of the racemic amide. The purified enzyme was digested with trypsin and the amino acid sequences of the N terminus and different tryptic peptides determined. These amino acid sequences were used to clone the encoding gene. Finally, a 9330 bp DNA fragment was sequenced and the amidase gene identified. The deduced amino acid sequence showed homology to other enantioselective amidases from different bacterial genera. No indications of a structural coupling of the amidase gene with the genes for a nitrile hydratase could be found on the cloned DNA fragment. The amidase gene was encoded by an approximately 500 kb circular plasmid in A. tumefaciens d3. The amidase was heterologously expressed in Escherichia coli and, as well as 2-phenylpropionamide, was shown to hydrolyse α-chloro- and α-methoxyphenylacetamide and 2-methyl-3-phenylpropionamide highly enantioselectively. Some amino acids within a highly conserved region common amongst all known enantioselective amidases (‘amidase signature’) were changed by site-specific mutagenesis and significant changes in the relative activities with different amides observed.

Keywords: biotransformation, substituted phenylacetamides

INTRODUCTION

The enzymic hydrolysis of nitriles represents a very convenient synthetic method for the production of amides and or carboxylic acids under mild reaction conditions. Furthermore, these enzymic reactions also allow the enantioselective synthesis of optically active amides and carboxylic acids from racemic precursors (Bunch, 1998; Kobayashi & Shimizu, 1994; Sugai et al., 1997).

We have recently described the isolation of bacterial strains with the ability to produce enantiomerically almost pure (S)-2-arylpropionic acids from the corresponding racemic nitriles and amides. These cultures were obtained from enrichments with different nitriles as sole nitrogen source (Layh et al., 1997). Thus, the isolate Agrobacterium tumefaciens d3 was obtained after enrichment with 2-phenylpropionitrile which converted racemic 2-phenylpropionitrile, 2-phenylbutyronitrile, naproxen nitrile, ketoprofen nitrile, ibuprofen nitrile and 2-aminophenylacetamide to the corresponding S-acids with enantiomeric excess (ee) values above 99% (Bauer et al., 1994; Layh et al., 1997). The enantioselectivity of these reactions was due to the coupled effects of an enantioselective nitrile hydratase and an enantioselective amidase. We have reported the purification of the enantioselective nitrile hydratase from A. tumefaciens d3 and shown that this enzyme converted different 2-arylpropionitriles with a rather low enantioselectivity to the corresponding S-amides (Bauer et al., 1998). This suggested that the observed...
high enantiospecificity for the conversion of the racemic nitriles to the corresponding S-acids was mainly due to a highly enantioselective amidase. Enantioselective amidases have been studied from bacterial strains (mainly from rhodococci and pseudomonads) (Gilligan et al., 1993; Kobayashi et al., 1993; Mayaux et al., 1990, 1991; Nishiyama et al., 1991) but the functional coupling of an enantioselective nitrile hydratase with an enantioselective amidase has not been described before.

In the present study the enantioselective amidase from A. tumefaciens d3 was purified and the coding gene cloned to further characterize this extraordinary combination of two enantioselective enzymes which allows the production of almost enantiopure S-2-arylpropionic acids from racemic nitriles. Furthermore, we tried to establish a structure–function relationship for the conversion of various amidases carrying different substituents adjacent to the amide group with respect to the reaction rate and the enantioclectivity of the amidase.

**METHODS**

**Bacterial strains, media and plasmids.** The isolation and characterization of A. tumefaciens d3 (DSM 9674) has been reported previously (Layh et al., 1997). For the purification of the amidase, strain d3 was grown in a mineral medium with succinate as sole source of carbon and energy and phenylacetonitrile (1 mM) as sole source of nitrogen as described previously (Layh et al., 1997). For the isolation of genomic DNA, strain d3 was routinely grown in Luria–Bertani (LB) medium. *Escherichia coli* DH5α and *E. coli* JM109 were used as host strains for recombinant DNA work. *E. coli* strains were routinely cultured at 37 °C in LB medium which was supplemented with ampicillin (100 μg ml⁻¹), if appropriate. pBluescript II KS(+) (Alting-Mees et al., 1992) was used for most cloning experiments and the t-rhamnose-inducible plasmid vector pJOE2702 (Vollf et al., 1996) for high levels of expression.

**Analytical methods.** Amides and acids were analysed by HPLC (HPLC pump 510, Autosampler 717 plus photo diode array detector 996, HPLC Millenium Chromatography Manager 3.05.01; Waters Associates).

If separation of enantiomers was unnecessary a reversed-phase column (Grom-Sil C8; Grom) was used. The aqueous solvent systems usually contained 30–60% (v/v) methanol plus 0.3% (v/v) H₃PO₄. Alternatively, for the separation of z-aminophenylacetamide and z-aminophenylacetic acid the solvent system consisted of 15% (v/v) methanol plus 83% (v/v) sodium phosphate buffer (pH 2.2, 10 mM) and 2% (v/v) of an ion-pair reagent according to the instructions of the manufacturer (PIC B6; Waters).

