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Molecular and biochemical characterization of the recombinant amidase from hyperthermophilic archaeon *Sulfolobus solfataricus*

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Abstract We have cloned, sequenced, and overexpressed in *Escherichia coli* the amidase gene from the hyperthermophilic archaeon *Sulfolobus solfataricus* (strain MT4). The recombinant thermophilic protein was expressed as a fusion protein with an N-terminus six-histidine-residue affinity tag. The enzyme, the first characterized archaeal amidase, is a monomer of 55,784 daltons, enantioselective, and active on 2- to 6-carbon aliphatic amides and on many aromatic amides, over the pH range 4–9 and at temperatures from 60° to 95°C. The *S. solfataricus* amidase belongs to the class of amidases that share a characteristic signature, GGSS(S/G)GS, located in the central region of the protein, and which show remarkable variability in their individual substrate specificities, can hydrolyze aliphatic or aromatic substrates, and share a large invariance of their primary structure.

Key words *Sulfolobus solfataricus* · Archaea · Amidase · Signatured amidase · Thermophiles

Introduction

Amidases are widely distributed in both prokaryotic and eukaryotic organisms. They act on C–N bonds other than peptide bonds (EC 3.5) and are particularly interesting for their potential industrial applications (Kobayashi et al. 1992; Nagasawa and Yamada 1989). Amidases catalyze the hydrolysis of various endogenous and foreign aliphatic and aromatic amides by transferring an acyl group to water with the production of free acids and ammonia. Acetamide (Hynes 1970), propionamide (Hashimoto et al. 1991; Mayaux et al. 1990), acrylamide (Kobayashi et al. 1992), 6-aminohexanoic acid cyclic dimer (Kinoshita et al. 1977), semisynthetic penicillin precursors (Valle et al. 1991), indolacetamide (Schroeder et al. 1984; Yamada et al. 1985), fatty acid amides (Cravatt et al. 1996), glutamine (Watford 1993), and asparagine (Milman and Cooney 1974) are all substrates of amidases.

Ninety-one amidases from different sources have been described and have been included in EC 3.5.1 and 3.5.2 categories, the first composed of 77 proteins acting on linear amides and the second composed of 14 proteins acting on cyclic amides. Only some of these have been subjected to molecular and biochemical characterization. The discovery of an amidase signature gene family (Mayaux et al. 1991) allowed grouping the primary structures of the codified enzymes into two classes: those sharing the characteristic GGSS(S/G)GS signature and those lacking this signature. The amidases that belong to the first class show a remarkable variance in their individual substrate specificities, can hydrolyze aliphatic or aromatic substrates, share a large invariance in their primary structures, and their quaternary structure, when characterized, is typically homodimeric.

The prototype of the second class of amidases (lacking the signature), "aliphatic amidases," was isolated from *Pseudomonas aeruginosa* (Ambler et al. 1987). This amidase, an hexamer, hydrolyzes only small aliphatic substrates, such as acetamide and propionamide. The amidases from *Brevibacterium* sp. R312 (Soubrier et al. 1992), *Methylophilus methylotrophus* (Silman et al. 1991), and *Helicobacter pylori* (Skouloubris et al. 1997) also belong to this family. They all have multimeric quaternary structures. Signatured amidase enzymes were found in archaeal genomes including *Methanococcus jannaschi* (Bult et al. 1996), *Archaeoglobus fulgidus* (Klenk et al. 1997), and *Pyrococcus horikoshi* (Kawarabayasi et al. 1998).

In this article, we describe the cloning, sequencing, and overexpression in *Escherichia coli* of the amidase gene from

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Sulfolobus solfataricus (strain MT4). The recombinant protein, a signatured amidase, has been characterized.

Materials and methods

Strains, plasmids, and phage vectors

Escherichia coli strains DH5α, C600, C600 Hfr, BL21(DE3), BL21(DE3)pLysS, and HMS174 (Stratagene. La Jolla, CA, USA) were used for DNA cloning and gene expression. λgt10 (Stratagene) vector was used to construct a genomic DNA library. pEMBL 130 plasmid (the kind gift of Dr. L. Dente, Laboratory of Genetics, University of Rome, Tor Vergata) was used to subclone the DNA fragments for the sequence, and pRSET A (Invitrogen, Carlsbad, CA, USA) and pTrcHis2 (Invitrogen) expression vectors were used to produce the recombinant proteins.

Enzymes and chemicals

Restriction enzymes were obtained from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden), modifying enzymes from New England Biolabs (Beverly, MA, USA), and DNA molecular weight and protein molecular markers from MBI Fermentas (Vilnius, Lithuania) and Bio-Rad (Hercules, CA, USA), respectively. Oligonucleotides were synthesized by Primm (Milan, Italy). ProBond resin, used for affinity chromatography purification of the recombinant protein, was purchased from Invitrogen. [α^{32}P]dATP was obtained from NEN Dupont (Brussels, Belgium). All other chemicals used were from commercial sources and of reagent grade. Most amides and free acids were obtained from commercial sources. Some amides not commercially available were synthesized starting from free acids, converted to the corresponding chlorides by reaction with $SOCl_2$, and then treated with gaseous NH_3 (Levene et al. 1930).

Polymerase chain reaction

A partially coding sequence of the amidase gene from *Sulfolobus solfataricus* MT4 was obtained by polymerase chain reaction (PCR). Taking advantage of the knowledge of the *Sulfolobus* codon usage, two degenerate oligonucleotides were designed on conserved regions of the published amino acid sequences of amidase. Forward primer, BC1, 5′-GG(T/A)GG(T/A)(T/A)(C/G)(T/A)(T/A)(C/G)(T/A)GG(T/A)GG(T/A)(T/A)(C/G)(T/A)GC-3′, was designed on a sequence coding for the amidase signature. Backward primer, BC2, 5′-(T/G)GA(A/T)GCGAT(T/C)AN(T/C)A(T/G/A)(T/G)A(A/C)ATC-3′, was designed on a sequence conserved in several, but not all, known amidase genes. Amplification was carried out in a 100-µl volume, using 4 ng *Sulfolobus solfataricus* genomic DNA, prepared according to the protocol described by Sambrook et al. (1989), using 0.25 µM each primer (Primm), 0.2 mM each dinucleotide triphosphate (dNTP) (Perkin-Elmer, Norwalk, CT, USA), and 2.5 U Taq DNA polymerase (Pharmacia, Uppsala, Sweden). The first cycle was performed at 95°C for 7 min, followed by 35 cycles of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C; the last cycle was 72°C for 7 min. The PCR product, when loaded on 1.2% agarose gel, showed a band of approximately 900 bp; this band was electroeluted and ligated to *Eco*RI-*Xba*I-digested pEMBL130.

