Directed evolution of amidase in *Methylophilus methylotrophus*; purification and properties of amidases from wild-type and mutant strains

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The obligately methylotrophic bacterium *Methylophilus methylotrophus* hydrolyses acetamide and acrylamide using a cytoplasmic amidase. In previous work, continuous culture was used to isolate spontaneous mutants which overexpressed either the wild-type amidase (strain MM6) or a mutant amidase with an apparently higher K_{cat} (strain MM8). We now report that NTG mutagenesis of strain MM8 followed by acrylamide-limited growth at low dilution rate (D 0.025 h⁻¹; 37 °C) led to the selection of a strain which continued to overexpress the amidase, but which exhibited an unexpectedly low amidase activity and a greatly decreased K_m for acrylamide (strain MM15). Amidases from the wild-type and mutant strains were purified and shown to be homotetramers (subunit M_r 38000, pI 4.1). The N-terminal amino acid sequence of the wild-type enzyme was 90% homologous with the aliphatic amidase from *Pseudomonas aeruginosa*, and Southern blotting using an oligonucleotide probe for this region showed that overexpression of the enzyme in the mutant strains was not due to gene amplification. Compared with the wild-type and MM6 enzymes, the MM8 enzyme exhibited a threefold higher K_{cat} and a slightly lower K_m for acrylamide, whereas the MM15 enzyme exhibited a similar K_{cat} and an eightfold lower K_m for acrylamide. The MM15 enzyme also reacted more extensively with the thiol group reagent DTNB, had a significantly lower sedimentation coefficient and exhibited a more relaxed substrate specificity, all of which were compatible with a looser tetrameric structure. It was also much more susceptible than the other three enzymes to inactivation by high temperature or by freezing and thawing (MM15 $\gg$ MM8 $>$ MM6/wild-type), both of which variably dissociated the enzyme into inactive dimers and monomers. The amidase activity of strain MM15 was almost 15-fold higher following growth at 25 °C than at 37 °C, since at this lower temperature the enzyme exhibited a similar K_{cat} to the MM8 enzyme and was not significantly dissociated. However, as strain MM15 readily outgrew the organism from which it was derived (strain MM8) during acrylamide-limited continuous culture at 37 °C, it is clear that under these conditions a low K_m was a greater selective advantage than a high K_{cat} .

Introduction

Methylophilus methylotrophus, a methylotrophic bacterium of commercial importance (see Vasey & Powell, 1984; Large & Bamforth, 1988), grows only on carbon sources such as methanol, methylamine and trimethylamine that contain no carbon-carbon bonds (Anthony, 1982). It also uses a very restricted range of nitrogen sources including ammonia, urea, methylamine, trimethylamine and aliphatic amides, all of which are initially converted to ammonia which is then assimilated using the glutamine synthetase/glutamate synthase (glutamine

synthetase/glutamine-oxoglutarate aminotransferase; GS/GOGAT) system (Windass *et al.*, 1980).

The hydrolysis of aliphatic amides to ammonia and the corresponding acid by *M. methylotrophus* is catalysed by a cytoplasmic amidase, the physiological regulation of which has been examined in detail (Silman *et al.*, 1989). The enzyme is induced by various short-chain amides, and repressed by ammonia but not by organic acids or methanol. It therefore differs from the extensively investigated aliphatic amidases of *Pseudomonas aeruginosa* and *Brevibacterium* sp. R312, both of which are induced by short-chain amides, and repressed by organic acids but not ammonia (Clarke, 1970, 1984; Maestracci *et al.*, 1984, 1988). As short-chain amides probably enter bacteria by simple diffusion, amidase is the first enzyme of amide metabolism.

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TNB, 5-thio-2-nitrobenzoic acid.

Previous work from this laboratory has shown that the amidase of *M. methylotrophus* undergoes the expected phenotypic response of such a first enzyme, being maximally expressed during growth under amide limitation. It also readily undergoes genotypic changes, and growth for even a relatively short period of time (as little as 10 generations) at low dilution rate in acetamide- or acrylamide-limited continuous culture led to the spontaneous appearance of hyperactive strains (Silman *et al.*, 1989). This approach enabled the isolation of two mutant strains of *M. methylotrophus* (strains MM6 and MM8), the amidase activities of which were respectively 4 and 12 times that of the wild-type organism. However, as both of the mutant strains contained approximately four times as much amidase as the wild-type organism, it was concluded that the catalytic activity (K_{cat}) of the MM8 enzyme was approximately three times higher than that of the wild-type and MM6 enzymes.

The appearance of these two hyperactive strains during growth under amide limitation was probably due to the selection of spontaneous mutants which exhibited enhanced specific growth rates (μ) at low amide concentrations and therefore readily took over the cultures under these strongly selective conditions (Silman *et al.*, 1989), i.e. they exhibited an increased 'biological fitness' (see Dykhuizen *et al.*, 1987). Although both of these strains exhibited an increased cellular amidase activity (increased enzyme concentration and/or K_{cat}), takeover would also have been expected to occur had the mutants alternatively exhibited an increased affinity (decreased K_m) for the growth-limiting amide, but no spontaneous mutants of this type were observed (although the large increase in the K_{cat} of the amidase from strain MM8 was accompanied by a small but significant decrease in the K_m).

We have therefore sought to isolate such a mutant using chemical mutagenesis followed by acrylamide-limited continuous culture. This paper reports the isolation of strain MM15, a mutant which contains an amidase with a low K_m for acrylamide, and describes the purification and properties of amidases from wild-type and mutant strains (MM6, MM8 and MM15) of *M. methylotrophus*.

