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Note

Effect of sodium hydroxide on the binding of NADP-isocitrate dehydrogenase to Matrex Gel Red-A

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The NADP-isocitrate dehydrogenase (E.C. 1.1.1.42) of *Azotobacter vinelandii* was first purified about 15 years ago[1]. Czerwinski *et al.*[2] reported the crystallization of this enzyme and initiated a crystallographic study. As the continuation of the structural analysis requires large amounts of highly purified enzyme, it became necessary to develop a rapid and reproducible purification procedure. Although other workers have used the same immobilized dye, Procion Red HE-3B, to purify NADP-isocitrate dehydrogenase from other sources[3,4], the procedure reported here does not require NADP and gives a highly purified enzyme. However, the method used to regenerate the resin can affect the binding of the enzyme to the immobilized dye.

EXPERIMENTAL

All chemicals were of analytical-reagent grade. Tris, $NADP^+$, DL-isocitrate and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). Triton X-114 was a gift from Rohm & Haas (Philadelphia, PA, U.S.A.). Matrex Gel Red A, Blue A, Orange A, Green A and Blue B were purchased from Amicon (Lexington, MA, U.S.A.) and Affi-Gel Blue from Bio-Rad Labs. (Richmond, CA, U.S.A.). Polybuffer 74 and polybuffer PBE-94 were obtained from Pharmacia (Uppsala, Sweden). CX-30 ultrafilters were supplied by Millipore (Bedford, MA, U.S.A.).

Azotobacter vinelandii (ATCC 9104) cells were grown, harvested and stored frozen (−70°C) as described by Chung and Franzen[1]. Twenty-five grams of the cells were suspended in 250 ml of 20 m*M* Tris–citrate (pH 7.0) containing 1 m*M* dithiothreitol and 1 m*M* magnesium chloride. Triton X-114 was added to give a 1% (v/v) solution. The suspension was then homogenized on ice in a Polytron homogenizer. Triton X-114 is a non-ionic detergent that is miscible with water at 0°C but separates into an aqueous and a detergent phase at 20°C[5]. The homogenate was warmed to 25°C and centrifuged for 10 min at 10,000 *g* to remove the cell debris and Triton X-114. The aqueous supernatant fraction was warmed to 30°C and centrifuged for 10 min at 10,000 *g* to remove residual Triton X-114. The aqueous phase was slowly brought to 45% of saturation with solid ammonium sulfate. After standing for 1 h, the suspension was centrifuged at 10,500 *g* for 15 min. The supernatant phase was then brought to 85% of saturation with ammonium sulfate and centrifuged as above after

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standing for 30 min. The precipitate was dissolved in water and then dialyzed against the Tris–citrate buffer to which glycerol had been added to make a 10% (v/v) solution at pH 6.8. The sample was concentrated using CX-30 ultrafilters. This fraction was stored frozen in aliquots at −10°C.

Preliminary experiments showed that of the Matrex Gels, only Red A would bind the isocitrate dehydrogenase. It was also determined that sodium chloride would elute the enzyme off the Red A resin. Affi-Gel Blue was found to bind the enzyme, but NADP was required to elute the enzyme off the resin.

A 15 × 2.5 cm affinity column of Matrex Gel Red A was prepared at room temperature. The column was eluted with 8 *M* urea and then washed with several bed volumes of the Tris–citrate–glycerol buffer. A 250-mg amount of the 45–80% saturated ammonium sulfate-precipitated fraction was loaded on to the Matrex Gel Red A column. After loading, the flow of the Tris–citrate–glycerol buffer was stopped and the column allowed to remain undisturbed for 2–5 h to maximize the binding of the enzyme to the Matrix Gel Red A ligand. The column was then eluted with the Tris–citrate–glycerol buffer. A step gradient of 0.4 *M* sodium chloride was applied to elute the enzyme when the absorbance returned to a value near to the baseline. The column was then regenerated by eluting the column with 8 *M* urea, and reused after washing with several bed volumes of the starting buffer. A constant flow-rate of 10 ml/h was maintained with an LKB Microperpex peristaltic pump. The effluent was continuously monitored at 277 nm with an LKB Uvicord S UV monitor equipped with a level sensor to isolate the peaks. Fractions of 5 ml were collected with an LKB Multi-Rac collector set with the appropriate time delay factor.

A chromatofocusing gel column (17 × 1.0 cm) was prepared at 5°C with the polybuffer exchanger PBE-94. The Glenco 24 × 1 cm column was maintained at 5°C by circulating chilled water through the water-jacket. The PBE-94 was packed and equilibrated with 25 m*M* histidine–HCl (pH 6.5) which contained 10% glycerol at a flow-rate of 99 ml/h. A 5-ml volume of polybuffer 74 at pH 5.0, diluted 1:10 with 10% glycerol–1 m*M* dithiothreitol, was added, followed by the sample, and then eluted with polybuffer 74 solution at 10 ml/h. The column was purged with 1.5 *M* sodium chloride solution prior to re-equilibration.

