Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Identification of many crystal forms of *Aspergillus nidulans* dehydroquinate synthase

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Received 29 May 2000
Accepted 1 December 2000

Extensive crystallization trials of *Aspergillus nidulans* dehydroquinate synthase, a potential novel target for antimicrobial drugs, in complexes with different ligands have resulted in the identification of nine crystal forms. Crystals of unliganded DHQS, binary complexes with either the substrate analogue, carbaphosphonate or the cofactor NADH, as well as the ternary DHQS–carbaphosphonate–cofactor complex, were obtained. The ternary complex crystallizes from ammonium sulfate and $CoCl_2$ in space group $P2_12_12$, with unit-cell parameters a = 133.8, b = 86.6, c = 74.9 Å. The binary carbaphosphonate complex crystallizes from PEG 6000 in space group $P2_12_12_1$, with a = 70.0, b = 64.0, c = 197.6 Å, and the binary cofactor complex crystallizes from PEG 3350 and sodium potassium tartrate in space group $P2_1$, with a = 83.7, b = 70.4, c = 144.3 Å, β = 89.2°. DHQS in the absence of ligands crystallizes in space group $P2_1$, with a = 41.0, b = 68.9, c = 137.7 Å, β = 94.8°. Each of these crystal forms are suitable for high-resolution structure determination. Structures of a range of DHQS–ligand complexes will be of value in the structure-based design of novel antimicrobial drugs.

1. Introduction

Dehydroquinate synthase is an enzyme of the shikimate pathway, the products of which are utilized in the biosynthesis of aromatic amino acids. DHQS catalyses the conversion of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate to dehydroquinate in an NAD^+-dependent reaction. The overall reaction pathway is complex and involves alcohol oxidation, phosphate β-elimination, carbonyl reduction, ring opening and intramolecular aldol condensation (Bartlett *et al.*, 1994; Bartlett & Satake, 1988; Bender *et al.*, 1989; Lambert *et al.*, 1985; Montchamp & Frost, 1997; Moore *et al.*, 1998; Srinivasan *et al.*, 1963; Widlanski *et al.*, 1989). This synthesis of aromatic amino acids is an essential metabolic function for most prokaryotic as well as lower eukaryotic cells, including plants. The pathway is absent in humans; therefore, DHQS represents a potential target for the development of novel and selective antimicrobial agents. Owing to the threat posed by the spread of pathogenic bacteria resistant to many currently used antimicrobial drugs, there is clearly a need to develop new anti-infective drugs acting at novel targets. A further potential use for DHQS inhibitors is as herbicides.

In fungi and other lower eukaryotes, DHQS is produced as part of the pentafunctional AROM protein which is active in the pre-chorismate section of the shikimate pathway. In filamentous fungi, the AROM protein is leaky and shares a common pool of dehydroquinate with the catabolic quinic acid utilization pathway (Lamb *et al.*, 1992; Wheeler *et al.*, 1996). DHQS is the N-terminal domain of AROM and retains enzymatic activity when expressed in the absence of other *A. nidulans* AROM functions (Moore *et al.*, 1994; van den Hombergh *et al.*, 1992).

Although the three-dimensional structure of *A. nidulans* DHQS in a ternary complex with the substrate analogue, carbaphosphonate and NAD^+ cofactor has been determined (Carpenter *et al.*, 1998), the crystallization conditions were not reported. Our work is aimed at extending structural studies of DHQS including the unliganded enzyme as well as different liganded states such as binary complexes with either carbaphosphonate or NADH. A further aim is the study of DHQS as complexes with novel inhibitors for use in structure-based inhibitor design. We have undertaken a wide-ranging study of the crystallization properties of DHQS that has resulted in a total of nine crystal forms being identified. These forms include examples of the unliganded enzyme, two binary complexes and a ternary complex which are suitable for generating high-resolution refined crystal structures. Such information will be of value in understanding conformational states of the

Table 1
Crystallization conditions, space group and unit-cell parameters and data-collection statistics.

(*a*) DHQS Crystallization conditions.

