AFFINITY CHROMATOGRAPHY OF CYTOSOLIC NAD-LINKED GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM NORMAL AND NEOPLASTIC MAMMALIAN TISSUES *

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1. Introduction

Affinity matrices employing NAD^+ or AMP as ligands have been designed for the purification of NAD-linked dehydrogenases [1–3]. We have designed an affinity matrix of 2, 4, 6-trinitrobenzene covalently bound to Sepharose through a hexamethylenediamine spacer, for the isolation of cytosolic NAD-linked glycerol-3-phosphate dehydrogenase (G3PDH) (EC 1.1.1.8) from homogenates of mammalian tissues with purifications ranging from 30 to 1500-fold. Recoveries were from 54 to 130% even when initial specific activities were as low as 0.015 (approximately 0.06 μg enzyme/mg total protein). This affinity matrix was effective in isolating G3PDH from rabbit muscle, brain, and mammary gland; from human kidney, muscle, and white blood cells; and from mouse liver and L1210 leukemia cells. The enzyme remained bound in the presence of 0.5 M KCl. Elution was effected by NADH at concentrations as low as 5 μM. The affinity matrix was successfully employed in either column or batch procedures.

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2. Methods

2.1. *Preparation of TNB-HMB-Sepharose*

Sepharose 4B was activated by CNBr (100 mg/ml settled gel volume) according to the method of Cuatrecasas [4]. Hexamethylenediamine (HMD) was coupled to the activated Sepharose by the method of Cuastrecasas [4] and the number of bound HMD residues was determined by titrating the gel to a thymolpthalein end-point with 0.1 N NaOH. We found 15–20 μmole of free amino groups per ml of settled gel.

Trinitrobenzenesulphonic acid (TNBS), 30 mg/ml settled gel volume, was dissolved in a volume of saturated sodium tetraborate ($Na_4B_2O_7$) equal to the settled gel volume of the HMD-Sepharose. The washed gel was suspended in the TNBS–$Na_4B_2O_7$ solution and the reaction mixture was stirred for 2 hr at room temperature. The reaction beaker was suspended above the stirring motor to prevent heating. When the reaction was complete, the TNB-HMD-Sepharose was washed with distilled water until no more color appeared in the wash water. The color of the resin was bright orange. Titration of TNB-HMD-Sepharose showed no free amino groups remaining.

2.2. *Preparation of homogenates*

Except where noted, all buffers contained 50 mM triethanolamine acetate, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol (TEA buffer). All operations were performed at 0–4°C.

Rabbit muscle, brains, and mammary glands were

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obtained frozen from Pel-Freez Biologicals (Rogers, Arkansas). Human muscle and kidney were obtained from autopsy specimens and immediately frozen. Human white blood cells were harvested from freshly drawn blood by the method of Skoog and Beck [5]. Mouse liver was obtained from BDF_1 mice and homogenized immediately. DBA-mouse-derived L1210 leukemia cells were harvested from the peritoneal cavities of BDF_1 mice six days after passage of 1×10^6 cells. Residual red blood cells in the human white blood cells and in BDF_1 mouse ascitic fluid were osmotically lysed and the remaining cells were washed twice with 0.85% NaCl prior to homogenization.

Finely-minced rabbit muscle and mammary glands, human liver and muscle were homogenized in 2–4 vol of 0.1 M phosphate, pH 7.5, 1 mM EDTA, 5 mM 2-merceptoethanol per g wet-weight tissue in three 30-sec bursts at high speed in a Waring blender. Rabbit brain was homogenized by stirring the frozen brains in 3 vol of TEA buffer per g wet-weight of tissue for 1.5 hr followed by homogenization using a Brinkman P10 Polytron homogenizer at low speed for 3 min. Human with blood cells or mouse L1210 leukemia cells (1 to 6×10^9 cells) were homogenized in 20 ml of TEA buffer by nitrogen cavitation [6] in a Parr nitrogen bomb at 900 and 1300 psi respectively. Finely minced mouse liver was suspended in 3 vol of TEA buffer per g wet-weight tissue, passed through a stainless steel wire mesh screen, and homogenized by nitrogen cavitation at 1200 psi. All tissue suspensions were centrifuged for 1 hr at 30 000 *g* and the supernatants poured through several layers of cheesecloth.

Homogenates were applied directly to the affinity column except for the human white blood cells and L1210 leukemia cell preparations which were concentrated to a maximum volume of 10 ml in an Amicon MMC ultrafiltration apparatus prior to application.