Construction and screening of genomic DNA library

A genomic DNA library from *S. solfataricus* MT4 strain (DSM 5833) was constructed as previously described (Sambrook et al. 1989). Briefly, genomic DNA was partially digested with *Eco*RI and ligated in *Eco*RI λgt10. Ligated DNA was packaged using Giga-Pack Gold II in vitro packaging (Stratagene), according to the manufacturer's instructions. Lambda particles were amplified on C600 hfl *E. coli* strain. Some 4.5×10^4 plaque-forming units (pfu) were screened using the 900-bp PCR product as a probe. Five positive clones were obtained. One clone, λ5C1, was studied by restriction analysis and Southern blot hybridization.

DNA sequencing

The amidase gene obtained from the λ5C1 clone was subcloned in pEMBL 130 plasmid. Sequencing reactions were performed with the PCR products as templates, using a Perkin-Elmer ABI 377 DNA Sequencer and the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, PE Italia, Monza).

Sequence alignment

Sequence analysis was carried out using programs from Wisconsin package, Version 10.0, Genetic Computer Group (Madison, WI, USA). FASTA (Pearson and Lipman 1988) was used for database searches. PILEUP was used for multiple sequence alignments (Feng and Doolittle 1987).

Expression of recombinant N-terminus His_6 tag amidase in *E. coli*

To obtain the amidase coding gene, two oligonucleotides were designed, AM3 and AM4: AM3 (5′-GAGAGGATCCATGGGAATTAAGTTACCCA-3′) having a *Bam*HI restriction site at the 5′-end and the ATG start codon of the gene and AM4 (5′-CTGGTACCTTATTTTTTGATTCTCTCA-3′) having a *Kpn*I restriction site at the 5′-end and the TAA stop codon. Amplification was carried out as described, using 4 ng of the purified λ5 C1 clone.

The amidase gene thus obtained was inserted into the *Bam*HI and *Kpn*I restriction sites of the expression vector pRSET A (Invitrogen), obtaining the recombinant plasmid pSeth12. The construct was sequenced to confirm the correct sequence of the gene after PCR and the correct insertion of the gene in frame with the ATG of the plasmid. The

recombinant protein is expressed as a fusion protein with an N-terminus six-histidine-residue affinity tag. To optimize gene expression, three different *E. coli* strains were transformed with pSeth12, using different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG), growing the transformed cells at different temperatures. Successful expression was achieved with BL21(DE3) pLysS strain at 37°C in LB ampicillin (100 μg/ml), adding 0.4 mM IPTG when the culture was at 1 OD_{600}. After induction, the culture was grown for another 3 h. Cells were harvested by centrifugation.

Expression of recombinant C-terminus His_6 tag amidase in *E. coli*

To express the amidase as a fusion protein with a six-His tag at the C-terminus, the amidase gene was cloned into the expression vector pTrcHis2 (Invitrogen). A PCR reaction was performed using two oligonucleotides, AMC1 and AMC2. AMC1 (5′-GTCCATGGGAATTAAGTTACCCA-3′) had an *Nco*I restriction site at the 5′-end overlapping the ATG start codon of the gene; AMC2 (5′-CTGGTAC-CTTTTTTGATTCTCTCA-3′) had a *Kpn*I restriction site at the 5′-end without a stop codon to allow the insertion of the gene in frame with the sequence coding for six histidine residues. Amplification was carried out as described previously, using 4 ng of the purified λ5C1 clone. The recombinant plasmid, pBal3, was transformed in *E. coli* Top10 strain. The expression was achieved as described for the pSeth12 construct.

Purification of the recombinant amidase

Recombinant protein was purified, under native conditions, by affinity chromatography, using the ProBond resin (Invitrogen). Cell paste was suspended (14 ml/g) in 20 mM sodium phosphate buffer, pH 7.8, 500 mM NaCl (NBB, native binding buffer), 100 μg/ml lysozyme, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and then sonicated (VibraCell; Sonics & Materials, Newton, CT, USA). DNase (5 μg/ml) and RNase (10 μg/ml) were added, and after 15 min in an ice bath the suspension was centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was then used for the enzyme purification. From the insoluble cell paste, more enzyme was extracted by NBB, 0.25% Tween 20, 0.1 mM ethyleneglycoltetraacetic acid (EGTA), followed by exhaustive dialysis against NBB. This solution was added to the supernatant and loaded onto a column containing ProBond resin equilibrated in NBB. The column was washed with NBB to an OD_{280} lower than 0.01, then with NBB at pH 6.5 (NWB, native wash buffer: 20 mM sodium phosphate buffer, pH 6, 5,500 mM NaCl) until the OD_{280} was less than 0.01. The column was washed with NWB containing 100 mM imidazole until the OD_{280} was less than 0.01.

The recombinant protein was finally eluted using NWB containing 300 mM imidazole (NIEB3; native-imidazole elution buffer, 300 mM) and exhaustively dialyzed against 25 mM bis-Tris propane, pH 7.5, 150 mM NaCl, 100 μM ethylenediaminetetraacetic acid (EDTA), and 200 μM dithiothreitol (DTT). The purity of the recombinant protein was checked by gel filtration on Superose 12, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and tested for activity; 2.2 g wet cell paste gave 2.5 mg of enzyme having a specific activity of 6.8 U/mg. The C-His-tagged enzyme, purified under the same conditions as the N-His-tagged enzyme, gave the same yield and exhibited the same features.

