The quaternary structure of the amidase from *Geobacillus pallidus* RAPc8 is revealed by its crystal packing

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The amidase from *Geobacillus pallidus* RAPc8, a moderate thermophile, is a member of the nitrilase enzyme superfamily. It converts amides to the corresponding acids and ammonia and has application as an industrial catalyst. RAPc8 amidase has been cloned and functionally expressed in *Escherichia coli* and has been purified by heat treatment and a number of chromatographic steps. The enzyme was crystallized using the hanging-drop vapour-diffusion method. Crystals produced in the presence of 1.2 M sodium citrate, 400 mM NaCl, 100 mM sodium acetate pH 5.6 were selected for X-ray diffraction studies. A data set having acceptable statistics to 1.96 Å resolution was collected under cryoconditions using an in-house X-ray source. The space group was determined to be primitive cubic \( P_{4}2_{1}3_{2} \), with unit-cell parameter \( a = 130.49 (\pm 0.05) \) Å. The structure was solved by molecular replacement using the backbone of the hypothetical protein PH0642 from *Pyrococcus horikoshii* (PDB code 1j31) with all non-identical side chains substituted with alanine as a probe. There is one subunit per asymmetric unit. The subunits are packed as trimers of dimers with \( D_{3} \) point-group symmetry around the threefold axis in such a way that the dimer interface seen in the homologues is preserved.

1. Introduction

Amidases (acylamide amidohydrolases; EC 3.5.1.4) catalyze the hydrolysis of amides to the corresponding carboxylic acids and ammonia. Amidases have been isolated from various sources and typically exhibit broad substrate specificities. Some amidases hydrolyze the amides of \( \alpha \)- or \( \omega \)-amino acids, are active on aromatic amides and demonstrate enantioselectivity (Kotlova et al., 1999). Amidases find applications as industrial catalysts in organic synthesis, in the treatment of industrial effluents containing toxic amides and also in the manufacture of therapeutic agents (Kobayashi & Shimizu, 1994).

A thermostable amidase was previously identified in a *Bacillus* isolate (RAPc8; Pereira et al., 1998), which is currently named *Geobacillus pallidus* on the basis of its 16S RNA sequence. The ORF which encoded a putative 348-amino-acid amidase (MW 38.6 kDa) was located immediately downstream of ORFs encoding the \( \omega_{1} \)- and \( \omega_{2} \)-subunits of a nitrile hydratase. This putative amidase ORF was cloned in pET21a(+) and overexpressed (Cameron et al., 2005).

Amidases are known to belong to at least two enzyme families having completely different folds (Novo et al., 2002). Sequence comparisons suggested that *G. pallidus* RAPc8 amidase belonged to the nitrilase superfamily (Pace & Brenner, 2001), with the characteristic glutamate, lysine and cysteine catalytic triad residues being conserved. Site-directed mutagenesis of an invariant Cys166 residue in the *Pseudomonas aeruginosa* amidase revealed that it acts as the nucleophile in the catalytic mechanism (Farnaud et al., 1999). The amidases from *P. aeruginosa* (Ambler et al., 1987), *Bacillus stearothermophilus* BR338 (Cheong & Oriel, 2000) and *Bacillus* sp. BR449 (Kim & Oriel, 2000) all belong to this group, the latter being identical.
to the amidase described here. To date, four crystal structures of nitrlase superfamily members have been published. Their three-dimensional structures show that the enzymes are multimeric and have a conserved \textit{afila} sandwich fold.

Farnaud \textit{et al.} (1999) have previously described crystals of the amidase from \textit{P. aeruginosa}, which belonged to either space group R3 or R32 but were unsuitable for data collection. In this paper, we describe the purification, crystallization and preliminary X-ray diffraction data analysis, space-group determination and oligomeric symmetry of the amidase.

2. Materials and methods

2.1. Expression of \textit{G. pallidus} RAPc8 amidase

The amidase gene from \textit{G. pallidus} RAPc8 was recombinantly expressed from plasmid pH223 as described by Cameron \textit{et al.} (2005). The recombinant \textit{Escherichia coli} strain BL21(DE3) (Stratagene) harboured the plasmid pH223 was grown in 1 L LB medium at 310 K containing 100 \mu g ml\(^{-1}\) ampicillin. At an optical density of 0.6 (at 600 nm), the culture was induced at 303 K with 0.2 mM IPTG. Cells were harvested by centrifugation 16 h post-induction and were washed once with 50 mM potassium phosphate buffer pH 7.4.

