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Crystallization and preliminary crystallographic analysis of cabbage histidinol dehydrogenase

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Abstract

Recombinant *Brassica oleracea* histidinol dehydrogenase (HDH) has been crystallized in various space groups using the method of vapour diffusion. The presence or absence of inhibitors and substrates as well as the use of different precipitants has enabled the growth of five different crystal forms. Extensive searches with the first crystal form (*A*) failed to produce any useful heavy-atom derivatives, mainly because of the instability of the crystals. This provoked the search for further crystal forms in the hope of finding more suitable crystals. At least two of these crystal forms are of interest for further study.

1. Introduction

Histidinol dehydrogenase (HDH; L-histidinol:NAD oxidoreductase; E.C. 1.1.1.23) catalyzes the final two steps in the biosynthesis of histidine in microorganisms and higher plants as follows (Miflin, 1980; Chiarotti, Alifano, Carlomagno & Bruni, 1986; Nagai & Scheidegger, 1991),

$$\text{L-histidinol} + \text{NAD}^+ \longrightarrow \text{L-histidinal} + \text{NADH}$$
$$\text{L-histidinal} + \text{NAD}^+ \longrightarrow \text{L-histidine} + \text{NADH}.$$

The enzyme consists of two identical subunits, each containing one active site where the two successive reactions are catalyzed. The active site contains one Zn atom and one NAD^+ binding site (Grubmeyer, Skiadopoulos & Senior, 1989). Histidine residues have been shown to be necessary for the ligation of Zn (Nagai & Ohta, 1994) and a loss of Zn, through mutation of the histidine (Nagai & Ohta, 1994) or through chelation with 1,10-phenanthroline (Lee & Grubmeyer, 1987), results in loss of enzymatic activity. Site-directed mutagenesis of conserved cysteine residues in *Salmonella* (Teng, Segura & Grubmeyer, 1993) and cabbage (Nagai, Kheirolomoom & Ohta, 1993) HDH has shown that the reaction mechanism does not proceed *via* thiohemiacetal formation as found for most other aldehyde dehydrogenases (Harris & Waters 1976; Tu & Weiner, 1988; Kitson, Hill & Midwinter, 1991). Despite extensive kinetic and other biochemical studies (Grubmeyer, Chu & Insinga, 1987, and references therein; Kheirolomoom *et al.*, 1994), the mechanism of the reactions of HDH remains unclear.

HDH has been purified to homogeneity from cabbage, *Brassica oleracea* (Nagai & Scheidegger, 1991) and cloned (Nagai *et al.*, 1991) and overexpressed in *Baculovirus* (Nagai *et al.*, 1992). The predicted mature protein is 438 amino acids long and has a calculated molecular weight of 47 474. Cabbage HDH shares approximately 50% amino-acid sequence identity with HDH from other species such as *Saccharomyces cerevisiae*, *Escherichia coli* and *Salmonella typhimurium* (Nagai *et al.*, 1991). Since histidine biosynthesis is required in plants, HDH is a target for rational herbicide design (Kishore & Shah, 1988). Crystallographic studies have been undertaken with the aim of providing a structural basis for the design of potent and selective inhibitors and also to clarify the reaction mechanism of this enzyme.

2. Methods

2.1. *Protein preparation*

A cDNA encoding a mature form of cabbage HDH was expressed using the baculovirus expression vector system as described previously (Nagai & Ohta, 1994) using the transfer vector pVL1393 (Invitrogen), the insect cell line Sf9 and the infectious BaculoGold linearized *Baculovirus* DNA (Pharmingen, San Diego CA). The expressed HDH was purified to apparent homogeneity as described by Nagai & Ohta (1994). Briefly, the infected insect cells (600 mg from 1 l of culture medium) were homongenized in 50 ml of 20 m*M* Tris–HCl (pH 7.4) containing 100 μ*M* EDTA, 1 μ*M* leupeptin, 1 μ*M* pepstatin A and 200 μ*M* phenylmethylsulfonylfluoride with a Branson sonicator (sonifier 450). After centrifugation at 10 000*g* for 15 min, HDH was purified from the supernatant through two-step anion-exchange chromatography using a DEAE-Toyopearl (5 × 6 cm) and then a Mono Q (HR 16/10) column on an FPLC system (Pharmacia). The amino-acid sequence of the N terminus of the purified protein was determined to be MKSYRLS. . ., which matches that deduced from the DNA sequnce (Nagai & Ohta, 1994).

