NAD+-Linked Isocitrate Dehydrogenase: Isolation, Purification, and Characterization of the Protein from Pea Mitochondria[1]

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ABSTRACT

The NAD+-dependent isocitrate dehydrogenase from etiolated pea (Pisum sativum L.) mitochondria was purified more than 200-fold by dye-ligand binding on Matrix Gel Blue A and gel filtration on Superose 6. The enzyme was stabilized during purification by the inclusion of 20% glycerol. In crude matrix extracts, the enzyme activity eluted from Superose 6 with apparent molecular masses of 1400 ± 200, 690 ± 90, and 300 ± 50 kD. During subsequent purification steps the larger molecular mass species disappeared and an additional peak at 94 ± 16 kD was evident. The monomer for the enzyme was tentatively identified at 47 kD by sodium dodecyl-polyacrylamide gel electrophoresis. The NADP+-specific isocitrate dehydrogenase activity from mitochondria eluted from Superose 6 at 80 ± 10 kD. About half of the NAD+ and NADP+-specific enzymes remained bound to the mitochondrial membranes and was not removed by washing. The NAD+-dependent isocitrate dehydrogenase showed sigmoidal kinetics in response to isocitrate ($S_{0.5}$ = 0.3 mM). When the enzyme was aged at 4°C or frozen, the isocitrate response showed less allosterism, but this was partially reversed by the addition of citrate to the reaction medium. The NAD+ isocitrate dehydrogenase showed standard Michaelis-Menten kinetics toward NAD+ (K_m = 0.2 mM). NADH was a competitive inhibitor (K_i = 0.2 mM) and, unexpectedly, NADPH was a noncompetitive inhibitor (K_i = 0.3 mM). The regulation by NADPH may provide a mechanism for coordination of pyridine nucleotide pools in the mitochondria.

Plants contain at least four forms of ICDH[2]. In addition to the NAD+-specific mitochondrial isoform (EC 1.1.1.41) associated with the tricarboxylic acid cycle, there are NADP+-specific isoforms (EC 1.1.1.42) in the cytosol, chloroplast, and mitochondria. The situation within the mitochondria is further complicated by the observation that the NAD+-dependent enzyme appears in both the membrane and matrix-associated fractions following rupture of the mitochondrial membrane, suggesting that there may be two different forms of this enzyme.

All of the isoforms of ICDH catalyze the oxidative decarboxylation of isocitrate:

$$threo\text{-}D_S\text{-isocitrate} + NAD(P)^+ \rightleftharpoons \alpha\text{-ketoglutarate}$$
$$+ CO_2 + NAD(P)H + H^+$$
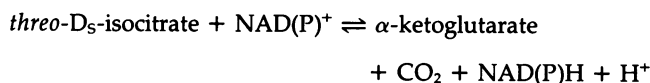
The NADP+-specific enzyme has received considerable attention (2). It appears to be universal in animal, bacterial, fungal, and plant systems. In peas (Pisum sativum L.),the cytosolic ICDH is a 82-kD dimer, and the plastid enzyme is reported to be a 154-kD dimer (2, 3). The mitochondrial NADP+-dependent enzyme has not been purified from plants, but in beef (18) and pig heart (4), it is a dimer of 90 to 115 kD. The gene for this enzyme has been cloned from yeast and has a calculated monomer size of 46.6 kD (15). Although they did not purify the enzyme, Rasmusson and Moller (20) have identified an ICDH from potato tuber mitochondria that shows high specificity for NADP+ and have presented evidence to suggest that this enzyme may be involved in the tricarboxylic acid cycle in these organelles.

The biochemical knowledge of the NAD+-dependent ICDH is much less advanced. This isoform appears to be exclusively mitochondrial and has been more difficult to work with than the NADP+ enzyme. The enzyme has been purified from several eucaryotes where it appears to be an octamer of 39-kD monomers in yeast (1), 41-kD monomers in beef heart (14), and 39- and 46-kD monomers in blowfly flight muscle (22) mitochondria. The enzyme from beef heart mitochondria was also observed in higher molecular mass forms apparently made up of two and four octamers (14). These enzymes have generally proven rather unstable and difficult to work with. Attempts to stabilize the enzyme have included the use of glycerol (7, 14), citrate (1), and propylene glycol (22).

