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Crystallization and preliminary X-ray analysis of the NADP-specific glutamate dehydrogenase from *Neurospora crassa*.

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Abstract

The NADP-linked glutamate dehydrogenase from *Neurospora crassa* has been crystallized by the hanging-drop method of vapour diffusion in the presence of 0.1 *M* glutamate. The crystals are trigonal and are in space group $P3_121$ with unit-cell dimensions of $a = b = 196.6$, $c = 102.0$ Å and with a trimer in the asymmetric unit. A full structure determination of this enzyme will lead to an understanding of the molecular basis of inter-allelic complementation observed with hybrid hexamers of naturally occurring mutants.

Introduction

The NADP-specific glutamate dehydrogenase (GluDH) from *Neurospora crassa* is a member of a widespread family of enzymes that catalyse the interconversion of 2-oxoglutarate and ammonia to glutamate.

$$\text{2-oxoglutarate} + \text{NADPH} + \text{NH}_4^+ \rightleftharpoons$$

$$\text{L-glutamate} + \text{NADP}^+ + \text{H}_2\text{O}$$

The role of the NADP-specific GluDH in *N. crassa* is to assimilate ammonia into α-amino groups (E.C. 1.4.1.4), the reverse reaction providing a catabolic route for the utilization of L-glutamate is fulfilled in this species by an NAD-specific GluDH (E.C. 1.4.1.2, Veronese, Nyc, Degani, Brown & Smith, 1974). The quaternary structure of the two enzymes from *N. crassa* fall into two oligomeric classes which are representative of all GluDH's. The NADP-specific GluDH is a hexamer containing six identical subunits of 48 kDa (Fincham, 1959) and the NAD-specific GluDH a tetramer of four identical subunits of 115 kDa (Veronese *et al.*, 1974). Recently the nature of the relationship between the oligomeric classes of GluDH has been established by analysing the alignment of their primary sequences against the three-dimensional structure of the hexameric NAD-linked GluDH from *Clostridium symbiosum* (Britton, Baker, Rice & Stillman, 1992).

The NADP-specific enzyme of *N. crassa* is of interest as it is allosterically regulated at the physiological pH of 7.2 by the substrate 2-oxoglutarate and other TCA (tricarboxylic acid) cycle intermediates (West, Tuveson, Barrat & Fincham, 1967; Ashby, Wootton & Fincham, 1974). Mutants of *N. crassa* defective in amination, and therefore termed *am* mutants, were first characterized from the Beadle and Tatum collection of auxotrophs (Fincham, 1950, 1951). The *am* mutations defined the structural gene for the *Neurospora* NADP-specific GluDH (Fincham & Pateman, 1957) which were later used to establish the first documented case of the phenomenon of inter-allelic complementation. There are available a large collection of characterized *am* mutants (Kinsey, 1977), a number of which are affected in enzyme function. To interpret sequence information determined from these mutants requires the three-dimensional structure of the enzyme. The structure of a related hexameric GDH, the NAD-specific enzyme from *C. symbiosum*, has recently been determined at 1.9 Å (Baker *et al.*, 1992; Stillman, Baker, Britton & Rice, 1993). Armed with this model structure it seemed timely, therefore, to initiate structural studies on the *Neurospora* NADP-specific enzyme with a view to providing a framework for the understanding of the molecular basis of the allostery and inter-allelic complementation occurring within hybrid hexamers.

Materials and methods

The *Neurospora* NADP-specific GluDH was purified using an adapted procedure following the rapid protocol described by Aguirre & Hansberg (1988). *N. crassa* (74A) was grown in shake culture from 10 l Vogel's medium, 1.5% sucrose. The mycelia were harvested by filtration, washed, dried and ground in sand with a pestle and mortar in the presence of 0.1 *M* phosphate buffer, pH 8.0. After centrifugation at 15 000*g* for 20 min to remove the cell debris, the cell extract supernatant was incubated at 323 K for 1 h, then on ice for 30 min before a repeat centrifugation. The cleared supernatant was then fractioned using $(NH_4)_2SO_4$, and NADP-specific GluDH was recovered between 30 and 55% saturation. This precipitate was resuspended in 10–20 ml of 0.1 *M* Tris–HCl buffer, pH 8.0 (buffer *A*) and dialysed twice against 5 l of the same buffer. The protein solution was then loaded onto a (2.5 × 33 cm) DEAE–Sephadex A-50 column (Pharmacia), pre-equlibrated with buffer *A*. GluDH activity was eluted with a linear gradient (0.1 *M*) of NaCl over 500 ml in buffer *A*. The pooled fractions were desalted by dialysis against buffer *A* prior to their loading onto a column (1 × 25 cm) of Reactive Blue-Sepharose (Sigma) pre-equilibrated with buffer *A*. The column was washed extensively and the enzyme eluted with a linear gradient (0.1 *M*) of NaCl in the same buffer over 60 ml. The pooled fractions were dialysed against buffer *A* and then loaded onto a Mono-Q HR 5/5 column (Pharmacia) equilibrated in the same buffer. GluDH activity was eluted with a linear gradient (0–0.5 *M*) of NaCl over 40 ml of buffer *A*. Electrophoretically pure fractions of GluDH were then desalted and concentrated to 10 mg ml^{-1} by successive washes with buffer *A* in a Centricon 30.

