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AFFINITY CHROMATOGRAPHY OF D-LACTATE DEHYDROGENASES FROM *LIMULUS POLYPHEMUS* (HORSESHOE CRAB) AND *HALIOTUS* SP. (ABALONE) VOLUNTARY MUSCLE ON 8-(6-AMINOHEXYL)-AMINO-ADENINE NUCLEOTIDE-SEPHAROSE

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An improved method of purification employing sequential isoenzyme elution on DEAE-cellulose at pH 6.5 and biologically specific elution with the reduced NAD-pyruvate adduct from 8-(6-aminohexyl)-amino-NAD⊕-Sepharose is described for *Limulus (horseshoe crab) muscle* D*-lactate dehydrogenase. The protein is judged as being at least* 98% *pure by its constant specific activity in the terminal purification steps, a molar extinction coefficient* (280 *nm*) *identical with that previously reported for the purified enzyme, and its protein and enzyme electrophoretic patterns on starch and polyacrylamide gel electrophoresis at three pHs. The binding of ammonium sulfate fractionated* D*-lactate dehydrogenase from crude cell-free Limulus* and *Haliotus* (abalone) muscle homogenates to 8-(6-aminohexyl)-amino-AMP- and 8-(6-aminohexyl)-amino-NAD⊕-Sepharose columns is demonstrated. A comparison of the binding properties of these two enzymes with those of vertebrate L-lactate dehydrogenases suggests that they may be significantly different in terms of their binding sites for the adenine portion of the coenzyme.

INTRODUCTION

Diphosphopyridine nucleotide-linked lactate dehydrogenases catalyze the following conversion:

$$\text{Pyruvate} + \text{NADH} + \text{H}^{\oplus} \rightleftharpoons \text{lactate} + \text{NAD}^{\oplus}$$

The enzyme is the catalyst for the terminal step in the glycolytic pathway. Lactate dehydrogenase (EC 1.1.1.27) from several vertebrates has been characterized by Kaplan et al. (1–4). Lactate dehydrogenase from the East Coast lobster, *Homarus americanus*, has also been studied extensively (5–7). Both the vertebrate and the lobster dehydrogenase ($M_r \simeq 140{,}000$) are composed of four polypeptide subunits and are stereospecific for L-lactate. Lactate dehydrogenase (EC 1.1.1.28) from the skeletal muscle of the

horseshoe crab, *Limulus polyphemus*, however, was reported to be notably different, existing as a dimeric protein ($M_r = 70,000$) and having an absolute specificity for D-lactate (8).

The purification and subsequent characterization of horsehoe crab lactate dehydrogenase suggested that the enzyme was homologous with the L-stereospecific enzymes (9,10). This interpretation was based upon remarkably similar values for the following parameters: subunit molecular weight, stoichiometric binding of *para*-hydroxymercuribenzoate and reduced coenzyme, apparent Michaelis constants and maximum velocities for substrates, inhibitory properties of oxamate and the reduced NAD-pyruvate adduct, hydride transfer from the 4-A position of NADH, tissue specific isoenzymes in the host, and amino acid composition of the reactive sulfhydryl peptide.

The observed differences and similarities enumerated above have been useful in the study of the evolution and comparative biochemistry of lactate dehydrogenases as well as in evoking additional physicochemical studies. However, the reported purification procedure for *Limulus* muscle lactate dehydrogenase was relatively tedious and unproductive (9,11). Five column steps were used, resulting in only 25 mg of purified protein from 1 kg of muscle (13% of initial enzyme activity). This paper reports an improved isolation procedure, employing ion exchange chromatography on DEAE cellulose at pH 6.5 and affinity chromatography on 8-NAD$^{\oplus}$-Sepharose.[1] The above modifications have resulted in a yield of 44 mg from 500 g tissue and at 36% recovery.

