Purification and Characterization of NADP-Specific Isocitrate Dehydrogenase from Ammonia-oxidizing Chemoautotrophic *Nitrosomonas europaea* ATCC 25978

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Ammonia-oxidizing chemoautotrophic *Nitrosomonas europaea* exhibited the NADP-specific isocitrate dehydrogenase (ICDH) activity. Isocitrate-oxidizing activity was found in two fractions (ICDH-FI and ICDH-FII) on a DEAE-Sepharose CL-6B. These enzyme proteins with isocitrate-oxidizing activity were further purified as electrophoretic homogeneity using gel filtration (Cellulofine GCL-2000-m) and then ion exchange chromatography (Mono Q). These enzymes were purified about 69-fold for ICDH-FI and about 140-fold for ICDH-FII. The molecular weights were determined as both 82,000 for both, using gel filtration and SDS-PAGE of enzymes. The isoelectric points of ICDH-FI and ICDH-FII were pH 5.8 and 6.2, respectively.

The pH and temperature optima for ICDH-FI and ICDH-FII were pH 8.5 and 8.0, respectively, and about 55°C for both enzymes. The enzymes were stable up to 45°C and acted specifically on D- or DL-isocitrate and not on any other organic acids tested. The apparent K_m values of ICDH-FI for D- and DL-isocitrate were 15.0 μ M and 17.5 μ M, respectively and K_m values of ICDH-FII for D- and DL-isocitrate were 13.0 μ M and 11.5 μ M, respectively. Both enzymes were strongly activated by Mn^{2+} and Mg^{2+} , and inhibited by Cu^{2+} , Ni^{2+} , Zn^{2+} and Hg^{2+} . They were strongly inhibited by *p*-chloromercuribenzoate.

Key words: *Nitrosomonas europaea*, ammonia-oxidizing bacteria, isocitrate dehydrogenase

Introduction

Nitrosomonas europaea is an obligate chemoautotrophic bacterium which oxidizes ammonium to nitrite via a pathway that includes hydroxylamine as an intermediate²⁾. Most studies on *N. europaea* have dealt with the ammonium oxidation pathway. However, little attention has been paid to the anabolism of bacterium.

NADP-specific isocitrate dehydrogenase [EC 1.1.1.42] catalyzes the oxidative decarboxylation of

isocitrate to 2-oxoglutarate in the presence of NADP and is commonly known as ICDH⁶⁾. Although heterotrophic bacterial ICDH has been extensively studied^{9,14,17)}, no information is available about this enzyme in *N. europaea* and its possible role in the bacterium. We have previously demonstrated the effectiveness of isocitrate and 2-oxoglutarate on the growth of *N. europaea*, a representative chemoautotroph¹⁰⁾. We investigated the activity of dehydrogenases involved in the tricarboxylic acid cycle in cell-free extract from *N. europaea*. Results of the study showed the prominent metabolic activity of ICDH. This finding

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was consistent with the results obtained by Hooper on *N. europaea* ATCC 19718⁷⁾.

In this paper, we described the purification and some of the properties of NADP-specific ICDH from *N. europaea* ATCC 25978.

Materials and Methods

Microorganisms and culture conditions

Nitrosomonas europaea ATCC 25978 was used throughout this study¹⁹⁾. The culture medium (P-medium) was composed of Na₂HPO₄, 13.5 g; KH₂PO₄, 0.7 g; (NH₄)₂SO₄, 2.5 g; MgSO₄·7H₂O, 0.1 g; NaHCO₃, 0.5 g; CaCl₂·2H₂O, 5 mg; chelated iron (Fe-EDTA, Dojindo Laboratories Co. Ltd., Japan), 0.1 mg and deionized water, 1 liter¹²⁾. The initial pH of the medium was 8.2±0.5, it was not further adjusted. The stock culture was kept at 5°C after aerobic growth in the P-medium. The seed culture was prepared in a 3-liter Erlenmeyer flask containing 1 liter of the medium by rotary shaking of the culture (200 rpm) for 7 days after inoculation of 100 ml of the stock culture. The main culture was done in a 10 liter flat-bottom flask with an aeration apparatus containing 10 liters of the medium¹⁶⁾.

All cultivation was performed at 29°C. Cells from 110 liters of liquid culture prepared using the procedure described above were sedimented by continuous flow at 12,000×g in a refrigerated centrifuge with a flow rate of 7 to 8 liters per hour at 5°C.