The NAD+-specific ICDH has not been purified to homogeneity from a plant source, although some work has been done on the enzyme from peas (6–9, 11, 12) and potato (Solanum tuberosum) (17, 20, 21). Cox and Davies (7) managed to partially stabilize the pea enzyme in glycerol and purified it about 46-fold. This enzyme had an apparent molecular mass of 535 and 260 kD as measured in the absence and presence of citrate by density gradient centrifugation (8).

The kinetic properties of the enzymes from pea and potato mitochondria were complex. They showed standard Michaelis-Menten kinetics for NAD+, and NADH was a competitive inhibitor. The enzyme's kinetics are sigmoidal with respect to isocitrate, and the degree of allosterism de-

[2] Abbreviations: ICDH, isocitrate dehydrogenase; FPLC, fast protein liquid chromatography.

pended on pH (8) as well as citrate and metal ion concentrations (11).

During the course of our studies of developmental changes in plant mitochondria, we were interested in the structure and metabolic regulation of this important protein. To begin this investigation, we have purified the pea NAD$^+$ ICDH to near homogeneity and have begun studying some of its molecular and kinetic characteristics.

MATERIALS AND METHODS

Supplies and Chemicals

The FPLC system and chromatographic supplies including PBE-94, PB-74, Superose 6, and Mono Q were from Pharmacia (Almeda, CA). Centriprep-30, Centricon 30 and 100, and Matrix Gel dye-binding columns were from Amicon (Beverly, MA). Electrophoresis supplies were from Bio-Rad. Other chemicals were from Sigma.

Peas (*Pisum sativum* L. var Scout) were imbibed in a pipet washer overnight and grown in vermiculite in the dark. Etiolated seedlings were harvested after 7 to 10 d. Mitochondria were isolated by grinding and differential centrifugation as previously described (23). The mitochondria were further purified on self-generating Percoll gradients (23), washed to remove Percoll, resuspended in 20 mM Mops (pH 7.2), and frozen at −20°C to break the membranes and release the matrix proteins.

Enzyme Isolation and Purification

Frozen, lysed mitochondria were thawed at room temperature and centrifuged for 20 min at 27,000g at 4°C to pellet the membranes. Glycerol was added to the supernatant to a final concentration of 20%, and the matrix fraction was concentrated in a Centriprep-30. The enzyme was stored for 24 h or less at 4°C or for longer periods at −20°C in 40% glycerol. All subsequent steps were at 4°C.

Dye-Ligand Chromatography

Binding to Matrix Gel dye columns Blue A, Blue B, Red A, Green A, and Orange A was tested according to manufacturer's instructions using 20 mM Mops, 5 mM β-mercaptoethanol, 20% glycerol (pH 7.5) as binding and washing buffer and eluted with NaCl in the same buffer. The NAD$^+$-specific ICDH from pea mitochondria bound to Blue A, Red A, and Green A but not to Blue B or Orange A. NAD$^+$ and NADH would not elute the protein from any of these columns. For purification purposes, an 8.0-mL Matrix Gel Blue A column was used. A linear salt gradient gave a broad peak of activity from 50 to 500 mM NaCl; therefore, a step elution with 500 mM NaCl in the wash buffer was used as the purification step. More than 65% of the enzyme activity eluted in 1.5 column volumes of elution buffer. For kinetic analysis, the active fractions were pooled, concentrated, and desalted using a Centriprep-30. Approximately 70% of the NADP$^+$-ICDH is removed at this stage.

