GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS*

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1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase has been isolated in pure crystalline form from a number of different sources including muscle, yeast and bacteria (for references see [1]). Unique among NAD-linked enzymes, crystalline muscle glyceraldehyde-3-phosphate dehydrogenases contain up to four equivalents of firmly bound NAD and active enzyme-NAD complex is thus directly amenable to study by X-ray crystallographic methods. The apoenzyme, which is obtained by treating the holoenzyme with charcoal, is less stable and does not readily crystallise in the absence of added NAD [2].

The enzyme from a given species is composed of four polypeptide chains of essentially identical primary structure [1] and X-ray crystallographic studies with holoenzyme from lobster muscle [3,4] have shown that the individual polypeptide chains could be related in pairs within the tetramer. The holoenzyme crystals are orthorhombic (space group $P2_12_12_1$) and the unit cell was found to consist of four tetrameric molecules. Subsequently, apolactic dehydrogenase was shown to possess a unit cell consisting of only one tetrameric molecule (with the monomer forming the crystallographic asymmetric unit) [5], and it therefore seemed desirable to attempt to prepare crystals of NAD-free glyceraldehyde-3-phosphate dehydrogenase so that the crystallographic properties of apoenzyme crystals could also be investigated. Numerous attempts to crystallise apoenzyme from pig and lobster muscle have, however, been unsuccessful due, at least in part, to the instability of the apoenzyme in solution even at 4° (cf. [6]). We have therefore sought to prepare suitably stable crystals of apoenzyme from a thermophilic source. The crystalline holoenzyme from *Bacillus stearothermophilus* was first prepared and studied by R.E.Amelunxen [7–9] and we now report an improved procedure for preparing crystals of the thermophilic enzyme with and without NAD. Some of the properties of the holo and apoenzymes are also described.

2. Methods and results

2.1. *Isolation and crystallisation of holoenzyme*

Bacillus stearothermophilus (strain NCA 1503) was grown at the Microbiological Research Establishment, Porton, by an improved procedure (K.Sargeant, D.N.East, A.R.Whitaker and R.Elsworth, to be published) and stored at −20°. Frozen cells (550 g) were resuspended in 550 ml buffer A (10 mM tris-HCl/5 mM EDTA/5 mM mercaptoethanol, pH 7), allowed to thaw at 4° and disrupted in a French Pressure Cell. The homogenate was centrifuged and to the supernatant (735 ml), a 10% solution of streptomycin sulphate (230 ml) was added slowly and with stirring. The resulting precipitate was removed by centrifugation and to the supernatant solid ammonium sulphate (43.6 g per 100 ml) to 70% saturation was added. After 12 hr at 4° the precipitate was recovered by centrifugation (and subsequently utilised for the isolation of triose-phosphate isomerase, lactic dehydrogenase and aldolase (Class II), unpublished results of E.Kolb, R.Jack and J.I.Harris); the supernatant fraction (860 ml), that contained 80–90% of the glyceraldehyde-3-phosphate dehydrogenase activity, was brought to 100% saturation by addition of a further quantity (20.9 g per 100 ml)

of solid ammonium sulphate. After 4–5 hr the enzyme precipitate was centrifuged, dissolved in 50 ml buffer B (5 mM tris-HCl/mM EDTA/mM mercaptoethanol, pH 7.5), dialysed, and applied to a column of DEAE-cellulose (2.5 × 30 cm) equilibrated with the same buffer. The enzyme was eluted with a gradient of sodium chloride (1,800 ml 0.5 NaCl in buffer B + 1800 ml buffer B); effluent fractions were analysed for absorbance at 280 nm and enzyme activity. Fractions that possessed a specific activity greater than 60 units/mg were pooled (total volume 250 ml) and brought to about 95% saturation with solid ammonium sulphate (63.0 g per 100 ml of enzyme solution). The enzyme precipitate was collected by centrifugation, redissolved in 10 mM tris/5 mM EDTA/mM DTT, pH 7.0, and allowed to crystallise at room temperature from a solution containing 70–72% ammonium sulphate. The crystalline enzyme possessed a specific activity of 100 units/mg when assayed at 22° [cf. 7] and was pure as judged by electrophoresis, on cellulose acetate at several different pH values, as well as on polyacrylamide gels with and without 0.1% SDS [10, 11]. In the presence of SDS the molecular weight of the subunit was estimated to be 36,000, a value that is in excellent agreement with that found previously [1] for the muscle and yeast enzymes. The ratio of $A_{280}:A_{260}$ was 1.05 indicating that the crystals contained their full complement (i.e. 4 equivalents/mole) of NAD [cf. 12]. The yield of pure enzyme was 400 mg. The holoenzyme crystals are orthorhombic with space group $P2_12_12$ and the unit cell (a = 133 Å, b = 126 Å, c = 98 Å), like that of the lobster muscle enzyme, contains four tetrameric molecules (unpublished results of A.J.Wonacott and R.M.Sweet).

