UNSUBSTITUTED AGAROSE AS AN AFFINITY MATRIX FOR ISOLATION OF NAD-DEPENDENT α-GLYCEROPHOSPHATE DEHYDROGENASE FROM THE TRYPANOSOMATID CRITHIDIA FASCICULATA

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SUMMARY. A soluble, NAD-dependent α-glycerophosphate dehydrogenase from the flagellate Crithidia fasciculata was purified to near homogeneity by substrate elution from DEAE cellulose then affinity chromatography on unsubstituted agarose. This two-step procedure permitted 124-fold purification. The crude enzyme did not bind to unsubstituted agarose, but was retained by n-alkyl-amino agarose derivatives. A molecular weight of 78,000 was obtained from the native enzyme by sucrose gradient ultracentrifugation and 66,000 for the polypeptide chain by SDS gel electrophoresis. The apparent K_m for dihydroxyacetone phosphate was 1.17 mM. The enzyme is stimulated by Mg^{++}; low (1 mM) concentrations of spermidine or spermine replaced Mg^{++} as activator.

Use of substituted agarose derivatives as affinity matrices for rapid purification of enzymes is amply documented (1,2); in such studies unsubstituted agarose served as control matrix. We describe here a procedure for isolating soluble NAD-linked α-glycerophosphate dehydrogenase from Crithidia fasciculata with unsubstituted agarose as an affinity matrix.

NAD-linked α-glycerophosphate dehydrogenase (EC 1.1.1.8) has been linked functionally to glycolysis and phospholipid synthesis (3-5). Pathogenic mammalian trypanosomes of the Trypanosoma brucei subgroup contain the enzyme as part of a nonmitochondrial α-glycerophosphate oxidase system for terminal respiration (6,7). The cytosol fraction from the non-pathogenic insect trypanosomatid Crithidia (ATCC 11745) contains the soluble enzyme in high amount compared to other soluble dehydrogenases (8); although it is not dependent on the enzyme for terminal respiration (9,10).

The soluble NAD-linked α-glycerophosphate dehydrogenase from trypanosomatids has been neither extensively investigated nor purified despite its likely pivotal role in morphogenesis of the salivarian trypanosomes and the ease of obtaining the enzyme from Crithidia. This paper details a two-step purification procedure involving substrate elution from DEAE-cellulose (11), then binding of the enzyme to, and its subsequent elution from, an unsubstituted agarose column. Similar enzymes from mammals, birds, and insects have been purified by several classical enzymological techniques, including $(NH_4)_2SO_4$ precipitation (12), polyacrylamide gel filtration (13), and Sephadex (14) and ion-exchange chromatography (15).

MATERIALS AND METHODS

General Methods: Crithidia grown to late log phase (48 h) in complex medium with aeration at 24-27 C (16), was harvested at 27,000 x g on a Sorvall SS-3 centrifuge with a Szent-Gyorgi continuous-flow apparatus at 0-4 C (flow rate: 1 1/5 min). Cells were washed in 50 mM Tris·HCl (pH 7.4), ground with neutral alumina (BioRad AG-7). A supernatant fraction containing soluble enzymes was isolated by centrifugation at 39,000 x g for 30 min.

Enzyme assay: Activity was assayed in a recording spectrophotometer (340 nm) at 25 C, in a reaction mixture (3.0 ml final volume) containing supernatant fraction protein, 50 mM Tris.HCl buffer, pH 7.4, 0.125 mM NADH and 10 mM $MgCl_2 \cdot 6H_2O$. Dihydroxyacetone phosphate (Sigma, prepared according to their instructions) at a final concentration of 1.91 mM started the reaction. Reactions proceeded for 1 min with the initial linear portion (30 sec) taken as rate of NADH oxidation. One unit of enzyme activity was defined as the amount that converted 1 µM NADH/min at 340 nm using 6.22 $cm^{-2}M^{-1}$ as ϵ for NADH; specific activity was calculated as µM NADH converted/min/mg protein.

