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NADP-SPECIFIC ISOCITRATE DEHYDROGENASE FROM *ESCHERICHIA COLI*

V. MULTIPLE FORMS OF THE ENZYME

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Two forms of NADP-specific isocitrate dehydrogenase (*threo*-D_S-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) in *Escherichia coli* have been resolved by polyacrylamide gel isoelectric focusing and electrophoresis. Incubation of the enzyme with Mn^{2+} plus isocitrate prior to focusing resulted in the formation of an additional form of the enzyme, presumably the enzyme-manganese-isocitrate complex. Glycerol, a cryoprotectant used to stabilize the enzyme during purification and storage, also stabilized it during focusing, but was not necessary during electrophoresis. Thin-layer gel filtration did not reveal any differences in molecular weight between the different species of isocitrate dehydrogenase.

Introduction

Both NAD and NADP-specific isocitrate dehydrogenase are present in animal and plant tissues, whereas only the NADP-specific isocitrate dehydrogenase (*threo*-D_S-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) occurs in most bacteria, including *Escherichia coli*. Regulation of the activity of the NADP-specific enzyme, unlike the allosterically controlled NAD-specific form [1], is not well understood. When *E. coli* adapts to growth on acetate, the glyoxylate cycle enzymes are induced and isocitrate dehydrogenase activity declines [2,3]. Recent studies [4] have shown that the enzyme in *E. coli* is phosphorylated concomitant with this reduction in enzymatic activity. The enzyme is also subject to concerted inhibition by glyoxylate and oxaloacetate under these growth conditions [5]. Few studies have been reported, however, on the regulation of the activity of this enzyme under conditions where the glyoxylate cycle is not induced. Isozymes of isocitrate dehydrogenase could provide a mechanism for the metabolic control of enzymatic activity.

There have been few reports of isozymes of isocitrate dehydrogenase in bacteria. Two forms of the NADP-specific enzyme were reported in *Acinetobacter lwoffi* [6] and in *Acinetobacter calcoaceticus* [7]. The *Acinetobacter* enzyme is the only reported case of a bacterial NADP-specific isocitrate dehydrogenase subject to allosteric control [8]. Electrophoretically separable forms of the enzyme were also observed in crude extracts of the marine bacterium, *Leucothrix mucor*, with as many as three active bands present in a given strain [9]. Further characterization of these different forms of isocitrate dehydrogenase has not been reported.

Resolution of two forms of isocitrate dehydrogenase from *E. coli*, grown on limiting glucose, by ion-exchange chromatography prompted the suggestion that the metabolic control of the enzyme might be mediated by these different forms of the enzyme [10]. Isoelectric focusing, a powerful technique for the resolution of proteins with slight differences in net charge, was chosen as an analytical tool to further characterize these multiple forms of the enzyme in *E. coli*.

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Materials and Methods

Materials. Chemicals were obtained from the indicated suppliers. Acrylamide (electrophoresis grade), *N,N'*-methylenebisacrylamide (BIS), *N,N,N',N'*-tetramethylethylene diamine and diallyltartardiamide (DATD) from Eastman; NADP, ammonium persulfate, Bistris, TES, phenazine methosulfate, Coomassie brillant blue G-250, DL-isocitrate (trisodium salt), nitroblue tetrazolium, ribonuclease, myoglobin, pepsin and human transferrin from Sigma Chemical Co.; 40% (w/v) pH 4–6 range ampholines from Bio-Rad Laboratories, 40% (w/v) pH 3–6 and pH 6–8 range ampholines from LKB Produktor; glycerol (certified A.C.S.) from Fischer Scientific; riboflavin, citric acid and bromophenol blue from Baker; Sephadex G-75 Superfine and G-200 Superfine from Pharmacia Fine Chemicals; bovine serum albumin and ovalbumin from Mann Research Laboratory. Inorganic salts were of reagent grade. Distilled, deionized water (Hydro Service and Suppliers, Inc.) was used to prepare all aqueous solutions.

Enzyme purification. The methods for growing *E. coli*, strain F143/KL259, for purifying the NADP-specific isocitrate dehydrogenase and the criteria employed for homogeneity were reported previously [11]. Protein was determined by the method of Bradford [12] with bovine γ-globulin as standard.