The columns and solvent systems used for the enantioselective separation of various chiral amides and carboxylic acids are shown in Table 1.

**Calculation of the enantiomeric excess and the enantiomeric ratio.** The enantiomeric excess (ee) of the products was defined as:

\[
\text{ee}^\text{p} = \frac{(S-R)}{(S+R)}
\]

where \( p \) = product. The enantiomeric ratio (E) for the acids formed was calculated from the conversion (c) and the ee value of the acid according to Chen et al. (1982) as follows:

\[
E = \frac{\ln[1-(1+\text{ee}^\text{p})]}{\ln[1-(1-\text{ee}^\text{p})]}
\]

**Preparation of cell-free extracts.** Cell suspensions of *A. tumefaciens* d3 and *E. coli* in 50 mM Tris/HCl buffer (pH 7.5) or 50 mM sodium/potassium phosphate buffer (pH 7.4), respectively, were disrupted by using a French Press (Aminco) at 80 or 125 MPa. Cell debris was removed by centrifugation at 100000 g for 30 min at 4 °C. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

**Standard assay for the determination of enzyme activities with cell-free extracts and purified enzyme preparations.** Amide hydrolysing activity was assayed routinely in reaction mixtures (0.5 ml) composed of 25 μmol Tris/HCl buffer (pH 7.5) or 25 μmol sodium/potassium phosphate buffer

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**Table 1.** Columns and solvent systems used for chiral HPLC analysis of various \( \alpha \)-substituted arylacetamides, \( \alpha \)-substituted arylpropionamides and the corresponding acids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column*</th>
<th>Mobile phase</th>
<th>Modifier</th>
<th>Retention volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Phenylpropionamide</td>
<td>AGP</td>
<td>NaH₃PO₄, 10 mM, pH 4.6</td>
<td>–</td>
<td>3.5</td>
</tr>
<tr>
<td>2-Phenylpropionic acid</td>
<td>AGP</td>
<td>NaH₃PO₄, 10 mM, pH 4.6</td>
<td>–</td>
<td>8.8 (R)</td>
</tr>
<tr>
<td>( \alpha )-Chlorophenylacetamide</td>
<td>HSA</td>
<td>Na₃PO₄, 100 mM, pH 7.0</td>
<td>2-Propanol (7%)</td>
<td>2.7</td>
</tr>
<tr>
<td>( \alpha )-Chlorophenylacetic acid</td>
<td>HSA</td>
<td>Na₃PO₄, 100 mM, pH 7.0</td>
<td>2-Propanol (7%)</td>
<td>6.9</td>
</tr>
<tr>
<td>Mandeloamide</td>
<td>HSA</td>
<td>Na₃PO₄, 10 mM, pH 6.0</td>
<td>2-Propanol (2%)</td>
<td>1.8</td>
</tr>
<tr>
<td>Mandelic acid</td>
<td>HSA</td>
<td>Na₃PO₄, 10 mM, pH 6.0</td>
<td>2-Propanol (2%)</td>
<td>6.3 (S)</td>
</tr>
<tr>
<td>( \alpha )-Methoxyphenylacetamide</td>
<td>HSA</td>
<td>Na₃PO₄, 100 mM, pH 6.0</td>
<td>2-Propanol (2%)</td>
<td>2.0</td>
</tr>
<tr>
<td>( \alpha )-Methoxyphenylacetic acid</td>
<td>HSA</td>
<td>Na₃PO₄, 100 mM, pH 6.0</td>
<td>2-Propanol (2%)</td>
<td>5.0 (R)</td>
</tr>
<tr>
<td>2-Methyl-3-phenylpropionamide</td>
<td>AGP</td>
<td>Na₃PO₄, 10 mM, pH 6.0</td>
<td>–</td>
<td>5.6</td>
</tr>
<tr>
<td>2-Methyl-3-phenylpropionic acid</td>
<td>AGP</td>
<td>Na₃PO₄, 10 mM, pH 6.0</td>
<td>–</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* AGP column: column size 100 × 4 mm, 5 μm silicagel particles with \( \alpha \)-acid glycoprotein (ChromTech). HSA column: column size 150 × 4 mm, 5 μm silicagel particles with human serum albumin (ChromTech).
(pH 7.4), 2.5 µmol phenylaceticamide (stock solution, 100 mM in methanol) and different amounts of protein (1–600 µg). The reaction was performed at room temperature in a plastic reaction tube. After different time intervals aliquots were taken (100 µl each), the reaction stopped by the addition of 10 µl 1 M HCl and the precipitated protein removed by centrifugation (5 min, 14000 r.p.m.). The formation of the corresponding acid was determined by HPLC. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 µmol product min⁻¹.