Protein was determined by the method of Lowry et al. (17) with bovine serum albumin (fraction V, Sigma) as standard.

Linear sucrose-gradient centrifugation: This was according to Martin and Ames (18) with Escherichia coli DNA polymerase I (mw 110,000) and equine hemoglobin (mw 64,000) as standards.

SDS gel electrophoresis: The procedure of Weber and Osborn (19) was used. Molecular-weight markers were ovalbumin and bovine serum albumin (Type F, Sigma)

RESULTS

Purification of α-glycerophosphate dehydrogenase: Crude enzyme (20-50 ml) was directly applied to an 2 X 18 cm or 2 X 40 cm column of DEAE (BioRad Cellex D) previously equilibrated with 50 mM Tris.HCl, pH 7.4 (Buffer A). The column was washed until no further protein was detected (Fig. 1). Usually, $<4\%$ of the applied activity was recovered from this wash. The column was then successively washed with Buffer A + 60 mM NaCl (Buffer B), followed by Buffer B + 40 mM DL-α-glycerophosphate (20 mM L-α). The latter wash fractions (4 ml) were collected into tubes containing 1 ml glycerol + 0.05 ml 0.5 M Tris.HCl buffer (pH 7.4). From DEAE columns of varying sizes, 20-30% of enzyme activity was eluted in the plain buffer-salt wash. The final (glycerophosphate) wash yielded 36-43% of the total enzyme activity in pooled fractions, with up to 31-fold purification. Control experiments with 50 mM Tris.HCl + 40 mM $NaPO_4$ buffer (pH 7.4) as eluent in place of 40 mM DL-α-glycerophosphate yielded only 11% of total activity.

Pooled post-DEAE column fractions were dialyzed in a cellulose hollow-fiber device (BioRad HF1 1/20 minibeaker) against 350 volumes of Buffer A + 25% glycerol. This preparation (5-30 ml) was then layered on a 1 X 24 cm or 2 X 20 cm column of agarose (Bio Gel A-0.5m, 100-200 mesh) equilibrated with Buffer A + 25% glycerol The column was first washed with the equilibration buffer (Fig. 2): $>95\%$ of the activity layered on the column remained bound. A linear gradient of 0-0.5 M

