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Crystallization and preliminary crystallographic analysis of glyceraldehyde 3-phosphate dehydrogenase from *Sacchromyces cerevisiae* (baker's yeast)

Rotem Gilboa, Alan Joseph Bauer and Gil Shoham* *at Department of Inorganic Chemistry and The Laboratory for Structural Chemistry and Biology, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: gil@vms.huji.ac.il*

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Abstract

Two related and not thoroughly resolved issues in biochemistry concern the role, if any, of enzyme surfaces in routine metabolism and the method by which metabolic intermediates move between enzyme active sites during multi-step degradation or synthesis. An important enzyme for which a detailed three-dimensional structural analysis has been initiated is yeast glyceraldehyde 3-phosphate dehydrogenase (yGAP-DH). This enzyme is active as a tetramer of total molecular weight of 145 kDa and requires nicotinamide adenine dinucleotide (NAD^+) as cofactor. In this report, the crystallization and preliminary crystallographic characterization of several crystal forms of yGAP-DH are described. Of the five distinct crystal forms, the most suitable was found to contain the holo-enzyme, and the crystals were grown by the vapor-diffusion method using polyethylene glycol 6000 as precipitant, sodium acetate as buffer (pH 4.6), and NAD^+ and dithiothreitol as additives. The crystals belong to the orthorhombic space group $P2_12_12$, with cell dimensions of $a = 87.33$, $b = 96.11$ and $c = 115.34$ Å. These crystals are mechanically strong, relatively stable in the X-ray beam and diffract X-rays (from a normal rotating-anode radiation source) to better than 2 Å resolution. A full 2.1 Å resolution diffraction data set (98% completion) has been measured. The three-dimensional structures of related GAP-DH enzymes from several other sources have been determined and reported, and are available for a molecular replacement structure solution.

1. Introduction

D-Glyceraldehyde 3-phosphate dehydrogenase (E.C. 1.2.1.12, GAP-DH) catalyses the oxidative phosphorylation of D-glyceraldehyde 3-phosphate to form 1,3-diphosphoglycerate in the presence of NAD^+ and inorganic phosphate. GAP-DH thus generates two molecules of NADH per molecule of glucose during glycolytic degradation. GAP-DH is one of 16 known enzymes in the ENZYME data bank (Bairoch, 1996) which recognize glyceraldehyde 3-phosphate (GAP) as a substrate. The yeast version of the enzyme (yGAP-DH) has characteristics similar to those reported for GAP-DH from other sources (for a review, see Harris & Waters, 1976). All known NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenases are composed of four identical subunits, have molecular weights of around 145 000 and reversibly bind four moles of the NAD^+ cofactor. This enzyme shows ordered kinetics, as NAD^+ must bind prior to the substrate, GAP. Though the yeast enzyme was originally isolated in a crystalline form (Warburg & Christian, 1939), its structure has not yet been reported. The structures of GAP-DH with and without cofactor from other sources have been determined, reported in the literature (Moras *et al.*, 1975; Mercer *et al.*, 1976; Murthy *et al.*, 1980; Griffith *et al.*, 1983; Skarzynski *et al.*, 1987; Skarzynski & Wonacott, 1988; Vellieux *et al.*, 1993; Korndoerfer *et al.*, 1995; Tanner *et al.*, 1996; Dueé *et al.*, 1996) and deposited in the Brookhaven Protein Data Bank (PDB) (Bernstein *et al.*, 1977).

There are several reasons why we have undertaken to solve the structure of yeast GAP-DH. Firstly, this enzyme is isolated from mesophilic source (T_{opt} of 301 K), and we hope that our studies may complement others in determining the structural basis for thermostability of this enzyme. The resistance of proteins to denaturation in high temperature is of great importance for life in extreme environments and in biotechnology. Strategies to improve the thermostability of a given structure may be explored by comparing homologous proteins from mesophilic and extremophilic sources. In this context GAP-DH has been an extensively studied model system (Branlant & Branlant, 1985; Fabry *et al.*, 1989; Marcinkowska *et al.*, 1989; Menendez-Arias & Argos, 1989; Jaenicke, 1991; Korndoerfer *et al.*, 1995; Tanner *et al.*, 1996.)

