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PURIFICATION AND CRYSTALLIZATION OF NADP$^+$-SPECIFIC ISOCITRATE DEHYDROGENASE FROM *ESCHERICHIA COLI* USING POLYETHYLENE GLYCOL

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Summary

A simple and rapid method is presented for purifying the NADP$^+$-dependent isocitrate dehydrogenase (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), from *Escherichia coli*, which relies on fractionation of the enzyme with polyethylene glycol. The shortened preparation results in a 32% relative recovery of purified enzyme at a specific activity of 127 μmol/min per mg of protein. The K_m values for *threo*-D$_S$-isocitrate, NADP$^+$, NAD$^+$, Mg^{2+} and Mn^{2+} are 6.4, 36, 3000, 19.7 and 2.0 μM, respectively. The stability of the enzyme as a function of dilution and temperature are also reported. Recrystallization of the purified enzyme under different conditions readily produces a variety of single crystals. Crystals grown from ammonium sulfate solutions belong to monoclinic space group C2 with a = 125 Å, b = 111 Å, c = 83.5 Å and β = 108° 45′. Density measurements of these crystals indicate there are two 80 000-dalton dimers per asymmetric unit.

Introduction

The success of crystallizing enzymes in a form suitable for pursuing an X-ray crystallographic analysis of its mechanism of action is often dependent on the preparative procedure employed [1]. The isolation of an enzyme frequently requires several purification steps employing a variety of modern techniques, the quality and/or quantity of the final product is often lowered by such processes.

Purification of the NADP$^+$-specific enzyme (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) from *Escherichia coli* has been previously reported by Reeves and coworkers [2]. The original preparation procedure utilized $(NH_4)_2SO_4$ fractionation, ion exchange chromatography, gel

permeation chromatography and preparative polyacrylamide gel electrophoresis to obtain 64% recovery of an enzyme with a specific activity of 11.8 μmol/min per mg protein. Subsequent modification of this procedure using additional chromatography steps (Reeves, H.C. (1974) private communication) resulted in a purified enzyme with an improved specific activity of 125. Characterization of this protein by Reeves et al. [3] indicated that the enzyme exists as a catalytically active dimmer composed of apparently identical subunits of approximately 42 000 daltons. This protein was subsequently crystallized [4] according to the Jokoby method [5] to produce small crystals of octahedral habit about 0.02 mm in width.

These earlier procedures were duplicated in our laboratory in an effort to produce large single crystals of an isocitrate dehydrogenase enzyme suitable for high resolution X-ray diffraction analysis. Although crystals of sufficient size (0.3 mm) could be grown using various microdiffusion and vapor equilibration techniques [6,7], the resulting crystals did not produce satisfactory high-resolution diffraction patterns indicative of a well ordered single crystal. We present here a shortened procedure for isolation of a bacterial $NADP^+$-dependent isocitrate dehydrogenase that results in both high yields and high quality enzyme as judged by its specific activity, K_m values and its disposition readily to form large single crystals. The homogenous preparation is also stable for several hours at room temperature, a prerequisite for an enzyme used for cofactor regeneration in NADPH-requiring reactors [8].

Very recently Hy and Reeves [9] have also reported a shortened procedure for isolating the enzyme from milligram quantities of whole cells by employing an $NADP^+$-affinity column. It is not known whether suitable large single crystals can be obtained from this enzyme or if it is feasible to scale up this procedure to obtain the large amounts of enzyme necessary to pursue its crystallographic study.

Experimental Procedure

Materials. All reagents used were of analytical grade and can be readily obtained from commercial sources.

Enzyme assay. Enzyme activity was determined by measuring reduction of $NADP^+$ at 340 nm at 25°C using a Gilford model 2400S spectrophotometer. The cuvettes contained 1 ml of 0.15 M Tris · HCl buffer at pH 7.6, 0.5 mM $MnCl_2$, 0.5 mM $NADP^+$ and 5 mM isocitrate. The reaction was initiated by the addition of enzyme. Specific activity is expressed as units/mg protein where one unit of enzyme activity is the amount of enzyme catalyzing the reduction of 1 μmol of $NADP^+$ per minute.

Protein determination. The protein content of the samples was estimated with the Folin reagent according to the method of Lowry et al. [10] or by the biuret method [11].

Sodium dodecyl sulfate gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed by the procedure of Weber and Osborn [12] in 7% acrylamide gels. The enzyme was heated for 4 min at 100°C in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS

and 1% dithiothreitol. The gels were stained with Coomassie Brilliant Blue and rinsed by the procedure described by Riley and Coleman [13].

Crystallization. Crystals of isocitrate dehydrogenase were grown at room temperature, pH 6.5 and pH 7.5, from both polyethylene glycol (PEG 6000) and $(NH_4)_2SO_4$ solutions. These agents could be mixed directly with protein, slowly increased by microdialysis techniques [6,14], vapor equilibrated in 2-mm diameter capillaries or layered to produce a temporary gradient [15].

