PURIFICATION BY AFFINITY CHROMATOGRAPHY OF THERMOSTABLE GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE FROM *THERMUS AQUATICUS*

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1. Introduction

Affinity chromatography involving the use of immobilized NAD has been explored as a means of purifying enzymes that utilise NAD(H) as coenzyme. Methods have been developed [1—3] to attach NAD covalently to suitable solid supports and a study of the properties of these materials has demonstrated their potential usefulness for separating mixtures of NAD(H)-linked dehydrogenases and for the partial purification of individual enzymes from crude extracts [1, 2, 4]. We now report the successful application of immobilised NAD linked to a benzamidohexyl derivative of Sepharose (NAD-Sepharose) to obtain pure glyceraldehyde 3-phosphate dehydrogenase from *Thermus aquaticus*, an obligate thermophile that grows optimally at 70°—75°C [5]. The same procedure has also been used to purify GPDH from *B. stearothermophilus* and, as far as we are aware, these are the first examples of the use of immobilised NAD as a routine step in a large-scale purification of a NAD-linked enzyme. A partial purification of phosphofructokinase was also achieved.

A preliminary account of this work has been given [6].

Abbreviations: GPDH = glyceraldehyde 3-phosphate dehydrogenase; PFK = phosphofructokinase; β-ME = β-mercaptoethanol; SDS = sodium dodecyl sulphate.

2. Methods and results

2.1. Preparation of NAD-Sepharose

NAD was attached to the Sepharose matrix by a modified procedure based on methods previously described by Porath et al. [7], Cuatrecasas [8], and Weibel et al. [3].

Finely ground cyanogen bromide (1 g/5 ml settled gel volume) was added to a vigorously stirred suspension of washed Sepharose 4B (Pharmacia) in an equal volume of deionised water. Crushed ice was added to keep the temperature at 20°—25°C and a pH of 11.0 was maintained by addition of 5 N NaOH with a Radiometer autotitrator. When the reaction was complete (10—15 min) the activated Sepharose was quickly cooled to 0°C with ice, washed with 25 vol of precooled 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.0), and collected by filtration on a sintered glass funnel. It was then stirred overnight at 4°C in 2 vol 1 M hexane-1,6 diamine previously adjusted to pH 10.0 with HCl. The aminohexyl derivative was thoroughly washed with deionised water, resuspended in 50% redistilled dimethylformamide (DMF) and stirred for 3—4 hr at room temp with a solution (2.5 vol) of 0.07 M *p*-nitrobenzyl azide in 40% DMF/60% 0.2 M borate, pH 9.3. The *p*-nitrobenzamidohexyl derivative was washed with 50% DMF (20 vol) and deionised water, and stirred at 37°C for 1 hr with 2—3 vol of 0.5 M $NaHCO_3$/0.1 M dithionite, pH 8.5. The resulting *p*-aminobenzamidohexyl derivative was washed with deionised water (30 vol) and diazotised by stirring for 10 min with 2.5 vol chilled 0.1 N $NaNO_2$ in 0.5 N HCl. The *p*-diazo derivative was washed successively with chilled de-

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ionised water (5 vol), 1% sulphamic acid (1 vol) and deionised water (5 vol), and was then allowed to react overnight at 4°C with 0.1 M NAD in 0.2 M borate, pH 8.3. The immobilised NAD derivative of benzamidohexyl Sepharose (NAD-Sepharose) was then collected by filtration and thoroughly washed.

2.2. *Purification of glyceraldehyde 3-phosphate dehydrogenase*

The initial steps were based on the methods used for the purification on a large-scale of enzymes from *B. stearothermophilus* [9]. GPDH was assayed at 25°C in 1 ml 40 mM sodium pyrophosphate, 4.5 mM sodium arsenate, 3 mM glyceraldehyde 3-phosphate, 1.5 mM NAD, pH 8.5, using a thermostated Guilford 222A spectrophotometer. PFK was assayed at 25°C by the method of Passoneau and Lowry [10].

