Purification, crystallization and preliminary X-ray diffraction analysis of thermostable nitrile hydratase

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Microbial nitrile hydratases are important industrial enzymes that catalyse the conversion of nitriles to the corresponding amides. Bacillus strain RAPc8 nitrile hydratase has recently been cloned and functionally expressed in *E. coli*. Here, the purification, crystallization and preliminary X-ray diffraction data of this nitrile hydratase are described. The heterotetrameric enzyme was crystallized using the hanging-drop vapour-diffusion method. Crystals produced in the presence of 30% PEG 400, 0.1 M MES (pH 6.5) and 0.1 M magnesium chloride were selected for X-ray diffraction studies. A data set complete to 2.5 Å was collected under cryoconditions at the in-house X-ray source at the University of the Western Cape. The space group was determined to be primitive tetragonal ($P4_1212$) with unit cell dimensions *a* = 106.61 Å, *b* = 106.61 Å, c = 83.23 Å, α = β = γ = 90°; with one dimer per asymmetric unit. Solution of the three-dimensional structure *via* molecular replacement is in progress.

Introduction

Nitrile-metabolizing enzyme systems are present in a variety of microorganisms from diverse ecosystems. These enzymes are of interest for the industrial production of amides and carboxylic acids. The nitrile hydratases (NHase; EC 4.2.1.84) catalyse the conversion of nitriles to corresponding amides. The amides are then converted to carboxylic acids by amidase (recently reviewed in ref. 1).

The interest in the nitrile hydratases is fuelled by their potential capacity to produce important commodity chemicals (including pharmaceuticals and their intermediates) under mild conditions, with the potential for regio-selectivity and stereo-selectivity. A key example of the fruits of such research is the commercially successful biotransformation of acrylonitrile to acrylamide using NHase-producing *Rhodococcus rhodochrous* J1 by the Japanese chemical company, Mitsubishi Rayon.[2,3]

Characterization of the known NHases has revealed that they are typically composed of two subunits, termed α and β. This group of enzymes can be divided into two different sub-groups based on their co-factor requirements. The iron-type NHases contain a single non-heme Fe^{III} ion per αβ dimer, while the cobalt-type NHases contain a single non-corrin Co^{III} per αβ dimer.[1,4] Several structural studies have been performed with the goal of defining the molecular basis of the functions of this family of proteins. Most revealing were the four reports describing crystal structures of the iron-type (*Rhodococcus* sp. R312, PDB accession number 1AHJ,[5] *Rhodococcus* sp. N-771[6]) and the cobalt-type NHases (*Ps. thermophila*, PDB accession number 1IRE,[7,8] *B. smithii*[9]). Comparative analysis of these structures reveals that the overall fold is conserved and that a characteristic metal-binding, claw-setting motif is similar between cobalt-type and iron-type NHases.

Many recent studies of nitrile-metabolizing enzymes have focused on themophillic microorganisms. This is, in part, due to the fact that the intrinsic lack of thermal stability of mesophile-derived enzymes is perceived as one of the factors that limit their successful industrial application.[10] To date, five reports describing isolation of thermostable NHases have been published.[10–14]

Moderately thermophilic *Bacillus* RAPc8 expresses a highly active, thermostable, cobalt-type NHase that exhibits very wide substrate specificity.[12] The gene encoding this enzyme has recently been cloned and constructs for its expression in *E. coli* prepared.[13] Here, the purification, crystallization and preliminary X-ray diffraction data of this NHase are described. This work forms a strong foundation for solution of the structure of *Bacillus* RAPc8 NHase and represents one of the first macromolecular X-ray crystallographic structural studies performed locally by a South African research group.