Yet, our main interest in yGAP-DH is in determining if the surface of the enzyme plays a role in correct metabolic function. As GAP is the substrate of such a large number of enzymes, we wish to know if the surfaces of GAP-metabolizing enzymes interact in order to aid in the transit of this intermediate; such directed transfer would prevent inefficiencies and losses during multi-step metabolic processing. Transfer of GAP through transient enzyme interactions has been implied for human and rabbit GAP-metabolizing glycolytic enzymes (Bauer *et al.*, manuscript in preparation). Our goal has been to perform all of our enzyme-surface studies (crystallographic, modeling, mutagenic, kinetic) on a single biological source, namely yeast. As the high-resolution structure of yeast triosephosphate isomerase (TIM) has been reported to the literature (Lolis *et al.*, 1990; Lolis & Petsko, 1990), our goal is to use the solved yGAP-DH structure to investigate any potential interactions which may facilitate the transfer of substrate GAP between the active sites of TIM and GAP-DH. The correct delivery of GAP from TIM to GAP-DH is a critical step in cellular energy realization from the degradation of glucose. The loss of the intermediate to other enzymes which recognize GAP as substrate or inhibitor would decrease the efficiency of NADH synthesis, and ultimately energy production. A docking algorithm (Katchalski-Katzir *et al.*, 1992) will be employed in order to determine if the enzymes potentially interact with each other through their surface regions.

In this report, we describe the crystallization and preliminary crystallographic characterization of several crystal forms of yGAP-DH, one of which seems to be of the quality and stability suitable for a detailed high-resolution structural analysis.

2. Experimental

GAP-DH from baker's yeast (*S. cerevisiae*) and all chemical reagents used for the crystallization solutions were purchased from Sigma-Israel. The enzyme as received from Sigma (yGAP-DH without the NAD^+ cofactor, or apo-yGAP-DH) was dialyzed overnight against a large excess of a buffer solution containing 50 m*M* HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)] and 1 m*M* EDTA, and adjusted to pH 7.0 with NaOH or KOH. In most of the crystallization trials, 5%(*v*/*v*) glycerol was added to this dialysis solution to compensate for the lost ammonium sulfate. The concentration of the protein after dialysis was generally 3–4 mg ml^{-1}. In some cases the dialyzed protein was further concentrated to approximately 8 mg ml^{-1} using Centricon centrifugal concentrators (Amicon). All of the crystallization experiments were performed by the hanging-drop vapor-diffusion method, where a wide range of conditions were tested using the factorial screen conditions (Jancarik & Kim, 1991). The protein drops were prepared by mixing the originally dialyzed protein solution with a specific precipitant/salt/buffer solution (see below) to a final volume of 10–20 µl. Each of these drops was then suspended over a 1 ml reservoir solution in 4 × 6 tissue-culture plates (Linbro) for a period of 2–30 d. Our efforts yielded five distinct crystal forms which were found to be suitable for crystallographic analysis. Crystals of the various forms obtained were characterized by both still and oscillation X-ray experiments. The diffraction images were measured on a Rigaku R-AXIS IIC imaging-plate area detector mounted on a Rigaku RU-300 rotating anode [nickel-filtered Cu $K\alpha$ radiation focused by nickel focusing mirrors; Shibata (1990); Sato *et al.* (1992)]. The raw diffraction data were processed with the *DENZO* and *SCALEPACK* software packages (Otwinowski, 1993).