Results and discussion

Crystals were grown by the hanging-drop method of vapour diffusion using Linbro crystallization plates. The protein was concentrated to 10 mg ml^{-1} in 0.1 *M* Tris–HCL buffer pH 8.5

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and glutamate added to a concentration of 0.1 *M*. 15 µl drops of this solution were then suspended over various $(NH_4)_2SO_4$ solutions made up in the range 30–60% saturation. Crystals were obtained in the range of 51–57% concentration wells after two or three months. The crystals appear as hexagonal bicapped prisms with dimensions up to 0.7 × 0.7 × 0.4 mm and on close inspection looking down the hexagonal axis they appear to have alternating large and small triangular faces.

The crystals were extremely sensitive to the touch and could not be stabilized and, therefore, for data collection the crystals were mounted directly from the drop. A preliminary data set was collected using the rotation method to 3.0 Å resolution (Fig. 1) with an MAR Research image plate and a wavelength of 0.92 Å at the DRAL Daresbury Laboratory SRS on station 9.5 with rotations of 2° per frame. The diffraction images were indexed using the *REFIX* program (Kabsch, 1988) in a primitive trigonal cell of dimensions of $a = b = 196.6$, $c = 102.0$ Å with $\alpha = \beta = 90$, $\gamma = 120°$. On analysis of the resulting data set after data reduction with the *MOSFLM* package (Collaborative Computational Project, Number 4, 1994, Leslie, 1992) it was seen that only axial reflections of the class $l = 3n$ were present. Furthermore, use of the program *HKLVIEW* (Collaborative Computational Project, Number 4, 1994) to look at the diffraction pattern in the *hkn* zones, indicated strong 32 symmetry in all layers. By noting the position of the mirror planes in the upper layers the space group was determined to be one of the two enantiomorphs $P3_121$ or $P3_221$.

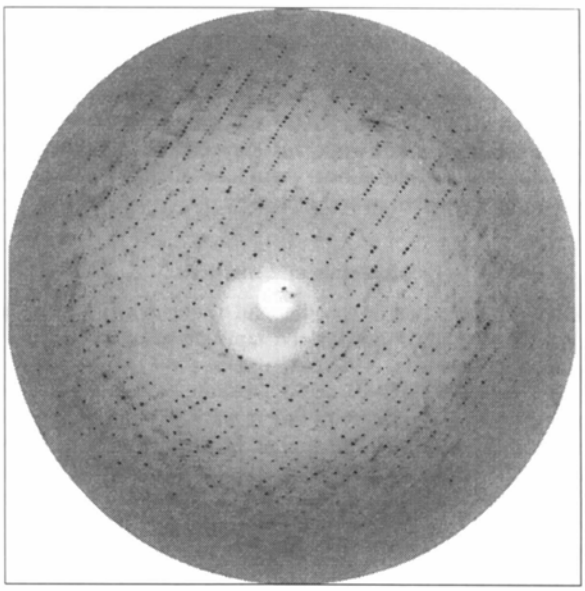


Fig. 1. A 2° rotation diffraction pattern from a crystal of GluDH from *N. crassa*. This image was taken on an MAR Research image plate on station 9.5 at the SRS at DRAL Daresbury Laboratory and is at a resolution of 3.0 Å at the edge of the plate.