We also wish to report the ability of 8-AMP-Sepharose to bind *Limulus* lactate dehydrogenase. The fact that the 8-AMP-Sepharose and 8-NAD$^{\oplus}$-Sepharose are effective binders of *Limulus* D-lactate dehydrogenase as well as several L-lactate dehydrogenases and the D-lactate dehydrogenase from the abalone (12) suggests that the enzymes from very diverse organisms may be highly homologous regarding the coenzyme binding domain. Such a homology has been shown for different pyridine nucleotide dehydrogenases (13).

MATERIALS AND METHODS

Materials

Live adult horseshoe crabs were purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. Whole animals were frozen

[1]Abbreviations used in this paper are: 8-NAD$^{\oplus}$-Sepharose: 8-(6-aminohexyl)-amino-NAD$^{\oplus}$-Sepharose; 8-AMP-Sepharose: 8-(6-aminohexyl)-amino-5'-AMP-Sepharose; EU: enzyme unit; DEAE: diethylaminoethyl.

overnight, thawed, and the skeletal muscle removed and stored frozen at −15°C. Abalone was collected off the coast of La Jolla, California, and the foot muscle stored frozen. The 8-AMP-Sepharose and 8-$NAD^{\oplus}$-Sepharose affinity chromatography resins were a generous gift of Dr. C.-Y. Lee, Department of Chemistry, University of California, San Diego, and had been prepared as described by Lee et al. (12,14). Ammonium sulfate, β-mercaptoethanol, chicken heart lactate dehydrogenase, $NAD^{\oplus}$, disodium NADH, and sodium pyruvate were purchased from the Sigma Chemical Company, Saint Louis, Missouri.

General Methods

Lactate dehydrogenase activity and protein content were measured as described by Long and Kaplan (9). One enzyme unit is defined as the amount of enzyme that results in a change of one absorbance unit per minute at 340 nm and room temperature, with a light path of 1 cm. The final reaction mixture is 3.0 ml and contains concentrations of 0.14 mM NADH, 6.7 mM sodium pyruvate, and 50 mM potassium phosphate (pH 7.5). Starch gel at pH 7.0 and polyacrylamide gel electrophoresis at pH 9.5 and 8.0 were done by the methods of Fine and Costello (15), Davis (16), and Gabriel (17), respectively. All steps in handling the enzyme were at 0°–3°C unless otherwise noted.

Purification of Limulus *Muscle Lactate Dehydrogenase*

Step 1. Cell-Free Extract. Five hundred grams of freshly thawed *Limulus* skeletal muscle was homogenized in 1.2 liters cold 0.05 M potassium phosphate buffer (pH 6.5) containing 1 mM disodium EDTA and 14 mM β-mercaptoethanol in two 15-sec bursts of a Waring blender. The resulting homogenate was centrifuged 30 min at 10,000 rpm in a GSA rotor driven by a Sorvall RC2 refrigerated centrifuge. The pellet was discarded and the supernatant was assayed for protein and enzyme activity.

Step 2. 40–65% Ammonium Sulfate Fractionation. Solid ammonium sulfate crystals were slowly added with stirring to the above supernatant to 40% saturation.[2] The suspension was allowed to stir 30 min and was then centrifuged as above. The resulting supernatant was brought to 65% saturation and then centrifuged. Pelleted material was suspended in a minimum of fresh extraction buffer and dialyzed against 2 liters of buffer for 4 h and repeated for 12 h. After dialysis the sample was clarified by centrifugation as above.

[2]Percentage of saturation was determined from Table I, GREEN, A. A., and HUGHES, W. L. (1955). *In* Methods in Enzymology, Vol. 1, COLOWICK, S. P., and KAPLAN, N. O. (eds.), Academic Press, New York, pp. 67–90. No correction was made for temperature.