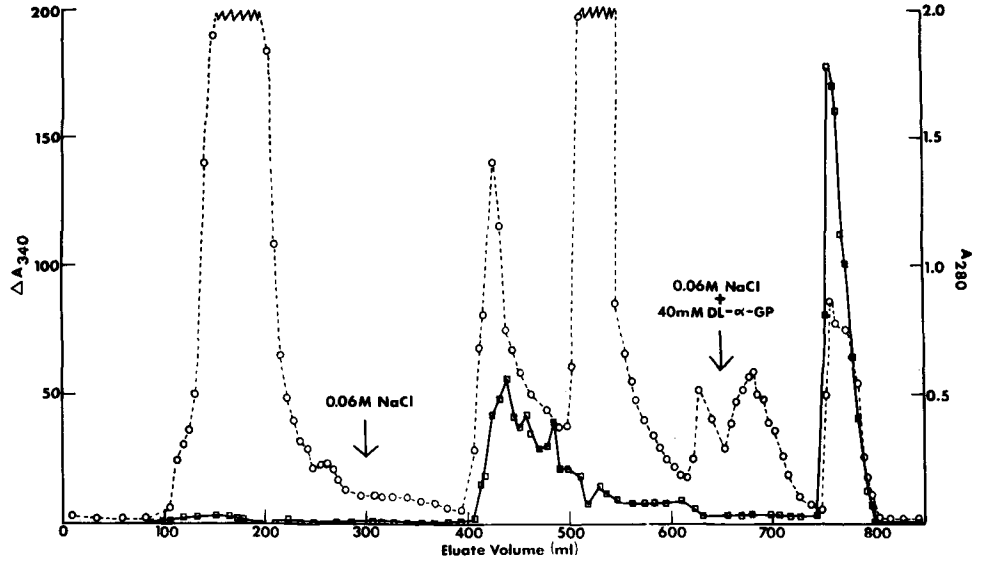

Fig. 1. DEAE-cellulose chromatography of α-glycerophosphate dehydrogenase (2 X 40 cm column). A total of 50 ml (565 mg protein) crude extract was applied. Initial wash was 300 ml 0.05 M Tris.HCl (pH 7.4); arrows indicate subsequent NaCl and NaCl + 40 mM DL-α-glycerophosphate washes. ΔA_{340} indicates enzyme activity as total change in absorbance/min/fraction; volume of fractions was 6.5 ml for the initial buffer and NaCl washes, and 4.0 for the NaCl + α-glycerophosphate wash. □—□ , ΔA_{340} (enzyme activity); 0 ---- 0, A_{280}

NaCl in equilibration buffer was applied. Fractions (2.5 ml for column in Fig. 2) were collected into tubes containing 0.5 ml glycerol + 0.025 ml 0.5 M Tris·HCl (pH 7.4). The peak containing most of the enzyme activity eluted at 60 mM NaCl (Fig. 2). Various preparations yielded up to 124-fold purification and a yield of 8.6% of initial activity (Table 1).

Enzyme from the crude fraction did not bind to unsubstituted agarose; but was retained by diaminoalkyl derivatives of agarose -- a binding seemingly not due to hydrophobic interactions since an NaCl gradient alone effected elution (20,21); moreover, the enzyme bound almost as well to 1,3-diaminopropyl agarose, spermidine-agarose, and 1,8-diaminooctyl agarose.

Properties of the enzyme: Assays of the final preparation were negative for: glyceraldehyde phosphate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, and malate dehydrogenase. The final enzyme preparation (Table 1) was homogenous on SDS gel electrophoresis, yielding 1 band, R_m 0.22 (Fig.3) A corresponding band was present in crude and post-DEAE fractions. The apparent molecular weight of the polypeptide chain was 66,000 (Fig. 4) while that of the native enzyme was 78,000 as determined by linear sucrose-gradient centrifugation.

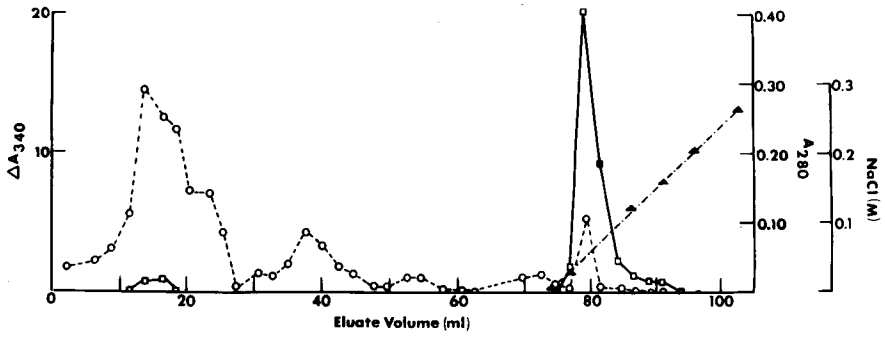

Fig. 2. BioGel A-0.5 m agarose chromatography of α-glycerophosphate dehydrogenase (1 X 24 cm column). Sample applied was 5 ml (1.0 mg of pooled peak DEAE fractions). Initial column wash was 50 ml 0.05 M Tris.HCl + 25% glycerol (pH 7.4); a linear NaCl gradient was then applied (25 ml 0.5 M NaCl in wash buffer + 25 ml wash buffer). Volumes of fractions were 3 ml (initial wash) and 2.5 ml (gradient). ΔA_{340} indicates enzyme activity as total change in absorbance/min/fraction. □——□ , ΔA_{340}; 0 ---- 0, A_{280}; ▲—·—·▲, M NaCl.

