CRYSTALLIZATION PAPERS

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Crystallization of NAD$^+$-dependent phenylalanine dehydrogenase from *Nocardia sp239*

A. Pasquo,[a]† K. L. Britton,[a] P. J. Baker,[a] G. Brearley,[b]‡ R. J. Hinton,[b] A. J. G. Moir[a] T. J. Stillman[a] and D. W. Rice[a] *at* [a]*Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, England, and* [b]*CAMR Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, England. E-mail: d.rice@sheffield.ac.uk*

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Abstract

The NAD$^+$-dependent phenylalanine dehydrogenase from *Nocardia sp239* has been crystallized by the hanging-drop method of vapour diffusion using ammonium sulfate as the precipitant. Two crystal forms were obtained in the presence and absence of the enzyme substrates phenylpyruvic acid or phenylalanine and its coenzyme NADH. Crystals of the native protein belong to the hexagonal system, with the space group being one of the enantiomorphic pair $P6_122$ or $P6_522$. The cell dimensions are $a = b = 111.0$, $c = 174.5$ Å, $\alpha = \beta = 90$ and $\gamma = 120°$. Crystals grown from the protein co-crystallized with its substrates all belong to the trigonal system, space group $P3_121$ or $P3_221$, with unit-cell dimensions of $a = b = 88.1$, $c = 112.6$ Å, $\alpha = \beta = 90$ and $\gamma = 120°$. Preliminary protein-sequencing experiments have established that this enzyme is related to the octameric PheDH's which are members of the wider superfamily of amino-acid dehydrogenases. However, gel-filtration studies suggest that this enzyme is active as a monomer. The full determination of the three-dimensional structure of this phenylalanine dehydrogenase will add to the understanding of the molecular basis of the differential substrate specificity within this enzyme superfamily. In turn this will contribute to the rational design of an amino-acid dehydrogenase which could be used for the diagnosis of phenylketonuria and for the chiral synthesis of high-value pharmaceuticals.

1. Introduction

The enzymatic amination of keto acids to the corresponding amino acids provides a route for the incorporation of ammonia into organic compounds thereby linking the metabolism of carbohydrates and amino acids. Previous studies have established that a number of enzymes catalysing this reaction form a superfamily with differential specificity for the amino-acid substrate. These include the enzymes leucine dehydrogenase (LeuDH) (Nagata *et al.*, 1988), glutamate dehydrogenase (GluDH) (Britton *et al.*, 1993), phenylalanine dehydrogenase (PheDH) (Takada *et al.*, 1991) and valine dehydrogenase (ValDH) (Turnbull *et al.*, 1998; Leiser *et al.*, 1996). The three-dimensional structures of species variants of two members of this enzyme superfamily, the hexameric GluDH (Stillman *et al.*, 1993) and the octameric LeuDH (Baker *et al.*, 1995) have been determined. While the quaternary structures of these two enzymes are different, there is a strong resemblance between the fold of their subunits and in the nature of the subunit interactions across the twofold axis to form dimers. Assembly of the dimers to form the higher order structures is however, quite different. Comparison of the structures of GluDH and LeuDH has shown that the residues involved in the catalytic mechanism are very similar. The differential substrate specificity displayed in this family is thought to be due to modifications of a restricted number of residues in the specificity pocket accommodating the side chain of the amino acid coupled to subtle differences in the shape of the pocket caused by differences in the position of the main chain which may be associated with the change in quaternary structure (Baker *et al.*, 1995). Homology-based modelling studies have suggested that a deletion of 15 residues at the C terminus of ValDH with respect to LeuDH account for the dimeric structure of this enzyme (Turnbull *et al.*, 1997).

PheDH's catalyse the reversible oxidative deamination of L-phenylalanine to phenylpyruvate and ammonia in the presence of NAD$^+$. The enzyme has been isolated and purified from a limited number of bacterial sources including *Brevibacterium*, *Bacillus*, *Sporosarcina*, *Nocardia*, *Thermoactinomyces* and *Rhodococcus sp. M4* (Hummel *et al.*, 1987, and references therein). The reported oligomeric states for the members of the PheDH family seem to vary from species to species (Ohshima *et al.*, 1991; Asano, Nakazawa & Endo, 1987; Asano, Nakazawa, Endo *et al.*, 1987; Misono *et al.*, 1989) but are most commonly reported as octamers. However, analytical gel-filtration studies suggest that the PheDH from *Nocardia sp239* is active as a monomer of molecular weight 42 000 (de Boer *et al.*, 1989). To our knowledge, this is the only reported case of a monomeric amino-acid dehydrogenase. The amino-acid sequence of this PheDH has not been determined and therefore, at present, it is not known whether this enzyme is a true member of the family.

