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PURIFICATION OF NAD-SPECIFIC ISOCITRATE DEHYDROGENASE FROM PORCINE HEART

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SUMMARY

An NAD^+-dependent isocitrate dehydrogenase (*threo*-D_S-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41) has been purified more than 500-fold from porcine hearts. Disc gel electrophoresis reveals that protein with NAD^+-specific isocitrate dehydrogenase activity accounts for 80% of the total protein; one major and two minor activity bands are observed. As compared to the $NADP^+$-specific isocitrate dehydrogenase isolated from the same species and organ, the NAD^+-dependent enzyme differs in molecular weight, isoelectric point and reaction to antisera prepared against the purified $NADP^+$ enzyme.

Most mammalian tissues contain two isocitrate dehydrogenases: one specifically using NAD^+ as coenzyme (*threo*-D_S-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41); the other, $NADP^+$ (*threo*-D_S-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42). As isolated from a variety of species[1-6], the enzymes have been shown to differ in their molecular weights[7,8], as well as in their response to metabolic regulation. In general, the NAD^+-dependent enzyme has been reported as more sensitive to activation by citrate and nucleotides, although the activator varies with the source of the enzyme[1,9,10]. Curiously, the two enzymes have never been purified from the same source; therefore, comparisons between the NAD^+ and $NADP^+$ enzymes have been complicated by species differences. A homogeneous preparation of the $NADP^+$-dependent isocitrate dehydrogenase from pig heart has been under intensive investigation in this laboratory; its physical properties have been examined and amino acid residues in the active site have been identified[6,11-14]. This paper presents the purification of the corresponding NAD^+-dependent isocitrate dehydrogenase from pig heart.

Assay

Isocitrate dehydrogenase activity was assayed spectrophotometrically at 340 nm. The standard assay contained 0.99 mM NAD^+, 1.33 mM $MnSO_4$, 20 mM DL-isocitrate, 33.0 mM Tris–acetate buffer (pH 7.19) and enzyme in a total volume of 1.0 ml. One enzyme unit is defined as the amount of enzyme which catalyzes the

formation of 1 μmole NADH/min at 22°. Protein was determined by the method of WARBURG AND CHRISTIAN[15].

Disc gel electrophoresis

Disc electrophoresis was conducted using the buffer system of WILLIAMS AND REISFELD[16], with the separating gel at pH 7.5. All other solutions were prepared in accordance with the Canalco Co. formulation except that glycerol was used in the stacking gel and the 5% separating gel was diluted from a stock solution containing 1% bisacrylamide (G. FAIRBANKS, unpublished data). The gels were stained for protein with Amido schwarz and for enzymatic activity in the dark with a solution containing 8.63 mM isocitrate, 0.171 mM NAD^+, 4.49 mM $MnSO_4$, 16.4 mM sodium citrate, 0.170 mM phenazine methosulfate, 0.630 mM nitro blue tetrazolium, 35.9% glycerol, and 285 mM Tris–acetate (pH 7.19).

Purification procedure

Whole porcine hearts obtained in ice from Pel-Freez Biologicals, within 48 h after slaughter were used as starting material. All Tris–acetate buffers were diluted from a 1.0 M solution (pH 7.19); citrate buffers were diluted from a 0.5 M sodium citrate solution (pH 6.5). Unless otherwise indicated, all procedures were conducted at room temperature and centrifugation was at 23 300 × *g* for 15 min at 18°.

40 cold hearts were cut into small pieces and homogenized in a Waring blender in 0.033 M Tris with 0.25 M sucrose for 2 min in the ratio of 200 g wet weight of tissue to 600 ml of solution. After centrifugation, the precipitate was discarded and the supernatant filtered through cheese cloth. The enzyme was precipitated by the addition of 15 l of saturated $(NH_4)_2SO_4$ solution to each 10-l enzyme solution (Step 2). The suspension was stirred for 30 min, centrifuged, and the precipitate was dissolved in 1.7 l of a 0.045 M Tris buffer, containing 4.5 mM Mn^{2+}, 9% glycerol and 10% $(NH_4)_2SO_4$. The purification could be stopped here if the enzyme solution was frozen at −90°.

In the second $(NH_4)_2SO_4$ step, saturated salt solution was added in the ratio of 0.15 ml to 1.0 ml enzyme solution. After the suspension was stirred for 30 min and centrifuged, the precipitate was discarded and an additional 0.18 ml was added to precipitate the enzyme, which was dissolved in approx. 200 ml of 0.045 M Tris buffer containing 4.5 mM Mn^{2+}, 9% glycerol and 10% $(NH_4)_2SO_4$. This enzyme solution was aliquoted and frozen at −90°.