**Enzyme purification.** Protein was purified at room temperature (~ 24°C) by use of a fast-performance liquid chromatography system consisting of a LCC 500 controller, pump P-500, UV-1 monitor, conductivity monitor, REC-482 recorder and FRAC autosampler from Amersham Pharmacia Biotech.

Crude extract (76 mg protein in 8 ml 6 O Amidase activity) was applied in two subsequent batches (with 38 mg protein each) to a Resource Q column (Amersham Pharmacia Biotech). Protein was eluted with 60 ml of a linear gradient of Tris-HCl (50 mM, pH 7.5) to Tris/HCl (50 mM, pH 7.5)/1 M NaCl at a flow rate of 1 ml min⁻¹. All buffers used contained additionally 2 mM dithiothreitol. Fractions (1 ml) were collected and enzyme activity determined by HPLC. The amidase was eluted as a single peak at a concentration of about 0.25 M NaCl. The active fractions from both chromatographic steps were pooled (3–4 mg protein in 3–8 ml, 1 U amidase activity) and 1 M (NH₄)₂SO₄ was added. The solution was incubated for 15 min on ice and finally filtered (Minisart NML, 0.2 µm pore size; Sartorius). This filtrate was transferred to a Phenyl-Superose column (HR 5/5; Amersham Pharmacia Biotech) and eluted with 60 ml of a linear gradient of Tris/HCl (50 mM, pH 7.5)/1 M (NH₄)₂SO₄ to Tris/HCl (50 mM, pH 7.5) at a flow rate of 0.5 ml min⁻¹. Fractions of 1 ml were collected. The active fractions eluted at a concentration of about 0.45 M (NH₄)₂SO₄ (0.54 mg protein in 5–5 ml, 0.45 U amidase activity). The amidase was concentrated by ultrafiltration (Centricon 30; Amicon) and the concentrated sample applied to a Superdex 200 prep grade HIlod 16/60 column and eluted with 90 ml Tris/HCl (50 mM, pH 7.5)/0.15 M NaCl at a flow rate of 0.8 ml min⁻¹. Fractions with amidase activity were pooled (0.38 mg protein in 6–9 ml, 0.45 U amidase activity) and applied to a Mono-Q column (HR 5/5). Protein was eluted with 60 ml of a linear gradient of Tris/HCl (50 mM, pH 7.5) to Tris/HCl (50 mM, pH 7.5)/1 M NaCl at a flow rate of 0.5 ml min⁻¹. The fraction with amidase activity (0.21 mg protein in 2–0 ml) was eluted at an NaCl concentration of 0.3 M.

**PAGE.** SDS-PAGE was performed by the method of Laemmli (1970). Gels were silver-stained by the method of Merrif et al. (1981) using the Amersham Pharmacia Biotech silver stain kit.

**Determination of molecular mass.** The relative molecular mass of the native enzyme was determined by gel filtration as described previously (Hirrlinger et al., 1996).

**Protein cleavage, isolation of peptides, and sequencing of peptides and N terminus.** Digestion of the amidase by trypsin and subsequent separation of tryptic digests by reversed-phase HPLC were performed as described by Eulberg et al. (1997) and Stone et al. (1989). Amino acid sequences were determined by automated Edman degradation using an Applied Biosystems model 491 sequencer.

**DNA manipulation techniques.** The genomic DNA was prepared as described by Ausubel et al. (1987). Plasmid DNA from E. coli DH5α was isolated with the Flexi-Prep kit (Amersham Pharmacia Biotech) or the Qiagen Spin Miniprep kit (Qiagen). Digestion of DNA with restriction endonucleases (Gibco-BRL; New England Biolabs), electrophoresis, purification and ligation with T4 DNA ligase (Gibco-BRL) were performed according to standard procedures (Sambrook et al., 1989). Transformation of E. coli was done by the method of Inoue et al. (1990).

**PCR.** Oligonucleotides were custom-synthesized according to the known or deduced sequences of the N-terminal amino acid sequence and various internal peptides. PCR mixtures (50 µl) for the amplification of genomic DNA contained 50 pmol each primer, 0.1 µM each deoxynucleotide triphosphate, 0–7.5% (w/v) dimethylsulfoxide, 1.5 mM MgCl₂, 0.7 U Taq DNA polymerase and the corresponding reaction buffer (Gibco-BRL).

For the amplification reaction with the primers deduced from the N-terminal sequence and peptide P6198, the following PCR program was used: an initial denaturation (95°C, 3 min, addition of the Taq polymerase after 2 min) was followed by 29 cycles consisting of annealing at 50°C, 1.5 min, polymerization at 72°C, 2 min, and denaturation at 95°C, 40 s. The last polymerization step was extended to 10 min.