Step 3. Sephadex G-100 Chromatography. The above sample was applied to a Sephadex G-100 column (4.8 × 100 cm, 1.8 liters) that had been preequilibrated with extraction buffer. Effluent fractions with significant enzyme activity were pooled and concentrated by bringing the sample to 70% ammonium sulfate saturation and centrifuging. The resulting pellet was dissolved in a minimum amount of 0.01 M potassium phosphate buffer (pH 6.5) containing 1 mM disodium EDTA and 14 mM β-mercaptoethanol and dialyzed 4 h against 1 liter cold buffer. A second dialysis of 10 h was performed, and the sample was centrifuged in an SS-34 rotor at 15,000 rpm.

Step 4. DEAE-Cellulose Chromatography (pH 6.5). The clarified sample was applied at 120 ml per h to a DEAE-22 cellulose column (6.5 × 45 cm, 1 liter) preequilibrated with 0.01 M potassium phosphate buffer (pH 6.5) containing 1 mM disodium EDTA and 14 mM β-mercaptoethanol. A linear buffer gradient (0 → 0.2 M sodium chloride, 4 liter) was initiated. After an initial 400 ml of eluate was collected, 23 ml fractions were collected and assayed for 280 nm absorption and enzyme activity. Fractions showing activity were then electrophoresed on starch gels (pH 7.0) and analyzed by enzymatic staining before being pooled. Fractions containing only the most anodal isoenzymatic forms were pooled and concentrated with 70% ammonium sulfate as above. The resulting pellet was dialyzed 4 h against 500 ml 0.01 M potassium acetate (pH 6.0) containing 1 mM EDTA and 14 mM β-mercaptoethanol. The dialysis was repeated an additional 4 h and the sample centrifuged.

Step 5. 8-NAD$^{\oplus}$-Sepharose Affinity Chromatography. The above sample was eluted at 60 ml per h onto an 8-NAD$^{\oplus}$-Sepharose column (2.2 × 26 cm, 100 ml) that had been flushed with 1 M sodium chloride in buffer (0.01 M potassium acetate, pH 6.0, containing 1 mM disodium EDTA and 14 mM β-mercaptoethanol) and then preequilibrated with this buffer. The column was then left without flow for at least 30 min before washing with buffer until 280 nm absorption fell to a base level. At this point one column volume of 0.2 mM reduced NAD-pyruvate adduct in buffer, prepared as described by Everse et al. (18), was applied, followed by a buffer flush. Effluent fractions (16 ml) were monitored for enzyme activity and 280 nm absorption. The two fractions containing over 90% of the initial activity were combined, concentrated with ammonium sulfate, and dialyzed for storage against fresh initial extraction buffer.

Binding of Lactate Dehydrogenases to 8-AMP-Sepharose and 8-NAD$^{\oplus}$-Sepharose

Columns of either 8-AMP-Sepharose or 8-NAD$^{\oplus}$-Sepharose were prepared in disposable Pasteur pipettes (1.8 ml resin volume), washed with

1.0 M sodium chloride, 0.01 M potassium acetate (pH 6.0) containing 14 mM β-mercaptoethanol, and equilibrated by passing at least five column volumes of the acetate buffer without salt. Enzyme samples that had been dialyzed against the acetate buffer were applied in a volume of approximately 1 ml. After charging the column with the sample it was allowed to stand 15 min without flow. The column was then washed with at least 5 ml buffer and approximately 1 ml fractions were collected and assayed for enzyme activity. A flush of 0.8 mM NADH in buffer was then performed and the resulting fractions were assayed.

RESULTS AND DISCUSSION

Purification of Limulus *Lactate Dehydrogenase*

The results of a typical purification of *Limulus* muscle lactate dehydrogenase are shown in Table 1. The scheme represents a significant improvement over that reported earlier (9,11) in terms of yield, number of steps, and time required. The increased availability of purified enzyme makes certain physicochemical studies, such as amino acid sequencing and X-ray crystallography, more feasible.