Methods

Maintenance of bacterial cultures. Stock cultures of wild-type *Methylotrophus methylotrophus* (NCIB 10515) and strains MM6, MM8 and MM15 derived from it were stored in 20% (v/v) sterile glycerol at -70°C and were routinely maintained on methanol/minimal salts (SE II) agar plates (Silman *et al.*, 1989).

Growth. *M. methylotrophus* was grown in methanol/minimal salts (SE II) medium at 37°C in batch, fed-batch and continuous culture with ammonia, acetamide or acrylamide as the source of nitrogen (Silman *et al.*, 1989).

Growth was measured from the increase in optical density at 600 nm, and cell density (mg dry weight ml^{-1}) was calculated by multiplying OD_{600} by 0.53.

Mutagenesis with NTG. *M. methylotrophus* strain MM8 was grown overnight in batch culture on a methanol/acetamide/minimal salts medium, then harvested and resuspended to a cell density of 0.6 mg dry wt ml^{-1} in 0.1 M-citric acid/0.1 M-trisodium citrate buffer pH 5.5, containing 100 μg NTG ml^{-1} . The cells were incubated at 37°C for 15 min prior to reharvesting. The cell pellet was then washed, resuspended in sterile growth medium and grown overnight at 37°C in batch culture.

Harvesting and preparation of washed or broken cells for measurement of amidase activity. Cells (approximately 20 mg) were harvested by centrifugation, then washed and resuspended in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer pH 6.0 to a cell density of approximately 5 mg ml^{-1} and, if required, disrupted by sonication (Silman *et al.*, 1989).

Purification of wild-type and mutant amidases. Harvested cells were washed and resuspended in 20 mM-Bis-Tris buffer pH 6.8 to a cell density of 40–50 mg dry wt ml^{-1} , then disrupted by passage three times through an Aminco French pressure cell at 15000 p.s.i. (103.5 MPa). The broken cells were centrifuged at 18000 g for 30 min (4°C) to yield a cell-free extract which was finally centrifuged at 175000 g for 75 min to produce a high-speed supernatant essentially free of particulate material. The high-speed supernatant (50 ml; 20 mg ml^{-1}) was passed through an acrodisc filter (0.2 μm pore size; Gelman), then loaded on to an FPLC Mono-Q anion-exchange column (Pharmacia) equilibrated with 20 mM-Bis-Tris buffer pH 6.8 and eluted using a linear gradient of KCl (0 to 350 mM over 20 min) at a flow rate of 4 ml min^{-1} . Amidase eluted from the column at 280–300 mM-KCl and was judged by SDS-PAGE and scanning densitometry to be approximately 95% pure. Further purification was achieved by loading the peak fraction (10 ml; 5 mg ml^{-1}) on to an FPLC Superose-6 gel-filtration column equilibrated with 20 mM-Bis-Tris buffer pH 6.8 containing 100 mM-KCl, and then eluting with the same buffer at a flow rate of 0.3 ml min^{-1} . The amidase was judged by SDS-PAGE to be pure after this step and was stored at -20°C (4°C for the MM15 enzyme) until required.

Measurement of enzyme activities. Amidase activities were determined by measuring the rate of ammonia formation at pH 6.0 (Silman *et al.*, 1989). K_m values for acetamide and acrylamide were determined from plots of s/v versus s using substrate concentrations in the range 0.1–100 mM (Silman *et al.*, 1989). Glutamate synthase activity was measured from the rate of oxidation of NADH in the presence of glutamine and 2-oxoglutarate (Silman *et al.*, 1989). Protein was measured using the method of Lowry.

Inhibition of amidase activity by DTNB. Purified amidase (0.5 mg) was incubated with 2 mM-DTNB in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer pH 8.0 at 26°C . Aliquots were withdrawn at suitable intervals and assayed for amidase activity using acetamide as the substrate.

Quantification of cysteine residues in amidase using DTNB. DTNB-accessible cysteine residues were determined by incubating purified amidase (0.5 mg) with 2 mM-DTNB/1 mM-EDTA in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer pH 8.0 at 26°C to a final volume of 1 ml in a 1 ml cuvette (1 mM-EDTA had no effect on the inhibition of amidase activity by DTNB). The release of TNB^{2-} was followed spectrophotometrically at 412 nm until complete, and the number of cysteine residues ($-\text{SH}$ groups) present in the enzyme was calculated using an ϵ value of 13.7 $\text{mm}^{-1} \text{cm}^{-1}$. Total cysteine residues were determined using the same procedure except that SDS (2%, w/v) was included in the buffer.

Determination of isoelectric point. Isoelectric focussing was carried out using ultrathin 5% (w/v) polyacrylamide slab gels containing 2.2%

(w/v) ampholytes (pH range 4.0–6.5) on an LKB Multiphor II system. Electrode wicks were used with 0.1 M- β -alanine (cathode) and 0.1 M-glutamic acid/0.5 M-phosphoric acid (anode) as the electrode solutions. The gels were prefocussed for 20 min at constant power (15 W), then run under the same conditions for 2 h before being fixed in 0.8 M-trichloroacetic acid for 1 h, washed overnight in distilled water and stained for protein using Kenacid blue R.

Determination of amino acid composition and N-terminal sequence. The amino acid composition of the wild-type amidase was measured by Dr L. Packman (University of Cambridge, UK). The N-terminal amino acid sequence of the enzyme (approximately 1 nmol blotted on to a polyvinylidene difluoride disc) was determined using an Applied Biosystems model 470 gas-phase sequencer.

