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

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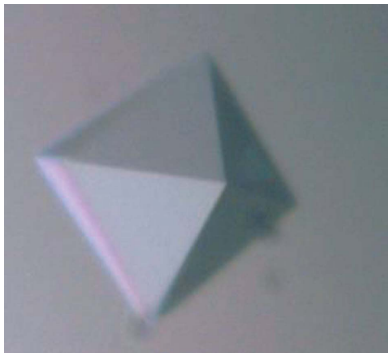
The amidase from *Geobacillus pallidus* RAPc8, a moderate thermophile, is a member of the nitrilase enzyme superfamily. It converts amides to the corresponding acids and ammonia and has application as an industrial catalyst. RAPc8 amidase has been cloned and functionally expressed in *Escherichia coli* 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 m*M* NaCl, 100 m*M* 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 $P4_232$, with unit-cell parameter a = 130.49 (±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 α- or ω-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 α- and β-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 nitrilase superfamily members have been published. Their three-dimensional structures show that the enzymes are multimeric and have a conserved $\alpha\beta\beta\alpha$ sandwich fold.

Farnaud *et al.* (1999) have previously described crystals of the amidase from *P. aeruginosa*, which belonged to either space group *R*3 or *R*32 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 *G. pallidus* RAPc8 amidase

The amidase gene from *G. pallidus* RAPc8 was recombinantly expressed from plasmid pNH223 as described by Cameron *et al.* (2005). The recombinant *Escherichia coli* strain BL21(DE3) (Stratagene) harbouring the plasmid pNH223 was grown in 1 l LB medium at 310 K containing 100 µg ml^{-1} ampicillin. At an optical density of 0.6 (at 600 nm), the culture was induced at 303 K with 0.2 m*M* IPTG. Cells were harvested by centrifugation 16 h post-induction and were washed once with 50 m*M* potassium phosphate buffer pH 7.4.

2.2. Protein purification

The washed cell pellet was suspended in 10 ml 50 m*M* potassium phosphate buffer pH 7.4 containing 10 m*M* DTT, 1 m*M* EDTA, 200 m*M* NaCl, 10% glycerol and disrupted by sonication. The cell debris was harvested by centrifugation at 15 000*g* (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 15 000*g* (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 15 000*g* (30 min, 277 K). The supernatant was applied onto a Hi-Load 16/10 Phenyl-Sepharose column (Amersham Biosciences) equilibrated with buffer *A* (1.7 *M* ammonium sulfate, 50 m*M* potassium phosphate pH 7.4, 5 m*M* DTT). Bound proteins were eluted with a linear gradient of ammonium sulfate (1.7–0 *M* ammonium sulfate in 50 m*M* potassium phosphate pH 7.4, 5 m*M* DTT). The fractions containing a high concentration of amidase were pooled, dialysed against buffer *B* (20 m*M* potassium phosphate pH 7.4, 5 m*M* DTT, 100 m*M* NaCl) and loaded onto a HiPrep 16/10 Q-Sepharose FF column (Amersham Biosciences) previously equilibrated with buffer *B*. The column was washed with 200 ml buffer *B* and protein was eluted with a linear 0.1–1 *M* NaCl gradient to buffer *C* (20 m*M* potassium phosphate pH 7.4, 2 m*M* 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 m*M* Tris–HCl pH 7.4, 2 m*M* DTT, 150 m*M* 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 µm 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 µl protein and 1 µl reservoir solution.

Once initial conditions had been resolved, 50 optimization conditions were carried out with drops containing 4 µl protein and 4 µl 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 m*M* NaCl, 100 m*M* 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^{+} 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

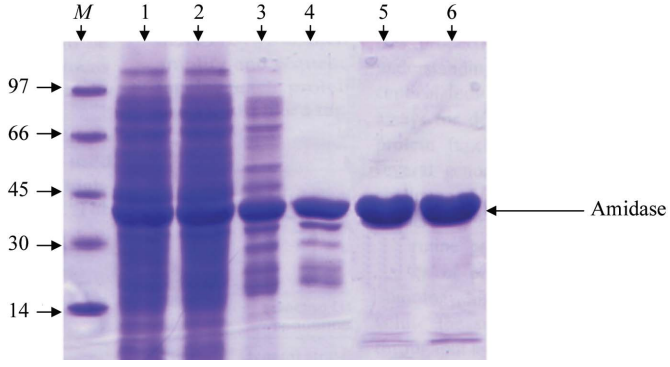


Figure 1
SDS–PAGE of amidase purification. Lane *M*, molecular-weight markers (kDa); lane 1, induced cells; lane 2, clarified cell extract; lane 3, heat-treated supernatant; lane 4, pooled fractions from Phenyl-Sepharose hydrophobic interaction chromatography; lane 5, pooled fractions from Q-Sepharose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