Chromatography on DEAE-cellulose at pH 6.5 has eliminated the requirement for the aminoethyl-cellulose and the DEAE-cellulose separations at several pHs. At pH 6.5, with the proper buffer and salt gradient, it is possible sequentially to elute each of the three major isoenzymic muscle forms. Figure 1 shows the enzyme elution profile on DEAE-cellulose. Electrophoresis on starch gels has proven to be a useful way of monitoring

TABLE 1. **Summary of *Limulus* D-Lactate Dehydrogenase Purification Procedure**

Step[a]	Volume (ml)	Total enzyme units	Total protein (mg)	Special activity (and fold purification)	Recovery (%)
Cell-free extract	1,400	39,200	39,200	1.00	100
40–65% $(NH_4)_2SO_4$ cut	130	34,400	7,400	4.64	88
Sephadex G-100 column	60	30,000	1,800	16.7	77
DEAE-22 column (pH 6.5)	20	15,000	160	94	38
8-$NAD^{\oplus}$-Sepharose column	3.6	14,000	44	318	36

[a] All values except for the cell-free extract are after concentration and dialysis of the sample.

the DEAE fractions for isoenzymic contamination, as shown in Fig. 1. Column cuts can be made on the basis of the electrophoretic enzymatic pattern, thereby assuring the isolation of unique isoenzymic proteins.

The second major improvement in the purification scheme is the incorporation of an 8-NAD$^{\oplus}$-Sepharose affinity column step. Elution with the reduced NAD-pyruvate adduct, a specific competitive inhibitor of lactate dehydrogenases, affords a separation of the enzyme on the basis of unique biological specificity. Figure 2 shows the elution profile on 8-NAD$^{\oplus}$-Sepharose. No additional purification was achieved using a linear elution gradient (0 → 0.2 mM) of the adduct. Two additional advantages of the affinity material are that the enzyme need not be concentrated before application, and the enzyme is remarkably stable on the resin.

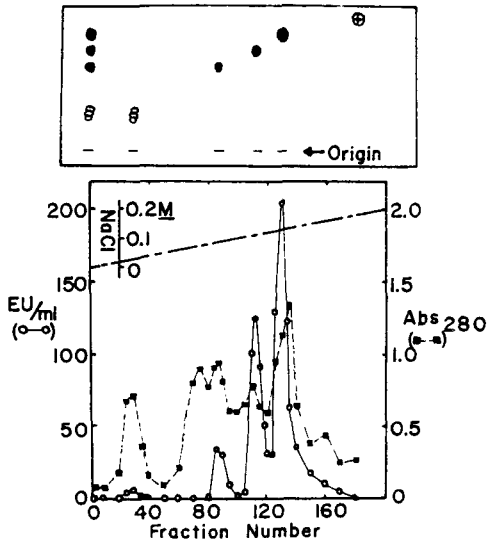


FIG. 1. A column elution and electrophoretic profile for *Limulus* muscle D-lactate dehydrogenase on DEAE-cellulose (pH 6.5). Experimental conditions are outlined in the Materials and Methods section. Bottom: Enzyme activity (EU/ml) and protein (Abs_{280}) are plotted against fraction number (after the initial collection of 400 ml) and sodium chloride gradient concentration. Top: the isoenzymic pattern resulting from the electrophoresis on starch gels (pH 7.0) and enzymatic staining of the pre-DEAE sample (extreme left) and enzyme peak fractions is shown. Fractions correspond to those shown directly below in the column elution profile.