Polyacrylamide gel electrophoresis. Discontinuous non-dissociating PAGE and SDS-PAGE were carried out as described previously (Silman *et al.*, 1989) using the procedures of Hames (1981). Gels were stained for protein with Kenacid blue R, and non-dissociating gels were also stained for acyl transferase activity using the method of Wyndham & Slater (1986).

Analytical ultracentrifugation. Sedimentation coefficients (0.7 mg amidase ml⁻¹) and M_r values (0.4 mg amidase ml⁻¹) were determined by sedimentation velocity and sedimentation equilibrium ultracentrifugation respectively in 20 mM-Bis-Tris pH 6.8 at 5 °C using an MSE Centriscan 75 analytical ultracentrifuge. Sedimentation velocity diagrams of the amidases in four multiplexed cells were recorded at 37 000 r.p.m. (113 000 g) by measuring the A_{280} of the cells at regular intervals. M_r values of the amidases were determined by allowing the enzymes to attain equilibrium by centrifuging at 13 000 r.p.m. (14 000 g) for 18 h in 1.6 mm columns and then measuring A_{280} (A_{350} was also measured in order to provide a base-line and hence minimize any effects of window contamination; Spragg, 1980). Data capture and analysis were effected using an Apple Graphics tablet and microcomputer. Sedimentation coefficients were corrected to standard conditions to give $s_{20,w}$ values, and M_r values were computed from the linear regression of $\ln(\text{absorbance})$ versus $(\text{radial distance})^2$, all using established procedures and local software (Rowe, 1984).

Preparation and restriction of chromosomal DNA. Chromosomal DNA was prepared from wild-type and mutant strains of *M. methylotrophus* essentially by the method of Chow *et al.* (1977) except that the lysate was digested overnight with proteinase K before being extracted three times with phenol/chloroform/isoamyl alcohol (50:50:2, by vol.) and then precipitated with 100% ethanol. Samples of the DNA (15 µg) were restricted overnight at 37 °C in a mixture containing 2 µl restriction buffer (Maniatis *et al.*, 1982) and 2 µl *Eco*RI, *Bam*HI or *Hind*III (10 U ml⁻¹). The resultant restriction fragments, together with samples of chromosomal DNA, were separated by electrophoresis at 100 V for 2.5 h in 1% (w/v) agarose.

Synthesis and labelling of an oligonucleotide probe for the amidase structural gene. A 17-mer oligonucleotide probe based on the N-terminal sequence of the amidase was synthesized by standard procedures using an Applied Biosystems 380B synthesizer, and was labelled with ³²P at the 5' end using T4 polynucleotide kinase (Maniatis *et al.*, 1982). Incorporation was monitored by chromatography of a small amount of the probe on DE81 paper with 0.3 M-ammonium formate as the solvent, followed by autoradiography of the dried chromatogram.

Southern blotting and hybridization. Southern blotting and hybridization (32 °C) was performed using standard procedures (Maniatis *et al.*, 1982) except that Hybond N membrane was used instead of nitrocellulose during the blotting, and the hybridization buffer contained 0.25 g Marvel dried milk powder, 3 g polyethylene glycol 600, 1.5 × SSPE buffer pH 7.4 and 1% SDS in a final volume of 50 ml

(1 × SSPE is 0.15 M-NaCl, 0.01 M-sodium phosphate, 0.001 M-EDTA). Following hybridization, membranes were washed three times in 3 × SSC buffer containing 1% SDS, and then three times in 0.5 × SSC buffer containing 1% SDS (all at 32 °C) (1 × SSC is 0.15 M-NaCl/0.015 M-trisodium citrate, pH 7.0). Washed membranes were wrapped in Saran Wrap and autoradiographed for 48 h at -70 °C.

Presentation of results. Where appropriate, results are presented as the mean ± SEM, with the number of independent determinations in parentheses.

Results

Selection of a mutant with a low- K_m amidase using NTG mutagenesis followed by growth in continuous culture under acrylamide limitation

M. methylotrophus strain MM8 was mutagenized using NTG and then grown in continuous culture at low dilution rate (0.025 h⁻¹) for approximately 23 generations under acrylamide limitation. Broken cells prepared from the resultant culture (culture C; for definition of cultures A and B see Silman *et al.*, 1989) exhibited a K_m for acrylamide of 2.0 mM, a value almost an order of magnitude lower than that of the wild-type organism or strains MM6 and MM8.

Culture C was streaked on to methanol/acetamide plates and six colonies were subsequently picked off at random and grown in acrylamide-limited fed-batch culture ($\mu = 0.3$ h⁻¹). All of these colonies exhibited a substantially lower K_m for acrylamide, but not for acetamide, than the wild-type organism or strains MM6 and MM8.

Bacteria from one colony, subsequently called strain MM15, were examined in more detail following growth in ammonia- or amide-limited fed-batch and continuous culture (Table 1). The results showed that the MM15 amidase still required an amide for induction, and that its maximum activity following growth in continuous culture was 2.1 µmol min⁻¹ (mg cells)⁻¹ (measured with acetamide as substrate). As this was achieved during growth at a dilution rate of 0.05 h⁻¹, the latter was taken to be the optimum dilution rate (D_{opt}) for amidase production in strain MM15. This activity was similar to that of the wild-type, but was approximately one-quarter that of strain MM6 and only approximately one-fifteenth that of strain MM8, from which strain MM15 was derived (Silman *et al.*, 1989). The K_m for acetamide (1.2 mM) of strain MM15 was similar to that of the other three organisms, whereas the K_m for acrylamide was much lower (2.0 versus 18.7, 19.4 and 12.3 mM); neither K_m value was affected by the growth conditions.