3. Results and discussion

3.1. *Crystallization*

The initial crystallization trials resulted in three promising crystal forms (forms I, II, III) for apo-yGAP-DH (without the NAD^+ cofactor) and two promising forms (forms IV, V) for holo-yGAP-DH (with NAD^+). The specific crystallization conditions are listed briefly in Table 1 and representative crystals of forms I–IV are shown in Fig. 1.

For crystal form IV, the most practical for diffraction studies so far, the specific crystallization procedure is as follows. The dialyzed apo-protein solution (3 mg ml^{-1}, as described above) was further concentrated to approximately 8 mg ml^{-1} with Centricon centrifugal concentrators (Amicon, 5000*g*, 20 min), and 1 m*M* of the NAD^+ cofactor and 1 m*M* dithiothreitol

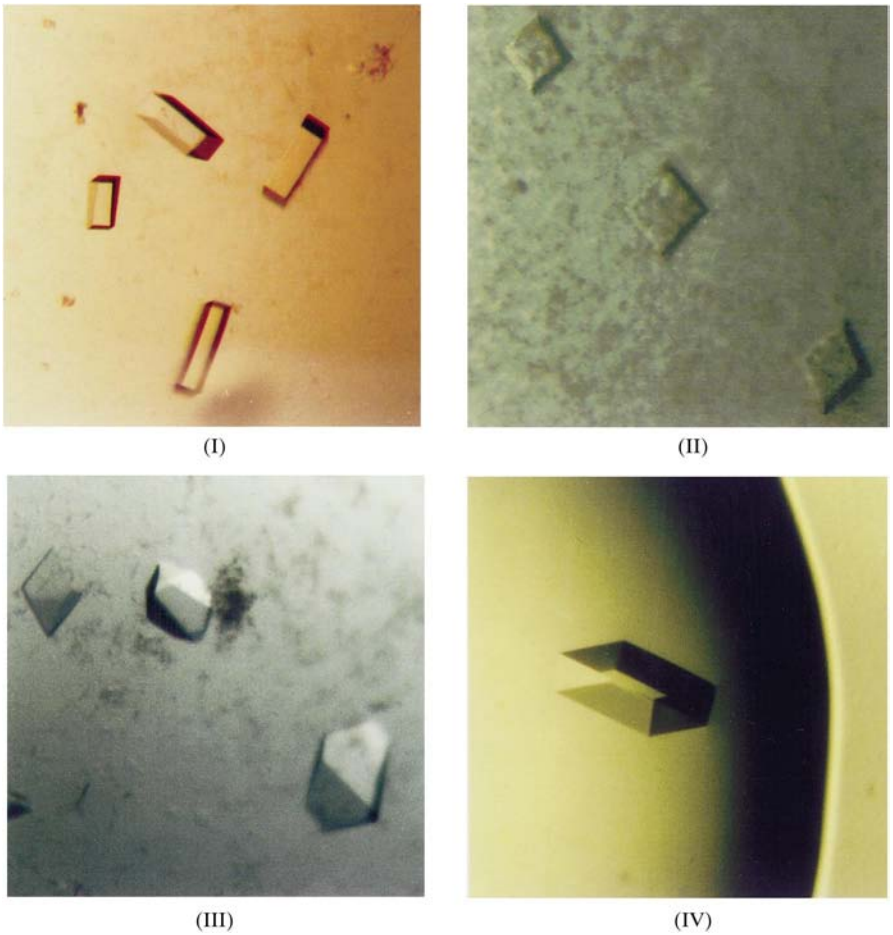

Fig. 1. Typical crystals of four of the five crystal forms obtained for yGAP-DH. (I) apo-yGAP-DH form I; (II) apo-yGAP-DH form II; (III) apo-yGAP-DH form III; (IV) holo-yGAP-DH form IV (the crystals used for full diffraction data measurement).