For further purification, the Matrix Gel Blue A pool was concentrated, filtered through a 0.45-μm filter, and applied to a 25-mL Superose 6 FPLC column previously equilibrated

in 20 mM Mops, 50 mM NaCl, 5 mM β-mercaptoethanol, and 20% glycerol (pH 7.5) (all FPLC buffers were filtered and degassed before use). The column was eluted at a flow rate of 0.1 mL/min (0.7 MPa), and 0.5-mL fractions were collected and tested for activity.

The concentrated peak activity fraction from Superose 6 was applied to a 1.0-mL Mono Q FPLC column previously equilibrated in 20 mM Mops, 5 mM β-mercaptoethanol, and 20% glycerol (flow rate 0.5 mL/min, 3.4 MPa). The column was eluted with a 30-mL gradient of 0 to 0.6 M NaCl in the same buffer. The NAD$^+$-ICDH eluted at 0.31 M NaCl.

Assays

The ICDH activity was measured in a 1-mL reaction containing 20 mM Mops, 5 mM MgSO$_4$, 5 mM β-mercaptoethanol, 1 mM NAD(P)$^+$, and 10 mM isocitrate (pH 7.5) by following the increase in A_{340}. A unit of activity was defined as 1 nmol NAD(P)H produced per min. Protein was estimated with the Bio-Rad Bradford protein reagent dye with BSA as the protein standard.

Molecular Mass Estimation and Gel Electrophoresis

Molecular mass estimations on the native protein were done on a Superose 6 FPLC column calibrated with bovine thyroglobulin (660 kD), apoferritin (443 kD), catalase (232 kD), goat γ-immunoglobulin (160 kD), BSA (66 kD), carbonic anhydrase (31 kD), and Cyt c (12 kD). SDS-PAGE was on 12.5 or 15% minigels using the Bio-Rad low molecular mass standards and stained with Coomassie blue or silver.

Native polyacrylamide minigels were 7% with a 110:1 acrylamide:bis-acrylamide ratio containing 0.38 M Tris (pH 8.8) and 17% glycerol. The stacking gels contained 3% acrylamide (16:1 ratio of acrylamide to bis-acrylamide) containing 0.12 M Tris (pH 6.4) and 17% glycerol. Bromophenol blue was used as a tracking dye. The gels were run for 4 h at 4°C (5 mA). A portion of the gel was stained for proteins using either Coomassie blue or silver stain. The remaining portion of the gel was stained for enzyme activity by immediately submerging the gel in a solution of 0.1 M Mops (pH 7.5), 5 mM MgSO$_4$, 1 mM NAD$^+$ (or NADP$^+$), 10 mM isocitrate, 0.1 mM phenazine methosulfonate, 0.38 mM nitroblue tetrazolium, and 20% glycerol in the dark at room temperature (14). The reaction was stopped by immersing the gel in 7% acetic acid.

RESULTS

Enzyme Stability and Purification

Before the NAD$^+$-specific ICDH from pea mitochondria could be studied in detail, it was necessary to find conditions under which the enzyme activity could be stabilized. The enzyme initially released from mitochondria was very unstable and lost all activity upon storage at 4°C for 3 to 4 h. As reported earlier, the addition of 20% glycerol stabilized the enzyme substantially (7, 14). The fraction recovered from Matrix Gel Blue A was much more stable, probably owing to the removal of some inhibitory protein or small molecule, and had a half life of 2 to 3 d at 4°C when stored in 20%

glycerol (Fig. 1). Most of the salts used during standard purification procedures including 0.1 M potassium phosphate or NaCl greatly accelerated the loss of activity. Citrate and 40% glycerol, however, were shown to increase the stability of the enzyme, both providing for <25% loss of activity during storage for 5 d (Fig. 1). Although there were substantial changes in the structure of the enzyme occurring during this time, glycerol provided sufficient stability to allow purification of the protein. All enzyme purification steps were run in 20% glycerol because the higher concentration interfered with the chromatography steps.