A 30-ml aliquot of the above solution was dialyzed overnight against 18 l of a 0.02 M citrate buffer containing 5 mM Mn^{2+} and 10% glycerol. The dialyzed enzyme was diluted with 40 ml of a solution containing 40% glycerol and 5 mM Mn^{2+} and applied to a DEAE-cellulose column (H. Reeve Angel, DE52) as described in Fig. 1. The positively charged $NADP^+$ isocitrate dehydrogenase was eluted in the wash and peak NAD^+ enzyme fractions were precipitated with 70% $(NH_4)_2SO_4$, dissolved in 1.0 ml of 0.05 M citrate buffer containing 40% glycerol and 5 mM Mn^{2+} and dialyzed against the same buffer overnight at 4°.

The solution from Step 5 was applied to a Sepharose 6B column (1.8 cm × 62 cm) equilibrated at 4° with 0.05 M citrate buffer containing 20% glycerol and 5 mM Mn^{2+} and eluted at a rate of 5.0 ml/h. Fractions of peak enzymatic activity

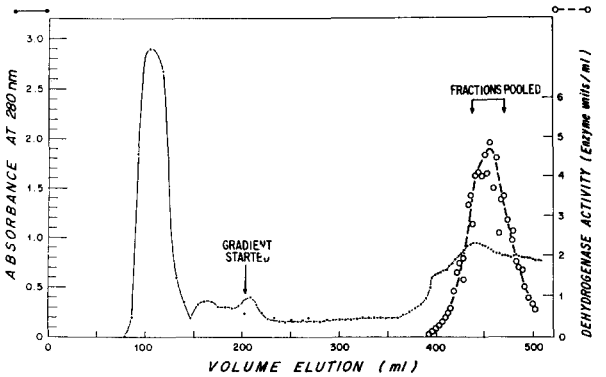

Fig. 1. Chromatography on DE52. The DE52 column (2.5 cm × 38.8 cm) was equilibrated with 0.012 M citrate (pH 6.5), 5 mM Mn^{2+} and 10% glycerol. After washing with 200 ml of the starting buffer at 80 ml/h, a linear gradient to 0.05 M citrate was started. Both reservoir and mixing flasks contained 160 ml of citrate buffer with 5 mM Mn^{2+} and 10% glycerol.

were pooled, precipitated with 70% $(NH_4)_2SO_4$, redissolved in 1 ml and dialyzed as above.

The purification scheme outlined in Table I results in a 500-fold increase in specific activity with an overall yield of 10.6%. Although the NAD+ enzyme was isolated from the supernatant fraction after homogenization of the pig hearts in a Tris buffer with 0.25 M sucrose, its mitochondrial origin[3,5,17,18] is not contradicted since the mitochondrial enzymes succinate–cytochrome *c* reductase and glutamate dehydrogenase[19] can also be detected in this supernatant fraction. The large increase in total enzyme units after Step 2 may be due to the removal of an inhibitor or another enzyme which interferes with the assay. Intact hearts frozen at −90° yielded no activity even when purification procedures were carried through Step 2. Although NAD+ isocitrate dehydrogenases have been notorious for their instability, the addition of glycerol and Mn^{2+} to buffers greatly enhanced the stability of the pig heart

TABLE I

PURIFICATION OF NAD+-SPECIFIC ISOCITRATE DEHYDROGENASE FROM PORCINE HEART

The data given for Steps 1–3 have been normalized to the 30 ml of enzyme solution applied to the DE52 column in Step 4.

	$\frac{A_{280\ nm}}{A_{260\ nm}}$	*Total protein (mg)*	*Total enzyme units*	*Specific activity (enzyme units/mg)*
Tris–sucrose homogenate (Step 1)	0.49	6290	56.9	0.0250
First $(NH_4)_2SO_4$ precipitation (Step 2)	1.13	6060	356	0.0638
Second $(NH_4)_2SO_4$ precipitation (Step 3)	1.35	961	322	0.334
Enzyme solution applied to DE52 (Step 4)	1.30	876	230	0.259
Concentrated enzyme after DE52 column (Step 5)	1.42	21.1	83.4	3.96
Pooled fractions from Sepharose 6B column (Step 6)	1.85	2.92	38.1	13.05