Density determination. Large single crystals of isocitrate dehydrogenase were fixed in 0.5% glutaraldehyde for 2 h and then rinsed in water overnight. The density of these salt free crystals was then measured by the gradient method described by Low and Richards [16] using the amino acids L-valine and L-leucine as markers. The density was also measured in aqueous media by employing a 52.5 to 75% (wt./vol) gradient of Ficoll 400 similar to the procedure reported by Westbrook [17]. Small droplets of nitrobenzene and 1-bromopentane, as well as crystals of anthracene, were used as markers.

Results

Enzyme purification

Isocitrate dehydrogenase enzyme was purified in this laboratory from the cell-free extract supernatant resulting from the initial isolation of the pyruvate and α-ketoglutarate dehydrogenase complexes [18]. However, the steps given below outline the entire procedure.

Step 1. E. coli (Crookes' strain) cells were grown in a Biogen continuous culture apparatus at 35° C as described previously by Reed and Mukherjee [19]. Frozen cells were suspended in 0.02 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA using 50 ml of buffer/20 g of frozen cells. This suspension was homogenized in a small Waring blendor and then lysed by passing twice through a precooled Manton-Gaulin continuous flow homogenizer at 6000 lb/inch^2. The lysed cell homogenate was centrifuged at 20 000 rev./min in a Beckman 21 rotor and Model L ultracentrifuge for 1 h. The protein content was estimated by the method of biuret and then diluted to 20 mg/ml with 0.02 M phosphate buffer.

Step 2. Protamine sulfate cut. The fractionation requires a fresh 2% aqueous protamine sulfate solution, pH 6.2, at 20° C. The extract was adjusted slowly to pH 6.2 with 1% acetic acid and centrifuged to clarity if necessary. Maintaining the extract at 0—4% with an ice bath, sufficient (12% v/v) 2% protamine sulfate solution was slowly added to make the final solution contain 0.12 mg of protamine sulfate per mg of protein. The turbid solution was immediately centrifuged at 27 000 × *g* for 15 minutes at 4° C. The pellets were inactive and discarded. The protein content of the supernatant fraction was estimated by biuret and then diluted to 12 mg protein/ml with 0.02 M phosphate buffer, pH 6.2, containing 5 mM $MgCl_2$.

Step 3. Polyethylene glycol fractionation. The fractionation was carried out by additions of a 50% (w/v) aqueous solution of 6000-dalton polyethylene glycol. The polyethylene glycol solution was added slowly to the cooled extract until the final solution reached 7% (w/v) in polyethylene glycol. Additional $MgCl_2$ was added to bring the total Mg^{2+} concentration to 14 mM and the

mixture was centrifuged at 27 000 × *g* for 15 min. The supernatant solution was assayed for isocitrate dehydrogenase activity at this point and additional polyethylene glycol solution added. The fraction precipitating between 12 and 19% polyethylene glycol (w/v) contains the isocitrate dehydrogenase activity.

Step 4. DEAE column. DE 52 (Whatman) was suspended in 5 M acetic acid, deaerated under vacuum, poured in the column and washed with 1 M sodium acetate solution, pH 7.3, until the pH of the effluent was greater than 6.0. The column was then equilibrated with 1 mM sodium acetate, pH 7.3. The polyethylene glycol pellet was resuspended in this same buffer and added to the column. Under these conditions the enzyme adsorbs to the DE 52 facilitating its separation from polyethylene glycol. The enzyme was taken off the column with a sodium acetate gradient from 0.001 to 0.5 M, pH 7.3.

Step 5. $(NH_4)SO_4$ fractionation. The active enzyme fractions from the DE 52 column were combined and fractionated by slow additions of a saturated ammonium sulfate solution. The fraction precipitating between 65 and 80% $(NH_4)_2SO_4$ saturation contains the active enzyme.

Step 6. Polyethylene glycol recrystallization. The ammonium sulfate pellet was resuspended and then dialyzed against 0.05 M acetate solution, pH 7.3, to remove excess ammonium sulfate. This solution was diluted to 10 mg ml and polyethylene glycol was slowly added to make the solution 25% (w/v). The initial precipitate was removed by centrifugation and the supernatant was allowed to stand at room tempreature until microcrystal formation occurred (about 2 h).

Table I summarizes the results of a typical preparation. The yield was 32% of purified enzyme with specific activity of 127—131 μmol/min per mg of protein when referenced to the supernatant obtained from the α-keto acid dehydrogenase isolation (see Step 3). The result of the SDS-gel electrophoresis run is shown in Fig. 1. The presence of only one band is indicated when the gels are stained with Coomassie Brilliant Blue and destained as described in Materials and Methods. In addition to serving as a criteria of purity for this procedure, the presence of a single band supports earlier work on the subunit composition of this enzyme [3].