Materials and methods

Expression of Bacillus RAPc8 NHase

Bacillus RAPc8 nitrile hydratase was recombinantly expressed in *E. coli* BL21 (DE3) pLysS (Stratagene), a protease-deficient strain of *E. coli* that expresses T7 lysozyme. Cells were transformed with expression vector pNH14K. This plasmid carried the coding sequences for the α and β subunits of NHase as well as the coding sequence for P14K under control of the T7 DNA polymerase promoter (details are reported in ref. 15). An 800-ml LB culture containing 50 μg ml^{-1} ampicillin and 30 μg ml^{-1} chloramphenicol was grown at 37°C, 220 rpm to an optical density (at 600 nm) of 0.4, at which point expression was induced with 0.4 mM IPTG. Cobalt chloride was added to a final concentration of 0.1 mM some 15 to 30 min prior to induction. Cells were harvested by centrifugation 4 h after induction and washed with 50 mM potassium phosphate buffer (pH 7.2).

Protein purification

The washed cell pellet was resuspended in 25 ml potassium phosphate buffer (pH 7.2). Because of the presence of T7 lysozyme to facilitate lysis, cells were easily disrupted by one cycle of freezing at –80°C and thawing at 37°C followed by brief sonication on ice. The lysate was centrifuged at 5000 × *g* for 20 min and the supernatant collected. The relative thermostability of *Bacillus* RAPc8 was exploited for the first step of purification. Heat treatment was used to precipitate a good proportion of heat-sensitive *E. coli* proteins. The cell-free extract was heated at 55°C for 45 min and centrifuged at 7000 × *g* for 20 min and the supernatant collected.

Solid ammonium sulphate was added to the heat-treated sample to achieve 20% saturation and left on ice for 1 hour. Precipitated proteins were removed by centrifugation at 7000 × *g* for 30 min at 4°C. The supernatant was loaded onto a HighLoad 16/10 Phenyl-Sepharose column (Amersham Biosciences) equilibrated with Buffer A (1.0 M ammonium sulphate, 50 mM potassium phosphate; pH 7.2). Bound proteins were eluted with a linear gradient of decreasing ammonium sulphate concentration generated with 50 mM potassium phosphate buffer; pH 7.2 (5 column-volumes, 1.0 M–0 M ammonium sulphate). Fractions containing NHase were pooled, dialysed against Buffer B (25 mM potassium phosphate; pH 7.2) and loaded onto a HiPrep 16/10 Q-Sepharose FF column (Amersham Biosciences) equilibrated with Buffer B. Bound proteins were eluted with a linear gradient of increasing sodium chloride concentration generated with Buffer C (500 mM sodium chloride, 25 mM potassium phosphate; pH 7.2) (5 column-volumes, 0 M–500 mM sodium chloride). Fractions containing NHase were pooled.

Samples from all stages of purification were analysed by SDS-PAGE

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using the well-documented method of Laemmli.[16] Gels containing 15% acrylamide were used. Samples were assayed for protein concentration using the Biorad protein determination kit (Biorad) with BSA as standard, and for nitrile hydratase activity using the ammonia detection assay as previously described.[10]

Crystallization

Prior to crystallization, pooled fractions from Q-Sepharose chromatography were dialysed against 20 mM Tris (pH 7.2), filtered through a 0.22-μm filter and concentrated to 10 mg ml^{-1} (as determined by absorbance at 280 nm) using Whatman VectorSpin (30-kDa molecular weight cutoff) tubes. The molar extinction coefficient of NHase as calculated by the method of Gill and von Hippel[17] was 78090 M^{-1} cm^{-1}. NHase was crystallized using the hanging-drop vapour diffusion method. Initial crystallization conditions were tested using Hampton Crystal Screen 1 (Hampton Research) and JBScreen 1 (JenaBioscience). A total of 74 individual conditions were tested. Parallel screens at 20°C and 4°C were prepared; 24-well VDX plates were used. The hanging drop was mounted on square siliconized cover slips and contained 1 μl protein and 1 μl reservoir solution.