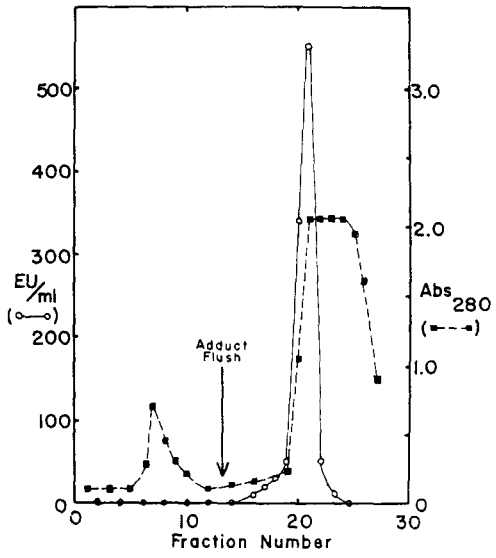
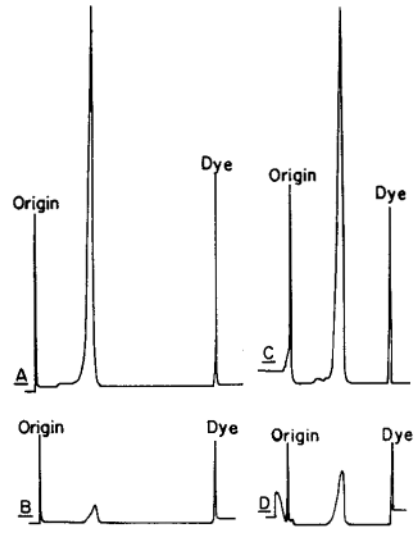


FIG. 2. A column elution profile for *Limulus* muscle D-lactate dehydrogenase on 8-(6-aminohexyl)-amino-NAD$^{\oplus}$-Sepharose. Elution conditions are given in the Materials and Methods section. The absorbance at 280 nm after the addition of the NAD-pyruvate adduct (arrow) (0.24 mM) is primarily due to the adduct.

Affinity chromatography is employed as a *terminal* step primarily because of the very large amounts of initial protein (39 g) and a limited quantity of the affinity material. Also, studies initially reported by Lee and Kaplan (19) showed that ammonium sulfate fractionation and affinity chromatography alone resulted in only a tenfold purification (15% purity), necessitating additional isolation steps. Another reason for the DEAE-cellulose step is the resulting resolution of the isoenzymic forms, which is not accomplished with the affinity chromatography.

The enzyme appears to be at least 98% pure, as based upon polyacrylamide disc gel electrophoresis at pH 9.5 and 8.0. Densitometry tracings and photographs of protein and enzyme-stained gels are shown in Fig. 3. Electrophoresis on starch gels at pH 7.0 reveals only one protein band, which corresponds to the enzymatically active band. The molar extinction coefficient of the pure enzyme at 280 nm, based on Lowry protein analyses, is 0.50×10^5, which agrees with that reported previously (9,11). Additionally, material passed over a DEAE-cellulose, pH 6.0 column (the terminal



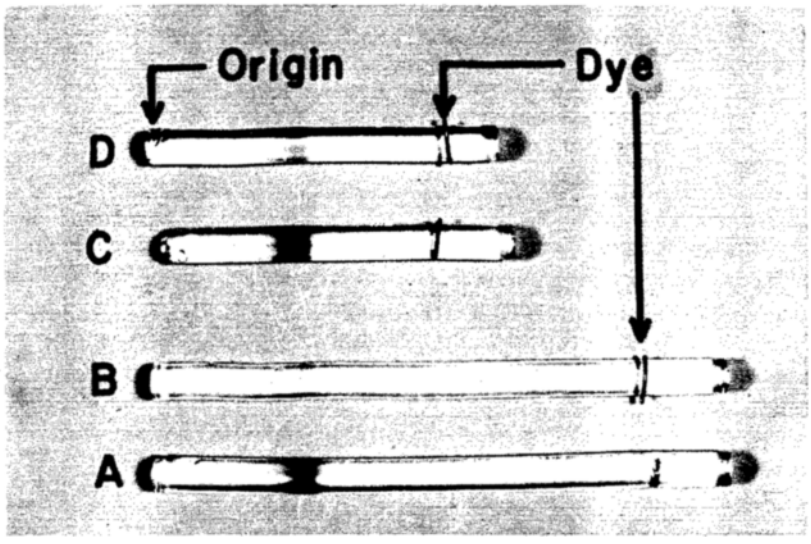


FIG. 3. Densitometry tracings and photographs of *Limulus* D-lactate dehydrogenase on polyacrylamide gels. A: Electrophoresis at pH 8.0, amido black stain of protein. B: Electrophoresis at pH 8.0, enzyme stain. C: Electrophoresis at pH 9.5, protein stain. D: Electrophoresis at pH 9.5, enzymatic stain. Stainless steel pins denote the position of the marker dye, bromophenol blue. The gels were overloaded with protein (35 μg) in order to see possible protein contaminants and enzyme activity.