T. aquaticus (strain ATCC 25104) was grown at 75°C in a 400 ℓ fermentor at M.R.E. Porton. Frozen cells (500 g) were thawed overnight at 4°C in 500 ml buffer A (10 mM Tris-HCl/10 mM β-ME/1 mM EDTA, pH 7.5) and disrupted in a French Pressure cell (16,000–20,000 p.s.i.). The homogenate was stirred for 30 min at room temp. in the presence of $MgCl_2$ (10 mM) and DNAase (3 mg), and then for 30 min with DEAE-cellulose (2 ℓ settled volume of DE 23, Whatman Biochemicals) equilibrated with buffer A. The suspension was filtered through a sintered glass funnel and the DEAE-cellulose was extracted with 1 ℓ washes of buffer A containing, successively, 0 (4 washes), 100 mM (2 washes), 400 mM (3 washes) and 1 M (2 washes) NaCl. The fraction eluted with 400 mM NaCl was adjusted to pH 6.8 with NaH_2PO_4 and absorbed batchwise with stirring onto hydroxyapatite (600 ml settled volume equilibrated with 10 mM sodium phosphate, pH 6.8). The slurry was poured onto 200 ml of fresh hydroxyapatite in a column (8 cm diameter) and was eluted successively with 1500 ml 60 mM, and 200 mM sodium phosphate/5 mM β-ME, pH 6.8. The first extract contained phosphoglycerate kinase, triosephosphate isomerase and malate dehydrogenase (details to be published) and the second extract contained GPDH and PFK. The latter fraction was brought to 1.2 M with solid ammonium sulphate and after centrifugation the supernatant fraction was run onto a column of NAD-Sepharose (200 ml bed volume). The column was eluted with 200 ml buffer B (100 mM sodium phosphate/1 mM EDTA/3 mM β-ME, pH 6.8) containing 700 ml NaCl and 1 mM ATP; with 3 to 5 ℓ of the same solution but without ATP, 500 ml buffer B containing 500 mM NaCl, and finally with the same

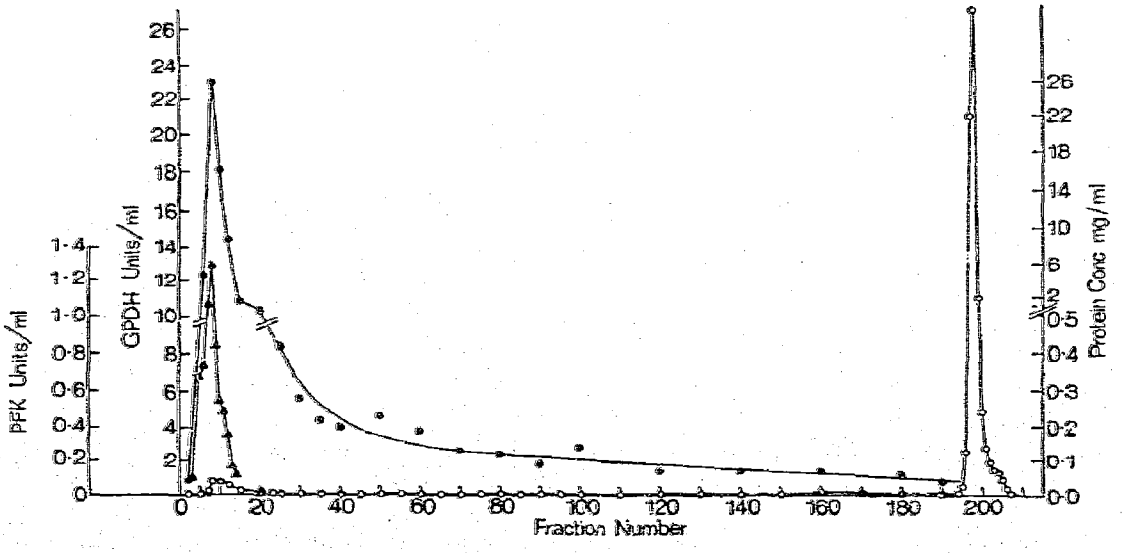

Fig. 1. Elution of phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase from NAD-Sepharose. The column was washed with 200 ml of 100 mM sodium phosphate, 3 mM β-ME/1mM EDTA/700 mM NaCl/1 mM ATP, pH 6.8; 5 ℓ of the above buffer without ATP; 200 ml of 100 mM sodium phosphate/3 mM β-ME/1 mM EDTA/500 mM NaCl, pH 6.8; and finally 200 ml of the above buffer plus 10 mM NAD. Fractions of 27–28 ml were collected. (o—o—o) GPDH activity (I.U./ml); (▲—▲—▲) PFK activity (I.U./ml); (●—●—●) protein concentration (mg/ml by Lowry test [11]).