Once initial conditions were resolved, optimization was carried out with drops containing 2 μl protein and 2 μl reservoir solution. Chemicals of the highest available purity were used. Protein concentration, incubation temperature, precipitant concentration and reservoir solution pH were varied. Diffraction quality crystals were finally grown at 20°C in 30% PEG 400, 100 mM magnesium chloride, 100 mM MES (2[*N*-morpholino]ethanesulphonic acid); pH 6.5 (40 mg ml^{-1} protein).

X-ray diffraction data collection and processing

X-ray diffraction data were collected at the in-house X-ray source in the Department of Biotechnology, University of the Western Cape [comprising a Rigaku RUH3R copper rotating-anode X-ray source operated at 40 kV, 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 under cryoconditions (at a temperature of 100 K) with a crystal-to-detector distance of 160 mm. (It was not necessary to add cryoprotectant to the crystals.) Data frames covering an oscillation angle of 0.5° per frame were collected for 10 min.

Space group determination was performed with Crystal Clear (d*TREK).[18] Molecular replacement was done with EPMR.[19] Solvent content and Matthews coefficient were calculated using Matthews from the CCP4 suite.[20,21]

Results and discussion

Recombinant NHase was purified in four steps to apparent homogeneity as determined by SDS-PAGE (Fig. 1). A final yield of 60.5% was achieved with 8.9-fold purification. Native gel electrophoresis showed that pure NHase was eluted from the final step of purification in one oligomeric state (not shown); a heterotetramer as previously observed with the native protein.[12] This indicated that a gel filtration chromatography step was not necessary prior to crystallization. It was possible to concentrate the protein to beyond 50 mg ml^{-1}. Purified protein was successfully used for crystallization experiments even after several weeks' storage at 4°C or at –80°C.

At the initial screening phase of crystallization, crystals were observed in three different conditions. Crystals of promising quality as determined by external morphology were seen in 1.4 M sodium citrate, 100 mM Hepes (pH 7.5); and in 30% PEG 400, 100 mM magnesium chloride, 100 mM MES (pH 7.5). Finer optimization experiments around these two conditions were performed. Protein concentration, incubation temperature, precipitant concentration and reservoir solution pH were varied. Ultimately, crystals produced from filtered protein solution at 45 mg ml^{-1} in 30% PEG 400, 100 mM magnesium chloride, 100 mM MES (pH 7.5) were selected for use in X-ray diffraction experiments. These crystals grew to final dimensions of up to 0.7 mm after incubation for four to five weeks at 20°C

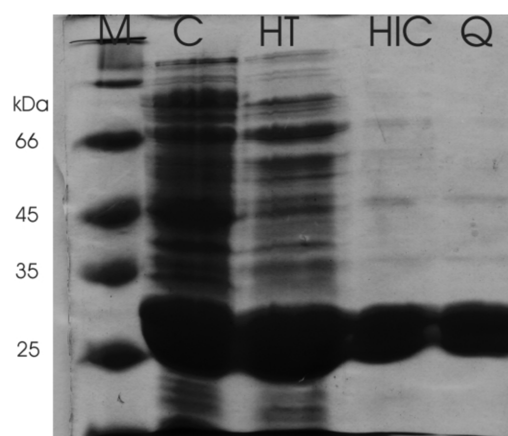

Fig. 1. SDS-PAGE gel of purification of NHase. Lane M: molecular weight markers; lane C: crude extract; lane HT: heat-treated extract; lane HIC: pooled fractions from Phenyl-Sepharose hydrophobic interaction chromatography; lane Q: pooled fractions from Q-Sepharose ion exchange chromatography.

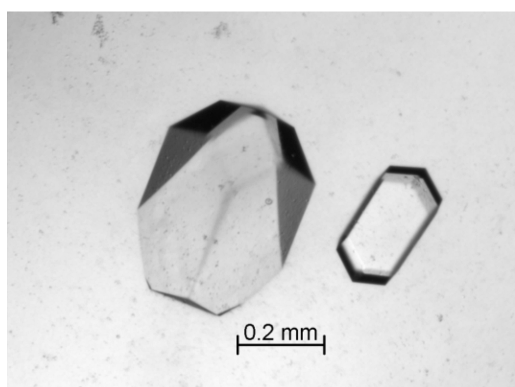

Fig. 2. Crystals of *Bacillus* RAPc8 NHase. Typical dimensions of crystals selected for diffraction studies were approximately 0.2 × 0.1 × 0.1 mm.