Assay for ICDH activity.

The ICDH activity was assayed using the method of Cook *et al.*⁵⁾. The rate of NADP reduction by isocitrate was measured by following the increase of absorbance at 340 nm using a Simadzu 160A spectrophotometer with a thermostated cell compartment at 40°C. The standard reaction mixture contained, in a total volume of 3.0 ml, 0.5 mM MnCl₂, 0.15 mM NADP, 0.17 mM DL-(or D-) isocitrate, 20 mM phosphate buffer, (pH 8.0, P-buffer) and enzyme.

One unit of the enzyme was defined as the amount producing one μ mole of the product per min. Specific activity was expressed as units per mg protein. The protein concentration was determined using a dye-binding assay method as described by Bradford³⁾, or by measuring the absorbance at 280 nm¹¹⁾. The latter method was used for monitoring protein during column chromatography.

Purification of ICDH

Step 1: Preparation of cell-free extract. Cells (5.0 g wet wt.) of the bacteria from a 110 L culture were washed once with chilled P-buffer and resuspended in 20 ml of same buffer. The cells were then disrupted by treatment with a French press under a pressure of approximately 500 kg/cm². The homogenates were centrifuged at 12,000×g for 30 min to remove unbroken cells and cell debris. The supernatant was centrifuged at 140,000×g for 30 min and the resultant supernatant was used as the cell-free extract. All purification procedures were performed at 0–4°C.

Step 2: Ammonium sulfate fractionation. Solid ammonium sulfate was added to the cell-free extract. The 40–60% ammonium sulfate precipitate was collected by centrifugation (9,500×g, 10 min) and dissolved in a small volume of 10 mM Tris-HCl buffer (pH 7.5). The enzyme solution was dialyzed against the same buffer for overnight.

Step 3: DEAE-Sephacel CL-6B column chromatography. The dialyzed enzyme solution was applied on a DEAE-Sephacel CL-6B (Pharmacia Fine Chemicals, Sweden) column (1.6×30 cm) equilibrated with the 10 mM Tris-HCl buffer (pH 7.5). After the column was washed with the same buffer, the elution was performed with a linear gradient of NaCl in the same buffer (0 to 0.7 M) at a flow rate of 12 ml/h, and the elute was collected in 1-ml fractions. The active fractions were collected and dialyzed against the same buffer, overnight. The enzyme solution (15 ml)

was concentrated 2 ml by osmotic ultracentrifugation using Ficoll 400 (Pharmacia).

Step 4: Gel filtration on a Cellulofine GCL-2000-m column. The concentrated enzyme solution was applied on a Cellulofine GCL-2000-m (Seikagaku kogyo Co. Ltd., Tokyo) column (0.9×60 cm) equilibrated with P-buffer. The elution was done with the same buffer at a flow rate of 6 ml/h and the enzyme fractions were pooled. The enzyme solution (5 ml) was concentrated to 0.5 ml by Ficoll 400.

Step 5: Mono Q column chromatography. The enzyme solution (0.5 ml) was applied on to FPLC (Fast Protein Liquid Chromatography, Pharmacia) with a Mono Q column (0.5×5 cm) buffered previously with P-buffer. The elution of the enzyme was performed by a linear gradient of NaCl (0 to 0.1 M) in the same buffer. The active fractions were collected, and the enzyme was dialyzed against P-buffer.

Polyacrylamide gel electrophoresis

The homogeneity of the enzyme preparation was examined by polyacrylamide disc gel electrophoresis (PAGE). Electrophoresis was done in 7.5% polyacrylamide gel in Tris-glycine buffer (pH 8.6) with a current of 2.0 mA per tube for 1.2 h at 0°C by the method of Hames⁸⁾. Gels were stained for protein with Coomassie brilliant blue G-250.

Determination of molecular weight

The molecular weight of the purified enzyme was estimated by gel filtration using a Superose 12 (Pharmacia) according to the method by Andrews¹⁾. The column (1.0×30 cm) was equilibrated with P-buffer containing 0.1 M sodium chloride. The purified enzyme was eluted in 0.25-ml fractions at a flow rate of 15 ml/h. β -Amylase (MW 200,000), alcohol dehydrogenase (MW 150,000), albumin; bovine serum (MW 66,000), carbonic anhydrase (MW 29,000) and cytochrome *c* (MW 12,400) were used as marker proteins.

The molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis as described by Weber and Osborn²⁰⁾ using SDS-PAG PLATE (Daiich Pure Chemicals Co. Ltd., Tokyo) with Tris-glycine buffer (pH 8.4) containing 0.1% SDS. The electrophoresis was performed at 60 mA for 60 min. The marker proteins used were glutamate dehydrogenase (53,000), transferrin (76,000), β -galactosidase (116,000), α_2 -macroglobulin (170,000) and myosin (212,000). The proteins were detected by the silver stain method¹³⁾.

Isoelectric focusing

Isoelectric focusing¹⁸⁾ was performed using AMPHOLINE PAG PLATE (pH 3.5-9.5, Pharmacia) gels on an ATTO AE-3230 apparatus. The gels were prefocused at 300 V for 60 min and focused at 300 V for 120 min at 4°C. Anode and cathode solutions were 1.0 M H_3PO_4 and 1.0 M NaOH, respectively. After electrofocusing, the protein in the gels was stained with silver stain. pI marker proteins with values of 3.5-9.5 were used as references.

Results and Discussion

Purification of ICDH

The results of the purification are summarized in Table 1.

In the first column chromatography using DEAE-Sepharose CL-6B, eleven peaks (A-K) of protein were fractionated (Fig. 1). The two fractions of ICDH were detected only when the isocitrate-oxidizing activity was assayed with NADP as a coenzyme in peaks E to G. The first fraction eluted (peak E) from the DEAE-Sepharose column, under these experimental conditions, was designated as ICDH-FI, and the second, peak G, was designated as ICDH-FII.

The ICDH-FI and ICDH-FII fractions were pooled and purified further. Successive chromatographies on gel filtration and then FPLC using a Mono Q column resulted in single sharp peaks

Table 1. Purification of isocitrate dehydrogenase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	352.2	55.8	0.16	1.0	100
Ammonium sulfate ppt (40-60% saturation)	55.0	36.5	0.66	4.1	65.4
DEAE-Sepharose CL-6B					
FI	1.14	8.15	7.15	44.7	14.6
FII	1.38	9.14	6.62	41.4	16.4
Cellulofine GCL-2000-m					
FI	0.33	3.45	10.45	65.3	6.2
FII	0.55	2.43	4.42	27.7	4.4
Mono Q					
FI	0.01	0.11	11.0	68.8	0.2
FII	0.004	0.09	22.5	140.6	0.2

Fig. 1. DEAE-Sepharose CL-6B column chromatography of ICDHs from *N. europaea*. The experimental details are described in the text.
Line: —, absorbance at 280 nm
Symbol: ○, ICDH activity

(Fig. 2).

Homogeneity of the purified enzyme solutions was examined by disc electrophoresis at pH 8.6. In Fig. 3, a disc electrophoretic pattern of the enzyme is shown, indicating that the preparation is electrophoretically homogeneous. As shown in Table 1, the specific activity of ICDH-FI and ICDH-FII increased 69-fold (11 units/mg protein) and 140-fold (22.5 units/mg protein).

Molecular weight

The molecular weights of both native enzymes were estimated at 82,000 by gel filtration. The

Fig. 2. Mono Q column chromatography of ICDHs from *N. europaea*.
Line: —, absorbance at 280 nm
Symbol: ○, ICDH activity
A: ICDH-FI B: ICDH-FII

molecular weight of the both denatured enzymes were approximately 82,000 by SDS-PAGE (Fig. 4). This molecular weight is almost same as that

Fig. 3. Polyacrylamide disc gel electrophoresis of the purified ICDHs from *N. europaea*. Electrophoresis was carried out on 7.5% gels as described in Materials and Methods. A: ICDH-FI B: ICDH-FII

reported for enzymes obtained from *Escherichia coli*¹⁵⁾ and *Azotobacter vinelandii*⁴⁾ (80,000) but were somewhat smaller than that reported for the enzyme obtained from *Bacillus stearothermophilus*³⁾ (92,500).