Table 1

PURIFICATION OF SOLUBLE NAD-LINKED α-GLYCEROPHOSPHATE DEHYDROGENASE

Step of Purification	Total protein (mg)	Total Activity	Specific Activity	Purification	% Yield
A. Crude extract	290	448.57	1.55	-	-
B. DEAE cellulose	3.58	172.14	48.03	31.03	38
C. BioGel A-0.5m	0.202	38.80	192.0	124.1	8.6

Variations in the molecular weight of the enzyme from various sources have been reported: 65,000 to 79,000, depending on the procedure (13,23).

Specific activities of the final preparations were up to 124-fold those of the crude extract; however the lability of the enzyme in this state indicated that higher purification was probably obtained: pooled fractions from agarose columns lost 45% of activity after 2 days at 0-4 C. Indeed, the most active fractions from agarose columns began losing activity soon after elution. Increased glycerol concentrations (up to 50%) and addition of sulfhydryl reagents

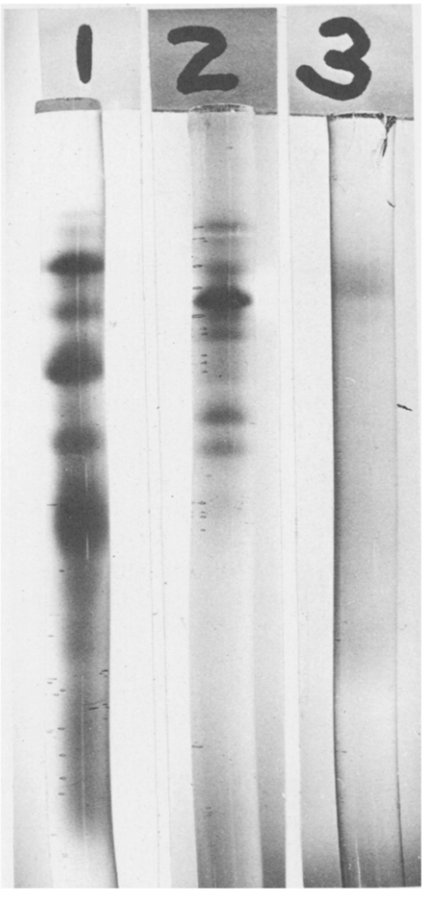

Fig. 3. SDS gel electrophoresis of α-glycerophosphate dehydrogenase. Gel contents: 1,162 μg crude fraction protein; 2, 31 μg post-DEAE fractions: 3, 10 μg post-agarose fractions (124-fold purified). Migration was toward the bottom of the picture (+ pole).

(1 mM dithiothreitol) or cofactor (1 mM NAD^+) did not enhance stability. The rate of activity loss after initial isolation decreased sufficiently to allow activation and kinetic studies.

The pH optimum was between 6.0 and 6.3 in 50 mM $NaPO_4$, with an apparent K_m of 1.2 mM for dihydroxyacetone phosphate. Preliminary results suggested a K_m value for NADH well below 10^{-5}M. The enzyme apparently lacks bound Mg^{++}: 10 mM EDTA in the absence of added Mg^{++} did not decrease activity.

The crude enzyme appears totally dependent on Mg^{++} or the polyamines spermidine or spermine for activity. Dialysis of crude fractions against 50 mM

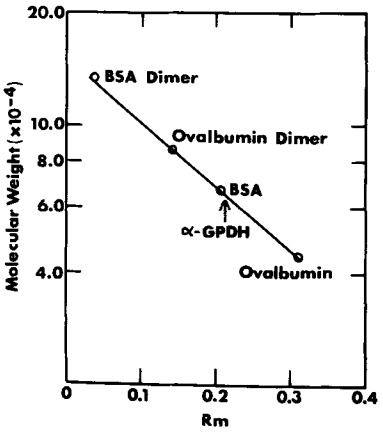

Fig. 4. Molecular weight estimation of α-glycerophosphate dehydrogenase by SDS gel electrophoresis (19). R_m values were calculated relative to the bromophenol blue dye front. Points were averaged from duplicate determinations. Molecular weights of standard proteins were: 68,700 (BSA, bovine serum albumin); 43,000 (ovalbumin) (22).