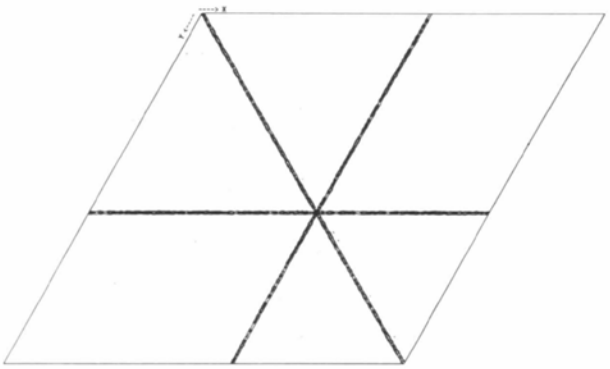
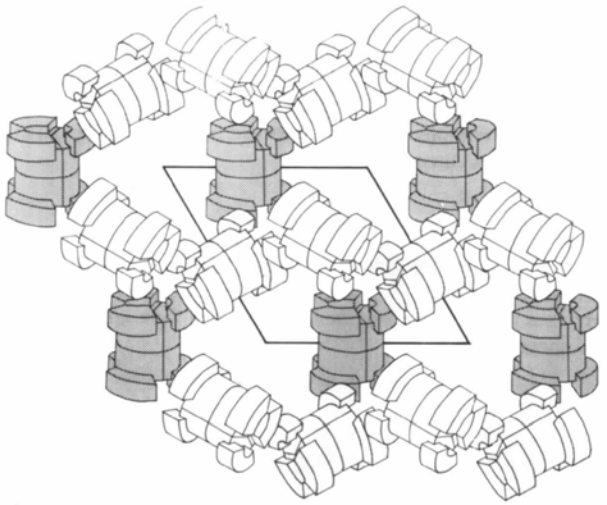
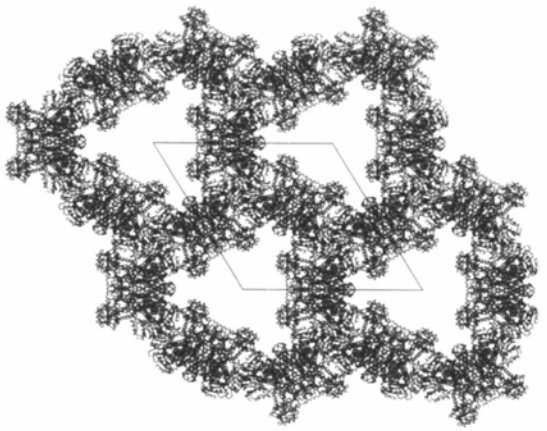


Fig. 2. A section through the translation-function solution for GluDH from *N. crassa* using a trimer of GluDH from *C. symbiosum* as the search model. This section is at $Z = 0$ and shows the whole unit cell, it was produced by the program *TFFC* (Collaborative Computational Project, Number 4, 1994).



(*a*)



(*b*)

Fig. 3. Packing diagrams to show the molecular arrangement of the GluDH hexamers in the unit cell. (*a*) A projection onto the *ab* plane down the crystallographic threefold axis. The hexamers are represented schematically with the ones unshaded being centred in the *ab* plane. Those which are lightly and more heavily shaded are translated in the threefold direction by $\frac{1}{3}$ and $\frac{2}{3}$ *c* cell lengths, respectively. (*b*) A C_α chain trace of the GluDH model obtained from the molecular-replacement solution. This trace covers the same area as in (*a*) and was produced using the program *FRODO* (Jones, 1978).

The volume of the unit cell is 3.42×10^6 Å^3 and V_m values for a trimer or a hexamer in the asymmetric unit (3.95 and 1.98 Å^3 Da^{-1}, respectively) lie in the range given by Matthews (1977). Because of the fragile nature of the crystals it was felt that a trimer in the asymmetric unit would be most likely, giving a high solvent content in the cell (69%). The hexamer of GluDH would, therefore, be constructed using one of the crystallographic twofold axes.

A cross-rotation function was calculated on data from 10 to 5 Å resolution using the program *POLARRFN* (Kabsch, W. unpublished work; Collaborative Computational Project, Number 4, 1994) against a hexameric model of GluDH from *C. symbiosum* with a 30 Å radius of integration. This appeared to give a clear solution and a translation function was then calculated with the program *TFFC* (Collaborative Computational Project, Number 4, 1994) using a trimer of GluDH in both the enantiomorphic space groups. A clear solution was obtained only with space group $P3_121$ (Fig. 2).

The packing of the hexamers within the unit cell was examined using *FRODO* (Jones, 1978) and this is shown schematically in Fig. 3(*a*), and as a C_α chain trace in Fig. 3(*b*). Large holes, with a minimum diameter of 95 Å, and which run through the entire lattice in the direction of the z axis can be seen and explain the high V_m for these crystals. A full refinement of this structure in now underway and should enhance our understanding of the molecular basis of interallelic complementation observed with hybrid hexamers of naturally occurring mutants.

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