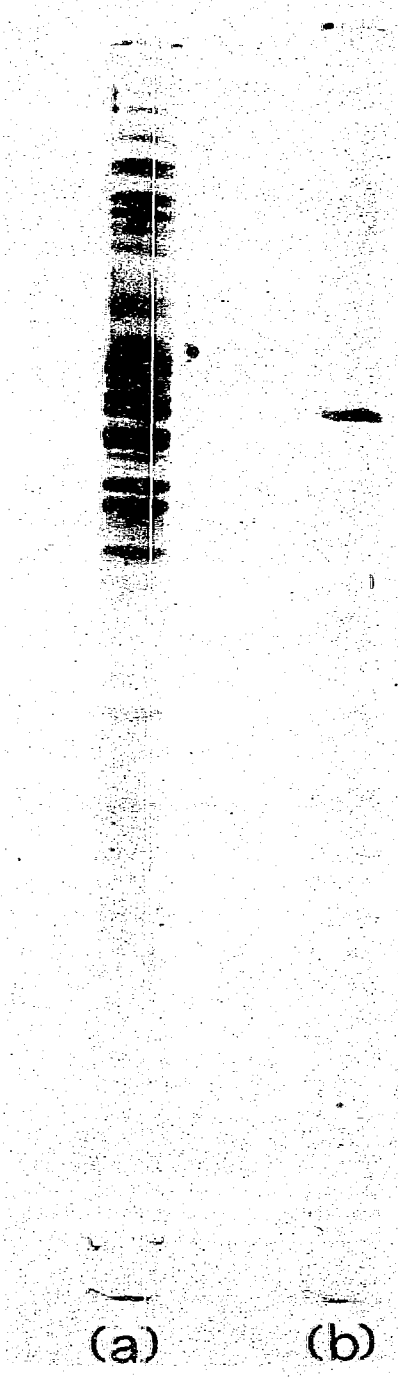

Fig. 2. SDS-gel electrophoresis [12] of fractions containing *T. aquaticus* glyceraldehyde 3-phosphate dehydrogenase (a) before and (b) after affinity chromatography on NAD-Sepharose.

solution (500 ml) containing 10 mM NAD (Sigma AA grade). As shown in fig. 1 PFK was eluted as a sharp peak, albeit in the presence of other proteins, with 1 mM ATP. The bulk of the proteins was eluted with the high salt wash but GPDH was not eluted in significant amount until NAD was included in the eluting buffer. Fractions containing GPDH were pooled (approx. 150 ml) dialysed against buffer A (3 changes each of 5 ℓ), and concentrated to about 20 ml by pressure dialysis. The enzyme solution was then dialysed against 20 mM Tris-HCl, 1 mM EDTA, 2 mM dithiothreitol, pH 7.5 and the enzyme was crystallised at room temp. from a 4 to 5 mg/ml solution by adding solid ammonium sulphate to 2.4 M. An overall yield of 55–60% has been obtained consistently from several preparations starting with 500 g frozen cells. The actual content of GPDH varies with different batches of cells but is usually between 100–200 mg per 500 g cells.

The material eluted from the NAD-Sepharose column gave a single band when examined by gel-electrophoresis with and without 0.1% SDS, and the pattern of protein bands present in an SDS-gel [12] before and after the NAD-Sepharose is shown in fig. 2. The pure enzyme contains four moles of firmly bound NAD (cf. [13]) and it gave a single N-terminal residue, methionine, when examined by the dansyl procedure [14].

2.3. Properties of GPDH

T. aquaticus GPDH is less active at 25°C than its counterpart from mammalian muscle (15 units/mg as compared with 40 units/mg for enzyme from pig muscle), but the specific activity increases with temperature and an Arrhenius plot is approximately linear to at least 60°C. The enzyme is unusually stable to heat and to denaturing solvents such as urea and SDS. The percentage activity remaining after heating for 20 min at various temperatures (1 mg/ml enzyme in 20 mM sodium phosphate/5 mM β-ME/mM EDTA, pH 7.0) is shown in fig. 3. The enzyme is table at 90°C (by contrast with the *B. stearothermophilus* enzyme which is totally inactivated under the same conditions [13]) and the half-life at 98°C is greater than 30 min. It is inactivated by iodoacetate, and in 1% SDS at 100°C the native enzyme (M.W. ≃ 150,000) dissociates to an inactive subunit with a molecular weight of 36,000. Details of the chemical and enzymatic

