PROPERTIES OF A PURIFIED NAD-LINKED α-GLYCEROPHOSPHATE DEHYDROGENASE FROM *LEPTOMONAS* SP.; ACTIVATION BY POLYAMINES

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SUMMARY. Cytosolic NAD-linked α-glycerophosphate dehydrogenase (EC 1.1.1.8) from a *Leptomonas* sp. was purified to homogeneity (up to 125-fold) by affinity chromatography on unsubstituted agarose. The purified enzyme with dihydroxyacetone phosphate as substrate required Mg^{++} for full activity; this was replaceable completely by spermidine and spermine. Other aliphatic amines were not stimulatory. K_m's were: 0.083 mM (spermidine), 0.3 mM (spermine), 10 mM (Mg^{++}); for dihydroxyacetone phosphate, 0.2 mM with spermidine, 0.29 mM and 1.25 mM with spermine and Mg^{++} as activators respectively. K_m's for NADH were also lower in the presence of polyamines than with Mg^{++}. The molecular weight of the polypeptide by SDS-gel electrophoresis was 43,000; the value for the native enzyme obtained by chromatography on agarose and ultracentrifugation through a linear sucrose gradient was 76,000 and 63,000 respectively.

Terminal respiration in bloodstream forms of *Trypanosoma brucei* subgroup is mediated by an L-α-glycerophosphate oxidase system (1) in which the cytoplasmic NAD-linked α-glycerophosphate dehydrogenase (EC 1.1.1.8) participates (2). This enzyme has also been implicated in glyceride metabolism (3). Considering its pivotal role in trypanosomatid biochemistry, α-glycerophosphate dehydrogenase has neither been isolated nor characterized in detail. Our laboratory has been the only one reporting success with its isolation (4). In that study, unsubstituted agarose served as an affinity matrix for purification of the enzyme from *Crithidia fasciculata*, a lower flagellate; enzyme activity was stimulated by polyamines.

We undertook further investigation of other trypanosomatid α-glycerophosphate dehydrogenases to determine whether a) unsubstituted agarose could serve as the isolation matrix and b) stimulation of the enzyme by polyamines is common to other Trypanosomatidae. A *Leptomonas* sp. was used because of the high enzyme activity in crude preparations (5) and the remarkable susceptibility of the organism to several antitrypanosomatid agents (4,5).

We report here that affinity chromatography on unsubstituted agarose, preceded by DEAE-cellulose chromatography, serves for purification of α-glycerophosphate dehydrogenase from Leptomonas sp. Kinetic data presented indicate polyamines may be natural activators for the enzyme.

MATERIAL AND METHODS

Organism; isolation of supernatant fraction: Leptomonas sp. (ATCC 30250), isolated by F.G. Wallace, University of Minnesota, was mass cultured in 10 l bottles containing complex HY medium (6), aerated and incubated 4-6 days at 30-32 C. Final pH's were 5.6-6.2. Isolation of the soluble fraction was as previously described (4). Crude supernatant collected from ~150 g of cells totalled 150-200 ml.

Enzyme assays: Reactions were monitored (final volume 3.0 ml) as change in absorbance at 340 nm for formation or oxidation of NADH or NADPH. Enzyme assays (at 25 C) were as before (4). One unit of enzyme activity was the amount of enzyme to convert 1 μM NADH/min; specific activity as μM NADH converted/min/mg protein.

Protein was estimated by the method of Lowry et al. (7) with bovine serum albumin (Type F, Sigma) as standard.

Sucrose-gradient centrifugation: The procedure of Martin and Ames (8) was followed with equine hemoglobin (MW 64,000) and E. coli DNA polymerase I (MW 110,000) as standards.

SDS-gel electrophoresis: The method of Weber and Osborn (9) was used with bovine serum albumin and ovalbumin standards.

RESULTS

Enzyme purification: Attempts were made to determine whether crude supernatant bound to unsubstituted agarose. Two agarose columns (Bio Gel A-0.5m, 100-200 mesh), 0.7 X 19 cm were constructed; one equilibrated with 50 mM Tris-HCl, pH 7.3, the other with same buffer + 20% glycerol. In both instances 100% of the activity was regained in the eluate. To the crude supernatant (40-75 ml), crystalline NaCl was added to a final concentration of 15 mM. This was then applied to a 2.1 X 26 cm DEAE-cellulose (Bio Rad Cellex-D) column pre-equilibrated with 50 mM Tris-HCl + 15 mM NaCl, pH 7.3; flow rate 40 ml/h. Much protein was eluted along with the enzyme (Fig. 1). The most active fractions (49% of the total activity) were pooled and dialyzed 2X against 1 l of 50 mM Tris-HCl + 20% glycerol, pH 7.3.