The PCR products were initially cloned into the T-tailed EcoRV-site of pBluescript II KS(+) (Marchuk et al., 1991).

**Hybridization procedures.** A DIG DNA labelling and detection kit was used according to the instructions of the supplier (Boehringer Mannheim). The hybridization temperature was set to 68°C.

**DNA sequencing and nucleotide sequence analysis.** The DNA sequence was determined by dideoxy-chain termination with double-stranded DNA of clones and overlapping subclones in an automated DNA-sequencing system (ALFexpress-sequencer; Amersham Pharmacia Biotech) with fluorescently labelled primers or nucleotides.

Sequence analysis, database searches and comparisons were done with the PCGene software package, release 6.85 and the BLAST search facilities at NCBI. The alignment of amidases was obtained with the program CLUSTAL using the default parameters.

**Expression of amidase in E. coli.** For expression in E. coli, the amidase gene was inserted in the plasmid vector pJOE2702 (Volf et al., 1996) under the control of an L-rhamnose-inducible promoter. The DNA segment encompassing the amidase gene was amplified by PCR with simultaneous introduction of an NdeI site upstream and a HindIII site downstream of the amidase gene. The amplified product was cleaved with NdeI and HindIII and ligated into pJOE2702. E. coli DH5α was transformed with the resulting plasmid (pST2WT) and the intactness of the amidase gene was verified by DNA sequencing. The plasmid was subsequently isolated and introduced into E. coli JM109 by transformation.

For induction, 0.2% (w/v) L-rhamnose was added to the culture (OD₆₀₀ 0.2–0.3) in LB/ampicillin medium. Induction was performed for 6 h at 30°C.

**Site-specific mutagenesis.** The mutants were produced from pST2WT by using a QuikChange site-directed mutagenesis kit from Stratagene. The generated mutants were verified by sequencing of the mutated gene.

**Preparation of plasmids for the detection of megaplasmids.** The isolation of plasmids from strain d3 was basically performed as described by Barton et al. (1995). Cells were embedded in low-melting-point agarose (0.5%) and treated with lysozyme, Brij58, N-lauroylsarcosinate and proteinase K. These agarose plugs (about 9 × 4 × 1.2 mm) were incubated for different time intervals (2–5 min) at 37°C with 1 U S1.

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nuclease and directly used for contour-clamped homogeneous field (CHEF) electrophoresis (Chu et al., 1986). The electrophoresis was performed by using the CHEF mapper system of Bio-Rad. The agarose slab gels (0.8%, 12.7 × 14 × 0.5 cm) were run in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 14°C at 6 V cm⁻¹ for 19 h with a constant pulse time of 80 s. Chromosomes from *Saccharomyces cerevisiae* YNN295 (Amersham Pharmacia Biotech) were used as high-molecular-mass DNA standards.

**Chemicals.** α-Chlorophenylacetamide was synthesized from mandeloamide and thionylchloride as described by Bergmann et al. (1934) and methanolic ammonia according to Ault (1934). 

**RESULTS**

**Purification, molecular mass and subunit structure of the enantioselective amidase**

The amidase was purified from cell extracts by a combination of anion-exchange chromatography, hydrophobic interaction chromatography and gel filtration as described in Methods. The enzyme was purified about 15-fold, giving a specific activity with phenylacetamide (5 mM) as substrate of 1.2 U (mg protein)⁻¹. The purified enzyme gave a single band by SDS-PAGE (0.34 μg enzyme) with a molecular mass of approximately 63,000 Da. By gel filtration the molecular mass of the holoenzyme was estimated to be 490,000 Da. Therefore it can be assumed that the enzyme is a homo-octamer.

**Enantioselectivity of the purified amidase**

Previously, the enantioselective hydrolysis of racemic 2-phenylpropionamide was demonstrated with whole cells of *A. tumefaciens* d3 (Bauer et al., 1994). This reaction was also catalysed by the purified amidase. From racemic 2-phenylpropionamide, S-2-phenylpropionic acid was preferentially formed. After 30% conversion the ee value of S-2-phenylpropionic acid was above 99% and after 44% conversion it was still 97%. Thus about the same degree of enantioselectivity was observed as with resting cells of strain d3.

**Determination of the N-terminal and some internal amino acid sequences**

The N-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation and compared with the N-terminal amino acid sequences of other (enantioselective) amidases. It did not show any significant sequence similarity to other known amidase sequences. To obtain more sequence information, the purified protein was digested with trypsin and the fragments separated by HPLC. Thus the sequences of four fragments were determined. One of these fragments (P6198; Table 2) showed significant homologies with conserved regions within other enantioselective amidases (e.g. aa 452–468 in the enzymes *Pseudomonas chlororaphis* B23 and *Rhodococcus* sp. R312).