2.2. Protein purification

The washed cell pellet was suspended in 10 ml 50 mM potassium phosphate buffer pH 7.4 containing 10 mM DTT, 1 mM EDTA, 200 mM NaCl, 10\% glycerol and disrupted by sonication. The cell debris was harvested by centrifugation at 15 000g (20 min, 277 K). The cell-free extract was diluted with an equal amount of suspension buffer containing no EDTA and heated to 318 K for 20 min. The debris was removed by centrifugation at 15 000g (30 min, 277 K).

The heat-treated protein sample was mixed with 3.5 M ammonium sulfate to achieve a final concentration of 1.7 M in the supernatant and left on ice for 1 h. Precipitated proteins were removed by centrifugation at 15 000g (30 min, 277 K). The supernatant was applied onto a Hi-Load 16/10 Phenyl-Sepharose column (Amersham Biosciences) previously equilibrated with buffer \(A\). The column was washed with 200 ml buffer \(A\) and protein was eluted with a linear 0.1–1 M NaCl gradient to buffer \(C\) (20 mM potassium phosphate pH 7.4, 2 mM DTT, 1 M NaCl). Highly purified protein fractions were pooled, concentrated (Vivaspin concentrator) and passed through an S-300 HR gel-exclusion chromatography column (GE-Healthcare) equilibrated with buffer \(D\) (20 mM Tris–HCl pH 7.4, 2 mM DTT, 150 mM NaCl).

Amidase purity and molecular weight was determined at all stages by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 15\% gels stained with Coomassie blue R-250. Samples were assayed for protein concentration using the Biorad protein-determination kit (BioRad) with BSA as a standard.

2.3. Crystallization

The highly purified enzyme was concentrated to 10 mg ml\(^{-1}\) (as determined from the absorbance at 280 nm) and filtered through a 0.22 \mu m filter. The amidase was crystallized using the hanging-drop vapour-diffusion method. Initial crystallization conditions were tested using Hampton Crystal Screens 1 and 2 (Hampton Research). A total of 148 individual conditions were tested. Parallel screens at 295, 298 and 277 K were prepared. The hanging drop was mounted on round siliconized cover slips which contained 1 \mu l protein and 1 \mu l reservoir solution.

Once initial conditions had been resolved, 50 optimization conditions were carried out with drops containing 4 \mu l protein and 4 \mu l reservoir solution. Protein concentration, incubation temperature, precipitant concentration and reservoir solution pH were all varied during the optimization trials. Diffraction-quality crystals were finally grown at 295 K in 1.2 M sodium citrate, 400 mM NaCl, 100 mM sodium acetate pH 5.6.

2.4. X-ray diffraction data collection and processing

X-ray diffraction data were collected using a Rigaku RUH3R copper rotating-anode X-ray source operated at 40 kV and 22 mA, a Rigaku R-AXIS IV\textsuperscript{+} image-plate camera, an X-stream 2000 low-temperature system and an AXCO PX50 glass capillary optic with a 0.1 mm focus. Data from crystals mounted on a cryoloop (Hampton Research) were collected at a temperature of 100 K with a crystal-to-detector distance of 157.1 mm. Data frames covering an oscillation angle of 0.5° per frame and spanning a range of 48° were collected for

![Figure 1](image1.png)

Figure 1

SDS–PAGE of amidase purification. Lane 1, molecular-weight markers (kDa); lane 2, induced cells; lane 2, clarified cell extract; lane 3, heat-treated supernatant; lane 4, pooled fractions from Phenyl-Sepharose hydrophobic interaction chromatography; lane 5, pooled fractions from Q-Sepharose ion-exchange chromatography; lane 6, pooled fractions from gel-exclusion chromatography.

![Figure 2](image2.png)

Figure 2

A crystal of the amidase from \textit{G. pallidus} RAPc8.
55 min per frame. Laue group determination, integration, scaling, merging and absorption correction were performed with both CrystalClear (dt*TREK; Plugrath, 1999) and HKL (Otwinowski & Minor, 1997). The determination of the space group is described in §3.

### 2.5. Sequence and structural alignments

A search for structural homologues of *G. pallidus* RAPc8 amidase was carried out using mGenTHREADER (Jones, 1999).