A number of genetic diseases of amino-acid metabolism including phenylketonuria, homocystinuria and maple syrup urine disease are almost unique in that their early detection can lead to a simple and effective treatment through the adoption of a diet low in the offending amino acid. This reduces the severe clinical symptoms associated with these crippling diseases. Upon reaching maturity this diet can then be relaxed as high levels of the particular amino acids can be tolerated. Thus, neonates are routinely screened for the genetic disease phenylketonuria and much research is being directed towards the production of a simple and sensitive test for the detection of elevated levels of phenylalanine associated with this disorder. It has been suggested that PheDH could be used to measure

† Present address: IRBM Istituto di Ricerche Biologia Molecolare P. Angeletti Via Pontina 00040 Pomezia-Roma, Italy.

‡ Present address: Baxter Health Care Corporation, 1700 Rancho Conejo Boulevard, Thousand Oaks, CA 91320, USA.

phenylalanine in biological samples, although some species of this enzyme display high levels of activity with other amino acids, such as tyrosine, which is potentially limiting. The current tests, therefore, require the use of a correction procedure to eliminate the potential for misleading positive results arising from the activity of PheDH's towards these other amino acids (Cooper *et al.*, 1989). Therefore, the availability of a PheDH with a high substrate specificity for phenylalanine but also low activity to tyrosine is highly desirable.

This paper reports the crystallization of the PheDH from *Nocardia sp239* as the first step in obtaining molecular insights into the precise factors that control specificity in this enzyme.

2. Protein purification

Nocardia sp239 was grown on a minimal salts medium containing the following: 4.0 g l^{-1} K_2HPO_4, 1.0 g l^{-1} KH_2PO_4, 1.0 g l^{-1} NH_4Cl, 0.01 g l^{-1} $CaCl_2.2H_2O$, 2.6 g l^{-1} K_2SO_4, 1.0 g l^{-1} NaCl, 16.5 g l^{-1} L-phenylalanine and 0.5 ml l^{-1} Mazu DF8005 antifoam. Following sterilization by autoclaving at 394 K and 103 kPa for 20 min the pH was adjusted to 7.0 and the medium supplemented by 1 ml l^{-1} 1 *M* $MgCl_2.6H_2O$, 10 ml l^{-1} trace element solution [composed of 3.9 g l^{-1} $FeCl_3.6H_2O$, 0.58 g l^{-1} $ZnSO_4.H_2O$, 1.0 g l^{-1} $CoCl_2.6H_2O$, 1.0 g l^{-1}, $NaMoO_4.2H_2O$, 1.16 g l^{-1} $CuSO_4.5H_2O$, 0.3 g l^{-1} H_3BO_3, 0.72 g l^{-1} $MnSO_4.7H_2O$, 7.2 ml l^{-1} HCl (conc) made up to 1 l with demineralized water] and 1 ml l^{-1} of vitamin solution (made up from 0.125 mg l^{-1} pyrodoxin, 0.125 mg l^{-1} biotin, 0.025 mg l^{-1} thiamine, 1.875 mg l^{-1} nicotinic acid and 0.125 mg l^{-1} riboflavin). The organism was grown as an 8 l batch culture in a stainless steel reactor at 310 K. The culture was stirred at 400–700 rev min^{-1} and the dissolved oxygen level was maintained above 40% by altering the air flow rate and impeller speed. The pH of the medium was controlled at 7.0±0.2 and the vessel was inoculated with a 200 ml seed culture prepared in the same medium in a 250 ml flask shaken at 200 rev min^{-1} for 16–24 h at 310 K. The 8 l culture was incubated for 18 h (optical density at 600 nm = 21) and harvested by centrifugation in 1 l pots. The enzyme yield was 360 units l^{-1}, where one unit of enzyme activity was defined as 1 µmol of substrate (phenylalanine) deaminated per min at 298 K and pH 10.8.

For the purification procedure all extraction buffers contained 100 µ*M* phenylmethylsulfonylfluoride (PMSF) and were stored at 277 K prior to use. *Nocardia sp239* cells were resuspended in 50 m*M* Tris–HCl pH 8.5 (buffer *A* using 5 ml of buffer per g of cells) and disrupted using an APV Gaulin Lab 60 homogeniser operated at 55 MPa. Cell debris was removed by centrifugation at 5000*g* at 277 K for 30 min. The cell-free extract was loaded onto a Procion Red-3B/Sepharose CL-4B column previously equilibrated with buffer *A* and 100 µ*M* PMSF. The resin was thoroughly washed with the same buffer to remove unbound proteins. The enzyme was then eluted from the column with 1 m*M* NADH in buffer *A*. The enzyme was concentrated to a final concentration of 5 mg ml^{-1} [as determined using the BCA assay system (Pierce Chemical Co, USA) using bovine serum albumin as the standard] and stored at 253 K. The enzyme purity was checked by SDS–PAGE on a precast gradient 10–15% polyacrylamide gel and SDS buffer strip on a Phast System (Pharmacia) in accordance with the manufacturers' instructions.

