Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray study of chloroplast glyceraldehyde-3-phosphate dehydrogenase

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Glyceraldehyde-3-phosphate dehydrogenase from spinach chloroplasts has been crystallized by vapour diffusion in the pH range 7–8.5 in $(NH_4)_2SO_4$ and Tris–HCl buffer or potassium phosphate buffer at room temperature. Crystals of the A_4 isoform, grown at pH 8.5 in Tris–HCl buffer, diffract to 3.0 Å (at 100 K) using synchrotron radiation. The crystals belong to the orthorhombic *C*222 space group, with unit-cell dimensions a = 145.9, b = 185.9 and c = 106.3 Å, and probably contain one tetramer per asymmetric unit. Structure determination by molecular replacement is in progress.

Received 14 August 1998
Accepted 8 October 1998

1. Introduction

Chloroplast glyceraldehyde-3-phosphate dehydrogenase [NAD(P)-GAPDH; E.C. 1.2.1.12] catalyzes the reaction of 1,3-bisphosphoglycerate to triose phosphate as the second reaction of the CO_2 reduction cycle. The enzyme is formed by subunits A (36 kDa) and B (39 kDa) in stoichiometric amounts, giving an $(A_2B_2)_4$ structure of 600 kDa (Pupillo & Faggiani, 1979; Cerff & Chambers, 1979). The two protein subunits, products of the *GapP* nuclear gene family, show considerable sequence homology. B subunits show 66 amino-acid substitutions with respect to A subunits in the spinach enzyme and are also characterized by the presence of a hydrophobic C-terminus domain of 31 amino acids with a putative regulatory role (Cerff, 1995).

In contrast, glycolytic glyceraldehyde-3-phosphate dehydrogenases (NAD-dependent, NAD-GAPDH) are tetramers of 37 kDa subunits (Russell & Sachs, 1991) and use NAD as the only coenzyme. These important enzymes are probably present in the cytoplasm of all eukaryotic cells and most bacteria and are encoded by a gene family *GapC*, although products of subfamily *GapCp* are targeted to plastids in some plants (Meyer-Gauen *et al.*, 1994).

Unlike NAD-GAPDH, the chloroplast enzyme can use both NADP(H) and NAD(H). NADPH is a photosynthetic product, and the intrinsic NADPH-dependent activity of the enzyme is enhanced by light and photosynthesis (Cseke & Buchanan, 1986; Scagliarini *et al.*, 1999). Indeed, certain substrates and effectors including NADP(H), ATP, Pi and reductants promote the *in vitro* dissociation of A_8B_8 oligomers to A_2B_2 protomers having increased NADP(H)-dependent activity (Baalmann *et al.*, 1995; Pupillo & Piccari, 1975; Trost *et al.*, 1993). The NAD(H)-dependent activity is not affected by these treatments and NAD^+ stabilizes the enzyme in a 600 kDa conformation with reduced NADP(H)-dependent activity (NADP-suppressed state).

In addition, chloroplasts contain other less abundant NAD(P)-GAPDH enzyme forms including an A_4 isoform of 150 kDa (Iadarola *et al.*, 1983; Scagliarini *et al.*, 1999). This latter isoform shows permanently high non-regulated NADP(H)-linked activity. Similar forms can be obtained by proteolytic cleavage of B subunits of the major regulatory isoform (Scheibe *et al.*, 1996; Zapponi *et al.*, 1993) by producing either free A subunits or truncated B subunits; the latter subunits are also potentially competent in catalysis.

The aim of our research is to investigate the structure of NAD(P)-GAPDH from chloroplasts. In the first study, the pyridine nucleotide binding site of the non-regulatory A_4 isoform (NADP preferring) will be compared with known structures of glycolytic NAD-GAPDH proteins (Skarzynski *et al.*, 1987; Diderjean *et al.*, 1997). Further investigations will address the structure of the major regulatory form $(A_2B_2)_x$, in particular the packing of A and B subunits and the inhibitory role of the latter. The overall study may thus help to unravel several previously unexplained features of this important enzyme.

2. Materials and methods

2.1. Purification and crystallization

Isoforms A_4 and A_2B_2 of GAPDH have been purified to homogeneity from spinach chloroplasts, as described in Scagliarini *et al.* (1999). Purified A_4 and A_2B_2 isoforms, at a protein concentration of 10 mg ml^{-1} in a buffer containing 20 m*M* potassium phosphate pH 7.0 and 1 m*M* $NADP^+$, were immediately used for crystallization experiments.

The A_4 isoform has been crystallized using the hanging-drop vapour-diffusion method at 293 K under the following conditions: the drop (5 µl) is obtained from one volume of protein solution (10 mg ml^{-1} in 20 m*M* potassium phosphate pH 7 and 1 m*M* $NADP^+$) plus one volume of reservoir solution (750 µl) containing 1.2–1.5 *M* $(NH_4)_2SO_4$ and 0.1 *M* Tris–HCl pH 8.5. Crystals suitable for diffraction studies (Fig. 1) appeared after four or five days, with approximate dimensions 0.3 × 0.1 × 0.1 mm.