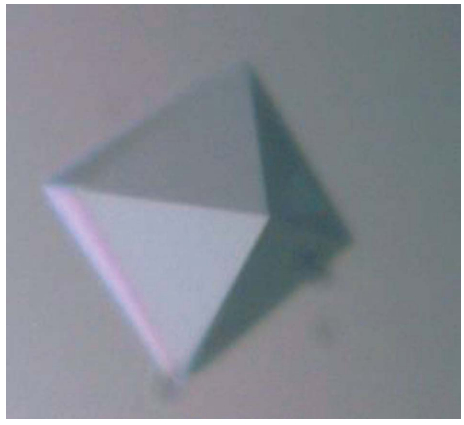


Figure 2
A crystal of the amidase from *G. pallidus* RAPc8.

55 min per frame. Laue group determination, integration, scaling, merging and absorption correction were performed with both *CrystalClear* (*d*TREK*; Pflugrath, 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

Table 1
X-ray data-collection statistics and crystallographic information.

Wavelength (Å)	1.5418
Space group	$P4_232$
Unit-cell parameters (Å, °)	$a = b = c = 130.49$ (±0.05), $\alpha = \beta = \gamma = 90$
Resolution range (Å)	30.00–1.93 (1.96–1.93)
Total No. of reflections	231265
No. of unique reflections	27777
Average redundancy	8.3 (4.7)
Completeness (%)	96.7 (96.4)
χ^2	0.98 (1.05)
R_{sym}	0.109 (0.301)
$\langle I/\sigma(I)\rangle$	5.4 (1.99)