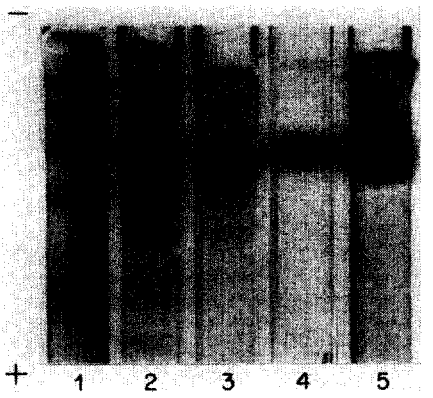

Fig. 2. Disc gel electrophoresis. Enzyme samples are as follows: gel 1, Step 4; gel 2, Step 5; gels 3–5, Step 6. Gels 3 and 4 contain $1.83 \cdot 10^{-7}$ mg of protein, gel 5 contains $7.34 \cdot 10^{-7}$ mg. Gels 1–3 are stained for protein, gels 4 and 5 are stained for enzymatic activity.

enzyme at 4° and 23°. Enzyme solutions after Step 6 remain stable at −90° for 6 months. In contrast, crude enzyme preparations lose 30% of their activity after storage under the same conditions, as reflected in Table I by the loss of activity between Steps 3 and 4. Because of the instability of the crude enzyme, attempts to increase the scale of the DE52 chromatography resulted in poor recoveries.

Disc gel electrophoresis of samples from Steps 4–6 are shown in Fig. 2. A comparison of gels 1–3 shows that most of the proteins of higher mobility are removed by the purification procedure. However, these gels do not adequately represent the 500-fold purification because many of the proteins originally present, such as the $NADP^+$-dependent isocitrate dehydrogenase, are positively charged and do not enter the gel in this buffer system. After Step 6, one major and two minor activity bands are observed in gel 5 which correspond to a major protein band and two minor protein bands of lower mobility in gel 3. The minor bands which are enzymatically active do not result from partial denaturation since enzyme solutions totally inactivated by incubation at room temperature exhibit the same protein band pattern. However, these minor activity bands may represent aggregation products of the pig heart enzyme, similar to those of the beef heart enzyme[1,8].

Determination of molecular weight by gel filtration

1 ml of each sample was applied to a Sepharose 6B column (2.3 cm × 35.5 cm) eluted with a 0.02 M citrate buffer containing 5 mM Mn^{2+} and 10% glycerol. The molecular weight of the NAD^+ isocitrate dehydrogenase was determined from the plot of the logarithm of molecular weight of protein standards *vs.* K_d. The molecular weight of the NAD^+ isocitrate dehydrogenase is estimated to be 340 000 if the protein is assumed to be similar in shape to the standards (Fig. 3). This value is close to the molecular weights of beef heart[8] and yeast[20] NAD^+ isocitrate dehydrogenases.

The NAD^+ isocitrate dehydrogenase uses *threo*-D_s-isocitrate exclusively and exhibits no activity when $NADP^+$ is substituted for NAD^+ in the standard assay. Rabbit antisera to homogeneous $NADP^+$ isocitrate dehydrogenase[14] forms a precipitin line of identity between the $NADP^+$ enzyme and crude preparations of the NAD^+

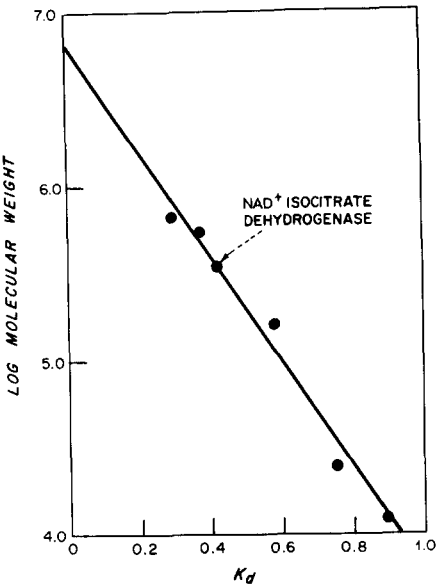

Fig. 3. Gel filtration on Sepharose 6B. The points represent the K_d values for the NAD+ isocitrate dehydrogenase and the following standards: thyroglobulin (670 000), β-galactosidase (540 000), aldolase (160 000), chymotrypsinogen (25 000) and cytochrome *c* (12 400).

activity. However, no line results after Step 5 of the purification scheme, indicating that the two proteins have no available antigenic determinants in common. Extensive kinetic studies to facilitate a more detailed comparison between the NADP+ and the NAD+ enzymes are now in progress and will be reported separately.

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