Kinetic parameters

The K_m values obtained from double reciprocal plots of enzyme activity

TABLE I

SUMMARY OF PURIFICATION RESULTS

Step	Vol. (ml)	Protein (mg)	Total activity	Specific activity	Yield	Purification
6.5% polyethylene glycol supernatant	1700	10 200	39 077	3.8	100	1
12—19% polyethylene glycol pellet	67	2 506	31 228	12.5	80	3.3
DE52 acetate-eluate	48	696	21 798	31.4	56	8.3
65—80% $(NH_4)_2SO_4$	6.9	210	16 531	78.9	42	20.8
25% polyethylene glycol Pellet	8	98	12 504	127.2	32	33.6
25% polyethylene glycol crystals	2	25.2	3 299	130.9	8	34.4

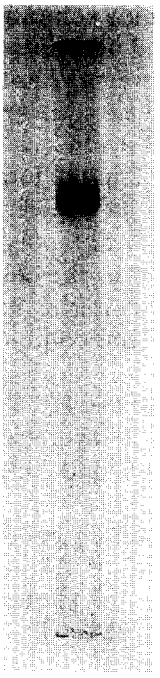

Fig. 1. SDS polyacrylamide gel electrophoresis of *E. coli* $NADP^+$-specific isocitrate dehydrogenase.

with respect to substrate, coenzyme and metals are listed on Table II. Previously reported values are also shown for comparison. The K_m values for Mn and Mg are smaller for the preparation isolated by the procedure described above. The maximal velocities in Tris buffer at pH 7.6 with 0.5 mM $MnCl_2$ or 5.0 mM $MgCl_2$ were 127 or 103 μmol/min per mg, respectively.

Enzyme stability

It had been previously reported that the enzyme was cold-labile [20] and that its stability was also influenced by pH and the nature of the buffer system [2]. The stability of the enzyme obtained by this procedure was observed to be markedly dependent on protein concentration. At concentrations above 5 mg/ml the homogeneous enzyme was stable at room temperature and could be frozen for extended periods (weeks) without appreciable loss of activity. Dilute solutions (< 0.1 mg/ml) of the enzyme lose activity in the cold (0—5°C), and

TABLE II

IMPROVEMENTS IN K_m VALUES

	K_m * (μM)	K_m ** (μM)
Threo-D_S-citrate	6.4	15.6
$NADP^+$	36	37
NAD^+	3000	—
Mg^{2+}	19.7	127
Mn^{2+}	2.0	12.9