Polyacrylamide gel isoelectric focusing. Polyacrylamide gel isoelectric focusing was performed by a method similar to that described by McCormick et al. [13]. Gels were either 5 or 7.5% T, 15% C with a combination of crosslinking agent (80% DATD/20% BIS). Ampholytes were used at a final concentration of 2% (w/v) and covered the pH ranges indicated in the figure legends. For polymerization mixtures that were to contain glycerol, 20% (v/v) glycerol was included in gel solutions before casting in 0.5 internal diameter × 15 cm glass tubes. Anolyte (lower chamber) and catholyte were 10 mM formic acid and 1 mM NaOH, respectively. Samples of isocitrate dehydrogenase were transferred from storage buffer, buffer A, (60 mM dibasic sodium phosphate/15.5 mM citric acid/10 mM $MgCl_2$/50 mM KCl/0.02% NaN_3/20% (v/v) glycerol, pH 62.) to a lower ionic strength buffer, buffer B, (7.0 mM dibasic sodium phosphate/1.8 mM citric acid/10 mM $MgCl_2$/0.02% NaN_3/20% glycerol, pH 6.0) by means of a Sephadex G-25 column. Samples were made 2% with respect to carrier ampholytes and were layered on top of the gels. Isoelectric focusing was conducted at 10°C at the voltages specified in the individual experiment. After focusing, the gels were removed from the tubes and stained for protein or enzyme activity (see below). At least one gel from each run was used to determine the pH profile. The gel was sliced into 2 mm discs and each disc eluted with 100 μl distilled, deionized water in a microtiter plate. The pH of the solution in each well was measured at room temperature.

The effect of preincubation of the enzyme prior to isoelectric focusing was also investigated. For conditions of preincubation and focusing, see Fig. 3. Stock solutions of NADP, isocitrate, $MnCl_2$ and citrate were 100 mM, pH 6.0.

Discontinuous polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed in multiphasic buffer system No. 3328 as described by Jovin et al. [14] except for the addition of 20% (v/v) glycerol to the gels. After electrophoresis at 1.5 mA/gel for 4 h at room temperature, gels were stained for protein or enzyme activity (see below).

Protein and activity stains. Gels were stained immediately after isoelectric focusing or electrophoresis with Coomassie brillant blue G-250 in 3.5% perchloric acid [15]. Enzyme activity was visualized by coupling the isocitrate-dependent reduction of NADP to formazan production [16].

Thin-layer isoelectric focusing. Isoelectric focusing was conducted in a thin-layer electrofocusing chamber as described by Radola [17]. Platinum ribbon electrodes were used in contact with electrode strips soaked in 2% carrier ampholytes. The gel bed was prefocused at 10°C for 4–8 h at 200 V, samples were than applied 8–9 cm from the cathode.

After focusing, replicate prints were taken with strips of Whatman 3 MM paper which were then dried, washed and stained for protein with Coomassie brillant blue G-250 [18]. The position of enzyme activity in the gel bed was also determined by a print technique. Strips of Whatman 3 MM paper, maintained in the dark, were saturated three times (air dried between applications) with the reaction mixture used to stain polyacrylamide gels for activity (see above). The dry strip was applied to the gel bed for 1–2 min to take up the enzyme and then suspended

in the dark for 5–10 min to permit the reaction to proceed. The reaction was stopped by placing the strip in 7% acetic acid and it was then air dried. The pH profile was determined at 25°C directly in a gel track containing enzyme employing a pH electrode.

For subsequent molecular weight determination by gel filtration (see below), isocitrate dehydrogenase was recovered from the Sephadex layer. The protein band focusing near pH 4.7, and the broad band above pH 5.0 were treated separately. A small amount of buffer A was added to the Sephadex, the slurries packed in small columns, and each eluted with 2 bed vol. buffer A. The eluants were concentrated in an Amicon Diaflo apparatus with a PM-10 membrane and dialyzed against three changes of 100 X volume of buffer A to remove carrier ampholytes.