Crystal form	Crystallization conditions†				
	Precipitant	Buffer (0.1 *M*)	Solvents	Additives	Ligands
A	5% PEG 6000	HEPES pH 7.0	20% ethylene glycol	—	NADH, CBP
B	7% PEG 6000	HEPES pH 7.0	20% ethylene glycol	—	CBP
C	50% $(NH_4)_2SO_4$	HEPES pH 8.0	—	1 m*M* $CoCl_2$	NADH, CBP
D	5% PEG 6000	HEPES pH 8.0	5% MPD	—	—
E	18% PEG 3350	HEPES pH 8.0	30% DMSO	0.2 *M* Na/K tartrate	NADH
F	7% PEG 6000	HEPES pH 8.0	30% DMSO	—	NADH
G	17% PEG 3350	HEPES pH 6.8	—	0.2 *M* NH_4 tartrate	NADH
H	9% PEG 6000	HEPES pH 7.5	20% ethylene glycol, 10% DMSO	5 m*M* MEGA7	ADP, CBP
I	1.125 *M* Li_2SO_4	HEPES pH 7.5	25% glycerol	—	NADH

(*b*) Space group and unit-cell parameters.

Crystal form	Space group	No. of subunits in asymmetric unit	Unit-cell parameters			
			a (Å)	*b* (Å)	*c* (Å)	*β* (°)
A	$P2_12_12_1$	2	68.5	80.2	142.2	90.0
B	$P2_12_12_1$	2	70.0	64.0	197.6	90.0
C	$P2_12_12$	2	133.8	86.6	74.9	90.0
D	$P2_1$	2	41.0	68.9	137.7	94.8
E	$P2_1$	4	83.7	70.4	144.3	89.2
F	$P2_12_12_1$	2	72.4	83.5	149.0	90.0
G	$P2_1$	2	42.1	67.1	148.6	92.2
H	$P2_12_12$	3	206.5	136.4	40.4	90.0
I	$P2_12_12_1$	2	65.2	70.6	154.1	90.0

(*c*) Data-collection statistics.§

Crystal form	Resolution (Å)	Completion (%)	R_{merge}¶	$I/\sigma(I)$	Redundancy
A	2.4 (2.49–2.4)	64.6 (65.1)	0.062 (0.272)	12.34 (2.12)	3.05 (1.72)
B	2.5 (2.6–2.51)	97.6 (99.0)	0.040 (0.078)	25.40 (16.07)	2.89 (2.89)
C	2.1 (2.18–2.1)	96.9 (96.2)	0.101 (0.412)	12.96 (2.10)	2.91 (2.61)
D	2.8 (2.85–2.8)	99.6 (95.9)	0.067 (0.377)	18.10 (2.32)	4.80 (3.38)
E	2.6 (2.64–2.6)	99.0 (96.5)	0.063 (0.367)	16.70 (2.50)	3.36 (2.68)
F	2.9 (3.0–2.9)	93.6 (90.1)	0.108 (0.354)	5.99 (1.55)	2.85 (2.19)
G	2.9 (3.0–2.9)	97.1 (95.7)	0.079 (0.402)	13.30 (0.82)	3.62 (3.09)
H	2.8 (2.9–2.8)	93.5 (80.4)	0.074 (0.217)	16.28 (4.17)	3.12 (2.37)
I	2.45 (2.54–2.45)	95.7 (81.3)	0.139 (0.541)	6.93 (1.46)	4.06 (3.03)

† MPD, methylpentanediol; MEGA7, heptanoyl-*N*-methylglucamide; CBP, carbaphosphonate. § Figures in parentheses are for the outer-shell data. ¶ $R_{merge} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$.

enzyme in the presence of different ligands, knowledge that can be applied to the rational design of novel antimicrobial drugs.

2. Materials and methods

The cloning and expression of the DNA encoding the dehydroquinate synthase domain of the pentafunctional AROM protein of *A. nidulans* has previously been described (Moore *et al.*, 1994; van den Hombergh *et al.*, 1992). The protein-isolation procedure was as described previously except that ceramic hydroxyapatite was used for the final chromatographic step (Moore *et al.*, 1994).

In order to reduce residual levels of NAD^+ cofactor for some experiments, DHQS was pre-incubated with 2 m*M* ADP and then subjected to gel filtration on either Sephadex G-25 or Sephacryl S-200; in the latter case, the column was also used to remove any aggregated DHQS prior to crystallization trials.