The dialyzed post-DEAE fractions were then layered (8-11 ml) onto a 0.9 X 47 cm agarose column equilibrated with Tris-HCl + 20% glycerol, pH 7.3; flow rate 10 ml/h. No activity was detected despite removal of protein (Fig.

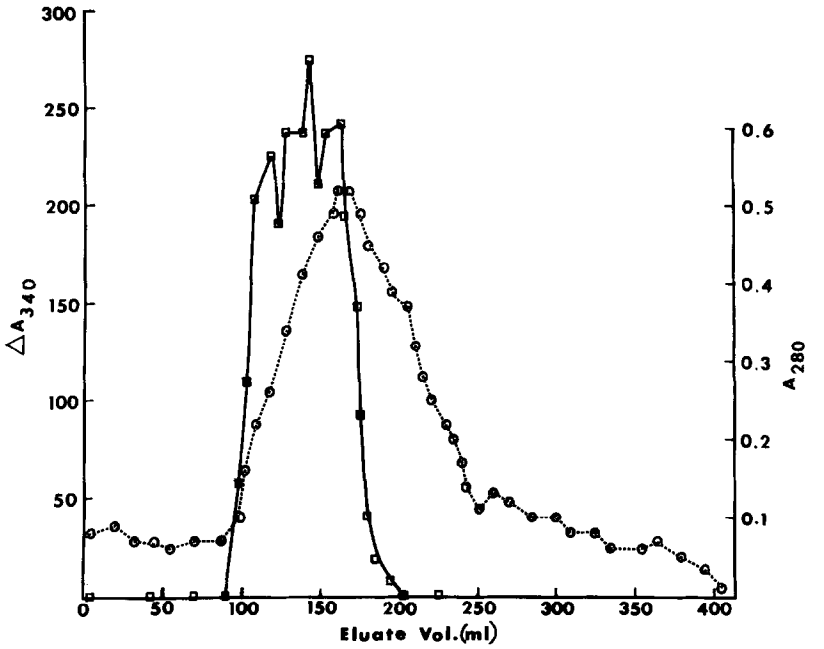

Fig. 1. DEAE-cellulose chromatography (column 2.1 X 26 cm) of α-glycerophosphate dehydrogenase. After 45 ml (200 mg protein) was applied, the column was then washed with 50 mM Tris-HCl + 15 mM NaCl, pH 7.3; fraction volumn was 5.0 ml. ΔA_{340} indicates enzyme activity as total change in absorbance/min/fraction. □—□, A_{340}; O----O, A_{260}.

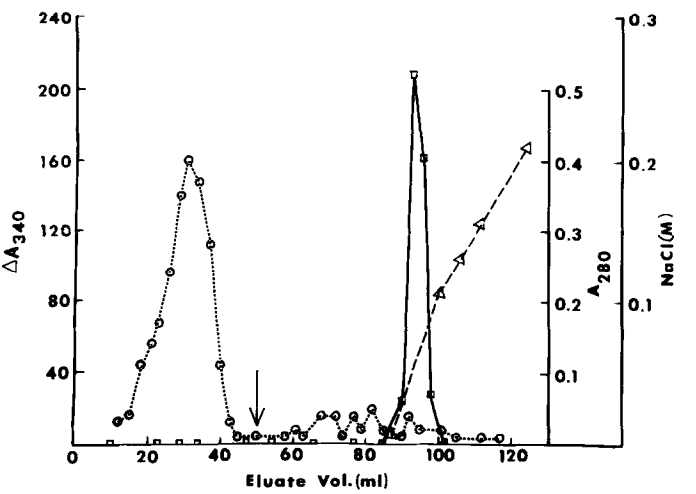

Fig. 2. Agarose chromatography (column 0.9 X 47 cm) of α-glycerophosphate dehydrogenase. A total of 11 ml (3.5 mg protein of pooled DEAE fractions) was applied and the column washed with 50 mM Tris-HCl + 20% glycerol, pH 7.3. Arrow indicates subsequent linear NaCl gradient (35 ml 0.25 M NaCl in starting buffer + 35 ml starting buffer); fraction volume was 2.5 ml. ΔA_{340} indicates enzyme activity as total change in absorbance/min/fraction. □——□, A_{340}; O---O, A_{260}; Δ------Δ, M NaCl.