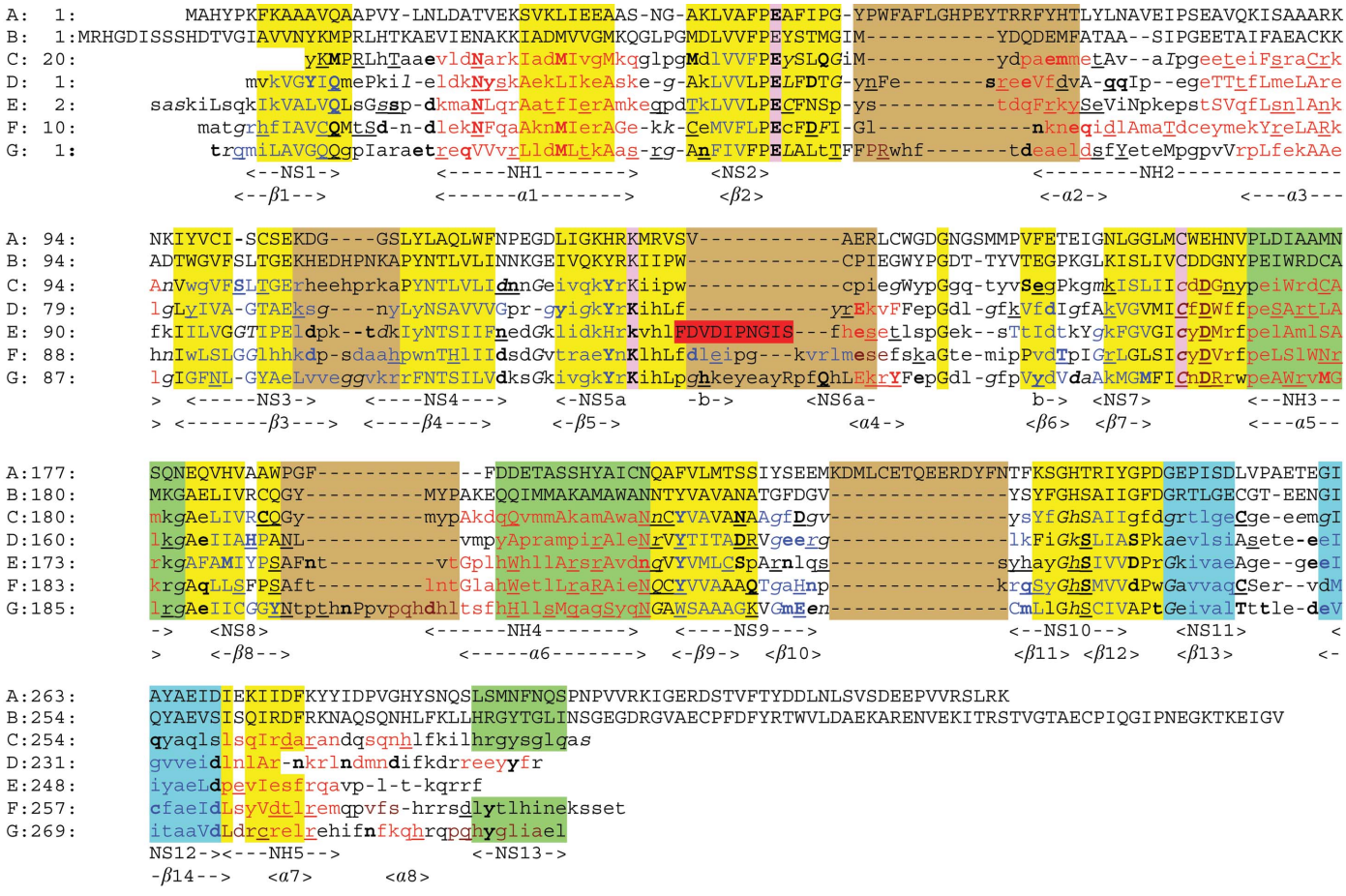


Figure 3
Alignment of the *G. pallidus* RAPc8 amidase sequence. A, cyanide dihydratase (*Pseudomonas stutzeri* AK61); B, amidase (*G. pallidus* RAPc8); C, *Pseudomonas aeruginosa* amidase model (PDB code 1k17); D, hypothetical protein PH0642 from *Pyrococcus horikoshii* (PDB code 1j31); E, putative CN hydrolase from yeast (PDB code 1f89); F, Nit domain of the NitFhit fusion protein (*Caenorhabditis elegans*; PDB code 1ems); G, *Agrobacterium* sp. strain KNK712 *N*-carbamoyl-D-amino-acid amidohydrolase (PDB code 1erz). The conserved Glu, Cys, Lys catalytic site is highlighted in pink. The central core of the enzymes is highlighted in yellow; the loop regions are highlighted in brown. The major contact surface leading to dimerization of the enzymes is highlighted in green. The second interacting surface occurring in 1ems and 1erz is highlighted in blue. The sequences of the proteins which have three-dimensional structures are indicated in *JOY* notation (Mizuguchi *et al.*, 1998). The amidase has the following sequence identities: 1k17, 80.1%; 1j31, 22.1%; 1f89, 21.4%; 1ems, 16.7%; 1erz, 18.2%.

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 m*M* NaCl, 100 m*M* 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°. A total of 231 265 reflections were collected in the resolution range 30.0–1.93 Å as shown in Table 1. $\langle I/\sigma(I)\rangle$ 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*–3*m*. Final assignment of the space group was only accomplished after finding a solution by molecular replacement. There were no reflections with $l \neq 2n$ along [00*l*]; this fact was first noted after analysis of the unmerged data using *POINTLESS* (Evans, 2006) and was subsequently confirmed by analysis with *CrystalClear*. This suggested that the space group was $P4_232$. The values of $R_{\rm sym\ (linear)}$ range from 0.05 to 0.3, reaching 0.1 at 2.9 Å resolution. The Matthews coefficient was 2.41 Å^3 Da^{-1}, which is consistent with a single subunit of 38 596 Da in an asymmetric unit of 92 644 Å^3 and a solvent content of 49%.

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 1f89) 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 amidohydrolase (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,