G3PDH activity was measured by the assay procedure of Fondy et al. [7]. Protein was determined by the method of Warburg and Christian [8].

2.3. *Affinity chromatography procedure*

Columns of 1.3 cm diameter containing 5 ml of TNB-HMD-Sepharose were equilibrated with TEA buffer prior to use. The homogenates were applied at a flow rate of 1 ml/min. After the sample addition, the columns were washed with TEA buffer until the OD_{280} of the eluent was less than 0.05. The enzyme was eluted from the column with 0.1 mM NADH in TEA buffer at a flow rate of 1 ml/min except for the mouse liver, human white blood cell and L1210 leukemia cell enzymes which were eluted with 0.3 mM NADH in order to minimize dilution of the small number of units. NADH was removed from the column eluents prior to protein determination either by gel-filtration on a 2.5 X 30 cm Sephadex G-25 column or by dialysis in an Amicon MMC ultrafiltration apparatus using a Diaflo PM10 membrane at 40 psi.

In separate experiments the enzyme was isolated from rabbit brain homogenates on 2.5 cm diameter columns with 50 ml bed volume of affinity matrix by elution either with a gradient of 0–1 M KCl or with an NADH gradient of 0–0.2 mM. Brain homogenate containing 328 units in 30 ml was applied to the columns, and, after buffer wash, the enzyme was eluted with a 400 ml gradient of NADH. Fractions of 20 ml were collected at a flow rate of 1 ml/min.

A batch procedure was also used with rabbit brain homogenates. In this procedure, 20 ml of gel suspended in 20 ml of TEA buffer were slowly added to 275 ml of brain homogenate containing 110 units. The mixture was stirred for 30 min, then poured into a 600 ml sintered glass funnel. The filtrate was collected by gravity and the gel was washed with five successive 100 ml buffer aliquots. The gel was then resuspended in 225 ml of TEA buffer and transferred to a 400 ml beaker. NADH was added to a final concentration of 0.1 mM and the reaction mixture was stirred for 30 min. The mixture was poured back into the sintered glass funnel, the eluted enzyme collected by gravity filtration, the matrix washed with 25 ml of buffer, and the washings added to the recovered enzyme.

3. Results

3.1. *Column procedure*

The results of the column procedure are summarized in table 1. Except for the human cell and the L1210 leukemia cell homogenates where very few enzyme units were present, all homogenates were applied to the columns until the enzyme activity in the eluents equaled the enzyme activity in the homogenates, thus indicating saturation of the matrix.

Table 1
Affinity chromatography of NAD-linked glycerol-3-P dehydrogenases on trinitrophenyl-hexamethylenediamine-Sepharose.

Tissue	Species	Bed volume (ml)	Units bound	Recovery of bound units (%)	Total recovery * of units (%)	Peak specific activity	Fold purification
Brain	Rabbit	5	37	97	98	20	200
Skeletal muscle	Rabbit	75	9750	54	55	26	30
Mammary (non-pregnant)	Rabbit	4	110	98	98	13	144
Skeletal muscle	Human	5	252	72	80	357	476
Kidney	Human	5	141	100	100	293	1270
Leucocytes	Human	1	1.0	58	57	2.7	135
Liver	Mouse	2	63	130	130	37	132
L1210 leukemia	Mouse	1	5.6	75	75	22	1470

* Including units not bound, units recovered in buffer wash, and units eluted with NADH.

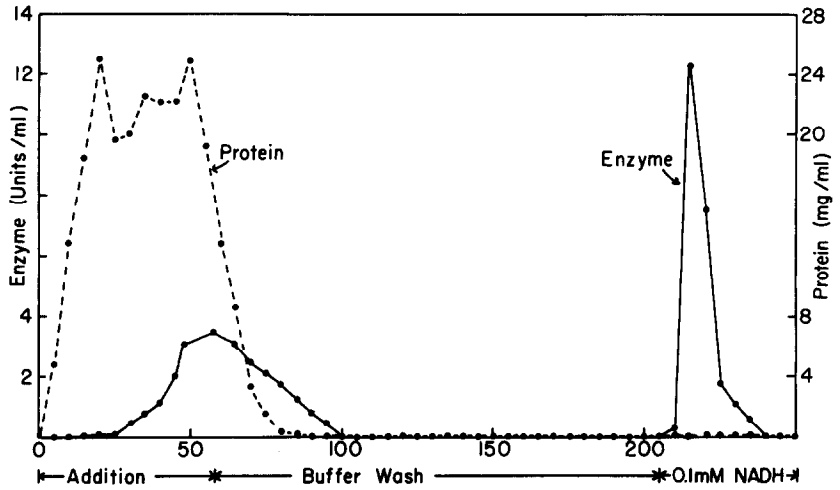

Fig. 1. Elution of glycerol-3-phosphate dehydrogenase from trinitrophenyl-hexamethylenediamine-Sepharose 4B. Homogenate of human kidney was added in a volume of 55 ml then washed with 150 ml of 50 mM triethanolamine acetate buffer, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.5. Enzyme activity was eluted with 45 ml of 0.1 mM NADH in the above buffer. Fractions of 5 ml were collected.