SDS-PAGE and scanning densitometry of cellular proteins from *M. methylotrophus* strain MM15 following growth in continuous culture at D_{opt} under acetamide

limitation showed that the amidase comprised 23% of the total cell protein (similar to that of strains MM6 and MM8 grown under the same conditions, and approximately four times that of the wild-type organism). The K_{cat} of the enzyme for acetamide hydrolysis, calculated from its cellular concentration and specific activity, assuming a native M_r of 155000, was 22 s^{-1} (i.e. approximately one-fifth that of the wild-type organism and strain MM6, and one-fifteenth that of strain MM8; Silman *et al.*, 1989).

Purification of the amidase from wild-type *M. methylotrophus*

M. methylotrophus was grown in continuous culture under acetamide limitation ($D = 0.15\text{ h}^{-1}$). Cells were harvested, sonicated and centrifuged to produce a high-speed supernatant fraction from which the amidase was purified using anion-exchange and gel-filtration FPLC (Table 2; Fig. 1). The 20.5-fold purification that was required to prepare the pure amidase from *M. methylotrophus* indicated that it constituted 4.9% of the cell protein during acetamide-limited growth, a value very close to the 4.3% previously reported from analysis of whole-cell polypeptide profiles using SDS-PAGE and scanning densitometry (Silman *et al.*, 1989).

SDS-PAGE showed that the dissociated enzyme consisted of a single protein of M_r 38000. Gel-filtration chromatography and sedimentation-equilibrium ultracentrifugation gave M_r values of 155000 and 150000 respectively for the native enzyme, indicating that the enzyme was a homotetramer. The amidase from *M. methylotrophus* therefore contains fewer subunits than the amidases from *P. aeruginosa* (6×38400) and *Arthrobacter* sp. J1 (8×40000) and is composed of slightly smaller subunits than the amidase from *Brevibacterium* sp. R312 (4×43000) (Asano *et al.*, 1982; Clarke, 1984; Maestracci *et al.*, 1988).

The purified amidase exhibited several kinetic properties which were quantitatively very similar to those previously determined with whole or broken cells of wild-type *M. methylotrophus* (Silman *et al.*, 1989), viz. a K_{cat} for acetamide and acrylamide of 128 s^{-1} and 151 s^{-1} respectively, and a K_m for acetamide and acrylamide of 1.1 mM and 16.1 mM respectively.

Amino acid composition and N-terminal sequence

Comparison of the amino acid composition of the *M. methylotrophus* and *P. aeruginosa* amidases based on a common chain length of 346 amino acids (subunit M_r 38000) indicated that the *M. methylotrophus* amidase contained substantially more glycine (+14), serine

Fig. 1. Purification of amidase from *M. methylotrophus*. Cells were disrupted by sonication, then centrifuged to produce a high-speed supernatant fraction from which amidase was purified using anion-exchange (Mono-Q) and gel-filtration (Superose-6) FPLC as described in Methods. Samples were subjected to SDS-PAGE and then stained with Kenacid blue. Tracks 1 and 6, M_r standards; 2, cells; 3, high-speed supernatant; 4, bulked Mono-Q fractions; 5, Superose-6 peak fraction.

Table 1. Amidase activities of *M. methylotrophus* MM15 grown in fed-batch and continuous culture

M. methylotrophus strain MM15 was grown in fed-batch culture ($\mu = 0.2\text{ h}^{-1}$) and continuous culture ($D = 0.05\text{ h}^{-1}$) in minimal medium supplemented with methanol and various limiting nitrogen sources as described in Methods. Amidase activity was measured with acetamide as substrate.

Growth conditions	Nutrient limitation	Amidase activity [$\mu\text{mol min}^{-1} (\text{mg cells})^{-1}$]
Fed-batch	Ammonia	0.64
	Acetamide	2.14
	Acrylamide	3.59
Continuous	Acetamide	2.06
	Acrylamide	1.51

(+13) and histidine (+10), but substantially less methionine (−8) and arginine (−10), than the *P. aeruginosa* amidase (Table 3). The two enzymes contained similar amounts of all the other amino acids ($\pm \leq 4$); the amount of tryptophan was not determined. The calculated numbers of negative/positive charges on the two enzymes at pH 7.0 were $-42/+35$ and $-45/+38$ respectively, giving each a net charge of -7 ; the calculated isoionic points for the two enzymes were 6.35 and 6.63 respectively.

N-terminal amino acid sequencing of the *M. methylotrophus* amidase showed that 17 of the first 19 residues

Table 2. Purification of amidase from wild-type *M. methylotrophus*

M. methylotrophus was grown in acetamide-limited continuous culture ($D = 0.15 \text{ h}^{-1}$). Washed cells were disrupted by sonication, then centrifuged to produce a high-speed supernatant from which the amidase was purified using anion-exchange (mono-Q) and gel filtration (superose-6) FPLC as described in Methods. Amidase activity was measured at 37°C with acetamide as substrate.