**Cloning of the amidase gene**

The N-terminal amino acid sequence and the sequence of peptide P6198 served for the design of oligonucleotide primers (Table 2). Thus with genomic DNA of strain d3 as template, a 1.4 kb DNA fragment was amplified. The PCR product was cloned into a pBluescript II KS(+) T- vector and the insert sequenced. In the deduced amino acid sequence the complete N-terminal amino acid sequence was found which was previously obtained by Edman degradation of the purified protein. It was therefore deduced that indeed a fragment of the amidase gene from strain d3 had been amplified. The PCR fragment was DIG-labelled and used as a probe to identify, by Southern hybridization, an EcoRI fragment of about 6 kb from the total DNA from strain d3, which had been cloned into pBluescript II KS(+). The plasmid with the amidase gene was designated pAAM1.

To obtain the genes immediately downstream of the amidase gene a probe was constructed by PCR from the terminal region of the insert of plasmid pAAM1 and used to clone a PstI fragment of about 3.5 kb from the genomic DNA in pBluescript II KS(+) (resulting in pAAM2).

**Determination of the nucleotide sequence of the amidase gene and the surrounding DNA fragments**

The DNA sequences of the inserts in plasmids pAAM1 and pAAM2 were determined. Thus a continuous DNA sequence of 9330 bp was obtained. The gene for amidase (*amdA*) was found almost in the middle of the sequenced

### Table 2. Sequences of the N terminus, tryptic peptides and deduced primers

Segments used for the design of oligonucleotides for PCR are underlined and uncertain amino acids are shown in square brackets.

<table>
<thead>
<tr>
<th>Protein or peptide</th>
<th>Amino acid sequence</th>
<th>Deduced primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6198</td>
<td>NTA[V] E DLTG [H/A] P [S] M [S/T] VPE [G/T] [K/L]</td>
<td>AGCTC (CT) TC (GCT) AC (GCT) GC (AGCT) GT (AG) TT</td>
</tr>
</tbody>
</table>

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Fig. 1. Genetic organization of the sequenced 9.3 kb DNA fragment. Plasmids pAAM1 and pAAM2 encoded the 6.2 kb EcoRI fragment and the 3.9 kb PstI fragment indicated, respectively. The positions and orientations of the different ORFs detected within the locus are shown by large arrows.

Table 3. Genes and gene products from the sequenced DNA fragment

<table>
<thead>
<tr>
<th>ORF</th>
<th>Position in sequence</th>
<th>Probable function of product</th>
<th>Size (aa)</th>
<th>Protein with homologous sequence</th>
<th>Source</th>
<th>Percentage identity*</th>
<th>GenBank accession no. for homologous proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1071</td>
<td>Membrane-associated</td>
<td>335</td>
<td>YIJE_ECOLI</td>
<td>Escherichia coli</td>
<td>26.3</td>
<td>P32667</td>
</tr>
<tr>
<td>2</td>
<td>1119</td>
<td>Lrp regulator</td>
<td>160</td>
<td>LRP_ECOLI</td>
<td>Escherichia coli</td>
<td>42.5</td>
<td>P19494</td>
</tr>
<tr>
<td>3</td>
<td>2654</td>
<td>1-Aminocyclopropane-1-carboxylate deaminase</td>
<td>349</td>
<td>IA1D_PSESP</td>
<td>Pseudomonas sp. ACP</td>
<td>66.9</td>
<td>Q00740</td>
</tr>
<tr>
<td>4</td>
<td>3012</td>
<td>Regulator</td>
<td>135</td>
<td>BKDR_PSEPU</td>
<td>Pseudomonas putida</td>
<td>25.2</td>
<td>P42179</td>
</tr>
<tr>
<td>5</td>
<td>5604</td>
<td>Amide/urea-binding protein</td>
<td>419</td>
<td>FmdD</td>
<td>Methylophilus methlyotrophus NCIMB 10515</td>
<td>27.2</td>
<td>CAA75186</td>
</tr>
<tr>
<td>6</td>
<td>6938</td>
<td>Amino acid transport</td>
<td>288</td>
<td>LIVH_SALTY</td>
<td>Salmonella typhimurium LT2</td>
<td>29.2</td>
<td>P30295</td>
</tr>
<tr>
<td>7</td>
<td>7804</td>
<td>Amino acid transport</td>
<td>346</td>
<td>BRAE_PSEA</td>
<td>Pseudomonas aeruginosa PAO</td>
<td>24.9</td>
<td>P21628</td>
</tr>
<tr>
<td>8</td>
<td>8841</td>
<td>Amino acid transport</td>
<td>171</td>
<td>BRAF_PSAE</td>
<td>Pseudomonas aeruginosa PAO</td>
<td>41.7</td>
<td>P21629</td>
</tr>
</tbody>
</table>

*Percentage of identical amino acids when sequences were aligned with sequences listed in the GenBank database.

region (Fig. 1). The gene encoded a protein of 517 aa which corresponded to a protein with a molecular mass of 55.9 kDa. An alignment of the deduced amino acid sequence of the amidase from *A. tumefaciens* d3 with previously published amidases demonstrated that the highest degree of sequence similarity was found to the enantioselective amidase from *P. chlororaphis* B23 (45% identity).