Tris·HCl buffer (pH 7.4) did not change this dependence. Fractions from DEAE and from agarose columns were stimulated 2-fold by 1 mM spermine or spermidine. Mg^{++} at 10 mM stimulated activity 1.6-fold. Higher Mg^{++} or spermidine concentrations or combinations of both did not increase activity. Activation by polyvalent organic cations seemed specific for spermidine or spermine since several other natural polyamines (eg. diaminopropane, putrescine, cadaverine) and cationic polyamino acids were not stimulatory. Sodium chloride at 20 mM was stimulatory; above 50 mM it inhibited. Enzyme activity appeared dependent on reduced-SH groups since addition of 100 μM p-chloromercuribenzoate lowered activity 90%.

DISCUSSION

The efficiency of the initial (DEAE) purification step points to a specific conformational change in the enzyme upon addition of substrate, since successful elution and purification was obtained with the same NaCl concentrations (60 mM) as the previous column wash. If substrate was omitted from the salt wash, 90 mM NaCl had to be used to elute a major enzyme peak; under these conditions purification was low -- 4- to 8-fold -- and activity spread over more fractions than with the salt-glycerophosphate wash.

Binding of the partly purified enzyme to unsubstituted agarose may set this enzyme apart from similar enzymes from mammals (13,14), birds (15), and insects (12). The Crithidia enzyme also appears unique in activation by polyamines as

well as Mg^{++}. Rat liver supernatant prepared by the alumina method has α-glycerophosphate dehydrogenase activity not stimulated by Mg^{++} or polyamines The reason for the stimulation of the Crithidia enzyme is unclear. Sanwall (24) reported a similar finding for E. coli glucose-6-phosphate dehydrogenase. Agarose, a linear polysaccharide with alternating D-galactose and 3,6-anhydro-L-galactose moieties (25), bears no structural resemblance to α-glycerophosphate or of dihydroxyacetone phosphate. Therefore the binding of the dehydrogenase is not attributable to a substrate or product-analog affinity. Since the enzyme in the crude extracts (unfractionated by DEAE-cellulose) did not bind to the unsubstituted agarose matrix, the enzyme may be loosely bound to a sugar-containing substance or polypeptide, or membrane structure in crude extracts. Purification with DEAE-cellulose may separate these components, so that the dehydrogenase is then free to bind to agarose. Bentley et al (26) have shown that rabbit muscle α-glycerophosphate dehydrogenase passed through DEAE lost a non-protein contaminant thought to be adenosine diphosphate ribose or a related compound. That the Crithidia enzyme has nonspecific affinity for multi-hydroxyl compounds seems unlikely for the agarose chromatography was done in the presence of 25% (v/v) glycerol. Affinity chromatography on unsubstituted agarose has been utilized to purify carbohydrate-specific lectins from Ricinis (27) and Bauhinia (28). Lectins are eluted from agarose by washing the column containing bound lectins with lactose-containing buffer; salt concentrations up to 0.2 M do not release the bound lectins (27,28). Elution of α-glycerophosphate dehydrogenase bound to unsubstituted agarose by 50 mM NaCl suggests that the mechanism of binding may differ from that of the plant lectins.

Replaceability of a divalent cation by polyamines for an enzyme associated with carbohydrate metabolism has been mentioned for E. coli glucose-6-phosphate dehydrogenase (24); here polyamine control of the enzyme may serve to regulate production of RNA precursors (29). Polyamine activation of the Crithidia dehydrogenase may denote means to regulate phospholipid biosynthesis; polyamine regulation of phospholipid metabolism in bacteria has been demonstrated (30).

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