Enterokinase cleavage of histidine tail

Recombinant protein, dialyzed against 50 mM Tris-Cl buffer pH 8, 1 mM $CaCl_2$, 0.1% Tween-20 was incubated for 16 h at 37°C in the same buffer with 1.5 units of enterokinase EkMax (Invitrogen) for 100 μg protein to remove the His tag. Tail cleavage was monitored by Western blot with an anti-his-tag antibody (Invitrogen) after electrophoresis on 15% SDS-PAGE of incubated sample and uncleaved protein.

The enterokinase-digested protein in 500 mM in NaCl was added to soybean trypsin inhibitor (STI) (Sigma, St. Louis, MO, USA). The resin, equilibrated with 50 mM potassium phosphate buffer, pH 8, 500 mM NaCl, was rocked slowly at room temperature for 2 h. The resin with bound enterokinase was then removed by centrifugation, and the recovered supernatant was freed from the tail by a further passage through a small ProBond column.

Protein determination

Protein concentration was determined at 280 nm using an E_{280} of 54,290 M^{-1} cm^{-1}, calculated according to Gill and von Hippel (1989), whereas in crude extracts quantitation was routinely performed using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) based on the method of Bradford (1976).

Enzyme assay

The standard enzyme assay was performed at 70°C for 10 min in 200 μl of a mix containing 50 mM bis-Tris propane buffer, pH 7.5, 7.5 mM benzamide, and the appropriate amount of amidase. At the end of the incubation time, 200 μl 20% acetic acid was added to stop the reaction, and 50 μl of this solution was injected onto a platinum EPS C18 column (Alltech, Deerfield, IL, USA), 53 × 7 mm. The elution was carried out isocratically with a mobile phase of 25/74/1 acetonitrile/water/acetic acid at a flow rate of 2.5 ml/min. The acid formation was monitored for aromatic acids at 254 nm with a Kontron D430 detector, whereas the aliphatic acids were detected at 210 nm with the same column eluted isocratically with a mobile phase, 25 mM orthophosphoric acid with 1% methanol. All the analyses were performed in 3–6 min. Quantification of the free acids was obtained by comparison with a calibration curve performed by injecting various amounts of the pure acid under the

same conditions. Activity with prolinamide, asparagine, and glutamine was determined by measuring the amount of amino acid formed and amide remaining with an amino acid analyzer. One enzyme unit is defined as the amount of the enzyme producing 1 μmol free acid per minute.

Stereoselectivity of the enzyme was checked with (+/-)-2-phenylpronamide. Formation of 2-phenylpropionic acid in the enzymatic assay was determined by HPLC by injecting samples into the chiral column Chirobiotic V (Astec, Whippany, NJ, USA) (150 × 4.6 mm) and eluting at 1 ml/min with a mobile phase, 20/80 methanol/20 mM triethylamine acetate, pH 4.1, monitored at 254 nm. Identification and quantification of *R*- and *S*-products was achieved by comparison with appropriate amounts of pure compounds injected into the column.

Hydroxamic acid and acid hydrazide formation was checked by incubating amidase for 10 min at 70°C in 50 mM phosphate buffer, pH 7.5, with 100 mM amide (benzamide was also checked at 10 mM) and 400 mM hydroxylamine or hydrazine (both at pH 7). The resulting hydroxamate was determined spectrophotometrically as reported by Fournand et al. (1998a), whereas acid hydrazide formed was detected by HPLC (Fournand et al. 1998b).

Molecular mass determination

The apparent molecular mass of the native enzyme was determined by analytical gel filtration on a Superose 12 column (Pharmacia) eluted with 20 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl. The elution volume (Ve) of each fraction was monitored using the absorbance at 226 and 280 nm and the enzyme activity.

The column was calibrated with the following standards: aldolase from rabbit muscle (158 kDa), horse liver alcohol dehydrogenase (80 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and ribonuclease A (13.7 kDa). The void volume (Vo) was determined by the elution volume of dextran blue exclusion. The molecular mass of native amidase was calculated by plotting the log of molecular mass versus the Ve/Vo ratio. The molecular weight of the denatured enzyme was determined by SDS-PAGE according to the method of Laemmli (1970) on discontinuous gel of 2%–12% SDS-PAGE using the low molecular weight calibration kit from Bio-Rad.

pH optimum, optimal temperature, and kinetic parameters

The pH optimum for the reaction was determined at 70°C using the following buffers: citrate for pH 4–5.8, sodium phosphate for pH 6–7.9, and bis-Tris propane for pH 6.5–9.5. The pH value of each buffer was checked at 70°C. All buffer concentrations were 50 mM. The substrate used was 7.5 mM benzamide.

The temperature dependence of the amidase reaction was studied under standard conditions over the range 40°–95°C. Enzyme thermostability was studied at 70°, 80°, or 90°C in eppendorf tubes (0.5 ml) using 70 μl at 0.2 mg/ml protein in 100 mM bis-Tris propane buffer, pH 7.5 (checked at the same temperature). The tubes were covered with a layer of mineral oil (Sigma), sealed, and incubated for the reported times. After incubation, a 50-μl sample was assayed for standard activity. Kinetic constants were determined at 70°C in 50 mM bis-Tris propane buffer, pH 7.5. Kinetic data were calculated by nonlinear regression analysis using ENZFITTER (Leatherbarrows 1987). For each calculation, at least seven velocity–substrate data pairs were used. Michaelis–Menten constants were determined from Lineweaver–Burk representation of data obtained by determining the initial rate of substrate hydrolysis using the range 0.1–30 mM.

Effect of metal ions

Chlorides of Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, V^{2+}, Mo^{5+}, and Co^{2+} were tested at 1 mM metal ion concentration. Samples containing the protein (dialyzed against 50 mM bis-Tris propane buffer, pH 7.5, to free from EDTA and DTT) and metal chlorides were incubated for 10 min at 70°C in 50 mM bis-Tris-propane buffer, pH 7.5, then 7.5 mM benzamide was added and the standard activity measured.

Isoelectric point determinations

pI was calculated using ExPASy Proteomics tools and measured by MonoP column chromatography (Pharmacia) following the manufacturer's instructions.