Almost all previously described genes for enantioselective amidases were physically connected to nitrile hydratase genes (Kobayashi et al., 1991, 1993; Mayaux et al., 1990, 1991; Nishiyama et al., 1991; Payne et al., 1997; Wu et al., 1998). It was therefore surprising that about 4 kb upstream and 4 kb downstream of the amidase gene, no indications of nitrile hydratase genes could be found. The putative ORFs that were found in the vicinity of the amidase genes encoded proteins with the highest sequence identities to transmembrane proteins (ORF1, ORF6, ORF7), of which the proteins encoded by ORF6 and ORF7 showed the highest degree of similarity to amino acid transport proteins. ORF5 and ORF8 encoded products which were most similar to an amide/urea-binding protein and an amino acid transport protein, respectively, which were both also considered to be part of membrane transport systems. The other ORFs showed the highest degree of similarity with AsnC regulator proteins (ORF2 and ORF4), an 1-aminocyclopropane-1-carboxylate desaminase (ORF3) and an amide/urea-binding protein (ORF 5) (Table 3).

Localization of the amidase gene on a plasmid

It is well known that several *Agrobacterium* strains possess large Ti plasmids and other plasmids (Barton et al., 1995) and it has been shown that amidases which are involved in auxin synthesis (indoleacetamide hydratases) of *A. tumefaciens* strains are encoded on the Ti plasmids.
Table 4. Conversion of different substituted aromatic amides by the enantioselective amidase from *A. tumefaciens* d3

The reaction mixtures contained in a total volume of 0.5 ml, 25 µmol Na/K phosphate buffer (pH 7.4), 0.25 µmol of the respective amide and 0.01–0.6 mg of cell extract from *E. coli* JM 109(pST2WT). The specific activity of the cell extract with 2-phenylpropionamide as substrate was 0.39 U (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structural formula</th>
<th>Relative activity</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Phenylpropionamide</td>
<td><img src="image" alt="2-Phenylpropionamide" /></td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Mandelic acid amide</td>
<td><img src="image" alt="Mandelic acid amide" /></td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>α-Methoxyphenylacetamide</td>
<td><img src="image" alt="α-Methoxyphenylacetamide" /></td>
<td>3</td>
<td>36</td>
</tr>
<tr>
<td>O-Acetylmandelamide</td>
<td><img src="image" alt="O-Acetylmandelamide" /></td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>α-Chlorophenylacetamide</td>
<td><img src="image" alt="α-Chlorophenylacetamide" /></td>
<td>56</td>
<td>&gt;100</td>
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<tr>
<td>2-Methyl-3-phenylpropionamide</td>
<td><img src="image" alt="2-Methyl-3-phenylpropionamide" /></td>
<td>37</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

(Paulus *et al.*, 1991; Schröder *et al.*, 1984). It was therefore attempted to localize the gene for the enantioselective amidase within the genome of strain d3. Thus the total DNA of strain d3 was prepared and analysed by pulse-field gel electrophoresis. It was found that strain d3 contained two (presumably circular) plasmids of 500 and 600 kb. The DNA was blotted and hybridized with the labelled 1.4 kb fragment of the amidase gene initially obtained by PCR (see above). Thus a hybridization signal was obtained with the 500 kb plasmid.

Expression of the amidase gene in *E. coli*

The amidase gene was amplified by PCR from plasmid pAAM1 using a set of primers which created new *NdeI* and *HindIII* restriction sites. The amplified fragment was then ligated into the *l*-rhamnose-inducible expression vector pJOE2702 (Volff *et al.*, 1996). The resulting expression plasmid (pST2WT) was used to transform *E. coli* JM109 and the amidase gene was induced by the addition of *l*-rhamnose. A comparison of SDS-PAGE gels from induced and uninduced cells of *E. coli* JM109(pST2WT) demonstrated the induction of an additional protein with a molecular mass of about 60 kDa. The amidase activity of these cell extracts with phenylacetamide as substrate was 0.23 U (mg protein)⁻¹. No activity could be found in the cell extracts from uninduced cells.