Table 1. *Crystallization parameters for y-GAP-DH crystal forms I–V*

Crystal form	I	II	III	IV	V
Protein concentration (mg ml^{-1})	3	3	3	8	8
Chemicals added to the crystal growth medium†	PEG 1500 18%(*w*/*v*) Cacodylate buffer 0.1 *M*, MPD 1%(*v*/*v*)	PEG 6000 18%(*w*/*v*) Acetate buffer 0.1 *M*	PEG 6000 25%(*w*/*v*) Citrate buffer 0.1 *M*	PEG 6000 8–12%(*w*/*v*) Acetate buffer 0.1 *M* NAD 1 m*M* DTT 1 m*M*	Same as form IV (crystals appeared together with crystal form IV in the same protein hanging drop)
pH	6.5	4.6	5.2	4.6	4.6
No. of crystals in a drop	2–6	5–10	10–20	2–8	1–2
Growth period (d)	30	3	4	7	7
Crystal dimensions (mm)	0.4 × 0.2 × 0.1 (Fig. 1, I)	0.25 × 0.25 × 0.1 (Fig. 1, II)	0.4 × 0.4 × 0.3 (Fig. 1, III)	0.4 × 0.4 × 0.2 (Fig. 1, IV)	0.2 × 0.2 × 0.2

† PEG, polyethylene-glycol; MPD, 2-methyl-2,4-pentanediol; DTT, dithiothreitol.

(DTT) were added to this solution to form the holo-protein crystallization solution. Protein drops were then prepared by mixing 5 µl of this protein solution with equal volume of a precipitating solution containing 8–12%(*w*/*v*) PEG 6000 and 0.1 *M* sodium acetate/HCl (pH 4.6), and were hung over 1 ml of the same precipitating solution. About two to eight distinct well shaped colorless crystals could usually be observed in the protein drop after about a week. These crystals appeared as flat 'rectangular boxes' with sharp edges and faces, and typical dimensions of about 0.4 × 0.4 × 0.2 mm (Fig. 1, IV).

3.2. *Crystallographic characterization*

Representative single crystals of the five crystal forms were mounted and sealed in glass capillaries, and used for the preliminary evaluation of their X-ray diffraction characteristics. The crystallographic parameters listed in Table 2 represent typical results obtained from these X-ray diffraction experiments (R-AXIS IIC imaging plate; Shibata, 1990; Sato *et al.*, 1992).

For crystal form IV, the specific diffraction data measurement and data-processing parameters are as follows. One crystal was used for a series of 45 min 1.0° oscillation frames at room temperature (293–295 K). The diffraction pattern of this crystal was significantly better than that of all the other crystal forms and could be observed beyond 2.0 Å resolution. Only slight radiation damage could be detected for this crystal during the few days of data collection. A total of 172 575 accepted reflections were measured in the 40.0–2.1 Å resolution range, and resulted in 56 193 independent reflections (98.3% completeness to 2.1 Å resolution, $R_{\rm merge}$ 6.4%). These data indicated a primitive orthorhombic unit cell with dimensions of a = 87.33, b = 96.11, c = 115.34 Å. The systematic extinctions along the diffraction axes suggested the orthorhombic space group $P2_12_12$. Assuming two monomers of holo-yGAP-DH in the asymmetric unit of the cell, a relatively high value of calculated V_m is obtained (3.4 Å^3 Da^{-1}). In contrast, if a full yGAP-DH tetramer per asymmetric unit is assumed a relatively low value of calculated V_m is obtained (1.7 Å^3 Da^{-1}). Unit-cell volume and symmetry considerations preferentially support the packing of two monomers, rather than one full tetramer, in each crystallographic asymmetric unit.

3.3. *Further studies*

Of the five yGAP-DH crystal forms described above, only the crystals of form IV showed the reproducibility, the radiation stability and the X-ray diffraction resolution (2.1 Å) which are most suitable for a complete crystallographic analysis. The other four crystal forms diffracted to only 3.5–3.1 Å resolution, and in addition three of them suffered from either considerable radiation damage (forms I and II) or crystallization difficulties (form III). The holo-yGAP-DH crystals of form IV were, therefore, selected for further crystallographic studies, and it is hoped that these crystals will enable a complete structure determination of this enzyme at high resolution. Efforts to use the full native data set collected from these crystals to solve the structure of holo-yGAP-DH by molecular-replacement techniques are now in progress.