* this procedure

** reported previously [2]

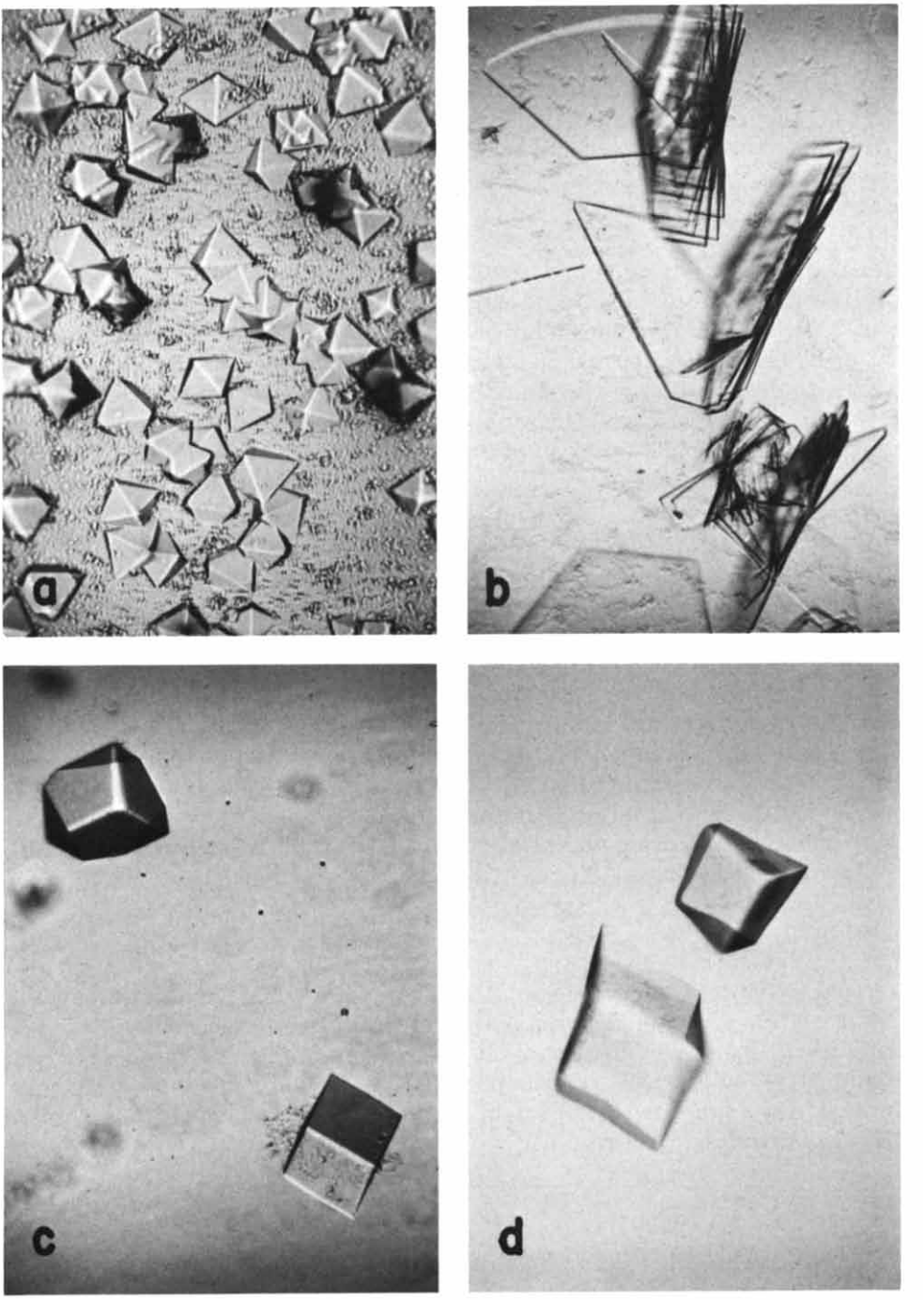

Fig. 2. Crystals of *E. coli* $NADP^+$-specific isocitrate dehydrogenase: (a) octahedral habit obtained from ammonium sulfate solutions with enzyme obtained by previous purification procedure; (b) polyethylene glycol grown crystals using current procedure; (c) and (d) crystalline habits obtained by dialysis and vapor equilibration with ammonium sulfate solutions.

presence of metal ion (Mn^{2+}) had no effect on its dilution lability. At 22—26° C dilute solutions (10—30 μg/ml) of the crystalline or 25% polyethylene glycol pellet preparation (Table I) lost little or no activity up to 6 h, and either preparation is satisfactory for NADPH generation in reactors of the type described by Sofer et al. [8] that use an insolubilized NADPH-requiring drug oxidase for generation of drug metabolites. Less pure fractions (i.e., the 65—80% $(NH_4)_2SO_4$ fraction, Table I) are not satisfactory since contaminants, probably one or more proteases, limit the stability of both the isocitrate dehydrogenase and the oxidase.

Crystallization

Crystals grown from enzyme obtained by the previous procedure are shown in Fig. 2a, while the different habits obtained from this procedure are shown in Fig. 2 b—d. The thin plates of Fig. 2b are readily obtained by using vapor diffusion cells to decrease the rate of equilibration to the higher polyethylene glycol concentrations. Figs. 2c and 2d represent two of the crystal forms obtained when this purified material is vapor-equilibrated against 60% $(NH_4)_2SO_4$ solutions. Both forms of these crystals belong to the monoclinic space group C2 with a = 125 Å, b = 111 Å, c = 83.5 Å and $\beta = 108° 45'$.

The average density of these crystals as measured by the gradient methods described under Methods was 1.225 g/cm. Assuming a partial specific volume $\bar{v}$ = 0.75 and n_r = 80 000, this implies that there are 7.4 dimers per unit cell. Thus Z = 8 for this unit cell and there are therefore two dimers per asymmetric unit. This would imply a packing parameter V_m = 1.71 $Å^3$/dalton which is at the lower end of the normal range reported by Matthews [21] of 1.68 to 3.4 $Å^3$/dalton for protein crystals.

The $NADP^+$-specific isocitrate dehydrogenase from *Azotobacter vinelandii* has recently been crystallized [22]. In view of the presumed identity of the two subunits, it is of interest that they also report having two molecules of 80 000 daltons comprising the asymmetric unit of their tetragonal unit cell, $P4_22_12$ with $a = b$ = 122. 1 Å and c = 163.9 Å.

Discussion

A rapid procedure applicable for the purification of large amounts of the $NADP^+$-specific isocitrate dehydrogenase of *E. coli* has been described. The advantages of this procedure are found in the savings of time, high yield (32%), high specific activity (127) with improved K_m values for substrate, coenzyme and metal, and a homogeneous protein that readily forms large crystals suitable for X-ray diffraction investigation.

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