For the crystallization trials, samples of the stored PheDH were dialysed overnight against 50 m*M* Bis-Tris propane buffer pH 9.0. The concentration of the protein was adjusted using an Amicon Centricon 10 microconcentrator by centrifugation at 4000*g* in a Beckman J2-21 refrigerated centrifuge, to give an optical density reading of 30 at 280 nm.

3. Crystallization of PheDH (form *A*)

Crystals were grown by the hanging-drop method of vapour diffusion using Linbro crystallization plates by mixing 5 µl of

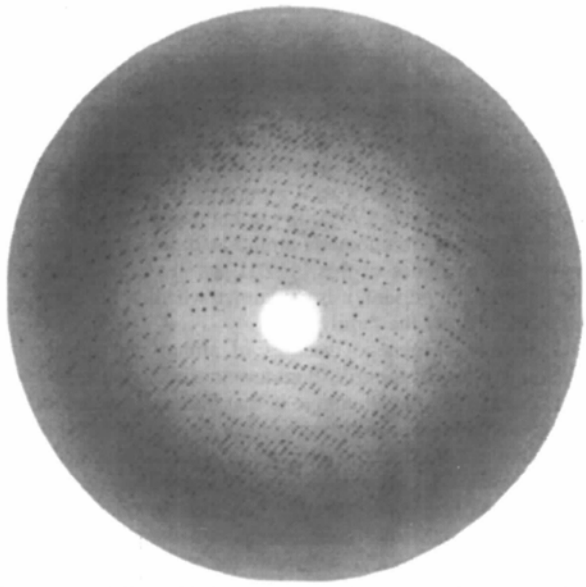

Fig. 1. A 2.0° oscillation image of the native *Nocardia sp239* phenylalanine dehydrogenase crystals (form *A*) taken using the MAR image plate on station PX9.5 at the SRS. The resolution at the edge of the image is 2.5 Å.

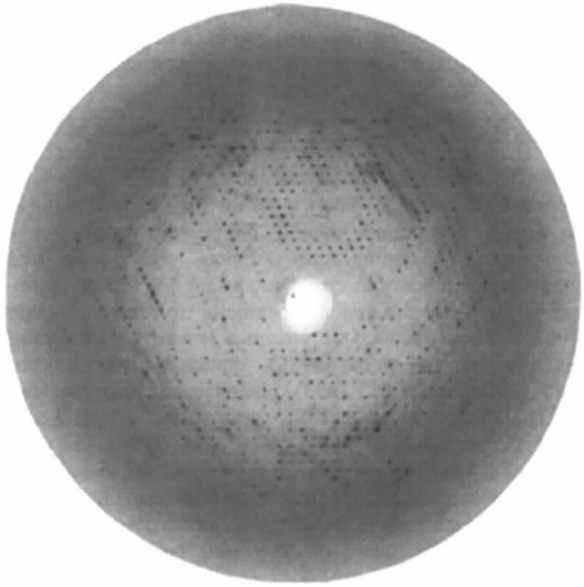

Fig. 2. A 1.3° oscillation image of the *Nocardia sp239* phenylalanine dehydrogenase crystals (form *B*) grown in the presence of phenylpyruvic acid taken using the MAR image plate on station PX9.5 at the SRS. The resolution at the edge of the image is 2.5 Å.

PheDH(*Noc*)	1	MEHEEVKVRRGPR-------SGLPVVVAIHSRAL**GPAVGGIR**	35
PheDH(*Bs*)	21	ANHEQIVFCNDPV-------SGLQAIIAIHDTTL**GPALGGTR**	55
PheDH(*Bb*)	20	SEHEQVVFCNDPA-------TGLRAIIAIHDTTL**GPALGGCR**	54
PheDH(*Ti*)	11	YGHEQVIFCRHPQ-------TGLKAIIALHNTTA**GPALGGCR**	45
PheDH(*Rh*M4)	9	WDGEMTVTRFDRE-------TGAHFVIRLDSTQL**GPAAGGTR**	43
LeuDH(*Bst*)	10	YDYEQVLFCQDKE-------SGLKAIIAIHDTTL**GPALGGTR**	44
GluDH(*Cs*)	52	VIPERVIEFRVPWEDDNGKVHVNTGYRVQFN**GAIGPYKGGLR**	93