Fraction	Total protein (mg)	Specific activity ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$)	Total activity ($\mu\text{mol min}^{-1}$)	Yield (%)	Purification (-fold)
Cells	4920	2.42	11906	100	1.0
Supernatant	1029	9.85	10136	85	4.1
Mono-Q	109	46.36	5053	42	19.2
Superose	34	49.52	1684	14	20.5

Table 3. Comparison of the amino acid composition of amidases from *P. aeruginosa* and *M. methylotrophus*

Amidase from *M. methylotrophus* was purified, and its relative amino acid content determined, as described in Methods. The amino acid composition of the enzyme was calculated on the basis of 346 residues per subunit M_r 38000) as reported for the *P. aeruginosa* amidase, the actual and derived amino acid sequence of which has been published (Ambler *et al.*, 1987; Brammar *et al.*, 1987). As the tryptophan content was not determined, the number of tryptophan residues was assumed to be 6 by analogy with the *P. aeruginosa* enzyme. Similarly as no distinction was made in the analysis between aspartate/asparagine and glutamate/glutamine, the ratios were also assumed to be the same as for the *P. aeruginosa* enzyme.

Amino acid	No. of residues		Difference
	<i>M. met.</i>	<i>P. aer.</i>	
Gly	48	34	+14
Ala	29	32	-3
Val	19	23	-4
Leu	17	20	-3
Ile	18	21	-3
Ser	29	16	+13
Thr	17	13	+4
Asp + Asn	28 (17 + 11)	32 (19 + 13)	-4
Glu + Gln	39 (25 + 14)	41 (26 + 15)	-2
Phe	11	9	+2
Tyr	15	17	-2
Trp	(6)	6	0
Cys	7	9	-2
Met	7	15	-8
Pro	13	17	-4
Lys	17	15	+2
His	17	7	+10
Arg	9	19	-10
No. of charges (pH 7.0)			
Asp (-1.0)	-17	-19	+2
Glu (-1.0)	-25	-26	+1
His (+0.5)	+9	+4	+5
Lys (+1.0)	+17	+15	+2
Arg (+1.0)	+9	+19	-10
Total	-7	-7	0

were identical to those of the *P. aeruginosa* amidase (Ambler *et al.*, 1987; Brammar *et al.*, 1987):

M. met. M I H(G) D I S S S Q D T V G V A V V (N)
P. aer. M R H G D I S S S N D T V G V A V V N

The only differences were the replacement of arginine by isoleucine at position 2 and the conservative replacement of glutamine by asparagine at position 10.

Southern blotting

The possibility that the approximately fourfold higher amidase concentration in strains MM6, MM8 and MM15 than in the wild-type organism was due to gene amplification was investigated using Southern blotting. A sequence of six amino acids at the N-terminal end of the amidase (MIHGDI-) was identified as being suitable for the synthesis of an oligonucleotide probe. A mixture of 17-mers (ATG AT[ACT] CA[CT] GG[ACTG] GA[CT] AT; 48 redundancies) was therefore synthesized and labelled with ^{32}P . DNA was prepared from the wild-type and mutant strains, digested with restriction endonucleases (*Bam*HI, *Hind*III and *Eco*RI) and subjected to Southern blotting using the ^{32}P -labelled probe. The resultant autoradiographs showed that the major hybridizing fragment in each digest (12.1 kb, 12.8 kb and 8.6 kb respectively) was present at approximately the same intensity in the wild-type and mutant strains, indicating that no significant amplification of the amidase gene had occurred.

Comparison of kinetic properties of wild-type and mutant amidases

Amidases were purified from the three mutant strains of *M. methylotrophus* (MM6, MM8 and MM15) using the method described above for the wild-type enzyme, and their kinetic properties were compared (Table 4). The purification factors for the wild-type, MM6 and MM8 enzymes were essentially as predicted from their cellular concentrations (see above; also Silman *et al.*, 1989) on the assumption that the purification factor should be equal to 100 divided by the amidase concentration expressed as a percentage of the total cell protein. In contrast, the purification factor for the MM15 enzyme was approximately five times the predicted value. More detailed analysis of this purification showed that approximately four-fifths of the total amidase was in the form of an inactive enzyme which ran slightly ahead of the active enzyme during anion-exchange FPLC. As the inactive form was discarded during the purification procedure, the resultant purification factor was consider-

ably higher than predicted from the measured concentration of amidase within the cell.

As expected from the above results, the K_{cat} values of the purified wild-type, MM6 and MM8 enzymes (128, 100 and 310 s^{-1} respectively) were very similar to those calculated from the specific activities of whole or broken cells (96, 96 and 323 s^{-1} respectively; see also Silman *et al.*, 1989). In contrast, the K_{cat} value of the pure MM15 enzyme was almost five times higher than that calculated for whole or broken cells (96 versus 22 s^{-1}). This K_{cat} value was therefore similar to that of the purified wild-type and MM6 enzymes, but only one-third that of the purified MM8 enzyme; K_{cat} values of the purified enzymes were therefore in the order wild-type/MM6/MM15 $\ll$ MM8 (Table 4). The K_{m} values of all four purified enzymes for acetamide were similar to the values obtained with broken cells and indicated that the K_{m} values for acetamide were essentially unchanged in all four strains. In contrast, the K_{m} values of all four purified enzymes occasionally showed slight differences from those obtained with broken cells, but taken overall were in the order MM15 $\ll$ MM8 $<$ wild-type/MM6.

Comparison of structural and physico-chemical properties of wild-type and mutant amidases

The amidases purified from strains MM6, MM8 and MM15 exhibited a subunit M_r of 38000–40000 (measured using SDS-PAGE and sedimentation-equilibrium ultracentrifugation respectively) and a native M_r of 150000–155000 (measured using sedimentation-equilibrium ultracentrifugation and gel-filtration chromatography respectively), and were therefore structurally similar to the wild-type enzyme. The inactive form of the MM15 enzyme also exhibited a subunit M_r of approximately 38000 as determined by SDS-PAGE, but gel-filtration FPLC showed that the native enzyme was composed of an approximately 3:1 mixture of monomers (M_r 38000) and dimers (M_r 76000).