Isoelectric point

From the results of analytical isoelectric focusing of both enzymes on an Ampholine polyacrylamide gel, the isoelectric point (pI) of ICDH-FI and ICDH-FII were pH 5.8 and 6.2, respectively. This value is similar to the pI of NADP-specific ICDH from *Azotobacter vinelandii*⁴⁾ (pH 6.1). However, the pI of the both enzymes were somewhat different from that of NADP-specific ICDH from *Escherichia coli*¹⁵⁾.

Fig. 4. Estimation of molecular weight of ICDHs by SDS-polyacrylamide gel electrophoresis. Symbols: ○, ICDH-FI or ICDH-FII

Effect of pH and temperature on enzyme activity

The reaction mixture adjusted to an appropriate pH with Britton-Robinson buffer, was incubated at 45°C. The optimum pHs of ICDH-FI and ICDH-FII were found to be 8.5 and 8.0, respectively (Fig. 5A).

The temperature optima of ICDH-FI and ICDH-FII were both 55°C (Fig. 5B).

Effect of temperature on enzyme stability

To examine the thermal stability of the ICDH-FI and ICDH-FII, each enzyme solution was incubated at various temperatures (20–70°C) for 10 min followed by assay of remaining activity. Both enzymes were stable up to 45°C (ICDH-FI) and 50°C (ICDH-FII) (Fig. 6).

Substrate and coenzyme specificity

ICDH activities on D- or DL-isocitrate, *cis*-aconitate, oxalacetate, citrate, 2-oxoglutarate, suc-

Fig. 5. Effects of pHs and temperature on the activity of ICDHs.
(A), Optimal pH; (B), Optimal temperature
Symbols: ●, ICDH-FI; ○, ICDH-FII

Fig. 6. Effects of temperature on the stability of ICDHs.
Symbols: ●, ICDH-FI; ○, ICDH-FII

cinat, fumarate, malate, pyruvate, and phosphoenol pyruvate were compared. Each component was added to the assay mixture to give a final concentration of 1.6 mM. ICDH-FI and ICDH-FII were active on D- and DL-isocitrate, only with NADP as a coenzyme and showed no activity on the other organic acids. The K_m value of both ICDH were calculated from a Lineweaver-Burk plot. The apparent K_m values of ICDH-FI for D- and DL-isocitrate were 15.0 μ M and 17.5

μ M respectively, and K_m values of ICDH-FII for D- and DL-isocitrate were 13.0 μ M and 11.5 μ M respectively. These K_m values were similar to that from of the *E. coli* (15.6 μ M)¹⁵⁾ and *A. vinelandii*⁴⁾. On the other hand, the K_m values for NADP were 26.8 μ M for ICDH-FI and 3.8 μ M for ICDH-FII.

Action of effectors

The effects of various metal ions (1.0 mM) and inhibitors (0.2 mM) on ICDH-FI and ICDH-FII activities were examined (data not shown). Mn^{2+} , Mg^{2+} and Co^{2+} increased the activity remarkably. On the other hand, some metals such as Ni^{2+} , Cu^{2+} , Zn^{2+} and Hg^{2+} significantly inhibited both enzymes. *p*-Chloromercuribenzoate (PCMB), an oxidant that is specific to SH terminals, strongly inhibited both enzymes. This is similar to that reported for the NADP-specific ICDH from *Bacillus stearothermophilus*³⁾.

The enzymatic and physicochemical properties of the purified both ICDHs were compared, and are summarized in Table 2. The table show that there are no significant differences between the two enzyme fractions, but the difference in coenzyme specificity were observed. These results suggest

Table 2. Comparison of properties for both isocitrate dehydrogenases

	ICDH-FI	ICDH-FII
Optimum pH	8.5	8.0
Optimum temperature (°C)	55	
Heat stability (°C, 10 min)	45	50
Coenzyme	NADP ⁺	NADP ⁺
pI	5.8	6.2
Molecular weight	82,000	82,000
K _m values (μM) for:		
D-isocitric acid	15.0	13.0
DL-isocitric acid	17.5	11.5
NADP	26.8	3.8
Effectors (1 mM)	Mn ²⁺ , Mg ²⁺	Mn ²⁺ , Mg ²⁺
Inhibitors (0.2 mM)	PCMB	PCMB

PCMB; *p*-chloromercuribenzoic acid

that both ICDHs exist as a monomer in the native state.

Further investigations on the partial amino acid sequence of ICDH-FI and ICDH-FII will be necessary to elucidate the difference of both ICDHs from *N. europaea*.

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