Thin-layer gel filtration. The molecular weight of isocitrate dehydrogenase was determined by thin-layer gel filtration as described by Radola [19] with a 0.8 mm layer of Sephadex G-200 Superfine swollen

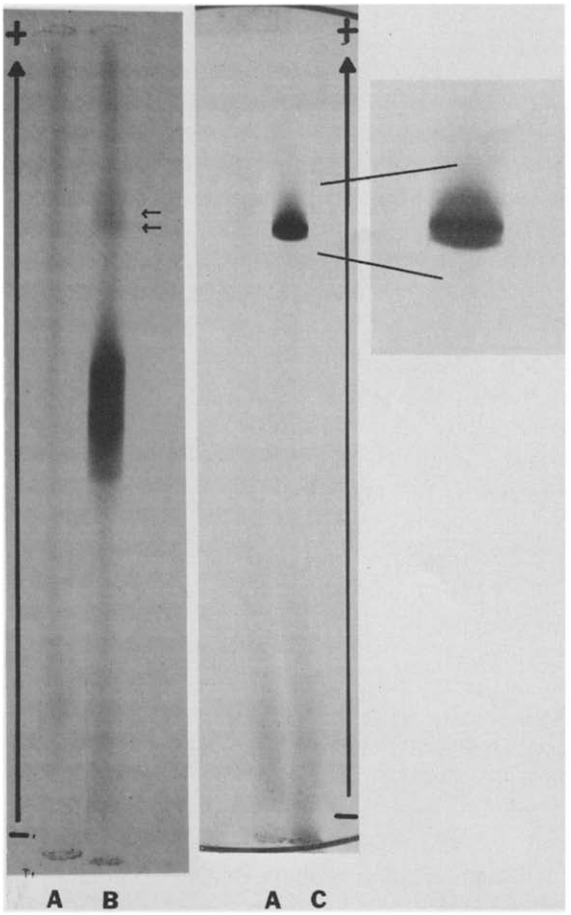

Fig. 1. Polyacrylamide gel isoelectric focusing. NADP-specific isocitrate dehydrogenase was subjected to isoelectric focusing in cylindrical polyacrylamide gels (5% T, 15% C) containing 2% ampholytes (1.6% pH 4–6, 0.4% pH 6–9). Gel A was a blank; Gel B received 100 μg enzyme; Gel C is identical to Gel B except 20% (v/v) glycerol was included in the gel solution before casting. A closeup of Gel C shows the two protein bands more clearly. Focusing was conducted at 10°C for 20 h at 100 V, 2 h at 200 V and 1 h at 400 V.

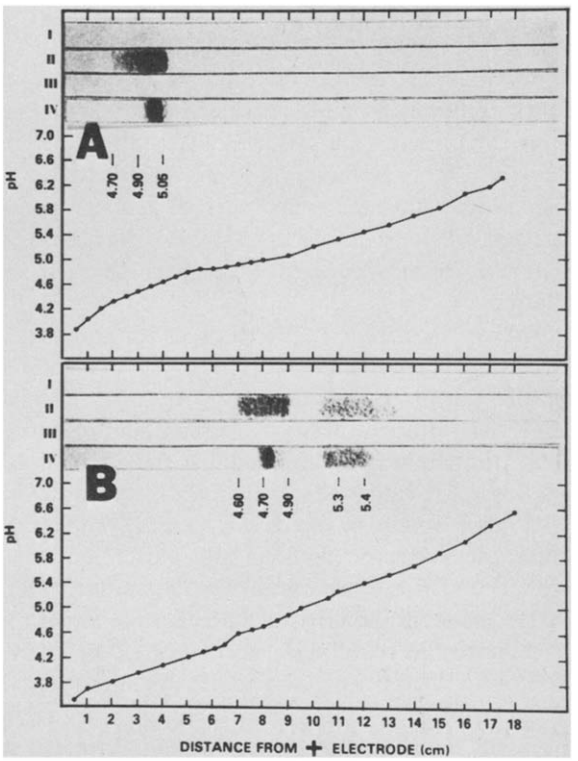

Fig. 2. Thin-layer isoelectric focusing. Samples of the enzyme were subjected to thin-layer isoelectric focusing in a 20 cm × 1 mm layer of Sephadex G-75 superfine containing 1.6% pH 4–6 and 0.4% pH 6–8 ampholytes with 20% glycerol (A) or 2.6% pH 3–6 and 0.4% pH 6–8 ampholytes without glycerol (B). Samples of enzyme were applied in 18 mm tracks (A: 0.50 mg/track, B: 0.58 mg/track). Focusing was conducted at 10°C at 200 V for 22 h and 600 V for 2 h (A) or at 200 V for 19 h and 800 V for 3 h (B). The pH profiles were read directly in the Sephadex layers. Paper prints of the gel layer were taken after focusing and stained for isocitrate dehydrogenase activity (I, II) or for protein (III, IV).