step in the previous purification procedure) showed no increase in specific activity and a constant specific activity across the enzymatic peak.

Binding of Lactate Dehydrogenases to 8-AMP-Sepharose and 8-NAD$^{\oplus}$-Sepharose

Lee et al. (12,19) were the first to report that AMP and NAD$^{\oplus}$ bound through the C_8 position of the nucleic acid ring to Sepharose were both effective for the binding and subsequent elution of *Limulus* and *Haliotus* (abalone) muscle lactate dehydrogenase. Enzymes at the stage of a dialyzed 40–65% saturated ammonium sulfate fraction and at apparent purity are both effectively bound, as shown in Table 2. These results establish the utility of the above ligands for the isolation of *Limulus* and *Haliotus* lactate dehydrogenases. We do, however, wish to point out that neither affinity material was effective in binding the *Limulus* enzyme in experiments in which the enzyme was gently mixed in a batchwise manner with the resin, followed by separation of the supernatant and beads by centrifugation. Under identical conditions, using 8-AMP-Sepharose, approximately 60% of the D-lactate dehydrogenase in an abalone cell-free extract and 85% of a purified chicken heart L-lactate dehydrogenase samples were bound. These results show that the extension of results from batch experiments may not be reliable indicators of actual column binding effectiveness. Amino acid sequence and X-ray crystallographic analyses suggest that the AMP binding domains of several dehydrogenases and kinases are homologous (13). Affinity binding studies using 8-AMP-and 8-NAD$^{\oplus}$-Sepharose on several vertebrate L-lactate dehydrogenases and the D-lactate dehydrogenases

TABLE 2. **Summary of *Limulus* (Horseshoe Crab) and *Haliotus* (Abalone) Muscle D-Lactate Dehydrogenase Binding to 8-AMP- and NAD$^{\oplus}$-Sepharose Columns**

Enzyme sample	Affinity ligand	Enzyme units applied	Enzyme units in buffer wash	Enzyme units in NADH flush
Limulus, crude	AMP	10.0	0	6.6
40–65% $(NH_4)_2SO_4$	NAD	126.0	11.2	102.0
Limulus, purified	AMP	10.0	0	7.5
	NAD[a]	270.0	0	252.0
Haliotus, crude	AMP	6.5	0	5.6
40–65% $(NH_4)_2SO_4$	NAD	8.0	1.0	6.2

[a]Values taken from the purification scheme described in this paper and adjusted for a column volume of 1.8 ml. Final elution was with the NAD-pyruvate adduct.

from the abalone (12) and *Limulus*, reported in this paper, also suggest a considerable degree of structural homology in the AMP binding domain.

Lee and Kaplan also report that N^6-(6-aminohexyl)-amino-5′-AMP Sepharose is as effective as 8-AMP-Sepharose for purifying dogfish lactate dehydrogenase. However, the former affinity material is ineffective in binding abalone and lobster lactate dehydrogenase (19). This has also been reported to be the case for *Limulus* lactate dehydrogenase (20). These results suggest that major differences in the AMP binding site may exist for different lactate dehydrogenases. The comparative results from batchwise binding reported above also point to this possibility. Further quantitative binding studies on a number of lactate dehydrogenases using modified immobilized AMP ligands will serve as a valuable complementary tool for the analysis of the evolution and degrees of homology of the pyridine nucleotide-linked enzymes.

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