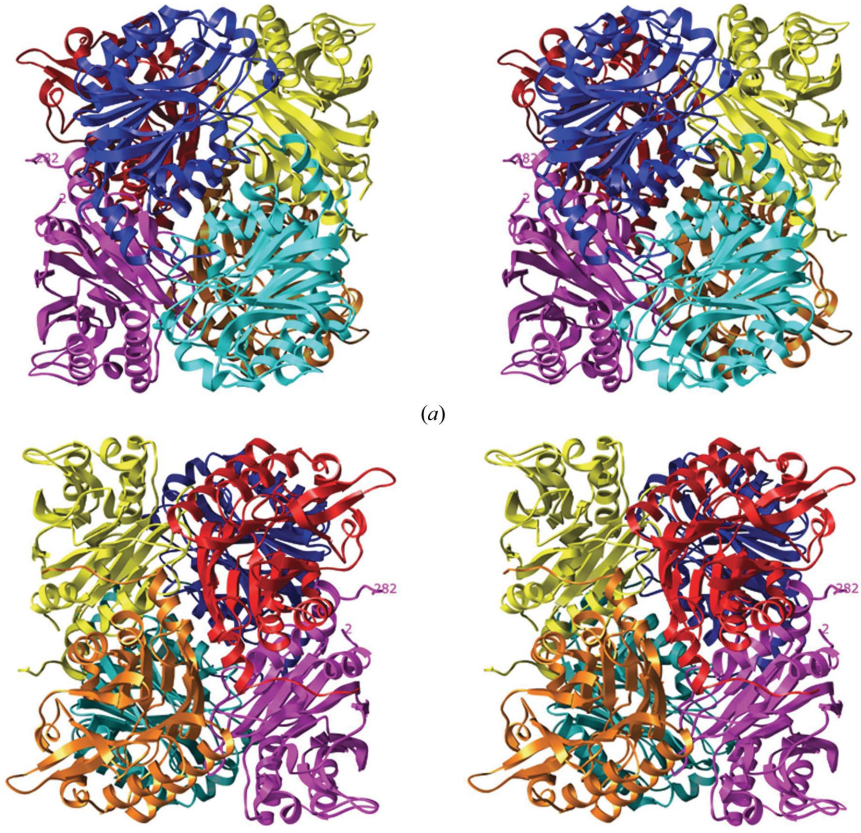


Figure 4
Stereoviews of the suggested solution hexamer viewed down one of the twofold axes from both directions. The preserved dimer surface is centred in (*a*). The interacting surfaces depicted in the foreground of (*b*) are based on the model of the putative CN hydrolase from yeast (PDB code 1f89). 66 C-terminal amino acids for each subunit are not depicted in this model.

1uf7 and 1uf8). The remaining structures were not determined in their enzymatic context, although recently an enzyme similar to 1j31 has been shown to have nitrilase activity (Mueller *et al.*, 2006). The versatility of the Glu, Cys, Lys catalytic triad in catalyzing a variety of reactions has been highlighted by Brenner (2002). However, detailed examination of a number of these enzymes will be necessary to understand the subtle changes responsible for both the differences in catalytic activity and substrate specificity.

An important issue in the case of these enzymes is the arrangement of the subunits in the oligomeric complex. The enzymes 1erz and 1ems are both tetramers having 222 point-group symmetry, while 1j31 and 1f89 are dimers. Homologous amidases are reported to be hexamers in solution (Brown *et al.*, 1973; Farnaud *et al.*, 1999), whereas members of the nitrilase branch of the superfamily are reported to have subunit numbers ranging from two to 18 (O'Reilly & Turner, 2003; Banerjee *et al.*, 2002). We have reported the quaternary structure of the cyanide dihydratase from *Pseudomonas stutzeri* AK61, which is an unusual 14-subunit terminating homo-oligomeric spiral (Sewell *et al.*, 2003). The sequence of the cyanide dihydratase differs from the crystallographically determined homologues by having two insertions (prior to α2 and β11) and a C-terminal extension. On the basis of a structure determined at low resolution by electron microscopy, Sewell *et al.* (2003) have proposed that the two insertions constitute an interacting surface, related by a pseudo-twofold axis, which is the basis for the elongation of the spiral. These insertions are present in all nitrilases which have been reported to have ten or more subunits, but are absent in the amidase. However, the amidase does have a deletion between β5 and α4 and the extended C-terminal domain in common with the cyanide dihydratase. The structural and functional roles of these elements remain unknown. A common feature of all known nitrilase homologue structures is the dimerization surface comprising α5 and α6. In addition, 1erz and 1ems have an interface comprising β13 and β14 which leads to the formation of the tetramer. Structural homology suggests that α5 and α6 exist in the amidase and therefore this interface, which is located on a twofold axis, is likely to be present in the crystal.

3.4. Molecular replacement

Molecular replacement using *Phaser* (McCoy *et al.*, 2005; Collaborative Computational Project, Number 4, 1994) with a probe comprising residues 1–242 of 1j31 with non-identical residues replaced with alanine and the resolution restricted to the range 3.5–20 Å yielded a single solution having a translation *Z* score of 7.2. An important feature of the solution was that the conserved dimer interface referred to above was indeed preserved by the crystallographic symmetry. In addition, three dimers, in which the subunits are related by crystallographic twofold axes, are packed around the $P4_232$ crystallographic threefold axis (Fig. 4). The closeness of packing clearly suggests that this may be the solution hexamer and shows that it has *D*3 point-group symmetry. The arrangement is such that the C-terminus is located opposite the face of closest packing, leaving ample space for the additional 66 residues per subunit.

4. Conclusion

The amidase from *G. pallidus* RAPc8 is confirmed to be a structural homologue of the nitrilases. Our crystallographic solution clearly implies that the biological hexamer has *D*3 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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