When the eluent from the sample addition containing unbound enzyme was reapplied to a fresh column, the enzyme was bound to the matrix indicating that the lack of initial binding was due to the saturation of the original column and not to the presence of a non-binding form of the enzyme. Unsubstituted Sepharose and hydroxymethyl-HMD-Sepharose did not bind G3PDH from the crude homogenates.

Elution with 0.1 mM NADH resulted in a sharp single peak of enzyme activity (fig. 1). The increase in specific activity varied from 30-fold for the human muscle to 1500-fold for the mouse L1210 leukemia cells. Recovery of bound enzyme units (table 1) varied from 54% for the rabbit skeletal muscle to

130% for the mouse liver. Peak enzyme fractions, containing 0.1 mM NADH, were very stable and could be stored in the cold room for 1–2 weeks without substantial loss of activity. El ution could also be effected by NAD^+, but in a zone less sharply defined than during elution with NADH.

The affinity matrix was stable for months at 0–4°C in distilled water or TEA buffer in the presence of a few drops of pentachlorophenol (0.5% in 95% ethanol), and could be reused many times with little loss in binding capacity if washed with 6 M urea between uses.

3.2. *NADH and KCl gradients*

When the rabbit brain enzyme was eluted with a salt gradient of 0–1 M KCl, no activity appeared in the eluent until the concentration of KCl had reached 0.5 M. When a 0–0.2 mM NADH gradient was used to elute the enzyme, a sharp peak of activity appeared in the eluate starting at 5 μM NADH.

3.3. *Batch procedure*

In the batch procedure, 90 of the 110 units applied bound to the matrix, and 76 units were eluted with 0.1 mM NADH. After concentrating and desalting the eluent, the specific activity was 2.89 units/mg of protein. Although the batch procedure was more rapid than the column procedure, the binding capacity of the gel and degree of purification were less. However, with very large scale preparations, the speed and simplicity of the batch procedure might justify its use.

4. Discussion

We have been investigating the properties, forms, and development of NAD-linked G3PDH in normal and neoplastic mammalian tissues. Because the level of this enzyme is very low in many normal [9] and most neoplastic tissues [10], classical isolation methods were in many cases impractical or impossible.

In initial attempts at affinity chromatography, glycerol-3-phosphate, a substrate for the enzyme, was bound to the Sepharose 4B by means of an HMD spacer. In order to eliminate ion-exchange properties conferred on the matrix by unreacted free amino groups, trinitrobenzenesulfonic acid (TNBS) was used as a blocking agent. The enzyme was bound to such matrices, but elution was effected not by glycerol-3-phosphate, but by low concentrations of NADH. Control matrices containing only TNB and no glycerol-3-phosphate ligand demonstrated that the affinity ligand was indeed the TNB group. The enzyme remains bound to the matrix in the presence of 0.5 M KCl, yet can be eluted with low concentrations of NADH (5–10 μM) indicating that the binding is both tight and specific. Even more striking is the ability of the matrix to bind the enzyme from homogenates where the G3PDH concentration and specific activity are as low as 4 μg/ml and 0.015 units/mg protein respectively. This efficiency of binding and specificity of elution has enabled us to study the properties, isoenzymic forms, and development of the enzyme in normal and neoplastic tissues even when G3PDH activity is extremely low.

The affinity matrix has been employed on a preparative scale to purify G3PDH from homogenates of rabbit muscle and rabbit mammary gland. Although there is some loss of enzyme yield when the column is scaled up, we have achieved purification to homogeneity with only one additional step, DEAE-Sephadex ion-exchange chromatography (unpublished data). The high specific activity obtained for the enzyme from human skeletal muscle and kidney indicate that at least with some tissues, purification to homogeneity may be possible with a single affinity column step.

It seems likely that the TNB-group functions as an affinity ligand for glycerol-3-P dehydrogenase by interaction with the coenzyme binding site. Studies on the mechanism of binding to the TNB ligand and its affinity for other dehydrogenases are in progress.

Acknowledgements

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