2.2. Data collection and reduction

Data collection was first attempted using a rotating anode at room temperature, but only a few diffraction intensities could be obtained. Therefore, a synchrotron source was employed – the Elettra X-ray diffraction beamline in Trieste, Italy. At 277 K the GAPDH crystals diffracted to 3 Å, but the intensities decayed very quickly after a few images owing to radiation damage. Therefore, cryocooling conditions (100 K) have been developed. The crystals were soaked for a few minutes in a solution containing 40%(*v*/*v*) glycerol and 1.3 *M* $(NH_4)_2SO_4$ as cryoprotectant, starting with a glycerol concentration of 10% and increasing the concentration in steps to the final concentration, thereby ensuring crystal-lattice integrity. The crystals were then rapidly exposed to a cold nitrogen stream (Oxford Cryosystems Cryostream) on the Elettra X-ray beamline. The quality of the data was greatly improved, but the resolution was the same. A typical X-ray diffraction pattern of GAPDH is shown in Fig. 2. The data with a resolution limit of 3 Å were recorded on an MAR Research 345 image plate using a radiation wavelength of 1 Å, an oscillation angle of 0.8° and a sample-to-detector distance of 350 mm.

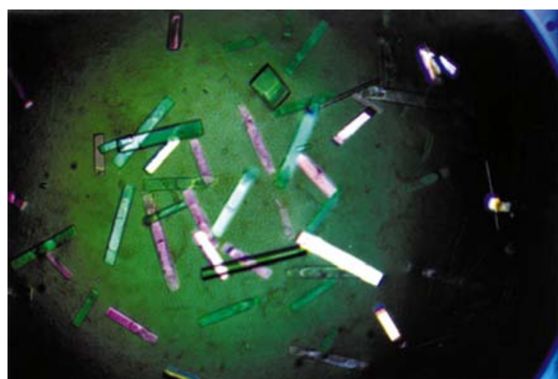

Figure 1
Crystals of isoform A_4 of chloroplast GAPDH grown from ammonium sulfate.

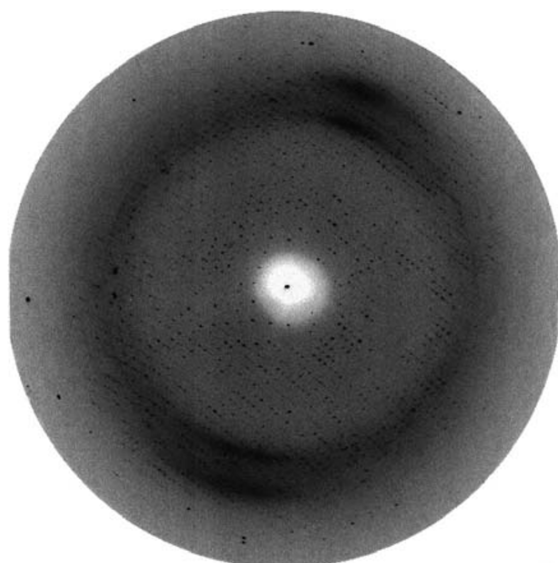

Figure 2
X-ray diffraction pattern of a single crystal of isoform A_4 obtained at the Elettra synchrotron X-ray diffraction beamline in Trieste with a MAR Research 180 image plate, a crystal-to-detector distance of 240 mm, a wavelength of 1 Å, an exposure time of 120 s, an oscillation angle of 1° and 2.8 Å resolution at the edge of the detector.

The data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystals belong to the space group *C*222 with unit-cell dimensions a = 145.9, b = 185.9, c = 106.3 Å. The presence of one tetramer per asymmetric unit could be hypothesized from the value of V_m, which is 2.5 $Å^3\,Da^{-1}$, giving a solvent content of about 51% per molecule calculated by the method of Matthews (1968). The number of observed reflections was 112404, which were merged into 26636 unique reflections with an R_{sym} of 0.074 (R_{sym} in the highest resolution shell of 0.286), a completeness of 91.3% and a multiplicity of around 4.

Structure determination by molecular replacement is now in progress. The structure of cytosolic GAPDH from *Bacillus stearothermophilus* (Skarzynski & Wonacott, 1988; PDB code 2GD1) is being used as a search model for structure solution using molecular replacement, because of the high homology (56%) of its sequence (Biesecker *et al.*, 1977) with GAPDH from chloroplasts (Ferri *et al.*, 1990). Preliminary data from molecular refinement indicates the presence of three monomers per asymmetric unit with a solvent content of about 62%: a monomer in proximity to the origin, which generates a whole tetramer using the 222 point group symmetry, and a dimer which generates a tetramer by means of a crystallographic binary axis.

The 'protomer' A_2B_2 was also crystallized in presence of $NADP^+$; the crystals grew in 1–2 weeks and reached a final size of about 0.2 × 0.1 × 0.1 mm. Studies concerning this latter species are now in progress.

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