(Fig. 2). The crystal selected for diffraction studies had dimensions of approximately 0.2 mm × 0.1 mm × 0.1 mm.

In total, 16 152 unique reflections were collected, giving a data set which was 93% complete at 2.5 Å. A typical diffraction image is shown in Fig. 3. Processing with Crystal Clear suggested the primitive tetragonal space group ($P4_1212$) with unit cell dimensions $a = b = 106.61$ Å, $c = 83.23$ Å; $\alpha = \beta = \gamma = 90°$. The ambiguity was removed by molecular replacement using 1IRE[7] as a search probe. $P4_1212$ gave a correlation of 35% and an R-factor of 56.2%, while $P4_3212$ gave a correlation of 13% and an R-factor of 60%. Data collection statistics are summarized in Table 1.

Based on the estimated molecular weight of a heterodimer (51 kDa), these unit cell dimensions allow a single heterodimer per asymmetric unit with 46.5% solvent content and a Matthews coefficient of 2.3 $Å^3$ Da^{-1}.[20] These values are within the range usually observed for protein crystals.

The availability of a complete data set from good, diffracting

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

Wavelength (Å)	1.54
Space group	$P4_1212$
Cell dimensions (Å)	$a = b = 106.61$, $c = 83.23$
Cell angles	$\alpha = \beta = \gamma = 90°$
Resolution range (Å)	20–2.5
Number of unique reflections	16 152
Completeness (%)	93
Last shell (2.59–2.52) (%)	77
R_{merge} (Last shell)	0.091 (0.391)

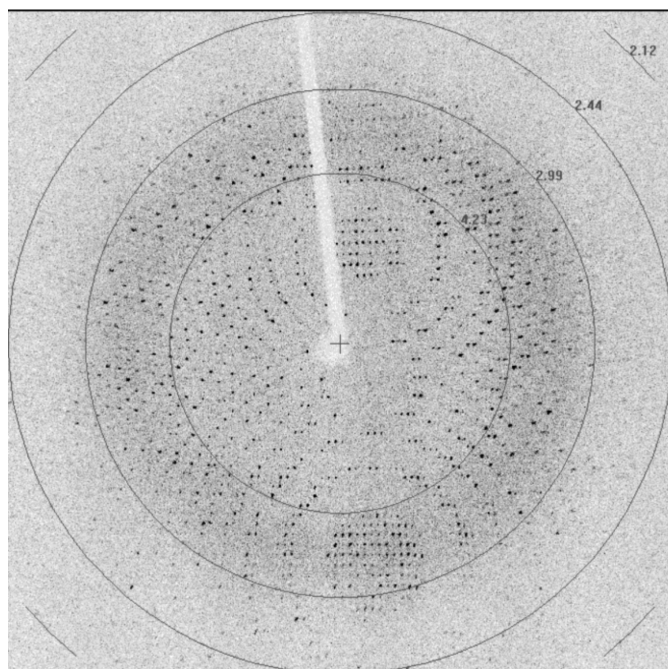

Fig. 3. X-ray diffraction image from NHase crystals. Spots were visible to beyond 2.5 Å. Resolution circles in Å.

crystals and a previously solved crystal structure of a related NHase (1IRE[7]) will allow determination of accurate phases *via* molecular replacement and hence the creation of a model structure based on the resulting electron density maps. This is the first published report of protein diffraction data being collected in South Africa. The goal of this work is to produce an engineered NHase that will demonstrate enhanced performance in industrial biotransformation. This will be accomplished by insight gained by solving the structures of a series of mutant NHases with and without bound substrate.

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