Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray study of pig liver dihydropyrimidine dehydrogenase

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Received 12 September 2000
Accepted 25 October 2000

Dihydropyrimidine dehydrogenase catalyzes the first and rate-limiting reaction in pyrimidine catabolism. The enzyme contains one FMN, one FAD and four Fe–S clusters per subunit of 1025 amino acids as prosthetic groups. It is also the major determinant of bioavailability and toxicity of 5-fluorouracil, a chemotherapeutic agent widely used in the treatment of solid tumors. Crystals of this enzyme diffracting to at least 2.5 Å have been obtained by the hanging-drop vapour-diffusion method and belong to space group $P2_1$ (unit-cell parameters a = 82.0, b = 159.3, c = 163.6 Å, β = 96.1°), with two homodimers per asymmetric unit.

1. Introduction

Dihydropyrimidine dehydrogenase (DPD; E.C. 1.3.1.2) catalyzes the NADPH-dependent reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively, the first and rate-limiting step in the pyrimidine catabolic pathway. In mammals, this pathway is the sole source of β-alanine (Wasternack, 1980), a putative neurotransmitter (Kontro, 1983) and final metabolite of uracil. A deficiency of DPD in humans is associated with congenital thymin-uraciluria, a complex hereditary disease often accompanied by growth and mental retardation (van Gennip *et al.*, 1997).

DPD is also the key enzyme in the degradation of the pyrimidine antimetabolite 5-fluorouracil (5FU), one of the most prescribed chemotherapeutic drugs used in the treatment of solid tumors (Heggie *et al.*, 1987). More than 80% of the administered 5FU is rapidly degraded to the neurotoxic agent α-fluoro-β-alanine (Woodcock *et al.*, 1980; Okeda *et al.*, 1990), not only necessitating the application of very high drug doses, but also causing severe toxic side effects. Individual as well as circadian variation in DPD activity is thought to be responsible for the highly variable and unpredictable bioavailability of 5FU (Cohen *et al.*, 1974; Diasio & Harris, 1989). Furthermore, the enzyme is a potential source of resistance to 5FU, since intratumoral DPD activity is inversely correlated with the response rate to 5FU in cancer patients (Etienne *et al.*, 1995). Thus, DPD is the major determinant in 5FU pharmacokinetics and toxicity; its controlled inhibition has become an important adjunct goal in the development of new anticancer drugs (Porter *et al.*, 1992; Mani *et al.*, 2000).

DPD is primarily found in the liver, but is also present in several other tissues (Ho *et al.*, 1986). Thus far, the enzyme has been purified from rat (Shiotani & Weber, 1981), pig (Podschun *et al.*, 1989), bovine (Albin *et al.*, 1996) and human liver (Lu *et al.*, 1992) as well as from *Alcaligenes eutrophus* (Schmitt *et al.*, 1996). The cDNA of porcine DPD was cloned and expressed in *Escherichia coli*, resulting in high yields of recombinant enzyme (Rosenbaum *et al.*, 1997). DPD is a homodimer of 2 × 111 kDa and contains an unusually large number of different redox cofactors. Each subunit of 1025 amino acids binds one FAD, one FMN, 16 non-haem Fe and 16 acid-labile S atoms (Rosenbaum *et al.*, 1998). Analysis of the DPD sequence suggests the assembly of the Fe and S atoms to four [4Fe–4S] clusters per subunit, two of them located in an N-terminal cysteine-rich region and two in a 50 amino-acid stretch at the C-terminus with sequence similarity to bacterial ferredoxins (Hagen *et al.*, 2000).

According to the non-classical two-site ping-pong mechanism of porcine DPD, separate binding sites are likely to be present for the NADPH–$NADP^+$ and pyrimidine-5,6-dihydropyrimidine substrates/products, respectively (Podschun *et al.*, 1990). Thus, NADPH reduces the flavin of the enzyme at site 1 and electrons are transferred, most likely *via* a yet unresolved number of FeS clusters, to the site 2 flavin, where the pyrimidine substrate is reduced by an *anti* addition across the 5,6 double bond. Sequence comparisons have revealed similarities to other proteins for some parts of the DPD polypeptide chain, allowing approximate localization of the domains harboring the two active sites. Based on fingerprints typical of FAD and NAD(P)H binding, the N-terminal part of the chain

Table 1
Diffraction, data collection and reduction statistics.

Values in parentheses are for the highest resolution shell (2.56–2.50 Å).

Space group	$P2_1$
Unit-cell parameters	
a (Å)	81.7
b (Å)	159.0
c (Å)	163.1
β (°)	96.1
No. of reflections	1338319
No. of unique reflections	143621
Resolution limits (Å)	25.0–2.5
R_{sym} (%)	11.4 (25.6)
$I/\sigma(I)$	9.7 (4.1)
Completeness (%)	97.7 (66.9)

(residues 1–520) was suggested to harbour the binding sites for these cofactors, *i.e.* functional site 1 (Yokota *et al.*, 1994). The FMN-containing uracil- and thymine-binding domain is localized in the central part of the DPD sequence (residues 521–851), which is related to dihydroorotate dehydrogenases (Rosenbaum *et al.*, 1998).