The NAD⁺-dependent ICDH was purified to near homogeneity. Starting with the crude matrix fraction of mitochondria purified from etiolated pea shoots by differential and Percoll gradient centrifugation, the maximum purification measured by increase in specific activity was about 200-fold following a combination of Matrix Gel Blue A and Superose 6 chromatography. At this point, the ICDH was 40 to 50% pure. Subsequent purification steps including Mono Q and additional gel filtration steps did remove most of the contaminating proteins but did not result in an increase in the specific enzyme activity.

The loss of activity during multiple purification steps correlated with a change in the apparent molecular mass of the protein. Crude mitochondrial matrix extracts chromatographed on Superose 6 revealed two peaks and a shoulder of NAD⁺-specific ICDH activity that eluted between the void volume and the bulk of matrix proteins, suggesting that the enzyme occurred in three different forms (Fig. 2, A and B). The largest form had an apparent molecular mass of 1400 ± 200 kD. The second form was 690 ± 90 kD with a shoulder at 300 ± 50 kD. After the crude enzyme was stored at −20°C (in the presence of 40% glycerol so that little loss of enzyme activity occurred), the highest apparent molecular mass peak disappeared, and the activity was predominantly
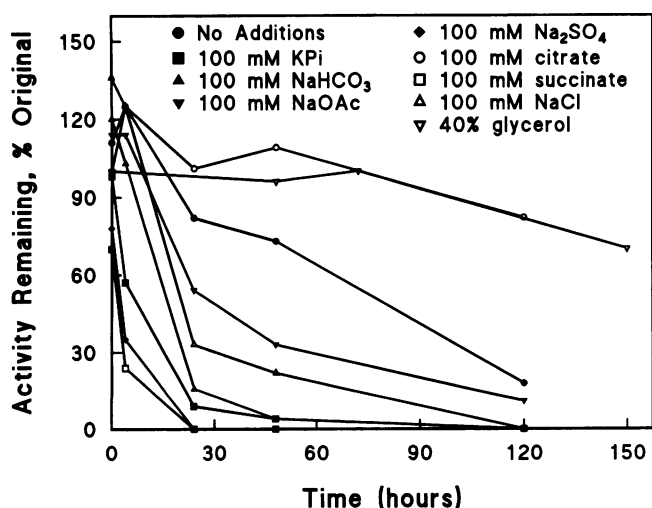
Figure 1. The effect of different additions on the stability of the NAD⁺-dependent ICDH from pea mitochondria. Mitochondrial crude matrix extract was stored at 4°C in the presence of 20% glycerol plus the different chemicals listed, and samples were taken and assayed at the times indicated.
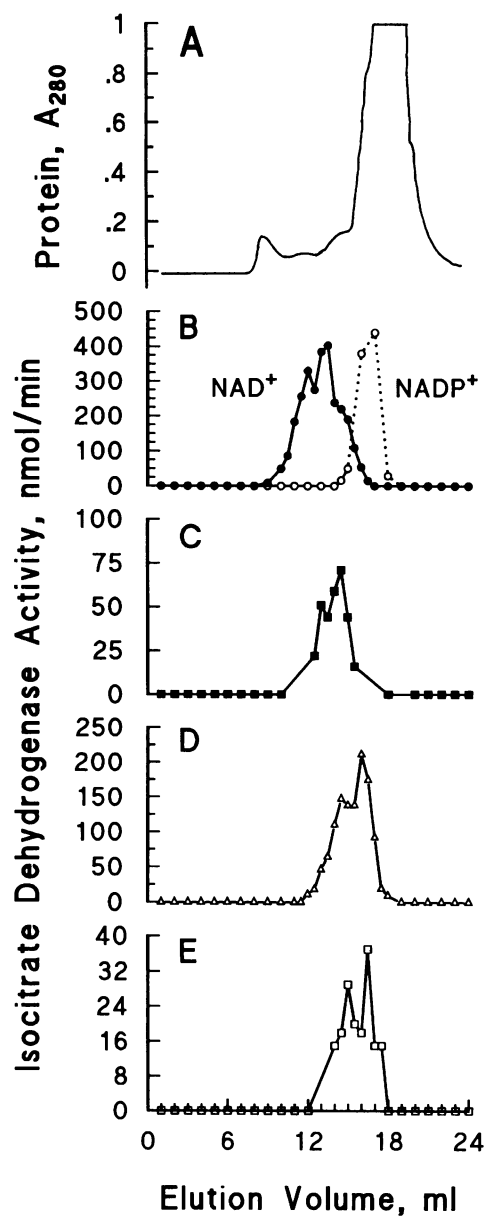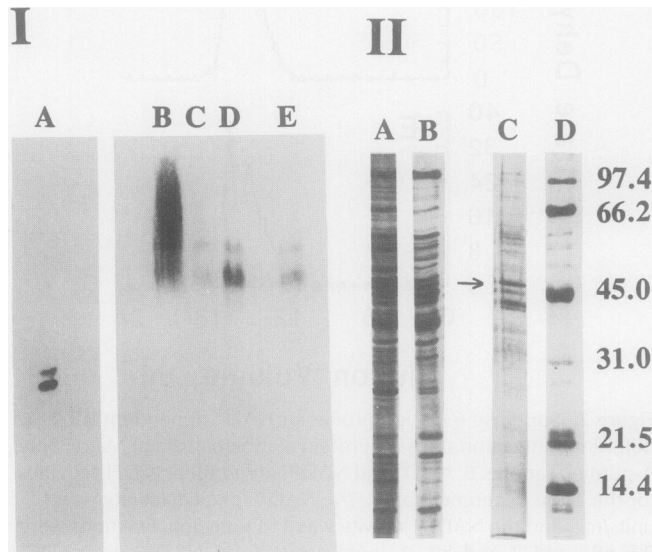