### 3. Results and discussion

#### 3.1. Expression and purification of amidase

*G. pallidus* RAPc8 was previously isolated in our laboratory (Pereira et al., 1998). The first ORF encoded a 348-amino-acid protein with 100% identity to the amidase from *Bacillus* sp. BR449 (Kim & Oriel, 2000). However, owing to six silent nucleotide substitutions, the DNA sequences shared a slightly lower identity (99%; Cameron et al., 2005). *E. coli* harbouring the recombinant plasmid pNH223 (Cameron et al., 2005) expressed the enzyme at high levels (Fig. 1).

The purification procedure comprises four steps: heat treatment, hydrophobic interaction chromatography (HIC), anion-exchange chromatography and gel filtration. SDS–PAGE (Fig. 1) indicated that HIC and anion-exchange chromatography was sufficient to yield >98% homogeneous enzyme. However, gel-exclusion chromatography was used as a final step to alter buffer conditions and salt concentrations. The amidase was concentrated to 10 mg ml⁻¹ using Vivaspin concentrators. Despite a buffer pH (7.4) that was distant from the theoretical amidase pI (5.6), aggregation was observed at protein concentrations above 10 mg ml⁻¹. SDS–PAGE analysis confirmed the presence of a 38.6 kDa band corresponding to the theoretical molecular weight of the amidase. The molecular weight of the amidase oligomer based on its elution from the calibrated S-300...
HR gel-filtration column was 208 kDa, consistent with it being a hexamer.

3.2. Crystallization and X-ray diffraction

Since the previously reported amidase crystals (Farnaud et al., 1999) were not of adequate quality for structure determination, a coarse screen was performed with Hampton Crystal Screens 1 and 2. Needle-shaped or branched crystals were observed in precipitants containing sodium citrate and PEG. Finer optimizations were performed around these conditions. Protein concentration, incubation temperature, precipitant concentration and reservoir solution pH were varied. Promising crystals, as determined by external morphology, were seen at a protein concentration of 10 mg ml$^{-1}$ in 1.2 M sodium citrate, 400 mM NaCl, 100 mM sodium acetate pH 5.6. The crystals grew to final dimensions of up to 0.7 mm after incubation for 8–10 d at 295 K. These crystals (Fig. 2), which had dimensions of approximately 0.2 × 0.1 × 0.1 mm, were selected for X-ray diffraction experiments. Data were collected from a single crystal which diffracted well, producing visible spots to 1.76 Å resolution and having a mosaicity of 0.50 m$^{-1}$. A total of 231 265 reflections were collected in the resolution range 30.0–1.93 Å as shown in Table 1. $R_{int}$ fell below 2 for averaged reflections between 1.96 and 1.93 Å resolution. A total of 27 777 unique reflections were collected, giving a data set which was 96.7% complete to 1.93 Å.

The unit cell was determined by the Fourier transform method (Steller et al., 1997). It was clearly primitive cubic and the Laue group was $m\overline{3}$. Only ten structures listed in the Protein Data Bank at the present time belong to this space group; of these, seven are viruses.

3.3. Sequence alignments

A search for structural homologues using mGenTHREADER produces four independent crystal structures (PDB codes 1ems, 1erz, 1j31 and 189) and a model of the amidase (PDB code 1k17). Fig. 3 shows alignments of the G. pallidus RAPc8 amidase sequence with these five proteins: the Nit domain of the NitFhit fusion protein (PDB code 1ems; Pace et al., 2000), N-carbamoyl-d-amino-acid amido-hydrolase (PDB code 1erz; Nakai et al., 2000), putative CN hydrolase from yeast (PDB code 1f89; Kumaran et al., 2003), hypothetical protein PH0642 from Pyrococcus horikoshii (PDB code 1j31; Sakai et al., 2004) and the amidase model structure (PDB code 1k17; Novo et al., 2002). Only the carbamoylase has been well characterized biochemically as well as structurally (Chen et al., 2003) and recently a number of structures of the carbamoylase having substrates bound in the mutated active site have become available (PDB codes 1uf4, 1uf5, 1uf6).
implies that the biological hexamer has D3 point-group symmetry with the C-terminal region located on the outside of the hexamer. This observation has implications for the biotechnological use of the enzyme. Detailed interpretation of the electron density is proceeding with a view to visualizing the fold of the C-terminal region and the detailed configuration of the active site.

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