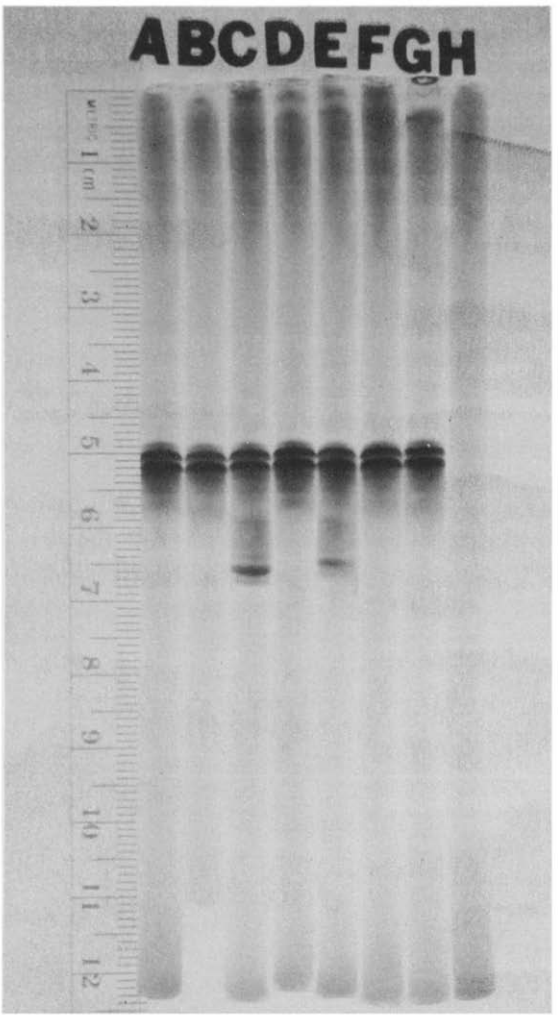

Fig. 3. Effect of substrates on isocitrate dehydrogenase focusing pattern. Isocitrate dehydrogenase (110 μg) applied to gels A-F had been dialyzed against buffer B, then preincubated for 30 min at room temperature with the indicated substrates or substrate analogs. A, no addition; B, 5 mM NADP; C, 5 mM isocitrate; D, 5 mM $MnCl_2$; E, 5 mM isocitrate and 5 mM $MnCl_2$; F, 10 mM citrate and 5 mM $MnCl_2$ and 5 mM NADP; G, no addition and H, blank gel. Enzyme applied to gel G was in buffer A, which contains citric acid and magnesium. Focusing was conducted as described in Fig. 2 except the pH 3–6 ampholytes used had been previously fractionated to obtain a narrower pH range (4–5) in these gels. Preincubation of the enzyme with isocitrate, either with (E) or without (C) added metal ion, resulted in the appearance of a new band (arrow) which has a p*I* approx. 0.2 pH units more acid than the isocitrate dehydrogenase doublet.

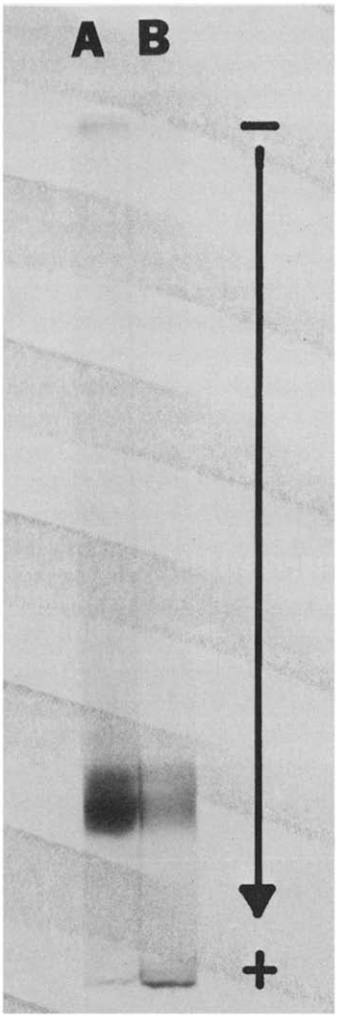

Fig. 4. Discontinuous polyacrylamide gel electrophoresis. Electrophoretic separation of two forms of isocitrate dehydrogenase was achieved in a multiphasic buffer system (see Materials and Methods). Gel A was stained for protein with Coomassie brillant blue G-250 and Gel B was stained for enzyme activity for 10 min.