Figure 2. Superose 6 elution profile for NAD⁺-dependent ICDH at different purification steps. A, Protein elution pattern of Matrix Blue A-purified sample. B, NAD⁺ and NADP⁺-dependent ICDH activities for the same experiment as A. The NAD⁺ specific activity was 475 units/mg, and the NADP⁺ activity was 114 units/mg. Fractions were 0.5 mL, and 2 and 80 μL were assayed for NAD⁺ and NADP⁺ activity, respectively. C, ICDH activity for a sample of the crude extract (specific activity 20 units/mg protein) that was frozen before chromatography on Superose 6. D, ICDH activity for a sample that was purified on Matrix Gel Blue A and then stored overnight at 4°C. E, Activity for the same sample as D except that the protein was subsequently chromatographed on Superose 6 and Mono Q before a final analysis by Superose 6.

found in the 690- and 300-kD peaks (Fig. 2C). Passing the crude extract over Matrix Gel Blue A column and overnight storage resulted in peaks at 300 and 94 ± 16 kD (Fig. 2D). Purification through Blue A, Superose 6, Mono Q, and back through Superose 6 yielded two clear peaks with apparent molecular masses of 210 ± 50 and 94 kD (Fig. 2E).

Although this data is only correlative, it does appear that as the enzyme dissociated during purification it lost activity. When the different fractions were analyzed by SDS-PAGE, the only bands that consistently appeared in all active fractions were a minor band at 45 kD and a major band at 47 kD (Fig. 3). The subunits of the yeast and mammalian ICDHs are reported to be 39 to 46 kD (1, 5, 14, 22). This indicates that the smallest active ICDH measured at 94 ± 16 kD is a dimer, most likely a homodimer of two 47-kD subunits. The 300 ± 50 kD form would be an octamer, the form that is considered to be the major active species (2), and the 690 ± 90 and 1400 ± 200 kD forms would be made up of two and four octamers, respectively.

The NADP⁺-dependent ICDH eluted from a Superose 6 column as a single symmetric band at 80 ± 10 kD (Fig. 2B). This NADP⁺-specific activity was readily purified away from the NAD⁺ enzyme under these conditions. As a result, following the Superose 6 step the NAD⁺-dependent ICDH contained no detectable NADP⁺-ICDH activity.