Among the published three-dimensional structures of GAP-DH from various sources, there is only one high-resolution model. This model is of GAP-DH from the moderate thermophile ($T_{\rm opt}$ = 325.5 K) *Bacillus stearothermophilus* which is determined to 1.8 Å resolution (Skarzynski *et al.*, 1987). This structure would be preferentially used as the reference model for the initial molecular-replacement studies. Another high-resolution three-dimensional structure which could be used for molecular-replacement trials is that of GAP-DH from the hyper thermophile ($T_{\rm opt}$ = 358 K) *Thermotoga maritima* which is determined to 2.5 Å resolution (Korndoerfer *et al.*, 1995). It is expected that once the structure of yGAP-DH is determined these two available structures could also be used as a reference for comparison of the various thermostable GAP-DH enzymes with respect to the degree of thermostability, the sequence homology and site-directed mutagenesis results (Biro *et al.*, 1990; Ganter & Pluckthum, 1990; Marbet *et al.*, 1992). Such potential comparison in the future is expected to give us additional clues for understanding the structural factors stabilizing proteins against heat denaturation.

The high-resolution structure of yeast GAP-DH, when determined, will also give us a great opportunity to investigate the potential role of surface residues in mediating intermediate transfer during multi-step metabolic conversions. This structure is required in order to complete the structural information of the yeast glycolytic pathway enzymes involving the triose-

Table 2. *Selected crystallographic parameters for the y-GAP-DH crystal forms*

Crystal form		I	II	III	IV	V
Crystal system		Primitive orthorhombic	*C*-centered orthorhombic	*I*-centered tetragonal	Primitive orthorhombic	Primitive orthorhombic
Unit-cell dimensions (Å)	*a*	80.5	98.7	117.8	87.33	96.1
	b	126.7	129.9	117.8	96.11	115.7
	c	278.5	124.6	120.9	115.34	162.2
Space group		ND†	ND	ND	$P2_12_12$	$P2_12_12_1$
No. of monomers in asymmetric unit		8	4	4	2	4
V_m (Å^3 Da^{-1})‡		2.43	2.72	2.82	3.4	3.1
Oscillation angle per frame (°)		0.6	0.5	1.0	1.0	0.5
X-ray time exposure per frame (min)		60	50	50	45	45
Number of frames		36	34	29	79	80
Data collection temperature (K)		271	293–295	293–295	293–295	293–295
Resolution (Å)		3.5	3.5	3.4	2.1	3.1
No. of accepted reflections		24398	6491	6679	172575	45205
No. of unique reflections used		16904	4235	5325	56193	25819
Completeness (%)		46.3	40.7	47.0	98.3§	77.1
$R_{\rm merge}$ (%)¶		9.3	12.6	7.2	6.4	7.8

† ND, not determined uniquely. ‡ V_m, specific protein molecular volume in the crystal (Matthews, 1968). § For the last resolution shell (2.1–2.2 Å): completeness = 90.6%, $R_{\rm merge}$ = 22.6%. ¶ $R_{\rm merge} = \sum(|I - \langle I\rangle|)/\sum I$.

phosphate isomerase (TIM), glyceraldehyde 3-phosphate dehydrogenase (GAP-DH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM) and enolase; all such structures other than that of GAP-DH are available for the baker's yeast enzymes. The expected yGAP-DH structure will be combined, for example, with the reported structure of yeast TIM (Lolis *et al.*, 1990) in order to determine specific surface interactions between these metabolically adjacent enzymes. Such novel interactions will be examined by docking calculations (Katchalski-Katzir *et al.*, 1992) and site-specific mutagenesis experiments involving the two enzymes.

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