Conversion of different α-substituted phenylacetamides by the amidase from strain d3

It was previously shown that whole cells and cell extracts from *A. tumefaciens* d3 converted 2-phenylpropionamide and α-aminophenylacetamide with a high degree
Recombinant E. coli strains were grown in LB medium supplemented with ampicillin (100 µg ml⁻¹) at 37 °C to an OD₆₀₀ of about 0.2–0.3 before 0.2% (w/v) l-rhamnose was added. After further incubation for 6 h on a rotary shaker at room temperature, cells were harvested by centrifugation and cell extracts prepared. The reaction mixtures contained, in a total volume of 0.5 ml, 25 µmol sodium/potassium phosphate buffer (pH 7.4) and 0.01–0.3 mg protein. The amides were added to an initial concentration of 5 mM (phenylacetamide) or 0.5 mM (all other substrates). At different time intervals, aliquots were taken, acidified by HCl and analysed by HPLC (see Methods). The specific activities of the cell extracts from E. coli JM109(pST2WT), E. coli JM109(pST2M1), E. coli JM109(pST2M2) and E. coli JM109(pST2M3) with phenylacetamide were 0.22, 0.21, 0.10 and 0.16 U (mg protein)⁻¹, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM109 (pST2WT)</td>
<td>E. coli JM109 (pST2M1)</td>
</tr>
<tr>
<td>Phenylacetamide</td>
<td>100</td>
</tr>
<tr>
<td>2-Phenylpropionamide</td>
<td>212</td>
</tr>
<tr>
<td>α-Chlorophenylacetamide</td>
<td>94</td>
</tr>
<tr>
<td>α-Aminophenylacetamide</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 5. Relative activities of the wild-type amidase and different mutants obtained by site-specific mutagenesis within the ‘amidase signature’ motif with different substituted amides

of enantioselectivity (Bauer et al., 1994; Layh et al., 1997). It was therefore tested whether the amidase also converted other α-substituted phenylacetamides enantioselectively. Different substituted phenylacetamides were chemically synthesized and tested as substrates for the amidase. Cell extracts from induced cells of E. coli JM109(pST2WT) were incubated with 2-phenylpropionamide, mandeloamide, O-acetylmandeloamide, α-methoxyphenylacetamide, α-chlorophenylacetamide or 2-methyl-3-phenylpropionamide. Thus it was found that the amidase showed good to excellent enantioselectivities with α-methyl-, α-methoxy- and α-chlorophenylacetamide and 2-methyl-3-phenylpropionamide (Table 4).

Site-specific mutagenesis of the ‘amidase signature’

All currently known (enantioselective) amidases contain a highly conserved glycine- and serine-rich sequence [GGSS(SAG)G] which is part of the ‘amidase signature’ (Chebrou et al., 1996a). Previously, the only known exception was the (R-specific) amidase from Comamonas acidovorans KPO-2771-4 which contained the sequence GASSSG (Hayashi et al., 1997). The corresponding sequence (GASSNG) from A. tumefaciens d3 (aa 174–179) was considerably different from the originally proposed amidase signature motif. Therefore, the corresponding DNA sequence was modified by site-specific mutagenesis towards the highly conserved ‘amidase signature’ motif. The two necessary mutations were introduced separately or simultaneously. The hydrolysis of different amides by cell extracts from E. coli strains synthesizing the three mutated forms and the wild-type form of the amidase were compared. These experiments demonstrated that the relative activities of the amidase for different substrates could be changed by mutations in the amidase signature motif (Table 5). These changes in substrate specificity did not result in a measurable change in the enantioselectivity of the mutant enzymes in comparison to the wild-type enzyme.