Results

Cloning of the amidase gene

A DNA fragment, 900 bp long, was obtained by PCR from *S. solfataricus* MT4 strain on the basis of conserved residues among known amidase sequences. The PCR product was inserted into pEMBL130 vector and sequenced. The deduced amino acid sequence was compared with the SwissProt database, finding high similarity to amidases. This DNA fragment, [α^{32}P]dATP labeled, was used as a probe to screen a λgt10 genomic library from MT4 strain previously constructed (Ammendola et al. 1998). Under stringent hybridization conditions, five positive clones were found. One clone, λ5C1, was studied by restriction analysis and Southern blot hybridization. It contained a genomic fragment of approximately 13 kb, which when digested by *Eco*RI produced three fragments of 10 kb, 1.7 kb, and 1.3 kb, respectively. DNA carried from the λ5C1 phage clone was analyzed by Southern blot analysis to identify the whole amidase gene. The sequence coding for the N-terminus part of the amidase and containing the upstream region of the gene mapped on the 1.3-kb fragment, whereas the sequence coding for the C-terminus part and containing the downstream region of the gene mapped on the 1.7-kb

fragment. The two fragments, of 1.3 and 1.7 kb, were subcloned in pEMBL130 plasmid and sequenced.

The *S. solfataricus* amidase gene codes for a polypeptide having a molecular weight of 55,784, corresponding to 504 aa. Upstream from the ATG start codon of the amidase gene are three sequences that share homology to the consensus of *S. solfataricus* promoter elements. A putative distal promoter element (DPE) TTTAAA sequence is present 37 bp upstream from the ATG start codon. A proximal promoter element (PPE) is present at –16 (TGCC). These sequences resemble the motifs described by Reiter et al. (1990) as TATA and ribosome-binding site (RBS) putative boxes, respectively. Furthermore, the sequence TTTATAA was found at –23 whereas its complementary sequence, AAATATT, was found at –9.

An open reading frame (ORF) is present starting 22 bp downstream from the amidase stop codon, running in the opposite direction with respect to the amidase gene reading frame. The corresponding amino acid sequence was compared to the SwissProt database and found to share identity with peroxiredoxin from several sources. The best matches were found with a putative peroxiredoxin from *Methanococcus jannaschii* (Bult et al. 1996) (58% identity) and with that from *Thermotoga maritima* (Nelson et al. 1999) (61% identity).

On the opposite DNA strand and 205 bp upstream from the amidase gene is an ORF coding for an unknown protein. Interestingly, database comparison of the translated sequence showed 27% identity with a transposase belonging to the IS605-TnpB family, from *T. maritima* (Nelson et al. 1999).

Sequence alignment

The amidase nucleotide sequence shares 100% identity with a sequence present in the GenBank database (accession number Y08256) deriving from sequencing of the genomic