Isoelectric focussing of the wild-type, MM6, MM8 and MM15 amidases yielded an isoelectric point (pI) of 4.1 ± 0.1 for all four enzymes. In contrast, the monomeric form of the MM15 enzyme exhibited a pI of 5.0, a value commensurate with its elution ahead of the native enzyme during anion-exchange FPLC.

Investigations of the thermostabilities of the wild-type and mutant amidases (Fig. 2) showed that the wild-type and MM6 enzymes were relatively stable at 60 °C ($t_{1/2}$ 3.2 and 4.3 h respectively), whereas the MM8 enzyme was significantly less stable ($t_{1/2}$ 1.5 h) and the MM15 enzyme was much less stable ($t_{1/2}$ 0.6 h). Similar relative differences were obtained when the enzymes were incubated at 50 °C, 70 °C and 80 °C, indicating that at all

Fig. 2. Thermostability of amidases from *M. methylotrophus*. Wild-type and mutant amidases were purified as described in Methods and then incubated at 60 °C at a concentration of 0.2 mg ml⁻¹. Samples were removed at intervals and immediately assayed for amidase activity at 37 °C with acetamide as substrate. Amidase activities of purified wild-type, MM6, MM8 and MM15 enzymes prior to heat treatment were 38.75, 35.92, 111.00 and 37.17 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ respectively. ●, Wild-type; ▲, MM6; ■, MM8; ▼, MM15.

Table 4. Purification and properties of amidases purified from wild-type and mutant strains of *M. methylotrophus*

Wild-type (WT) and mutant strains of *M. methylotrophus* were grown in continuous culture at D_{opt} under either acetamide limitation (wild-type and strain MM6) or acrylamide limitation (strains MM8 and MM15). Whole-cell amidase concentrations were measured using SDS-PAGE and scanning densitometry (see Silman *et al.*, 1989). Amidase was purified and assayed as described in Methods; the two purification factors shown are the measured value and, in parentheses, the predicted value based on the measured enzyme concentration in whole cells (100/amidase concentration as percentage of cell protein).

Strain	Concn of amidase in cells (% of cell protein)	Purification factor	Kinetic properties of pure amidase			
			Acetamide		Acrylamide	
			K_{cat} (s^{-1})	K_{m} (mM)	K_{cat} (s^{-1})	K_{m} (mM)
WT	4.3	20.5 (23.3)	128	1.1	151	16.1
MM6	19.8	4.5 (5.1)	100	1.2	131	19.0
MM8	24.6	4.1 (4.1)	310	1.7	419	12.1
MM15	23.0	21.2 (4.3)	96	1.3	127	2.1

of these temperatures the thermostabilities of the enzymes were in the order MM15 $\ll$ MM8 $<$ wild-type/MM6. Similarly, a single freeze-thaw cycle inhibited the wild-type, MM6 and MM8 enzymes by $<1\%$ compared with 85% for the MM15 enzyme.

When samples of the wild-type and mutant enzymes that had been incubated for various periods of time at high temperatures were analysed using gel-filtration FPLC, it was found that they had variably dissociated into dimers and monomers. The propensity of the enzymes to dissociate under these conditions was in the order MM15 $\gg$ MM8 > MM6/wild-type and thus paralleled their loss of amidase activity. Subsequent measurements of the amidase activities of heat-treated samples indicated that the loss of amidase activity was linearly related to the loss of tetrameric structure (Fig. 3). This was confirmed by purifying the dimeric and monomeric forms of the enzymes using gel-filtration FPLC and showing that neither form exhibited any amidase activity.

Effect of the thiol reagent DTNB

Exposure of the wild-type and mutant amidases to the thiol reagent DTNB (10:1 molar ratio of DTNB to cysteine residues; see Table 3) caused 15–18% inhibition of the wild-type, MM6 and MM8 amidase activities compared with 35% inhibition of the MM15 activity (Table 5). Spectrophotometric analysis of the reaction between DTNB and each of these enzymes indicated that the DTNB reacted with 4 cysteine residues in the wild-type, MM6 and MM8 enzymes, and with 8 cysteine residues in the MM15 enzyme. These values increased to 28 ± 2 for all four enzymes following exposure to SDS, thus confirming the cysteine content determined by amino acid analysis of the wild-type enzyme (7 cysteines per subunit; 4 subunits) and indicating that the mutations had not affected any of the cysteine residues. These results therefore showed that DTNB reacted with 13–15% of the total cysteine residues in the wild-type, MM6 and MM8 enzymes compared with approximately 31% in the MM15 enzyme.

The approximately doubled sensitivity and accessibility of the MM15 amidase to DTNB, together with its greater propensity to dissociate at high temperature or by freezing and thawing, suggested that this enzyme has a slightly more open structure than the other amidases. This possibility was investigated by subjecting all four enzymes to sedimentation-velocity ultracentrifugation. The MM15 enzyme sedimented significantly more slowly than the wild-type, MM6 and MM8 enzymes (sedimentation coefficient 5.26S versus 6.63, 6.81 and 6.75S respectively), thus confirming its less compact structure. The MM15 amidase also exhibited a more relaxed substrate specificity than the other three enzymes, as evidenced by its increased capacity to hydrolyse the longer-chain amides propionamide and butyramide (949% and 16% respectively of the rate with

Fig. 3. Relationship between activity and structural integrity of amidases from wild-type and mutant strains of *M. methylotrophus*. Amidases were purified as described in Methods and then incubated at 60 °C or 70 °C for up to 3 h. Samples were then assayed for amidase activity with acetamide as substrate, and analysed for loss of tetrameric structure using gel-filtration (Superose-6) FPLC (see legend to Fig. 2 and text). Amidase activities of purified wild-type, MM6, MM8 and MM15 enzymes prior to heat treatment were 38.75, 35.92, 111.00 and 37.17 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ respectively. ●, Wild-type; ▲, MM6; ■, MM8; ▼, MM15.