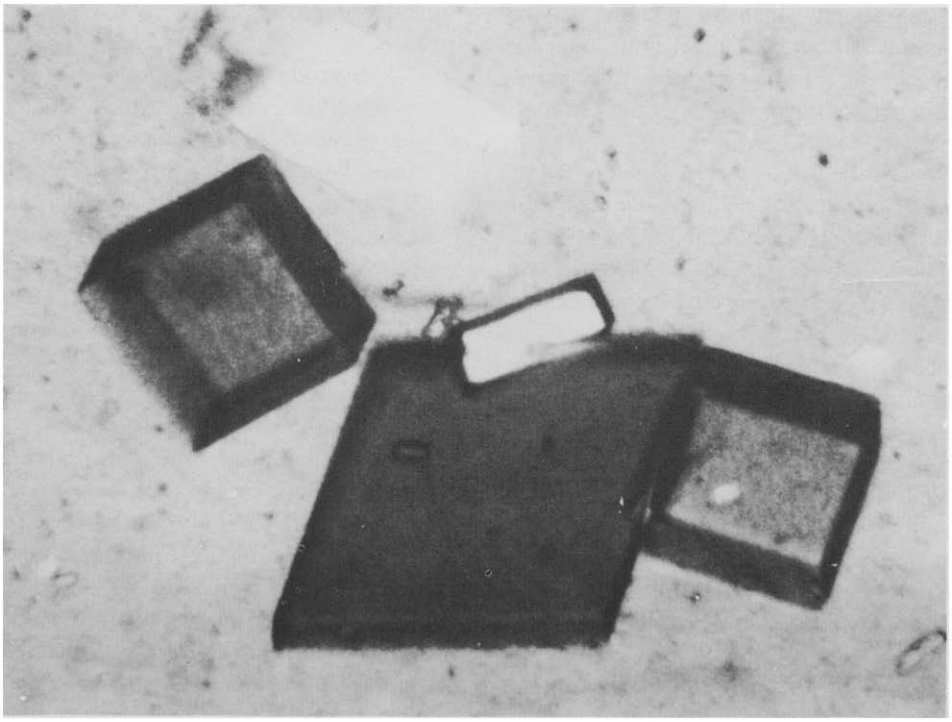

Fig. 1. Photomicrograph of crystals of NAD-free glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* (Scale 5 cm = 1 mm).

2.2. *Crystallisation of NAD-free enzyme*

The holoenzyme (14 mg) was dissolved in 10 mM sodium pyrophosphate/5 mM DTT/5 mM EDTA, pH 7.0, and stirred with acid washed charcoal (200 mg) at 0° for 60 min. The suspension was filtered and subsequently centrifuged at 18,000 rpm for 20 min. The enzyme solution had an A_{280}:A_{260} ratio of 2.05 showing that it was essentially free of NAD. The specific activity (100 units/mg in the presence of added NAD) was fully maintained and the recovery of apoenzyme was approximately 70%. The solution was dialysed against 2.33 M ammonium sulphate in 10 mM tris-HCl/2 mM EDTA/mM DTT (the 'pH' of the undiluted solution was 6.6); the protein concentration was adjusted to 5 mg/ml and crystals were obtained from approximately 2.5 M ammonium sulphate within three to four days at room temperature. These crystals (measuring up to 1 mm in each dimension and shown at a magnification of X60 in fig. 1) were inactive in the absence of added NAD. They are monoclinic with space group $P2_1$ and the unit cell (a = 83 Å, b = 130 Å, c = 84 Å) is composed of two tetrameric molecules (unpublished results of A.J.Wonacott and R.M.Sweet).

3. Discussion

Enzyme prepared as described above has an A_{280}:A_{260} ratio of 1.05 for the holoenzyme and 2.05 for the apoenzyme, values that are in excellent agreement with those reported previously [12] for glyceraldehyde-3-phosphate dehydrogenases from other sources. Amelunxen ([9]; R.E.Amelunxen and J.Clark, personal communication) report values of 0.75 and 1.07 respectively for their crystalline preparations of holo and apoenzymes, indicating that these preparations may have contained material absorbing at 260 nm that was not removed when the holoenzyme was treated with charcoal. Nevertheless enzyme prepared by our procedure has been found to possess the same remarkable stability towards heat and urea. Thus redissolved crystals of the holo and apoenzymes retained almost complete activity after heating at 80° for 5 min whereas the pig enzyme is inactivated at this temperature (fig. 2). Moreover, the *B. stearothermophilus* enzyme regains its activity fully following exposure to 8 M urea for up to 1 hr at 20° whereas the pig and

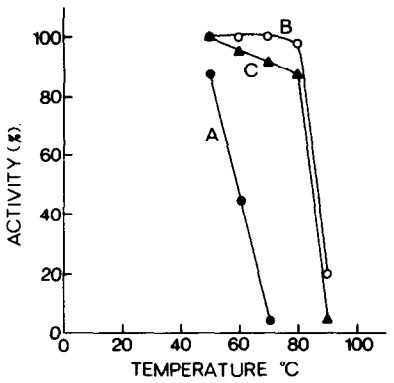

Fig. 2. Effect of temperature on the stability of glyceraldehyde-3-phosphate dehydrogenases. Enzyme solutions (3 mg/ml in 10 mM tris-HCl/5 mM EDTA at pH 7.0) were maintained at the indicated temperatures for 5 min: Pig muscle holoenzyme (A), *B. stearothermophilus* holoenzyme (B), apoenzyme (C).

lobster muscle enzyme are totally inactivated under the same conditions (unpublished results). It should also be noted that by contrast with the muscle enzymes the stability (and crystallisability) of the thermophilic enzyme is not significantly affected by removal of the NAD.

In many of its other properties (for example, molecular weight, and amino acid composition) thermophilic glyceraldehyde-3-phosphate dehydrogenase closely resembles its counterparts from mesophilic sources [cf. 9] and it is to be hoped that the detailed comparison of the primary and tertiary structures of the muscle and *B. stearothermophilus* enzymes will provide a structural basis for the mechanism of thermal stability in polymeric enzymes of this type.

The unusual thermal stability of enzymes from thermophilic sources aids in their purification and further study by chemical and physicochemical methods, and this greatly enhanced stability could be of particular advantage in the study of enzymes that are unstable and consequently difficult to purify and to crystallise from conventional mesophilic sources.

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