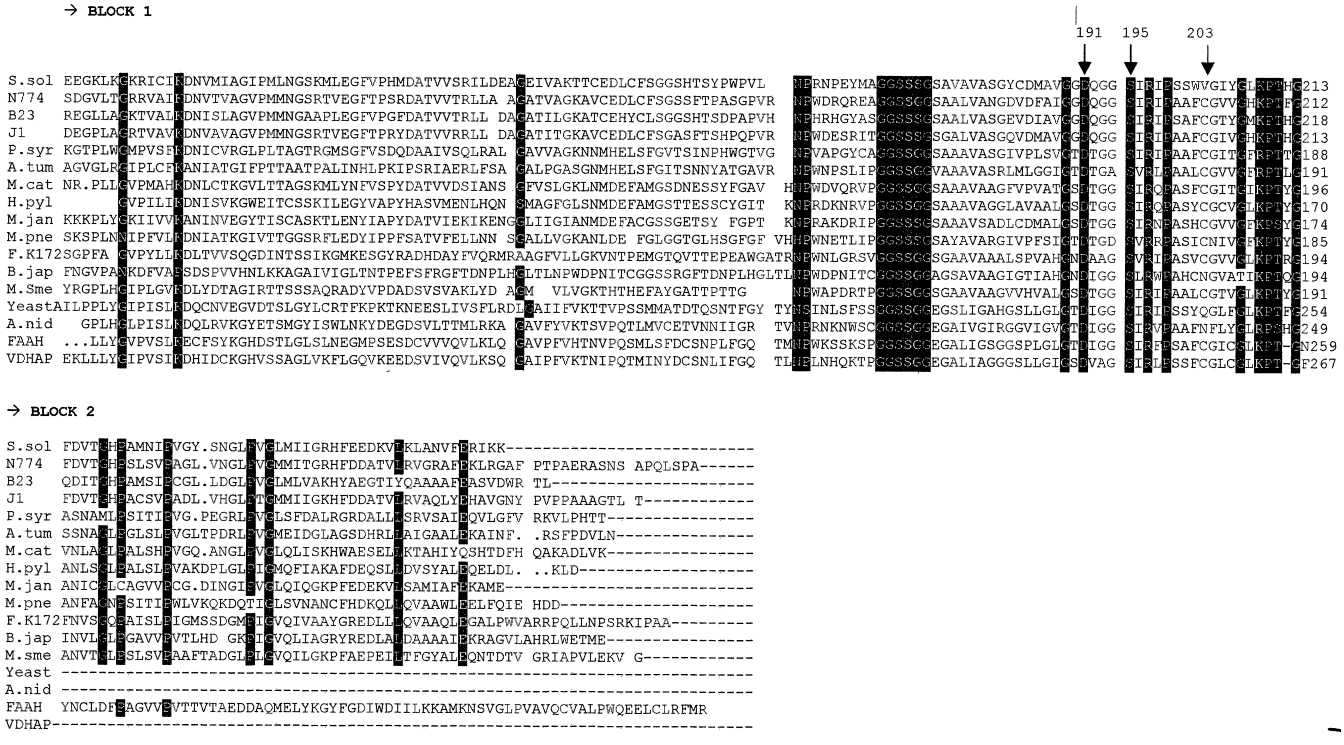

Fig. 1. Alignment over part of the overall *Sulfolobus solfataricus* amidase sequence of some amidase signature enzymes. From *top to bottom* (the database accession numbers are given in parentheses after each enzyme name): *S.sol*, amidase from *S. solfataricus* MT4 and P2 strains (504 residues, GenBank AF290611 and Y08256); *N774*, amidase from *Rhodococcus* sp. N774 (521 residues, GenBank X54074); *B23*, amidase from *Pseudomonas chlororaphis* B23 (506 residues, GenBank D90216); *J1*, amidase from *Rhodococcus rhodochrous* J1 (515 residues, GenBank D16207); *P.syr*, indolacetamide hydrolase from *Pseudomonas syringae* (*P. savastanoi*) (455 residues, GenBank M 11035); *A.tum*, indolacetamide hydrolase from *Agrobacterium tumefaciens* (467 residues, SwissProt P25016); *M.cat*, glutamyl-tRNA amidotransferase subunit A from *Moraxella catarralis* (492 residues, GenBankQ49091); *H.pyl*, glutamyl-tRNA amidotransferase subunit A from *Helicobacter pylori* (453 residues, GenBank P56114); *M.jan*, glutamyl-tRNA amidotransferase from *Methanococcus jannaschii* (434 residues, GenBank Q58560), *M.pne*, glutamyl-tRNA amidotransferase subunit A from *Mycoplasma pneumoniae* (478 residues, SwissProt P75534); *F.K172*, 6-aminohexanoate-cyclic-dimer hydrolase from *Flavobacterium* sp. KI72 (493 residues, GenBank M26953), *B.jap*, indole-3-acetamide hydrolase from *Bradyrhizobium japonicum* (465 residues, GenBank X15117); *M.sme*, nicotinamidase/pyrazin amidase from *Mycobacterium smegmatis* (468 residues, GenBank AF 058285); *Yeast*, putative amidase from *Saccharomyces cerevisiae* (549 residues, GenBank X 56043); *A.nid*, acetamidase from *Aspergillus nidulans* (548 residues, GenBank M 16371); *FAAH*, fatty acid amidohydrolase from rat liver (579 residues, GenBankU72497); *VDHAP*, vitamin D_3 hydroxylase-associated protein from chick kidney (464 residues, GenBankU00694). *Arrows* indicate active site functional residues Asp191, Ser195, and Cys203. Residues highlighted in *reverse type* are conserved. *Block 1*, N-terminus region of the proteins forming block 1 (Chebrou et al. 1996); *Block 2*, C-terminus region of the proteins forming block 2 (Chebrou et al. 1996)

DNA from *S. solfataricus* P2 strain (Sensen et al. 1996). The SwissProt database was searched using the deduced amino acid sequence of *S. solfataricus* amidase as query. The search identified several amidase enzymes from prokaryotic and eukaryotic sources, sharing a sequence identity ranging from 25% to 46%. These amidase enzymes share a very conserved sequence signature, GGSS(S/G)SA (Fig. 1). Moreover, higher identity levels, 45.8%, 44.6%, and 44.1%, respectively, were found with amidases from *Pseudomonas chlororaphis* (Ciskanik et al. 1995), *Rhodococcus rhodochrous* J1 (Kobayashi et al. 1993), and *Rhodococcus* sp. 774 (Mayaux et al. 1991), respectively.

Heterologous expression of recombinant amidase

PCR was used to obtain the amidase structural gene with theλ5C1DNA as template. The PCR product, a 1,515-bp fragment, was inserted into the *Bam*HI/*Kpn*I-digested pRSET A expression vector. The construct, pSETH12, was used to transform *E. coli* BL21(DE3) pLysS strain, and gene expression was induced by IPTG. The protein was expressed as a fusion with a peptide of 4 kDa, containing six histidine residues and a enterokinase cleavage site. The recombinant enzyme was mostly found as an insoluble fraction, but was also present in the soluble fraction (Fig. 2). To enhance the amount of soluble protein, the amidase gene was cloned in the expression vector pTrcHis 2 (Invitrogen), which produces a tagged protein with six histidines at the C-terminus. The recombinant plasmid, pBal3, was transformed in *E. coli* Top 10 strain; the expression was induced as previously described. The major fraction of this recombinant amidase was still insoluble.

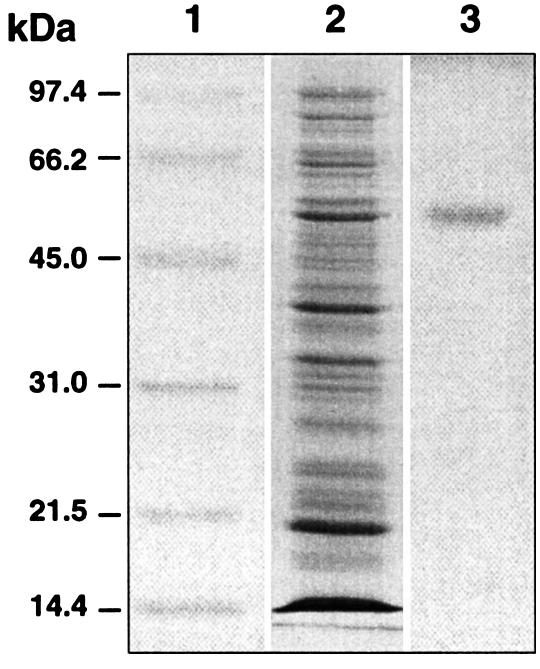

Fig. 2. SDS-PAGE gel, 15% acrylamide, stained with Coomassie blue, showing the purification product from the soluble fraction in the *Escherichia coli* transformant. *Lane 1*, low molecular mass standards; *lane 2*, soluble fraction; *lane 3*, purified N-His-tagged recombinant amidase of *S. solfataricus*

Physical properties

The N-His-tagged recombinant enzyme present in the soluble fraction added to the amount extracted from the pellet under native conditions was purified by the one-step affinity chromatography procedure previously described. The C-terminus His-tagged enzyme, purified under the same conditions as for the N-terminus His-tagged enzyme, gave the same yield and exhibited the same features.