The enzyme could be stored at 4°C in 20% glycerol for 30

Table I. *Summary of the Isocitrate Kinetic Constants for NAD⁺-Dependent Pea Mitochondrial ICDH*

The enzyme was purified by Matrix Gel Blue A and Superose 6 chromatography and either assayed immediately, frozen-thawed and assayed, or allowed to stand for 24 h at 4°C before it was assayed. The assays were performed with or without 1 mM citrate. The data was fit to the Hill equation using the Enzfitter program from Elsevir-BIOSOFT. The results are the average of three experiments.

Tissue	V_{max}	K	Hill Constant	$S_{0.5}$
	nmol/mg/min	mM		mM
Fresh	8788	0.025	3.1	0.3
Frozen	1332	0.182	1.8	0.4
Frozen + citrate	3220	0.010	2.8	0.3
Day old	9043	0.10	3.1	0.3
Day old + citrate	9615	0.064	1.2	0.3

h with only about 15% loss of activity. However, much higher losses in activity occurred during the chromatographic steps. Part of the activity loss resulted from the inclusion of salts to elute the enzyme from the Matrix Gel Blue A and Mono Q columns. A nondenaturing gel system and an in-gel activity stain were used to follow the dissociation of protein during purification. Figure 3 shows the results of analyzing the crude matrix extract, the eluate from the Matrix Gel Blue A, a sample from Superose 6, and a sample that was exposed to 500 mM NaCl on this gel system. The crude extract shows NAD⁺ and isocitrate-specific activity in about four bands (possibly representing the 1400-, 690-, 300-, and 210-kD forms). After the protein was processed by any of the chromatography steps or even just treated with the concentration of NaCl needed to perform the chromatography, all of the activity appeared in two minor and one major bands. The NADP⁺-dependent ICDH activity in the crude matrix fraction ran much farther into the gel and could clearly be separated from the NAD⁺ enzyme.

Enzyme Kinetics

A kinetic analysis was done on the 200-fold purified enzyme following Matrix Gel Blue A and Superose 6 chromatography. The enzyme response to increasing isocitrate concentration was clearly sigmoidal with a Hill constant of 3.1, a K value of 0.025 mM, and an $S_{0.5}$ of 0.3 mM (Table I). Unlike some earlier reports (7, 8), citrate had no effect on the freshly isolated enzyme (Table II). After the enzyme had been frozen and thawed, the activity, affinity for isocitrate, and the Hill constant all decreased. This decrease could then be partially reversed by the addition of citrate (Table I). These changes in the kinetic constants for this enzyme might be related to the dissociation that occurred under these same conditions (Fig. 2). Cox and Davies (7) reported that their crude NAD⁺-ICDH from peas was sigmoidal with respect to isocitrate but that the addition of 1 mM citrate caused the kinetics to change to Michaelis-Menten. Although we could not reproduce this with fresh enzyme, we could show the affect with enzyme that had aged at 4°C for 24 h. Under these conditions, the Hill number was 3.1 in the absence of citrate and 1.2 in the

Figure 3. Nondenaturing, SDS-PAGE, and activity gels of ICDH during purification. I, The protein samples were separated by nondenaturing PAGE, and NAD⁺ or NADP⁺-specific ICDH activity was detected as described in "Materials and Methods." A, Crude matrix extract stained for NADP⁺-ICDH. B to E, Stained for NAD⁺-ICDH. B, Crude matrix extract (360 µg protein). C, Crude matrix extract incubated 1 h in 0.5 M NaCl and then dialyzed (180 µg protein). D, Matrix Gel Blue A-purified sample (150 µg protein). E, Sample purified through Matrix Gel Blue A and Superose 6. II, Silver-stained 15% SDS-PAGE gel for different steps during ICDH purification. A, Crude matrix extract (9 µg protein). B, Matrix Gel Blue A-purified protein (5 µg protein). C, Superose 6 fraction (0.5 µg protein). D, Molecular mass standards (kD).