DISCUSSION

The preliminary biochemical characterization of the amidase from A. tumefaciens d3 revealed some unusual characteristics of the enzyme which justified a more detailed study. First, it was found that the amidase appeared to be composed of eight identical subunits. This was in contrast to the majority of known enantioselective amidases which generally consist of only one or two (identical) subunits (Giskanik et al., 1995; Hayashi et al., 1997; Kobayashi et al., 1997; Mayaux et al., 1990, 1991). The only known exception was an enantioselective amidase from Rhodococcus erythropolis MP50 which was studied previously by our group (Hirrlinger et al., 1996). Second, the N-terminal amino acid sequence of the enzyme from strain d3 showed no detectable sequence similarity to known amidases. Previously, no enantioselective amidase had been studied from any member of the β subclass of the Proteobacteria to which the genus Agrobacterium belongs. It was therefore initially assumed that the amidase from strain d3 could have evolved independently from previously known enantioselective amidases. This hypothesis was clearly disproved by the amino acid sequence of the enzyme deduced from the encoding gene, which demonstrated significant homology of the amidase from strain d3 with other amidases from various bacterial sources. Nevertheless, some surprising results were obtained from the molecular analysis of the location of amdA in the genome of strain d3. The first new observation was that amdA was structurally not connected with the genes encoding the two subunits of a nitrile hydratase. An adjacent localization of the genes encoding a nitrile hydratase and an amidase has been found in almost all genetically analysed nitrile hydratase/amidase systems (Kobayashi et al., 1992). In
strain d3, immediately downstream of \textit{amdA}, a gene
was found (ORF5) which showed significant sequence
similarity with a gene encoding a putative periplasmic
amide-binding protein (FmdD) from \textit{Methylphilus
methylotrophus} which is involved in the transport of
formamide (Mills et al., 1998). Furthermore, the same
structural coupling of genes encoding amidases and
putative periplasmic amide-binding protein was found in
\textit{A. tumefaciens} d3 and \textit{M. methylotrophus}. The
putative gene product of ORF5 also showed some
sequence similarities with cytoplasmic regulatory
proteins from \textit{Pseudomonas aeruginosa} (AmiC) and
\textit{Rhodococcus rhodochrous} J1 (NhlC) which are
involved in the regulation of the synthesis of the
amidases from these strains (Komeda et al., 1996; Wilson
et al., 1991; Wilson et al., 1993). Thus the protein
encoded by ORF5 may also serve one of these functions
in the metabolism of aromatic amidases by strain d3.
Participation in the transport of the amidases is more
likely because of the structural coupling of ORF5 with
ORFs 6, 7 and 8 which show sequence similarities to the
transmembrane proteins in various binding-protein
dependent leucine/isoleucine/valine transport systems.
A homologous system (FmdE, FmdF) has been found
connected to the formamide-binding protein (FmdD)
from \textit{M. methylotrophus} (Mills et al., 1998). Similarly,
it was suggested that the transport of short-chain aliphatic
amides by \textit{P. aeruginosa} and \textit{Rhodococcus} sp. R312 is
catalysed by an ABC transport system (Chebrou et al.,
1996b; Wilson et al., 1995). This may suggest that ORFs
5, 6, 7 and 8 together form a transport system for amidases
converted by AmdA.

Another novel observation was the localization of \textit{amdA}
on a plasmid. To the best of our knowledge this has
never been described previously for aliphatic or enantio-
selective amidases. The size of this plasmid (about
500 kb) was significantly larger than the mean size
(about 200 kb) of the Ti plasmids found in strains of
\textit{A. tumefaciens} (Hooykaas et al., 1994). Furthermore,
according to the amino acid sequence, the indo-
lecetamide amidases, which are encoded on Ti
plasmids, form a distinct group of amidases which are
clearly separated from the ‘enantioselective’ amidases
(Chebrou et al., 1996b). It is therefore suggested that the
amidase from strain d3 is encoded by a plasmid different
from Ti plasmids. The localization of the amidase gene
on a plasmid may facilitate the exchange of the \textit{amdA}
gene among different bacterial genera. This may explain
the observed sequence similarities between amidases
isolated from rhodococci and pseudomonads. In mul-
tiple alignments of the amino acid sequences no cor-
relation was found between the sequences of the
amidases and the taxonomic position of the strains from
which they were isolated.

Amidases with the ability to enantioselectively hydrolyse
2-phenylpropionamide and other 2-arylpropionamides
have been found in several rhodococci and pseudo-
monads (Ciskanik et al., 1995; Gilligan et al., 1993;
Hirrlinger et al., 1996; Kakeya et al., 1991; Kobayashi et
al., 1993; Mayaux et al., 1990, 1991). Also, the undefined
biocatalysts distributed previously by Novo Nordisk
(Denmark) obviously contained enantioselective
amidases (Beard et al., 1993; Cohen et al., 1992). The
major aim in these previous studies was the preparation of
\textit{S}-2-arylpropionic acids (such as \textit{S}-ibuprofen, \textit{S}-naproxen and \textit{S}-ketoprofen), which are the pharma-
cologically active enantiomers in these non-steroidal
anti-inflammatory drugs produced in large quantities by
the pharmaceutical industry. In the present study it was
tested whether the presence of a methyl group in 2-
substituted arylacetamides is indispensable for the high
enantioselectivity of the amidases or whether other
substituents also may permit a highly enantioselective
hydrolysis of amidase. These investigations clearly
demonstrated that amidases carrying different types of
substituents (like a chlorine atom or a methoxy group)
on the carbon atom in the direct neighbourhood of the
amide group are also converted with a high degree of
enantioselectivity. This observation may allow novel
applications of these enzymes in biotechnology.

It has been proposed previously that the amino acids
which constitute the ‘amidase signature motif’
\textit{GGSS(GAS)G} may form a hydrophobic pocket
(Chebrou et al., 1996a). The results of the mutagenesis
experiments performed in the present study demon-
strated that mutations within this ‘amidase signature
motif’ change the relative activity of the amidase with
different amidases and suggest a function of this motif as
substrate accommodation site. The pronounced changes
observed for the turnover rates of different amidases in the
mutant forms of the enzyme indicate that further
modification within this group may allow the con-
struction of mutants with highly modified substrate
specificities and presumably also modified enantio-
selectivities.

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