in buffer C (50 mM sodium phosphate/100 mM KCl/ 10 mM $MgCl_2$/0.02% NaN_3, pH 7.0). The layer was equilibrated overnight and developed (6 h) in the same buffer at room temperature at a 10° angle. Samples (10 μl) of isocitrate dehydrogenase and standard proteins (1% in buffer C) were applied to the layer from the edge of a glass coverslip. Protein standards used (and their molecular weights) were ribonuclease A (13 700), myoglobin (17 800), pepsin

(35 000), ovalbumin (45 000), bovine serum albumin monomer and dimer (67 000; 134 000) and human transferrin (81 000). A paper replicate print of the plate was taken, air dried and stained for protein [15]. The migration distance for each protein was normalized with respect to myoglobin. The reciprocal of the relative migration distance (1/R MYO) plotted as a function of the log molecular weight of the standard proteins generated a standard curve [20] from which the molecular weight of isocitrate dehydrogenase was determined.

Results

Isoelectric focusing of *E. coli* NADP-dependent isocitrate dehydrogenase in polyacrylamide gels (Fig. 1) revealed two faint bands (arrows) of protein with isoelectric points (p*I*) of 4.65 and 4.70 in addition to a very broad smear of protein with p*I* values ranging from 5.1–5.5 (Fig. 1, Gel B). The broad, high p*I* band was completely eliminated by incorporation of 20% (v/v) glycerol into the gels (Fig. 1, Gel C) and the enzyme focused into the two bands at 4.65 and 4.70 (Fig. 1, closeup).

In order to test for possible artifacts of isoelectric focusing in polyacrylamide gels, electrofocusing was also carried out in granular gels. Isoelectric focusing of the enzyme in thin layers of Sephadex (Fig. 2) yielded similar patterns of activity (II) and protein (IV) in the presence (A) and absence (B) of glycerol as were obtained by focusing in polyacrylamide gels.

When the enzyme was preincubated with manganese plus isocitrate and subsequently subjected to isoelectric focusing in the presence of 20% glycerol, an additional protein band was observed 0.2 pH units below the lower of the two isozymes (Fig. 3).

The improved separation of the the two isozymes, when compared to Fig. 1, results from the use of pH 4–5 ampholytes obtained by fractionation of pH 3–6 range ampholytes in a preparative 110 ml LKB column. Incubation of the enzyme solution (which contained 1 mM $MnCl_2$) with 5 mM isocitrate (Fig. 3C) or with 5 mM each isocitrate and $MnCl_2$ (Fig. 3E) produced similar patterns.

Thin-layer gel filtration did not reveal any differences in the molecular weight of the species separated by isoelectric focusing. The molecular weights (80 000–90 000) determined for isocitrate dehydrogenase recovered after thin-layer isoelectric focusing in the absence of glycerol (Fig. 2B) from the doublet region (p*I* 4.7) and from the broad band (p*I* 5) were indistinguishable. There was no evidence of protein or enzymatic activity at the position on the gel filtration plate where subunits or aggregates of the enzyme would be expected. Electrophoretic separation of isocitrate dehydrogenase in the multiphasic buffer system (Fig. 4) also resolved two forms of the enzyme.

Discussion

Denaturation during isoelectric focusing. The unusually broad band of protein (p*I* 5.1–5.5, Fig. 1, Gel A) obtained after isoelectric focusing of isocitrate dehydrogenase in the absence of glycerol represents forms of the enzyme which have undergone varying degrees of denaturation during focusing, or could be due in part to protein insolubility on approaching isoelectric pH. The changes involved must not have radically altered the active site, since catalytic activity is retained.