2.1. Crystallization

Dehydroquinate synthase was concentrated and buffer exchanged into 10 m*M* Tris pH 7.4, 40 m*M* KCl using Centricon-30 centrifugal concentrators (Millipore). For initial surveys of crystallization conditions, standard screening kits purchased from Hampton Research Corporation were used, *i.e.* Crystal Screen I, Crystal Screen II, PEG/Ion and Crystal Screen Cryo kit, as well as four different GRID Screens (*i.e.* ammonium sulfate, PEG 6000, PEG/LiCl and MPD). DHQS at 15–25 mg ml^{-1} was used, with typically 3 µl of protein solution mixed with 3 µl of reservoir solution set up as sitting-drop vapour-diffusion experiments using microbridges (Harlos, 1992). All crystallizations were carried out at 277 K. Crystallization conditions identified from screens were optimized by employing finer intervals of pH and precipitant concentration. In some cases, cryoprotectant [20%(*v*/*v*) ethylene glycol] and/or up to 30%(*v*/*v*) DMSO were included in the crystallization solution to aid the solubility of certain inhibitors.

2.2. Data collection and processing

X-ray diffraction data were mainly collected in-house using a MAR345 image-plate system on a Rigaku generator equipped with a Cu anode and Osmic multilayer optics to give *Kα* radiation (λ = 1.5418 Å). The data for crystal form *G* were collected at ESRF, Grenoble, beamline ID14.2 (λ = 0.933 Å) using a 165 mm MAR CCD detector. All data sets were collected at 100 K using an Oxford Cryosystems Cryostream, except for form *C* which was collected at 297 K. If cryoprotectant was not already present in the crystallization medium then crystals were transferred for a few seconds into reservoir solution with added ethylene glycol [20%(*v*/*v*)] prior to freezing.

Indexing and integration of data images were carried out with *DENZO* (Otwinowski & Minor, 1996) and the data were merged using *SCALEPACK* (Otwinowski & Minor, 1996). Initial choice of space group made on the basis of assignments in *DENZO* combined with systematic absence data from *SCALEPACK* were confirmed by the molecular-replacement studies outlined below. The size of the asymmetric unit was estimated from consideration of the normal range of packing densities for protein crystals (Matthews, 1968) and later confirmed by molecular-replacement studies.

2.3. Molecular replacement

Rotation and translation searches were carried out using *CNS* (Brunger *et al.*, 1998). The real-space Patterson search method was used for cross-rotation searches using data in the resolution range 15–4 Å. The coordinates of the DHQS dimer (PDB code 1dqs; Carpenter *et al.*, 1998) were used as the search model. The model Patterson map was calculated by placing the molecule in an orthogonal cell of *P*1 symmetry with cell edges corresponding to three times the distance from the geometric centre to the remotest atom of the molecule. The top 3000 Patterson function vectors between 5 and 47 Å in length were chosen for the rotation-function calculation. The Eulerian angle

asymmetric unit of the cross-rotation function was determined automatically by the program. The 1000 highest peaks of the rotation function were sorted by cluster analysis of 10° intervals. Initial cross-rotation function solutions were subjected to rigid-body Patterson correlation refinement. The translation function was carried out using the correlation coefficient grid-search method. All the molecular-replacement solutions were checked by displaying the transformed coordinates in *O* (Jones *et al.*, 1991) to confirm that lattice contacts appeared reasonable.

3. Results and discussion

Approximately 4000 crystallization conditions have been surveyed to date. Crystals of DHQS were obtained under four main conditions *viz.* (i) PEG 6000, HEPES pH 7.5, 5% MPD, (ii) ammonium sulfate with 10 m*M* cobalt chloride, HEPES pH 8.0, (iii) PEG 3350 with sodium/potassium tartrate, HEPES pH 8.0 and (iv) 1.125 *M* lithium sulfate, 0.1 *M* HEPES pH 7.5, 25% glycerol (Table 1). These crystallization conditions were derived from Crystal Screen II condition number 30 (10% PEG 6000, 0.1 *M* HEPES pH 7.5, 5% MPD), Crystal Screen II condition number 25 (1.8 *M* ammonium sulfate, 0.1 *M* MES pH 6.5, 10 m*M* cobalt chloride), PEG/Ion Screen condition number 37 (0.2 *M* sodium/potassium tartrate pH 7.2, 20% PEG 3350) and Crystal Screen Cryo number 16 (1.125 *M* lithium sulfate, 0.075 *M* HEPES pH 7.5, 25% glycerol), respectively. The nine different crystal forms identified, designated *A*–*I*, are the result of variation of the basic crystallization conditions described above together with different combinations of ligands (Table 1). For each type of complex investigated, crystals of sufficient quality to give data sets with resolution limits in the range 2.1–2.8 Å were obtained, *i.e.* ternary complex (form *C*), binary carbaphosphonate complex (form *B*), binary cofactor complex (form *E*) and unliganded (form *D*).