Fig. 3. An amino-acid sequence alignment of the N-terminal sequence of *Nocardia sp239* PheDH (*Noc*) against the corresponding regions of the published sequences for several PheDH's together with representative members of the amino-acid dehydrogenase superfamily. Each segment of sequence is labelled with the start and end numbers, respectively. The other sequences included are *Bacillus sphaericus* PheDH (*Bs*) (Okazaki *et al.*, 1988); *Bacillus badius* PheDH (*Bb*) (Yamada *et al.*, 1995); *Thermoactinomyces intermedius* PheDH (*Ti*) (Takada *et al.*, 1991); *Rhodococcus* M4 PheDH (*Rh*M4) (Brunhuber *et al.*, 1994); *Bacillus stearothermophilus* LeuDH (*Bst*) (Nagata *et al.*, 1988) and *Clostridium symbiosum* GluDH (*Cs*) (Teller *et al.*, 1992). The amino acids highlighted in bold are those which are identical across the superfamily of amino-acid dehydrogenases.

Nocardia sp239 PheDH protein solution with 5 µl of the precipitant and equilibrating the drops over the precipitant at 290 K. Trials with ammonium sulfate solution in the range 31–33% saturated in 50 m*M* Bis-Tris propane buffer at pH 8.8 (buffer *B*) resulted in crystals with the morphology of hexagonal pyramids (form *A*). The maximum dimensions of 1.0 × 0.8 × 0.5 mm were obtained for these crystals and they could be stabilized with 50% saturated ammonium sulfate in buffer *B*. X-ray precession photographs of the crystals show that the crystals belong to the hexagonal system. Axial reflections along *l* are systematically absent except when $l = 6n$ and combined with the presence of 6*mm* symmetry in the *hkl* zone identifies the space group as one of the enantiomorphic pair $P6_122$ or $P6_522$, with cell dimensions $a = b = 111.0$, $c = 174.5$ Å, $\alpha = \beta = 90$ and $\gamma = 120°$. The volume of the unit cell is 18.62×10^5 Å^3 and assuming a subunit molecular weight of 42 000 gives a V_m value of 3.78 Å^3 Da^{-1} for a monomer in the asymmetric unit which lies within the range given by Matthews (1977). Preliminary images have been collected with a MAR research image plate on station PX9.5 at the CCLRC Daresbury Synchrotron Radiation Source (SRS) which show diffraction to 2.5 Å (Fig. 1).

4. Crystallization in the presence of substrates (form *B*)

In parallel studies, crystals were grown in the presence of either the substrate phenylpyruvic acid (30 m*M*) or in the presence of both 30 m*M* phenylalanine and 10 m*M* NADH. Crystallization trials were carried out as above using ammonium sulfate as the precipitant in the range of 25–36% saturation in buffer *B*. This led to the formation of cuboid crystals of maximum dimensions 0.6 × 0.6 × 0.6 mm which could be stabilized in 50% saturated ammonium sulfate in buffer *A*, including the relevant substrates. Data to 3 Å were collected on the form grown in the presence of 30 m*M* phenylpyruvic acid at station PX9.5 of the CCLRC Daresbury SRS with a MAR research image plate (Fig. 2). Inspection of the diffraction images and analysis of the data using the auto-indexing algorithms in the program *REFLX* (Kabsch, 1988) show that the data belong to the primitive trigonal system, with cell dimensions $a = b = 88.1$, $c = 112.6$ Å, $\alpha = \beta = 90$ and $\gamma = 120°$. Axial reflections along *l* are systematically present except when $l = 3n$ and the *hk*1 zone shows 31*m* symmetry suggesting that these crystals belong to one of the enantiomorphic space groups $P3_121$ or $P3_221$. The volume of the unit cell is 7.56×10^5 Å^3 and the V_m value of 3.07 Å^3 Da^{-1} for a monomer in the asymmetric unit lies in the range given by Matthews (1977).

To establish whether this PheDH belongs to the superfamily of amino-acid dehydrogenases the sequence for the first 35 N-terminal residues was determined on an Applied Biosystems 476A sequencer. Alignment of this sequence against other sequences for members of the family showed a pattern of sequence similarity consistent with the identification of this enzyme as a member of this family (Fig. 3).

Both crystal forms obtained are suitable for structural studies and preliminary efforts are being directed towards the solution of the structure of the form *B* crystals grown in the presence of phenylpyruvic acid. An attempt will be made to solve the structure by isomorphous replacement and to that end a screen for heavy-atom derivatives is now under way. The results of this analysis may well provide the full understanding of the structure–function relationship in this enzyme and allow us to better refine our knowledge of the molecular basis of the differential specificity within the superfamily of the amino-acid dehydrogenases. In the long term this may well enable the engineering of an enzyme with the desired specificity for the diagnosis of phenylketonuria.

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