The recombinant protein, purified and depleted of its fusion partner, was used for enzyme characterization. The relative molecular mass of the native enzyme, estimated by gel permeation chromatography on a Superose 12 column, was approximately 55,000 (± 2,000) Da. SDS-PAGE analysis of denatured protein showed a similar size. Also, sedimentation velocity experiments and equilibrium sedimentation, carried out at pH 8 and at enzyme concentrations between 0.1 and 3 mg/ml, confirmed that the recombinant amidase was a monomer that did not aggregate up to the concentration used. The isoelectric point, determined by a computer program based on amino acid composition, is 5.94 for the protein without the His-tag tail and 6.05 with the tail. The latter value is in good agreement with the experimental value obtained by MonoP chromatography (Pharmacia).

Substrate specificity

Sulfolobus solfataricus recombinant amidase exhibited very broad activity (Table 1) with either aliphatic or aromatic amides. Tailed and untailed enzymes did not show any difference.

Among assayed substrates, the best aromatic substrate was *p*-toluamide with k_{cat} = 7.3 s^{-1}, although the enzyme showed activity on *o*- and *m*-toluamide, 2-phenylpropionamide, 3-phenylpropionamide, 2-thiophenecarboxamide, benzamide, nicotinamide, isonicotinamide, picolinamide, pyrazinamide, salicylamide, anthranilamide, and 3-indolacetamide. The best aliphatic substrate was isobutyramide with a k_{cat} of 21.9 s^{-1}, but the enzyme was also active versus acetamide, propionamide, pentanamide, hexanamide, cyclohexanamide, lactamide, prolinamide, glutamine, asparagine, acrylamide, and methacrylamide.

Enantioselectivity

The enantioselectivity of the enzyme was tested with 2-phenylpropionamide, evaluating its selectivity by chiral chromatography on a Chirobiotic T column (150 × 4.6 mm; Astec) with tecoplamin bound to a silicon matrix. The column was standardized with the pure *R*- and *S*-forms of 2-phenylpropionic acid (Aldrich, Milwaukee, WI, USA). The enzyme was able to hydrolyze 10 mM 2-phenylpropionamide to 5 mM of the *S*-form of 2-phenylpropionic acid in

Table 1. Kinetic properties of *Sulfolobus solfataricus* amidase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})×10^2	AS (μmoles min^{-1} mg^{-1})
Acetamide	3.6	1.6	4.4	3.8
Propionamide	1.3	3.2	24.6	3.9
Lactamide	3.0	10.7	35.7	12.8
Butyramide	1.2	9.9	82.5	11.9
Isobutyramide	1.0	21.9	219.0	26.2
Pentanamide	1.3	20.5	157.7	22.0
Hexanamide	5.8	5.3	9.1	6.4
Cyclohexanamide	5.0	16.3	32.6	19.5
Acrylamide	3.0	8.3	27.7	10.2
Metacrylamide	1.2	2.9	24.2	3.5
Prolinamide	6.7	3.1	4.6	3.4
Benzamide	0.3	6.4	213.3	6.8; 13.7[a]
o-OH benzamide	0.4	1.3	32.5	1.4
p-OH benzamide	1.5	1.1	7.3	1.2
o-NH_2 benzamide	0.3	0.9	30.0	1.0
p-NH_2 benzamide	0.3	0.8	26.6	0.8
o-Toluamide	0.3	0.3	7.5	0.3
p-Toluamide	0.3	7.3	243.3	8.1
Nicotinamide	0.3	1.6	53.3	1.7
Isonicotinamide	0.8	1.6	20.0	1.8
Picolinamide	2.5	1.8	7.2	2.1
3-Phenylpropionamide	4.0	7.1	17.7	7.6
Indol-3-acetamide	0.9	1.8	20.0	1.9

AS, specific activity
[a] Specific activity measured at 85°C

about 35 min at 70°C, without any formation of the *R*-form even if the incubation was prolonged, demonstrating the high selectivity of the enzyme.

Acyl transfer

The enzyme was found to transfer the acyl group also to hydroxylamine and hydrazine, as reported for other amidases (Fournand et al. 1998a, 1998b): the specific activities of the enzyme reported in Table 2 proved that, with hydroxylamine and hydrazine as acceptors, the efficiency of the acyl donors increased from acetamide to isobutyramide, as found with water as acceptor. Other amides also, such as isonicotinamide, nicotinamide, picolinamide, and prolinamide, were found to be good acyl donors to both hydroxylamine and hydrazine.

pH optimum, thermoactivity, and thermostability

Optimal pH enzyme activity was at pH 7.5 (Fig. 3A). However, 50% of maximal activity could be achieved at both pH 4 and pH 9.5, demonstrating a very broad pH dependence, a feature shared by most of the amidases studied so far (Fournand et al. 1998b; Kobayashi et al. 1993; Hirrlinger et al. 1996). The enzyme was active up to 95°C (Fig. 3B) and had good thermostability; its activity was constant for more than 72 h at 70°C; at 80°C and 90°C, the half-life was 25 h and 0.5 h, respectively (Fig. 3C).

Table 2. Specific activities for amidase-catalyzed acyl transfer reaction from amides to hydroxylamine and hydrazine, expressed as micromoles of hydroxamic acids or acid hydrazides per minute and per milligram protein

Amides[a]	Hydroxylamine	Hydrazine
Acetamide	8.4	1.4
Propionamide	18.4	3.0
Isobutyramide	25.0	22.7
Benzamide	9.2	6.1

Reactions were conducted in 50 mM sodium phosphate buffer, pH 7.5, for 10 min at 70°C
[a] Reagents were at the following concentrations: amides, 100 mM (benzamide, 10 mM); hydroxylamine and hydrazine, 400 mM; enzyme 0.9 μM

Activators

Fe^{3+}, V^{2+}, and Co^{2+} at 1 mM were slight activators of the enzyme, increasing the original activity by about 20%. Other metal ions, such as Cu^{2+}, Mn^{2+}, and Ca^{2+}, had no effect.

Storage

The enzyme may be stored at room temperature in the presence of 0.04% NaN_3 for 1 year without any loss of activity. NaN_3 does not alter the detection of enzyme activity.