Determining the molecular-replacement solution for crystal form *D* was the key to solving four further forms. As this and the solution for form *C* were not straightforward, we thus describe them in detail below. For form *D*, the cross-rotation function resulted in 68 peaks. Although the first peak is 5.3σ above the mean, it is not well separated from the rest of the peaks (0.4σ from the second peak and 1.0σ above the third peak). Following rigid-body Patterson correlation refinement, treating the two domains of each monomer as separate rigid bodies, peak 26 (θ_1 = 251.0, θ_2 = 7.1, θ_3 = 353.6°) had the highest correlation coefficient of 15.9%, which is 3σ above the second highest peak (number 22, at 11.8%). The translation search using the refined best rotation-function peak gave a solution with a correlation coefficient (E2E2) of 41.0% for x = 6.32, z = 1.35 Å. Visual checking of the molecular packing in the unit cell using *O* confirmed this to be the correct solution, which has now been refined to an *R* factor of 0.23 ($R_{\rm free}$ = 0.275). Solutions to crystal forms *E*, *F*, *G* and *I* were obtained using the coordinates from crystal form *D*.

For crystal form *C*, the rotation function gave 77 peaks; the top two peaks were significantly higher than the others *viz.* θ_1 = 351.8, θ_2 = 82.9, θ_3 = 224.9°, 15.7σ above the mean and 10.9σ above the third peak; θ_1 = 351.5, θ_2 = 82.9, θ_3 = 256.2°, 11.9σ above the mean and 7.1σ above the third peak. From the estimated size of the asymmetric unit, only one peak for a dimer would be expected. Nevertheless, both peaks were subject to the translation search, which resulted in correlation coefficients of 45.7% for x = 46.8, y = 9.3, z = 14.8 Å and 34.3% for x = 45.2, y = 26.6, z = 5.0 Å, respectively. When the two solutions were displayed in *O* it was clear that the dimers in each case were placed on crystallographic twofolds, in one case along the *c* axis and the other through (0, $\frac{1}{2}$, 0). The asymmetric unit is therefore composed of two monomers contributed from two separate biological DHQS dimers. We thus took monomer I from solution one and monomer II from the second solution and performed a third translation search which determined the relative position of these two molecules (II relative to I of x = l67.6, y = 0.05, z = −0.08 Å). Subsequent rigid-body refinement gave an *R* factor of 0.334 and the structure has been further refined to an *R* factor of 0.189 ($R_{\rm free}$ = 0.235).

Form *A* crystals of the ternary DHQS–carbaphosphonate–cofactor complex are related to those described by Carpenter *et al.* (1998), although we found that the crystals had a high mosaic spread. However, as noted above, good-quality crystals of this ternary complex were obtained in another space group (form *C*) under very different conditions. Molecular-replacement solutions for crystal forms *B* and *H* were obtained from coordinate set 1dqs.

The crystals of binary complexes of DHQS with cofactor (forms *D*–*G* and *I*) generally diffracted to a lower resolution limit than for ternary complexes, although for crystal form *G* data to 2.6 Å were collected. It was found that many of these crystal forms are able to tolerate high concentrations of organic solvents such as DMSO and ethylene glycol and thus appear suitable for soaking in inhibitors that might have low water solubility.

The fact that DHQS is able to crystallize in a wide variety of forms could reflect conformational variability of the protein or a distribution of surface charges that allows many ways of packing molecules with different lattice contacts. In the case of HIV-1 reverse transcriptase, we have observed a series of six crystal forms (Esnouf *et al.*, 1998; Stammers *et al.*, 1994) which are of all the same space group but show large variations in unit-cell parameters. This appears to be the result of flexibility in the protein, as significant relative domain movements between the different crystal forms are observed (Esnouf *et al.*, 1998).

The identification of many different crystal forms of various liganded states of DHQS holds out the prospect of understanding the ligand-binding properties and conformational states of this enzyme in some detail. Refinement of the nine crystal forms of DHQS reported here is under way and when completed results from the different liganded states of DHQS should provide a further stimulus for the structure-based design of novel inhibitors of the enzyme, which could in turn lead to drug candidates that would fulfil an important clinical need.

The Oxford Centre for Molecular Sciences is supported by the BBSRC, MRC and EPSRC. DKS is funded by the MRC. This work was funded by grants from the BBSRC and Arrow Therapeutics. We thank Dr J. Frost for the gift of carbaphosphonate, Dr Karl Harlos for help with the X-ray data collection and Dr Robert Esnouf for computing support.

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