Table 5. Sensitivity to DTNB of amidases purified from wild-type and mutant strains of *M. methylotrophus*

The inhibition of amidase activity by DTNB, and the reaction of DTNB with cysteine residues present in the enzyme ($\pm$ SDS), were determined as described in Methods. Amidase activity was measured with acetamide as substrate. DTNB was present at 10 times the molar concentration of the cysteine residues. The results are the means of up to four independent determinations, each of which did not vary by more than approximately $\pm 10\%$ of the mean value.

Source	Enzyme form	Inhibition of activity (%)	Reaction with cysteine residues [mol DTNB (mol enzyme) ⁻¹]		
			–SDS	+SDS	(%)
WT	Tetramer	15	4.2	27.3	15
MM6	Tetramer	16	4.0	26.0	15
MM8	Tetramer	18	3.9	30.5	13
MM15	Tetramer	35	8.5	27.3	31
	Monomer/dimer	ND	25.2	26.5	95

ND, Not determined (the monomer/dimer mixture did not exhibit amidase activity).

acetamide versus approximately 150% and 2% respectively).

Spectrophotometric analysis of the reaction between DTNB and the inactive form of the MM15 amidase (mainly monomers) indicated that essentially all of the cysteine residues were accessible to the inhibitor even in the absence of SDS. This suggested that the cysteines

were located mainly on the interfacial surfaces of the subunits and were thus rendered largely inaccessible to DTNB when the enzymes were in their tetrameric forms. In spite of the apparent importance of cysteine residues for amidase activity, dithiothreitol (2 mM) did not protect either the wild-type or the MM15 enzyme against denaturation by heat or freezing and thawing.

Physiological properties of strain MM15

The above results suggested that the lower than expected amidase activity of strain MM15 was caused by the high degree of dissociation of the enzyme *in vivo* into inactive monomers and dimers, and that this probably reflected the inherent thermolability of the enzyme. This hypothesis was tested by measuring the activity and concentration of the amidase following the growth of strain MM15 in continuous culture under acrylamide limitation at a series of different temperatures (25–37 °C; $D = 0.1 \text{ h}^{-1}$) (Fig. 4). The results showed that cellular amidase activity was inversely related to the temperature of the culture, and that following growth at 25 °C the activity was similar to that of strain MM8 grown at 37 °C [i.e. 27.9 versus 24.4 $\mu\text{mol min}^{-1} (\text{mg cells})^{-1}$ measured with acetamide as substrate]. As the amidase concentration remained almost constant under all of these growth conditions (22.4–24.3% of the cell protein), it was concluded that even at temperatures as low as the normal growth temperature (37 °C), the high thermolability of the enzyme has a strongly deleterious effect not only on the tetrameric structure of the MM15 amidase but also on its apparent K_{cat} .

Purification and properties of amidase from strain MM15 grown at 25 °C

The amidase was therefore purified from strain MM15 grown under acrylamide limitation at 25 °C ($D = 0.1 \text{ h}^{-1}$) using the procedures described above for the purification of the wild-type and other mutant amidases. Pure amidase was obtained after a purification of only 4.5-fold, a value completely commensurate with the measured cellular amidase concentration (22.4% of the cell protein) and which therefore indicated that all of the amidase was in the active, tetrameric form (compared with only approximately one-fifth following growth at 37 °C). The purified enzyme exhibited K_{cat} values of 310 s^{-1} and 329 s^{-1} , and K_{m} values of 1.2 mM and 2.5 mM, for the hydrolysis of acetamide and acrylamide respectively, thus confirming its high $K_{\text{cat}}/K_{\text{m}}$ ratio for acrylamide hydrolysis relative to that of the other enzymes. Gel-filtration FPLC and SDS-PAGE showed that the native and subunit M_r values (155 000

Fig. 4. Effect of growth conditions on the activity and concentration of amidase in *M. methylotrophus* strain MM15. The organism was grown in acrylamide-limited continuous culture ($D = 0.1 \text{ h}^{-1}$) over the temperature range 25–37 °C. Amidase activity (●) was measured at 37 °C with acetamide as substrate, and amidase concentration (▲) was determined by SDS-PAGE followed by scanning densitometry as described in Methods.

and 38 000 respectively) were the same as those exhibited by the wild-type and other mutant amidases. Sedimentation-velocity ultracentrifugation revealed that the enzyme had a sedimentation coefficient of 6.78S, virtually identical to that of the wild-type, MM6 and MM8 enzymes. Reaction of the enzyme with DTNB also yielded similar results to those obtained with the wild-type, MM6 and MM8 enzymes, i.e. 18% inhibition of activity and a stoichiometry of 5.2 mol DTNB (mol enzyme) $^{-1}$ [28.5 mol DTNB (mol enzyme) $^{-1}$ in the presence of SDS]. All of these properties were commensurate with the amidase being a tightly associated tetramer. This was partly reflected in the substrate specificity of the enzyme, which was midway between that of the MM15 (37 °C) enzyme and the other enzymes [the rates of hydrolysis of propionamide and butyramide were 326% and 7% respectively of the rate with acetamide, versus 949% and 16% respectively for the MM15 (37 °C) enzyme, and 139% and 1.4% respectively for the wild-type, MM6 and MM8 enzymes].