Table II. *The Effect of Divalent Cations, Nucleotides, and Organic Acids on the NAD-Dependent ICDH from Pea Mitochondria*

All assays used Superose 6-purified enzyme, and the reaction conditions were 10 mM isocitrate, 1 mM NAD, 5 mM $MgSO_4$, and 2 mM β-mercaptoethanol in 20 mM Mops (pH 7.5).

Compound	Concentration	Enzyme Activity	
	mM	*units/mg*	*% Control*
Experiment I			
Control		2.75	100
Citrate	0.01	2.34	85
	0.1	2.56	93
	1.0	2.82	103
ADP	0.01	2.93	107
	0.1	3.08	112
	1.0	2.78	101
	10.0	1.47	53
Experiment III			
Control		4.21	100
AMP	0.01	4.51	107
	0.1	4.03	96
	1.0	4.03	96
ATP	0.01	3.48	83
	0.1	3.99	95
	1.0	3.88	92
Experiment IV			
Control		3.67	100
α-Ketoglutarate	0.1	3.88	106
	1.0	3.92	107
	5.0	3.48	95
L-Glutamate	0.1	3.67	100
	1.0	3.74	102
	5.0	3.59	98

Table III. *Comparison of Mitochondrial NAD^+ and $NADP^+$-Dependent ICDH that Are Matrix and Membrane Associated*

Mitochondria were purified through a Percoll gradient and resuspended in 20 mM Mops (pH 7.5) and 2 mM β-mercaptoethanol. A portion of the mitochondria were dissolved in 0.5% Triton X-100 to yield total activity. The remaining portion was ruptured with two freeze-thaw cycles, and the matrix and membrane fractions were separated by centrifugation.

Enzyme Substrate	Enzyme Activity				
	Total mitochondria	Matrix		Membrane	
	nmol/min	*nmol/min*	*%*	*nmol/min*	*%*
NAD^+	17,362	7200	41	9,200	53
$NADP^+$	19,121	7100	37	12,015	63

Figure 4. Effect of NADPH on the NAD^+-dependent ICDH from pea mitochondria. The enzyme used was purified through Matrix Gel Blue A and Superose 6.

presence of 1 mM citrate, and there was little change in V_{max} or K value (Table I).

The enzyme showed standard Michaelis-Menten kinetics for NAD^+ with an apparent K_m of 0.2 to 0.8 mM between different enrichment fractions of the enzyme. As with the other mitochondrial decarboxylases, NADH is a competitive inhibitor with a K_i for NAD^+-ICDH of 0.2 mM (data not shown). It is interesting that, although $NADP^+$ had no effect on the enzyme either as a substrate or an inhibitor, NADPH proved to be a potent inhibitor (Fig. 4). Unlike NADH in which the inhibition was competitive with respect to NAD^+, the inhibition by NADPH was noncompetitive with respect to NAD^+ ($K_i = 0.29$ mM). This would suggest that there is a separate binding site for NADPH on the ICDH that is not the catalytic site for NAD^+ and NADH binding.