2. Crystallization

Recombinant pig liver DPD was purified following a previously described procedure (Rosenbaum *et al.*, 1997). The protein samples used for crystallization were pure according to SDS–PAGE and were stored at 193 K at a concentration of approximately 20 mg ml^{-1} in 25 m*M* HEPES pH 7.5, 10 m*M* DTT and 10%(*v*/*v*) glycerol. The same buffer was used for dilution of the protein samples to the concentrations used in the crystallization trials.

Hampton Research Screens (Hampton Research) were used for initial screening of crystallization conditions at 277 and 293 K in hanging drops. The conditions yielding small crystals were further optimized by variation of precipitant and protein concentration and buffer pH. The best crystals of DPD were obtained using vapour diffusion against 17–22%(*w*/*v*) polyethylene glycol 6000, 10 m*M* DTT and 100m*M* sodium citrate pH 4.7. Each drop was suspended on a silanized cover slip over 1 ml reservoir solution and contained 3 µl protein solution (4.5 mg ml^{-1} DPD) mixed with 3 µl reservoir solution. No cofactors were added to either solution. Crystals appeared within 2 d of equilibration at 293 K and grew to a size of approximately 0.1 × 0.1 × 0.7 mm within 7 d. The dark yellow crystals exhibit a long square pillar shape, characterized by a hollow space within them (Fig. 1). The diffraction from these crystals was rather poor, possibly because of this growth disorder. Despite numerous attempts, the crystal morphology could not be improved. However, if the beam was centred at the very edge of the crystal, which was usually well shaped (Fig. 1), the diffraction pattern improved and data of reasonable quality could be obtained.

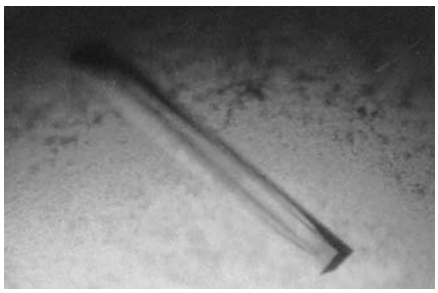

Figure 1
Crystal of recombinant pig liver dihydropyrimidine dehydrogenase. The approximate dimensions are 0.1 × 0.1 × 0.7 mm.

3. X-ray analysis

A crystal was transferred to a cryosolution containing 22%(*w*/*v*) polyethylene glycol 6000, 10 m*M* DTT, 100 m*M* sodium citrate pH 4.7 and 20%(*v*/*v*) glycerol. After soaking for at least 5 min, the crystal was flash-cooled to 100 K in a nitrogen-gas cold stream (Oxford Cryosystems Cryostream). X-ray data were collected as 0.8° oscillations at a wavelength of 0.995 Å at beamline 711 at MAX-lab (Lund Synchrotron, Sweden) using a MAR Research image plate.

Crystallographic data were collected to 2.5 Å resolution. Crystals of DPD belong to the monoclinic space group $P2_1$. The pseudo-precession image of the $hk0$ layer, calculated from the X-ray data with the program *PATTERN* (Lu, 1999), shows systematic absences of the reflections ($0k0$: $k = 2n + 1$). The unit-cell parameters are a = 81.7, b = 159.0, c = 163.1 Å, β = 96.1°. Processing and scaling of the crystallographic data with the program *HKL* (Otwinowski & Minor, 1997) resulted in an overall R_{sym} of 11.4% and an R_{sym} in the highest resolution shell (2.56–2.50 Å) of 25.6%. Complete data-collection statistics are shown in Table 1. The value of the Matthews coefficient (Matthews, 1968) is 2.38 for two dimers in the asymmetric unit, which corresponds to a solvent content of 50.5%.

Calculation of the native Patterson map did not reveal any significant peaks. A self-rotation function was calculated with the program *POLARRFN* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The κ = 180° section of the map, calculated with an integration radius of 30 Å using data in the resolution range 20–5 Å, shows two peaks of heights 50.5% (ω = 71.7, φ = 90°) and 37.5% (ω = 18.0, φ = 90°) of the origin peak, indicating the presence of non-crystallographic twofold axes.

Although parts of the DPD amino-acid sequence show significant similarity to several proteins, a determination of its three-dimensional structure by molecular replacement failed owing to the limited size of the search models compared with DPD. The full structure analysis based on an isomorphous heavy-atom derivative and the MAD phasing technique, utilizing the intrinsic anomalous signal of the iron ions of the FeS clusters, is now under way.

We are indebted to K. Schnackerz and K. Jahnke (University of Würzburg) for protein samples and to Mona Gullmert for excellent technical assistance. DD was supported by a fellowship from The Wenner-Gren Foundation and the project was supported by a grant from the Swedish Cancer Foundation. We gratefully acknowledge access to the synchrotron at the MAX-laboratory in Lund, Sweden.

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