Discussion

The isolation of mutant strains of bacteria using continuous culture is based upon selection for improved

'biological fitness', i.e. the ability of the mutant to outgrow the original organism during growth under a particular nutrient limitation. Such mutants are altered in their ability to use the growth-limiting nutrient, normally as a result of changes in the activity or affinity of the first enzyme of the metabolic pathway that utilizes this nutrient (see Kubitschek, 1974; Harder *et al.*, 1977; Mortlock, 1982; Dykhuizen & Hartl, 1983; Hartley, 1984; Dykhuizen *et al.*, 1987).

As small aliphatic amides probably enter cells rapidly by simple diffusion, amidase is the first enzyme of amide metabolism. Mutants which exhibit increased amidase activity at low amide concentrations are therefore potentially very susceptible to selection during growth at low dilution rate under amide limitation. We have recently used this type of approach with some success to isolate spontaneous mutants of *M. methylotrophus* which exhibit enhanced amidase activity as a result of synthesizing more of the wild-type enzyme (strain MM6) or more of an altered enzyme with a higher K_{cat} (strain MM8) (Silman *et al.*, 1989). The description in this paper of the subsequent isolation from a chemically mutagenized culture of strain MM8 of a mutant that synthesizes more of an altered enzyme with a lower K_m (strain MM15) thus confirms the selective advantage of such a phenotype. Furthermore, the ability of strain MM15 to synthesize more of an altered enzyme with both a high K_{cat} and a low K_m during growth at 25 °C rather than at the normal growth temperature of 37 °C completes the four general types of amidase mutants that might be expected to be isolated using this type of 'directed evolution' approach, i.e. overexpression (MM6), overexpression plus a high K_{cat} (MM8), overexpression plus a low K_m (MM15) and overexpression plus both a high K_{cat} and a low K_m (MM15 grown at 25 °C).

As the Southern hybridization experiments showed no evidence of gene amplification in the mutant strains, it is likely that overexpression of amidase in these strains occurs via an up-promotor mutation (perhaps similar to that previously reported for the *P. aeruginosa* amidase as a result of selecting for altered substrate specificity; see Clarke, 1970, 1984; Clarke & Drew, 1988), which is clearly retained during all stages of strain selection (MM6 → MM8 → MM15).

The necessity of having to use NTG mutagenesis to generate strain MM15 (in contrast to the spontaneous appearance of strains MM6 and MM8) suggests that the low- K_m phenotype may involve multiple and/or rare mutations in the amidase structural gene. It would also appear that the low- K_m phenotype is accompanied by increased thermolability, since the K_m for acrylamide and the $t_{1/2}$ (60 °C) of the enzymes are both in the order wild-type/MM6 > MM8 ≫ MM15.

The wild-type amidase is similar to several other

bacterial amidases (particularly the amidase from *P. aeruginosa*) in terms of its subunit M_r and/or N-terminal amino acid sequence, and in its sensitivity to inhibition by thiol reagents such as DTNB (which reacts with only one cysteine residue per subunit), but it differs in being a tetramer rather than a hexamer or an octamer (Clarke 1970, 1984; Asano, 1982; Thiery *et al.*, 1986; Clarke & Drew, 1988). Although the MM6, MM8 and MM15 enzymes are structurally similar to the wild-type enzyme, the MM15 enzyme is much more sensitive to inhibition by high temperatures or by freezing and thawing, and dissociates far more readily into inactive monomers and dimers, than any of the other enzymes. The greater propensity of the MM15 enzyme to undergo dissociation probably reflects its more open structure, as evidenced by its greater sensitivity to inhibition by DTNB (which reacts with eight cysteine residues per molecule; twice as many as in the wild-type, MM6 and MM8 enzymes, or in the MM15 enzyme synthesized during growth at 25 °C), by its lower sedimentation coefficient, and by its significantly enhanced relative activity with longer-chain amides. It is possible, therefore, that the mutation(s) which gives rise to the low- K_m phenotype renders the enzyme more unstable, perhaps by altering the charge distribution on the interfacial surfaces of the subunits.

The later conclusion resolves the paradoxical observation that strain MM15 grown under acrylamide limitation ($D = 0.1 \text{ h}^{-1}$) exhibits an amidase activity which is only approximately one-fifteenth that of strain MM8 (in spite of containing approximately the same amount of enzyme), since the K_{cat} is about two-thirds lower and approximately four-fifths of the enzyme is in the form of inactive monomers and dimers. As both of these properties are influenced by the temperature and retention time of the culture, it was concluded that the amidase activity of strain MM15 mainly reflects its very high thermolability, and this was confirmed by the observation that the amidase activity of strain MM15 grown at 25 °C was similar to that of strain MM8 cultured at the normal growth temperature.

The remarkable feature of strain MM15 is that it outgrew strain MM8 at 37 °C under acrylamide limitation in spite of having a much lower potential for acrylamide hydrolysis and of making up to approximately 18% of its cell protein as an inactive enzyme. It is clear, therefore, that a substantially increased affinity for the growth-limiting nutrient is an overwhelming selective advantage during growth at low dilution rates in continuous culture.

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