TABLE I. PURIFICATION OF LEPTOMONAS α- GLYCEROPHOSPHATE DEHYDROGENASE

Purification step	Total protein (mg)	Total activity	Specific activity	Purification	% yield	E_{280}/E_{260}
Crude extract	392.32	5279.5	3.23	-	100	0.95
DEAE-cellulose	20.86	2786.7	43.62	13.5	52	1.18
Agarose	0.198	1242.2	336.35	104.2	23.5	1.7

2). A 70 ml linear gradient of 0-0.25 M NaCl in equilibration buffer was then applied. Seventy-five per cent of the total activity was eluted at 5 mM NaCl (Fig. 2). The resultant preparation had 104-fold purity and a yield of 23.5% (Table I). The active fractions were pooled and dialyzed against several changes of equilibration buffer to remove any traces of salt which would interfere with cofactor stimulation. A progressive increase in the E_{280}/E_{260} ratio became evident (Table I).

Stability of α-glycerophosphate dehydrogenase: Crude enzyme preparations kept at 0-4 C or frozen in 0.25 M sucrose had no loss of activity up to 6 weeks. If kept frozen in the absence of sucrose, enzyme activity decreased after the first week with 1% of initial activity remaining after 4.5 weeks. Post-DEAE fractions kept in 20% glycerol at 0-4 C lost no activity up to 4 weeks; with no glycerol activity remained constant for 2 weeks. Bovine serum albumin (1 mg/ml) stabilized the post-agarose enzyme activity 100% for 1 week at 0-4 C. Purified enzyme frozen with bovine serum albumin lost 85% of initial activity within 2 days; neither lecithin, NAD^+, nor β-mercaptoethanol stabilized the enzyme kept frozen or at 0-4 C.

pH Optimum: This was 6.3 in 25 mM KPO_4 buffer. All kinetic studies were at this pH.

Mg^{++} requirement; replaceability by polyamines: The enzyme required Mg^{++} for full activity; rates without cofactor were <10% of Mg^{++}-activated rates.

TABLE II. EFFECTS OF DIVALENT CATIONS AND POLYAMINES ON ACTIVATION OF α-GLYCEROPHOSPHATE DEHYDROGENASE [a]

A. Divalent cation (25 mM)	% Activation relative to Mg^{++} (25 mM) [b]
$ZnSO_4$	5
$MnSO_4$	5
$CoCl_2$	5
$CdCl_2$	5
$NiCl_2$	75
B. Amine (2 mM)	**% Activation relative to spermidine (2 mM) [c]**
Diaminopropane	10
Diaminobutane	17
Cadaverine	37
Diaminohexane	24
Diaminoheptane	20
Diaminooctane	9
L-Arginine	2
Carbamylcholine	2
Spermine	96

[a] enzyme preparation of 100-fold
[b] specific activity of Mg^{++} control: 162.35
[c] specific activity of spermidine control: 235.41

Of other divalent cations tested, only Ni^{++} gave significant stimulation: 75% of the rate with Mg^{++} was attained (Table IIA). Spermidine could completely substitute for Mg^{++} (see control rates, Table II): at 2 mM it stimulated the enzyme 1.45-fold over the Mg^{++} rate; spermine (2 mM) stimulated activity 1.35-fold. Cadaverine was the only other polyamine which showed slight stimulation (Table IIB). Suboptimal Mg^{++} + polyamine combinations were not synergistic. Both NaCl and KCl at 25 mM inhibited activity. The reverse reaction (formation of NADH) was not affected by cofactors.