It might also be argued that aggregation of the enzyme during isoelectric focusing could mask some previously exposed dissociable groups and thereby alter the p*I* of the protein. Thin-layer gel filtration experiments failed to reveal differences in molecular weight for the multiple forms of the enzyme separated by isoelectric focusing. It would appear that molecular weight aggregates, such as those found for the *Acinetobacter* isocitrate dehydrogenase [6,7], are not the basis for the multiple forms of the *E. coli* enzyme.

Effects of glycerol. The incorporation of glycerol into the isoelectric focusing medium caused all of the *E. coli* isocitrate dehydrogenase to focus into two closely spaced bands of protein and activity (Fig. 1–3). It should be emphasized that the enzyme was focused for the time required to reach isoelectric equilibrium at the temperature, gel concentration and pH range employed. Under these conditions, the protein pattern and pH values obtained were consistent and reproducible. The glycerol appears to protect the enzyme against denaturation or precipitation in the low ionic strength focusing environment. This suggestion is supported by the observation that the enzyme precipitates upon dialysis against distilled water but

not when dialyzed against 20% glycerol [11].

Effect of Mn^{2+}-isocitrate. Incubation of *E. coli* isocitrate dehydrogenase with Mn^{2+}-isocitrate prior to isoelectric focusing produced a new species of the enzyme (Fig. 3C and E). This band presumably represents the ternary isocitrate dehydrogenase-Mn^{2+}-isocitrate complex previously identified by EPR studies of the pig heart enzyme [21]. The fact that all of the enzyme was not converted to the E.S. form and the appearance of a continuum of staining material between the E.S. band and the two isozymes would suggest that the substrate is dissociating during focusing. Although it has been shown that citrate stabilizes *E. coli* isocitrate dehydrogenase and is an inhibitor of the enzyme [16], it will not substitute for isocitrate in this effect, nor will NADP.

Isozymes vs. artifacts. Two distinct forms of NADP-specific isocitrate dehydrogenase were resolved by isoelectric focusing in polyacrylamide gels with glycerol added to stabilize the enzyme (Fig. 1 and 3). The resolution by isoelectric focusing and polyacrylamide gel electrophoresis of more than one species from a preparation that appears homogeneous by other criteria raises the question of artifacts. There is the potential for artifact formation in polyacrylamide gel systems [22]. Since isoelectric focusing of the enzyme in Sephadex (Fig. 2) and polyacrylamide (Fig. 1 and 3) gave very similar results, the observed heterogeneity could not be a function of the polyacrylamide gel system. Second, ampholytes have been implicated in the anomalous behavior of yeast isocitrate dehydrogenase on sucrose or glycerol gradient isoelectric focusing [23]. The author suggested that the multiple peaks observed were artifacts generated by exposure of the enzyme to ampholytes. The observed pattern of multiple activity peaks shifted with the stage of purification and depended upon the ratio of ampholyte to protein. This was not the case for *E. coli* isocitrate dehydrogenase since the patterns obtained by focusing identical samples of the enzyme in polyacrylamide gels containing 1,2 and 4% ampholine were indistinguishable (unpublished data). Third, the process of isoelectric focusing itself has been reported to cause changes in the amino acid composition of some proteins [24]. In order to verify that the two isozymes of isocitrate dehydrogenase separated by isoelectric focusing (Fig. 1 and 3) were not artifacts of the method, an alternate analytical procedure was sought. The presence of two isozymes of *E. coli* isocitrate dehydrogenase was confirmed by separation of two bands of activity and protein by polyacrylamide gel electrophoresis in a multiphasic buffer system (Fig. 4).

Molecular weight studies under denaturing and nondenaturing conditions [11,16,26] have demonstrated that isocitrate dehydrogenase is a dimer composed of subunits which have the same molecular weight. In contrast to the NADP-specific enzyme from beef heart [26], the *E. coli* enzyme yields a single band of protein after SDS electrophoresis in a continuous [25] or discontinuous [11] system. Although this does not exclude the possibility of nonidentical subunits which differ in net charge, two subunits would generate three, not two, isozymes of the protein. In order to ascertain the structural difference between the two isocitrate dehydrogenase isozymes and their possible role in metabolic regulation, preparative separation of the two isozymes will be required.

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