2.2. *Crystallization*

A systematic search for crystallization conditions produced small crystals which diffracted to 2.8 Å resolution and were suitable for further crystallographic study (crystal form *A*, Table 1). The protein (10 mg ml^{-1}) was first incubated at 277 K with the substrate NAD^+ (4 m*M*) and a potent inhibitor IRL 1654 (4 m*M*) for at least 10 min. This solution was then mixed with an equal volume (usually 4 μl) of a reservoir solution (1.4 *M* sodium sulfate, 0.1 *M* sodium citrate, 4 m*M* $ZnCl_2$, pH 4.8; 1000 μl) against which the drop containing the protein was equilibrated using the hanging-drop method of vapour diffusion at room temperature. The protein always precipitated before crystals began to form. Filtering of the protein mixture before mixing with the reservoir solution reduced the number of crystals found in each drop, however, filtering the solution after mixing with the precipitant did not improve the number or size of the crystals obtained. Crystals generally grew to a size of 0.3 × 0.1 × 0.1 mm over a period of 1 month. Characterization of the crystals using a complete data set (Table 2) from a FAST (Enraf) area detector mounted on a rotating-anode generator, showed that they belong to space group $I4_122$ and diffract to 2.8 Å resolution (Table 1). Efforts to increase the size of these crystals resulted in lengths of up to

Table 1. *Crystal data*

Crystal form	Size (mm) Best resolution (Å)	Unit-cell size (a, b, c, Å) Molecules in a.u.*	Space group	pH	Precipitant
A	0.6 × 0.1 × 0.1 2.5	143.8, 143.8, 101.9 1	$I4_122$	4.8	Na_2SO_4
B	0.4 × 0.3 × 0.2 3.3	103.3, 207.8, 340.2 6–8	$I222$ or $I2_12_12_1$	4.8	Na_2SO_4
C	1.0 × 0.15 × 0.1 2.8	119.8, 97.7, 76.6 2	$P2_12_12_1$	6.0	PEG 6000
D	1.0 × 0.15 × 0.1 3.2	48.9, 94.2, 189.2 2	$P222$, $P222_1$, $P2_12_12$ or $P2_12_12_1$	6.0	PEG 6000
E	0.15 × 0.05 × 0.05	Not yet characterized		4.7	PEG 20000

* Number of molecules in the asymmetric unit of the crystal.

Table 2. *Crystal characterization*

Crystal form	X-ray source	Resolution (Å)	Completeness (%)	Multiplicity	R_{merge}* (%)
A	Synchrotron	2.5	72.0	2.5	9.0
A	Laboratory	2.8	96.8	3.9	11.7
B	Laboratory	3.3	45.9	1.4	9.1
C	Laboratory	3.0	81.6	2.3	10.1
D	Laboratory	3.5	78.1	2.3	7.6

* $R_{merge} = \sum |I_i - \langle I_h \rangle| / \sum \langle I_h \rangle$, where I_i is the ith measurement of reflection h.

0.6 mm, but no corresponding increase in width and therefore, no increase in the diffraction power. These crystals diffract to a resolution of 2.5 Å resolution (Table 2) at a synchrotron radiation source (station D23 of the synchrotron at Lure in France). Crystal form *B* was obtained using the same crystallization conditions as for form *A* and the two different forms often appeared in the same drop. Form *B* crystals, which are favoured by higher concentrations of protein, are much larger, but diffract relatively poorly. The cell dimensions and large number of monomers in the asymmetric unit (Table 1) also limit the usefulness of these crystals for structure determination.

Crystal forms *C* and *D* were obtained using Jancarik and Kim's method of sparse-matrix screening (Jancarik & Kim, 1991). Both crystal forms are obtained under the same conditions, however, crystal form *C* requires the presence of NAD^+ while crystal form *D* is obtained in the absence of substrate. After optimization of the conditions, long, thin and often hollow needles were obtained with the hanging-drop method of vapour diffusion at room temperature. These orthorhombic crystals (Table 1) grow within 2 d after mixing the protein solution (20 mg ml^{-1}) with an equal volume of a reservoir solution (16–20% PEG 6000, 0.2 *M* magnesium acetate, 0.1 *M* Cacodylate, pH 6.0) and equilibrating the drop with 1000 µl of the latter solution. Experiments with additives such as β-octylglucoside, dioxane, glycerol, acetone, dithiothreitol, $ZnCl_2$ and other divalent cations did not improve the morphology or diffraction power of the crystals which is 2.8 Å in the best cases (Table 2). Low temperature or the addition of inhibitors produced very thin plates which were never big enough to be analysed properly. A fifth crystal form (*E*) was obtained when screening a large range of polyethylene glycols as precipitants *versus* pH using the *Pick&Mix* program with a Douglas Instruments robot (Chayen, Shaw Stewart & Baldock, 1994). Crystals up to 0.15 × 0.05 × 0.05 mm in size were grown after equilibration using the sitting-drop method of vapour diffusion. A drop consisting of 2 µl protein solution with an excess of NAD^+ and 2 µl of reservoir solution (8–12% PEG 20 000, 0.1 *M* sodium acetate, pH 4.7) is equilibrated against 60 µl of the latter solution. These crystals have a similar morphology to that of form *A* but are grown in the absence of inhibitors and have not been characterized further.