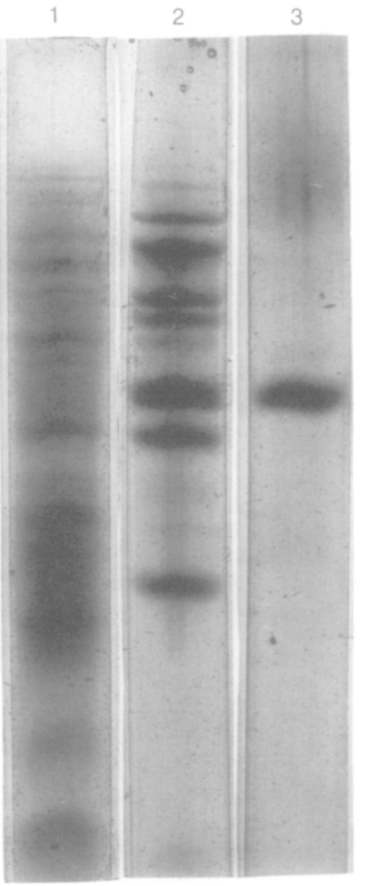

Fig. 3. SDS-gel electrophoresis of α-glycerophosphate dehydrogenase. Gels contained: 1, 24 μg crude supernatant; 2, 12.9 μg post-DEAE fractions; 3, 16.3 μg post-agarose fractions (104-fold purified).

Kinetic constants: K_m values were obtained by Lineweaver-Burke plots and the direct linear plot (10). K_m's for dihydroxyacetone phosphate, in the presence of spermidine (2 mM) and spermine (2 mM) were lower than that obtained with Mg^{++} (25 mM): 0.2 mM, 0.29 mM, and 1.25 mM respectively. Similarly, K_m for NADH was lower with polyamine compared with Mg^{++}: 6.3 μM (with spermidine), 8.3 μM (with spermine), and 11.9 μM (with Mg^{++}). K_m's for the individual cofactors were: spermidine, 0.083 mM; spermine, 0.3 mM; and Mg^{++}, 10 mM.

Purity: Post-agarose preparations were homogenous on SDS-gel electrophoresis, showing 1 band with an R_m of 0.31 (Fig. 3) corresponding to a molec-

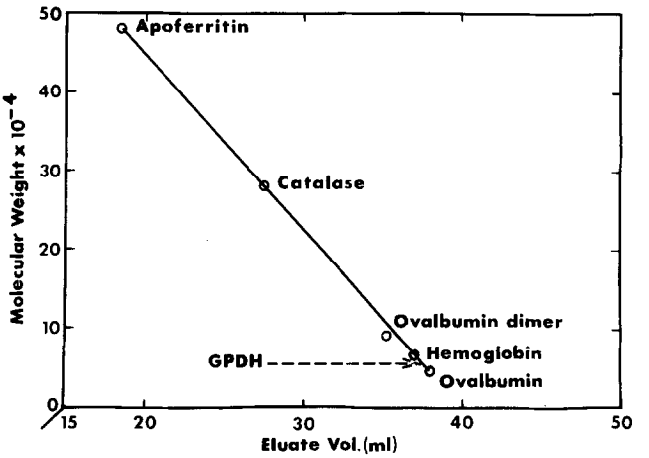

Fig. 4. Molecular weight determination (agarose column 1.3 X 52 cm) of α-glycerophosphate dehydrogenase. The column was equilibrated with 50 mM Tris-HCl-20% glycerol + 0.2 M NaCl, pH 7.3. Each standard (5 mg) was dissolved in equilibration buffer; fraction volumes were 1.0 ml. The post-agarose preparation contained 30 μg protein (100-fold purity).

ular weight of 43,000. Crude supernatant, assayed for other cytosol enzymes, had malic dehydrogenase, malic enzyme, and hexokinase activities; only α-glycerophosphate dehydrogenase was detected in purified preparations.

Molecular weight determinations: A 1.3 X 52 cm agarose column equilibrated with Tris-HCl-20% glycerol + 0.2 M NaCl was used. The high salt concentration was needed to prevent binding of purified enzyme to agarose. Standards used were ovalbumin (MW 45,000), hemoglobin (MW 67,000), catalase (MW 280,000), and apoferritin (MW 480,000); all dissolved in equilibration buffer. By this method the molecular weight of the native enzyme came to 63,000 (Fig. 4) while that obtained by linear sucrose-gradient ultracentrifugation was 76,000. The molecular weight by SDS-gel electrophoresis was 43,000 (Fig. 5).

Sulfhydryl reagents: p-Chloromercuribenzoate (100 μM) completely abolished activity with any activator, indicating dependence on reduced SH-groups. Iodoacetate (1 mM) inhibited the Mg^{++}-stimulated rate 50% but did not affect polyamine activated rates.