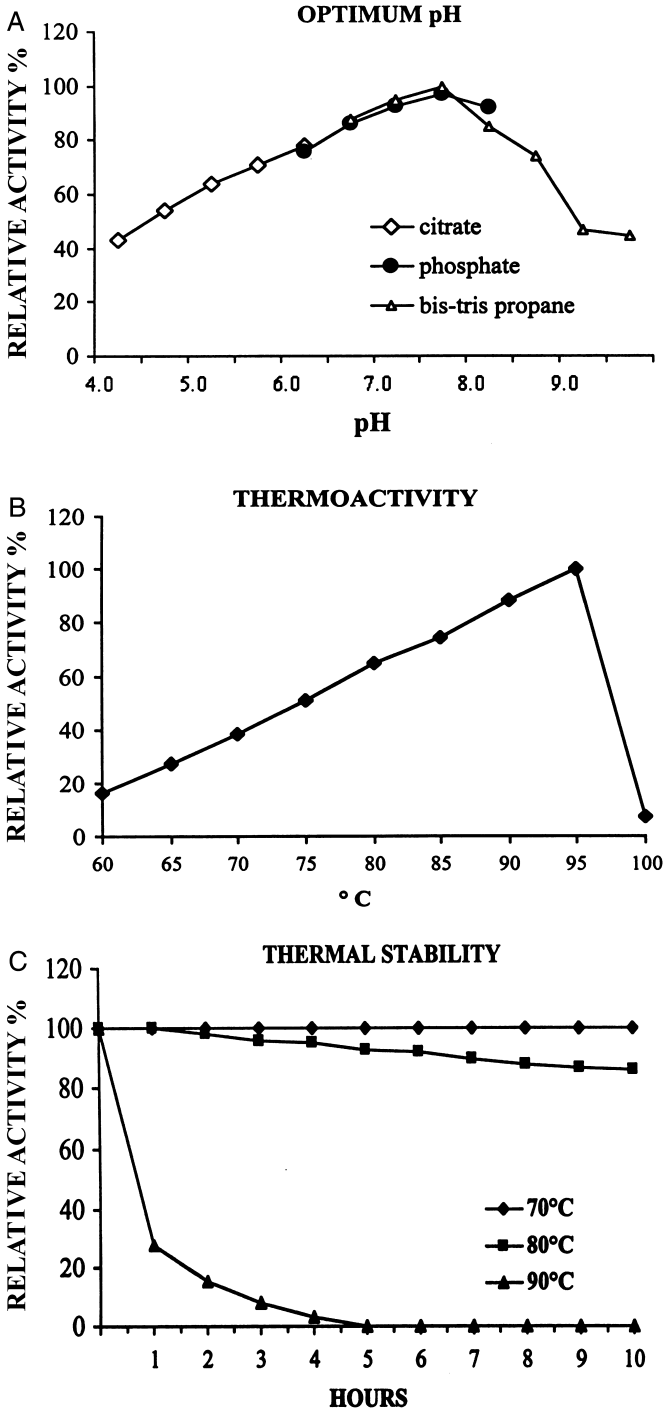

Fig. 3. Effect of pH (**A**) and temperature (**B**) on the activity of the purified amidase of *S. solfataricus*. **A** Enzyme activity was measured at 70°C for 10 min, using 7.5 mM benzamide in 50 mM citrate buffer at pH 4–5.8 (*squares*), sodium phosphate buffer at pH 6–7.9 (*circles*), and bis-Tris propane buffer at pH 6.5–9.5 (*triangles*). The pH value of each buffer was checked at 70°C; 100% of activity corresponds to 6.8 units/mg protein. **B** Enzyme activity was measured in 50 mM bis-Tris propane buffer at pH 7.5 for 10 min, using benzamide as substrate; 100% of activity corresponds to 6.2 units/mg protein. Each enzyme assay was performed with 50 µg/ml. **C** Thermal stability of the *S. solfataricus* amidase. The enzyme (70 µl at 0.2 mg/ml in 100 mM bis-Tris propane buffer, pH 7.5, checked at the same temperature) was placed in eppendorf vials (0.5 ml), covered by a layer of mineral oil, and preincubated at 70°C (*rhomboids*), 80°C (*squares*), and 90°C (*triangles*). Residual activity was measured in a 50-µl sample by standard assay; 100% activity corresponds to 6.5 units/mg protein. All reported values are the average of three measurements; parameter errors were less than 5%

Discussion

The amidase gene was cloned from the *S. solfataricus* MT4 strain. Search by homology comparison among archaeal promoter sequences strongly suggested that the untranslated 5′-end upstream from the amidase gene from *S. solfataricus* included several putative control elements of gene expression. These sequences shared homology with the transcription factor B (TFB) DNA-binding site (Littlefield et al. 1999). Recently, X-ray resolution of the archaeal DNA transcription factor B, TFB complex, has shown that a TTTANA sequence consensus, termed BRE, was involved in binding the helix-turn-helix domain of the TFB protein (Littlefield et al. 1999). The presence upstream of the *Sulfolobus* amidase gene of the repeats described here might account for the strictly regulated gene expression of the *S. solfataricus* amidase.

The amidase gene from several microorganisms, such as *Rhodococcus rhodochrous* J1, *Pseudomonas chlororaphis* B23, and *Rhodococcus* sp. N774, is localized upstream or downstream from the nitrile hydratase subunit genes, suggesting that in these microorganisms the two enzymes are coregulated (Yamada and Kobayashi 1996). Nitriles used as carbon or nitrogen sources are converted into amides and then into organic acids by a two-step process. In *S. solfataricus* MT4 strain, we were unable to detect a gene for nitrile hydratase close to the amidase gene. Furthermore, the amidase locus was not closely related to its putative regulators.