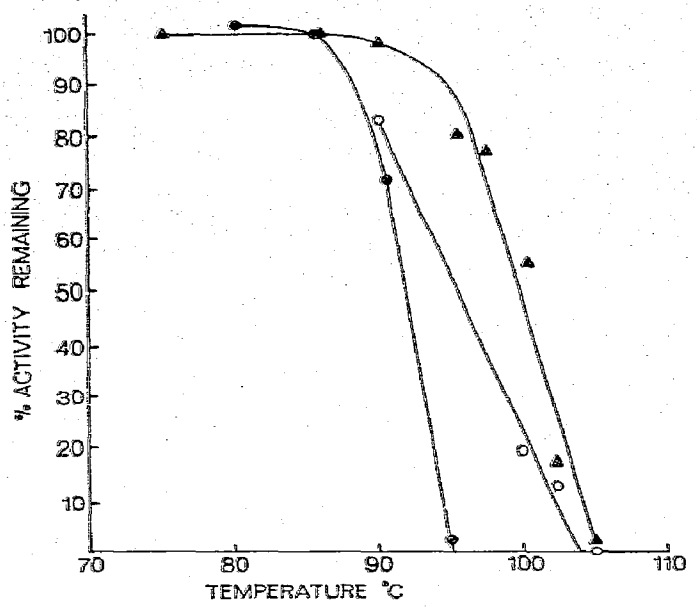

Fig. 3. Effect of temperature on the activity of *T. aquaticus* glyceraldehyde 3-phosphate dehydrogenase. Solutions of the enzyme 1 mg/ml in 20 mM phosphate/5 mM β-ME/mM EDTA, pH 7.0 were incubated for 20 min at the indicated temperatures and rapidly cooled in an ice bath before assay. (▲—▲—▲) Holo GPDH; (o—o—o) Apo GPDH; (●—●—●) PFK.

properties of *T. aquaticus* GPDH will be given elsewhere (unpublished results of J.D. Hocking and J. Ieuan Harris).

3. Discussion

Lowe and Dean [1] coupled NAD directly to CNBr activated cellulose and, by means of a water soluble carbodiimide to "spacer" molecules such as 6-amino-hexanoic acid attached to the cellulose matrix. Mossbach and his colleagues [2] have also used the carbodiimide method to couple NAD to 6-amino-hexanoyl-Sepharose. This mode of coupling is believed to link NAD to the terminal carboxyl of the "spacer" molecule through either the 6-amino group of the purine ring [1] or by esterification of the secondary hydroxyl groups of ribose [2]. We preferred to use the method of diazonium coupling [3] to the spacer molecule. This is thought to involve substitution at position 8 in the adenine nucleus of NAD and we considered that this linkage might be more resistant to cleavage by degradative enzymes present in bacterial extracts. NAD-Sepharose prepared in this way has proved to be adequately stable (if kept at −20°C in 50% DMF) over a period of several months. It has an acceptable capacity for GPDH (1.5–2.0 ml binds about 1 mg of enzyme) and the same batch has been re-used several times, albeit with progressively decreasing efficiency. The initial absorption of the enzyme is improved by the presence of ammonium sulphate (cf. [15]) and very little enzymatic activity was eluted until NAD was added to the eluting buffer.

B. stearothermophilus (strain 1503) and *T. aquaticus* grow optimally at 60–65°C and 70–75°C, respectively, and it is therefore of interest that *T. aquaticus* GPDH is even more stable to heat and to denaturing solvents such as SDS than its counterpart from *B. stearothermophilus* [13]. In many other respects the chemical and enzymatic properties of the two thermophilic enzymes are very similar to those of mesophilic GPDH's. Studies of the amino acid sequence and physicochemical properties of the two thermophilic GPDH's are in progress and it is hoped that the structural changes that confer stability in a thermophilic environment will be identified from a comparison of the three dimensional structures of the muscle [16] and *B. stearothermophilus* (Butler, Thierry and Wonacott, work in progress) enzymes.

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