2.3. *Heavy-atom derivative screening*

An extensive heavy-atom derivative search using crystal form *A* revealed that the crystals were extremely sensitive to heavy-atom compounds. After soaking attempts with 40 different compounds, 13 of these containing mercury and six containing platinum, the crystals were usually found not to diffract. Shorter soaking times and/or weaker concentrations led to a lack of binding or complete non-isomorphism with a significant reduction in the quality of the diffraction. Large metal clusters such as TAMM and PIP (O'Halloran, Lippard, Richmond & Klug, 1987) gave similar results. For these experiments, crystals were stored and soaked in a buffer containing the same ingredients as the crystallization reservoir, except that the concentration of the precipitant was usually increased from 1.4 to 1.75 *M*. Experiments with precipitant concentrations at 1.4 *M* or up to 2.0 *M* produced the same effects. Heavy-atom solutions were prepared freshly in the same buffer. The concentration of the heavy-atom compound was first tried at 5 or 10 m*M* and then decreased, according to the results obtained, down to 0.01 m*M* for some mercury compounds. Soaking times varied between 30 min and 2 months, but were initially always performed over 1 d or a week.

A further complication was the lack of isomorphism between different native crystals grown under the same conditions. For example, the merging *R* factors between different native data sets for crystal form A were as high as 30%, although the cell dimesions were virtually identical. During these experiments, attempts were made to find a better stabilization buffer using pH changes (variations from pH 4.7 up to pH 6.0), additives (MPD, PEG 400) and cross-linking

(gluteraldehyde) without success, as all of these changes reduced the quality of the crystals and often caused cracking. Other stabilization buffer additives such as glycerol, which has been shown to help unstable proteins such as this one which tends not to crystallize after overnight storage (Owen, Papageorgiou, Garman, Noble & Johnson, 1995) have not been tried, however. Crystals could be grown in the presence of glycerol, but were never large enough to be analysed with X-rays. The failure to find useful heavy-atom derivatives of crystal form *A* is what prompted the search for further crystal forms of HDH. The heavy-atom search for crystal forms *C* and *D* is still in the early stages. Experiments with five different compounds (Hg, Ir and Pt) show that these crystals of HDH are also sensitive to heavy-atom binding. If the heavy atom compound bound to the protein in the crystals, the crystals often cracked or the data collected was so noisy that any heavy-atom signal was obscured and not useful for structure determination. These results were also complicated by a lack of isomorphism between native crystals. Clearly, experiments to find (1) suitable stabilization buffers, (2) optimized crystallization conditions (seeding and many additives have not yet been tried) and (3) more suitable heavy-atom compounds, need to be performed.

Since crystal forms *A* and *B* will only grow in the presence of the inhibitor IRL1654, and large enough crystals of forms *C* and *D* only grow in the absence of inhibitors, it has also not been possible to obtain derivatives using heavy-atom substituted inhibitors. Soaking of crystal forms *C* and *D* with the latter inhibitors (which contain bromine and mercury, G. Iwasaki, unpublished data) also cracked the crystals. Attempts to co-crystallize the protein with several heavy-atom compounds were unsuccessful because of the fact that the compounds caused the protein to precipitate, although this was only tried with mercurial reagents. With the idea that the sensitivity of the HDH crystals might be due to the large number of potential binding sites on the protein [*e.g.* seven cysteines and 11 methionines per monomer), mutants where cysteine residues have been replaced by alanine [constructs made for the study of the reaction mechanism of the enzyme, Cys112 to Ala, Cys149 to Ala, or both (Nagai *et al.*, 1993)], were subjected to crystallization trials. The failure to produce crystals of these mutants may be because of their decreased solubility.

An unsuccessful search for heavy-atom derivatives is never complete as there are an almost unlimited number of experiments that can be performed. In this case, modification of protein amino groups (Mowbray & Petsko, 1983) and iodination of the protein are just two examples, and there are many more heavy-atom compounds that could be tried by traditional soaking methods or by co-crystallization. Further experiments to find a better stabilization buffer would be useful for crystal forms *A*, *C* and *D*, as might variations on the protein purification method to try and produce a more well behaved batch of protein. Crystal forms *C* and *D* grow quickly and could probably be improved. It may also be an advantage that they grow with a non-ionic precipitant (PEG 6000). The latter two crystal forms are therefore very promising for further attempts to find derivatives of HDH. Crystal form *E* needs to be characterized further.

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