Multialignment of the deduced amino acid sequence of *Sulfolobus* amidase with sequences present in the protein database showed that the archaeal protein belongs to the signatured GGSS(S/G)GS class of amidases previously identified (Mayaux et al. 1991). Furthermore, two conserved sequence blocks in the primary structure of the signatured amidase genes have been described (Chebrou et al. 1996) and are reported in Fig. 1. Block 1 includes the signature and is present in Eukarya and Prokarya. Block 2, localized at the C-terminus of the protein, is conserved only in Prokarya. Among eukaryotic amidase enzymes, which lack block 2, only fatty acid amide hydrolase (Cravatt et al. 1996) has a region containing two amino acids, which are conserved in the prokaryotic block 2 (Chebrou et al. 1996). Interestingly, as shown in Fig. 1, *Sulfolobus* amidase has both blocks, suggesting that it is evolutionarily correlated to prokaryotic enzymes. The absence of block 2 in eukaryotic amidase enzymes suggests that Eukarya lost some sequences in the genomic rearrangements after their divergence from Archaea.

The *S. solfataricus* recombinant amidase was expressed as a fusion protein with a 6-His tag at the N- or C-terminus. The yield of both fusion proteins was comparable, and their catalytic activity was identical to the catalytic activity of the enzymes after enterokinase cleavage. These findings suggest that the polypeptide ends of the enzyme in its three-dimensional structure are not close to the active site. The analysis of substrates efficiently hydrolyzed by the enzyme indicates that *S. solfataricus* amidase is very similar to *Rhodococcus* J1 amidase (Kobayashi et al. 1993); both are active on a large spectrum of substrates hydrolyzing many aliphatic and aromatic amides.

Among aliphatic substrates, *S. solfataricus* amidase is weakly active against acetamide (k_{cat} = 1.6 s^{-1}): the k_{cat} increases with increase in the carbon chain up to isobutyramide, which is the best substrate with a k_{cat} value of 21.9 s^{-1}. Among aromatic amides, the best substrate is *p*-toluamide, with a $k_{cat}$of 7.3 s^{-1}: nicotinamide, isonicotinamide, and picolinamide, with k_{cat} values of 1.6, 1.6, and 1.8 s^{-1}, respectively, have lower values compared to benzamide (k_{cat} = 6.4 s^{-1}), indicating that the substitution of one carbon of the ring with a nitrogen negatively affects the turnover number of the enzyme independently of the position of substitution with respect to the carboxamide group.

These results and those reported in Table 1 demonstrate a preferred interaction of the substrate with hydrophobic residues of the enzyme far from its catalytic site and are consistent with the hypothesis that the amidase molecule has a cleft where the carboxamide group of the substrate may be admitted while the ring or the aliphatic chain of the substrate remains outside. Hydrophilic substituents in the aromatic rings as OH and NH_2 have negative effects independently of their location with respect to the carboxamide group, whereas hydrophobic groups such as CH_3 have a negative effect in the ortho-position and a positive effect in meta- or para-positions with respect to the carboxamide group of the substrate. Saturated or unsaturated rings and aliphatic chains of the substrates should then undergo some interactions with the walls of the cleft, which depends on the presence of heteroatoms in the ring and of hydrophilic or hydrophobic group substituents and on hydrocarbon chain length.

Hydrophobic interactions with the aliphatic carbon chain of substrates are minimally achieved by propionamide. Acetamide, which is poorly degraded by the enzyme, has a chain too short for good interaction. The best aliphatic substrate is represented by the five carbon atoms of pentanamide. The enzyme is active also on substrates such as cyclohexanamide, where the carboxamide group is bound to a cyclohexane ring (the k_{cat} of cyclohexanamide is tripled with respect to that of hexanamide) or to a pyrrolidine ring as in prolinamide. Glutamine and asparagine are also hydrolyzed by the enzyme. The specific activity of *S. solfataricus* amidase with benzamide at 70°C is 6.8 μmol min^{-1} (mg protein)$^{-1}$ whereas at 85°C it is 13.8, very close to the value of 12.2 μmol min^{-1} mg^{-1} at 30°C reported for *R. rhodochrous* J1 amidase (Kobayashi et al. 1993).

Also, the apparent $K_m$values at 70°C of the hyperthermophilic enzyme for propionamide and benzamide, which are 1.3 mM and 0.3 mM, respectively, are comparable to those of the mesophilic amidase from *Rhodococcus* for the same substrates (0.48 mM and 0.15 mM, respectively). Furthermore, the *Sulfolobus* enzyme, similarly to the amidase from *Rhodococcus*, may transfer the acyl group of the substrate either to water, to give the free acid and ammonia, or to other acceptors, such as hydroxylamine, to give the corresponding hydroxamic acid or to hydrazine to give the corresponding acid hydrazides. This activity may be relevant for industrial applications.

A further use of this amidase could be to degrade some amides as acrylamide that are dangerous for the environment, or, if coupled with a thermophilic nitrile hydratase, in the production of organic acids starting from nitriles. These biocatalytic processes are actually carried out by mesophilic enzymes at temperatures ranging from 30° to 50°C (Kobayashi et al. 1991). *S. solfataricus* amidase exhibits good thermophilicity and good thermostability. typical of the enzymes from this microorganism. It may work at temperatures ranging from70° to 90°C and could be used to biotransform many amides with great efficiency. Finally, the enantioselective activity of the enzyme is of particular interest for its potential industrial use: only one of the enantioforms of most compounds of pharmaceutical interest has biological activity.

Recently it has been suggested that, in the *Rhodococcus rhodochrous* J1 amidase, the active site of the enzyme is close to the signature consensus (Kobayashi et al. 1997). In this enzyme, Asp191, Ser195, and Cys203 residues have been considered important residues for catalytic activity. Site-directed mutagenesis of Asp191 and Ser195 demonstrated the role of these residues in the enzyme activity, whereas Cys203 was less relevant. In the *S. solfataricus* amidase, Asp191 and Ser195, which have the same numbering as the rhodococcal counterpart, are present but a Val substitutes Cys203, strengthening the view that Cys203 has little or no involvement in the catalytic activity.

To investigate the biochemical features of the amidase and to broaden its industrial potential, site-directed mutagenesis experiments are in progress. Also, the availability of large amounts of the hyperthermophilic enzyme has allowed us to begin crystallographic experiments to gain insight into the three-dimensional structure of a signatured thermophilic amidase and to increase our knowledge of the protein thermostability strategy used by thermophilic microorganisms (Scandurra et al. 1998).

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