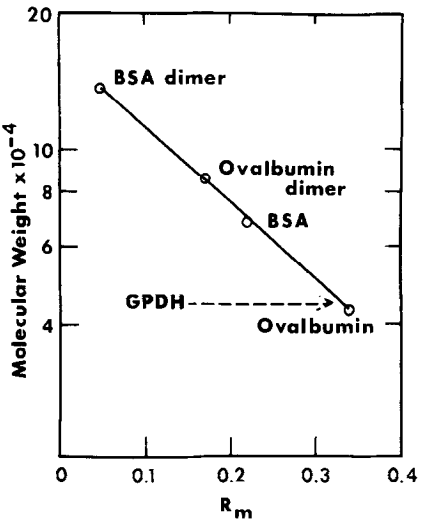

Fig. 5. Molecular weight determination of α-glycerophosphate dehydrogenase by SDS-gel electrophoresis. Method as previously described (4). Standard proteins were: bovine serum albumin (MW 68,700) and ovalbumin (MW 43,000).

DISCUSSION

Bacchi et al. (4) have shown that unsubstituted agarose serves as an affinity matrix for the isolation of soluble NAD-linked α-glycerophosphate dehydrogenase from C. fasciculata. This report has substantiated their finding through use of another member of the family Trypanosomatidae – Leptomonas sp. The initial steps in the purification procedures varied: for Leptomonas, direct application of 15 mM NaCl wash to DEAE-cellulose resulted in elution of the enzyme yielding 49% of the total activity, whereas elution of the enzyme from C. fasciculata required a preliminary salt wash followed by substrate elution from DEAE, yielding 36-43% of the total activity. It must be emphasized that a linear NaCl gradient be used for elution of the Leptomonas enzyme from agarose; direct application of 0.2 M salt produces a spreading of enzyme activity over many fractions and low purity (10- to 20-fold).

The removal of a non-protein component during DEAE-cellulose chromatography seems likely (4,11) since crude supernatant, even when dialyzed, did not bind to unsubstituted agarose (and because of our increased E_{280}/E_{260}

ratio). Other affinity methods have employed NAD^+, AMP, and their analogues as ligands for purification of NAD-linked dehydrogenases (12,13). Kornbluth et al. (14) have shown binding of α-glycerophosphate dehydrogenases from crude organ homogenates to 2,4,6-trinitrobenzene bound to Sepharose through a hexamethyldiamine spacer (binding to unsubstituted agarose was not evident) and subsequent elution of the bound enzyme with NADH; elution was not affected by α-glycerophosphate. McGinnis and deVellis (15) designed an affinity column with 6-phosphogluconic acid as the ligand. This compound was found to act neither as an inhibitor nor substrate of α-glycerophosphate dehydrogenase when in solution; when immobilized onto agarose, specific binding of the enzyme occurred with subsequent elution by α-glycerophosphate. Again, crude rat muscle enzyme did not bind to unsubstituted agarose.

The molecular weight of purified Leptomonas α-glycerophosphate dehydrogenase as determined by linear sucrose gradient ultracentrifugation is similar to the C. fasciculata enzyme (4), but a discrepancy exists as ascertained by SDS-gel electrophoresis: for C. fasciculata the polypeptide chain was 66,000 compared to 43,000 for Leptomonas. The reason for such variance is not clear, but differences in enzyme structure may be inferred as noted by the variation of DEAE retention patterns in the initial preparative step.

Trypanosomatid α-glycerophosphate dehydrogenase may be unique in activation by Mg^{++} or polyamines. Interaction between Mg^{++} and polyamines in aminoacylations has been proposed (16,17), although the nature of activation by polyamines in such systems is in question (18). Sanwall (19) demonstrated spermidine replacement of Mg^{++} as activator of E. coli glucose 6-phosphate dehydrogenase and suggested that it may be the natural activator. The C. fasciculata enzyme behaves similarly (4). That spermidine or spermine may be natural cofactor(s) for Leptomonas α-glycerophosphate dehydrogenase is a possibility for a) these are the most active polyamines; b) they have been detected in crude extracts of the organism (Bacchi, unpubl.); c) the enzyme did not respond to other amines; and d) K_m's for spermidine and spermine are far lower than that of Mg^{++}.

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