Isocitrate dehydrogenase concentration and activity were determined spectrophotometrically. The assay was initiated by the addition of 1–10 μl of enzyme to 0.5 ml of a solution containing 0.1 m*M* NADP, 4 m*M* isocitrate, 5 m*M* magnesium chloride and 0.1 *M* Tris–HCl (pH 7.8). After mixing, the increase in absorbance at 340 nm with time at 22°C was followed. One enzyme unit is equivalent to 1 μmole of NADP reduced per minute. Concentrations of isocitrate dehydrogenase were measured at 280 nm using an absorptivity of 0.89 ml mg^{-1} cm^{-1} (ref. 1).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using standard procedures[6,7]. The gels were visualized with ultrasensitive silver stain[8].

RESULTS AND DISCUSSION

The elution profile of a typical experiment is shown in Fig. 1. Unbound enzyme is eluted in the first bed volume (peak I). At 1.7 bed volumes (130 ml) the ionic strength of the Tris–citrate–glycerol buffer is increased with 0.4 *M* sodium chloride.

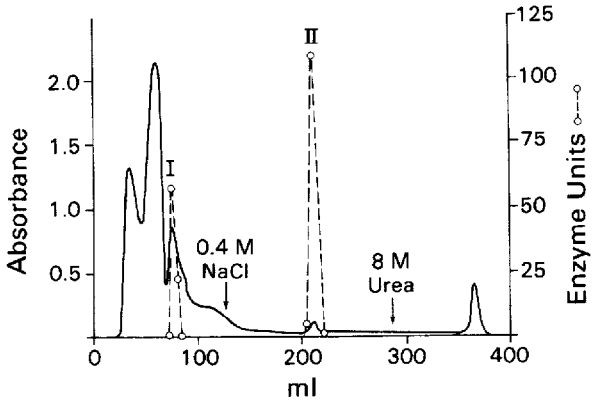

Fig. 1. Elution of the ammonium sulfate-precipitated fraction on the Matrex Gel Red A affinity column. Absorbance determined at 277 nm.

At 2.8 bed volumes (210 ml) most of the enzyme activity elutes off the column (peak II). At 3.8 bed volumes (290 ml) the column is regenerated with 8 *M* urea. Peak I has a specific activity of 5 units/mg, which is nearly the same as the starting material. The specific activity of peak II is 169 units/mg, which represents a 45-fold increase in purity (Table I). The isocitrate dehydrogenase peak (II) was dialyzed against the Tris–citrate–glycerol buffer to remove the sodium chloride and concentrated using the CX-30 ultrafilters. SDS–PAGE of peak II showed a major band with a molecular weight of 77,000. A minor band of molecular weight 13,500 is also present but is barely visible even though the gel was stained with ultrasensitive silver stain. When peak II is applied to the chromatofocusing column, the isocitrate dehydrogenase activity elutes as a sharp peak at pH 5.8. SDS–PAGE of this peak shows a single band also of molecular weight 77,000. These molecular weight values are in agreement with values previously reported for the enzyme isolated from this and other strains of *A. vinelandii*[1,9,10]. The p*I* of 5.8 is slightly less than the value of 6.1 reported for the Strain O enzyme, but this may be due to the differences in the methods used to determine the p*I*[9].

The usual method of regenerating the Matrex Gel Red A is to include a small amount of sodium hydroxide with the urea[4]. This procedure was found to reduce drastically the binding of the isocitrate dehydrogenase from *A. vinelandii* on this type of affinity resin. Resin which has been regenerated with urea–sodium hydroxide binds

TABLE I

SUMMARY OF PURIFICATION OF NADP-ISOCITRATE DEHYDROGENASE

Purification step	*Total protein (mg)*	*Specific acitivity (units/mg)*	*Total units*	*Yield per step (%)*	*Overall yield (%)*
Crude extract	5700	1	5700	100	100
45–85% saturated ammonium sulfate	1160	4	4280	75	75
Affinity chromatography	568	169	2097	49	37

less than 5% of the applied enzyme. Peak I in this instance contains 95% of the enzyme with no increase in purity. Exhaustive washing with the eluting buffer of the urea–sodium hydroxide-regenerated resin does not increase the binding capacity. Regeneration with only urea restores the binding capacity of the resin and the results shown in Fig. 1 are again obtained.

The reason for this effect of sodium hydroxide on the binding of *A. vinelandii* NADP-isocitrate dehydrogenase to Matrex Gel Red A is not clear. As the binding capacity of the resin can be restored, a non-destructive process must be involved. The simplest explanation is that the sodium ions are tightly bound to the sulfonic acid groups on the Procion Red HE-3B ligand. This would neutralize the negative charges on the ligand, which are necessary for the enzyme to bind to the resin. Crystal structures of other dehydrogenases have shown that the negatively charged pyrophosphate moiety of the dinucleotides binds to a positively charged arginine or lysine residue on the protein[11]. As the isocitrate dehydrogenase from *A. vinelandii* is physically different from the single-chain isocitrate dehydrogenases isolated from other sources[1,3,4], it is reasonable that the *A. vinelandii* enzyme would have a slightly different conformation around its nucleotide binding domain. Conceivably the small differences could be sufficient to cause this particular isocitrate dehydrogenase to behave differently in the binding activity to affinity column ligands.

It is apparent from these results that the method employed in regenerating an affinity resin can greatly influence the binding capacity of the resin for a particular protein. If the protein under investigation does not bind to the resin in the desired or expected manner, then different methods of regeneration should be tried before abandoning a particular resin.

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