The effect of a range of potential modifiers were tested on the NAD^+-dependent ICDH from pea mitochondria (Table II). The pea enzyme, as with all other forms of the enzyme studied (2), requires a divalent cation. Mg^{+2} or Mn^{+2} can fill this requirement, whereas Zn^{+2} and Sr^{+2} are inhibitory (data not shown). Unlike the situation with the yeast, blowfly, and bovine enzymes, AMP, ADP, and ATP have no effect on the pea enzyme. Similarly, α-ketoglutarate and L-glutamate, inhibitors of the *Neurospora crassa* (5) and *Rhodosporidium toruloides* (13) enzymes, do not affect the pea protein.
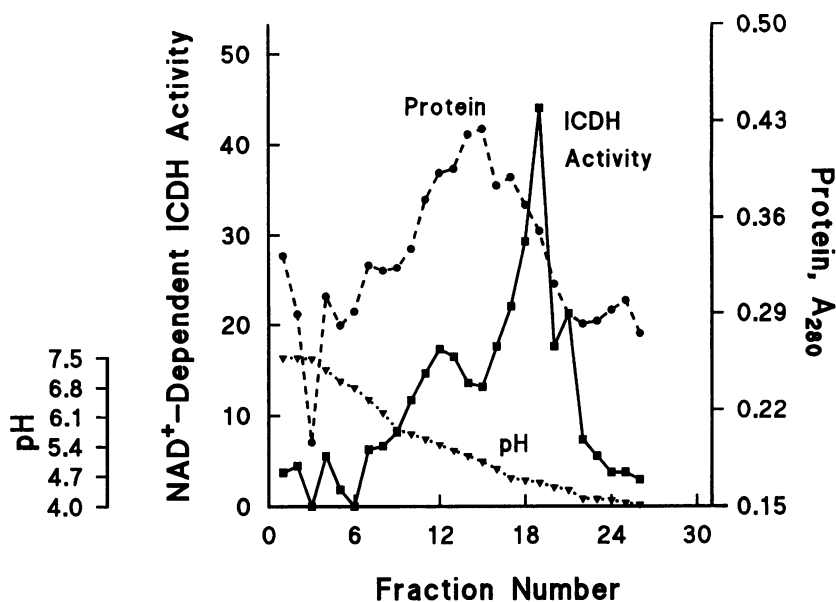
Pea mitochondria contain two different ICDH enzymes with specificity for NAD^+ and $NADP^+$. In addition, both enzymes occur in a soluble and membrane-associated form (Table III). The amounts of the NAD^+- and $NADP^+$-specific enzyme activities in isolated purified mitochondria were nearly equal. After the mitochondria were broken by freezing and thawing, about 60% of the $NADP^+$-dependent enzyme was still associated with the membrane fraction. With the NAD^+ enzyme, 53% of the initial activity and 56% of the activity recovered was membrane associated. Repeated washings did not release any more enzyme from the membrane. At this point, we are not able to determine whether the matrix and membrane-associated forms of the enzyme are different molecular forms or the same forms in different environments.

The soluble mitochondrial NAD^+-dependent ICDH has a pH optimum of 7.5 to 8.0 whether measured with the crude enzyme or the enzyme purified through Matrix Gel Blue A,
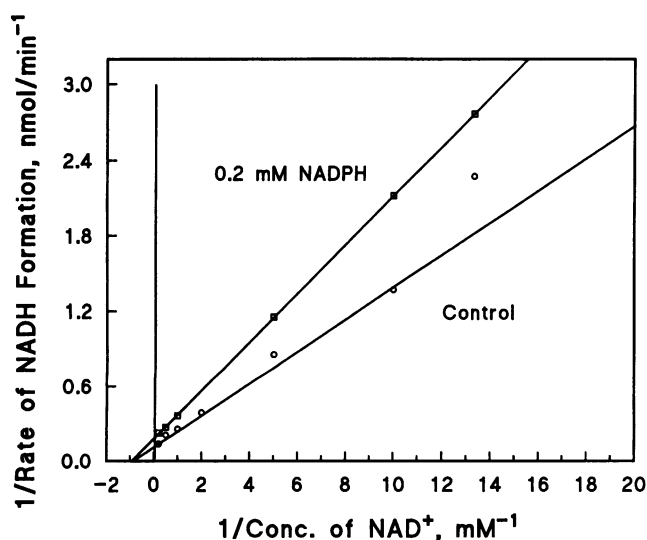
Figure 5. Elution of pea mitochondrial NAD$^+$-dependent ICDH off a PBE-94 chromatofocusing column. The enzyme was purified through the Matrix Gel Blue A column and applied to and eluted from the PBE-94 column according to the manufacturer's instructions. After the pH of each fraction was measured, the pH was adjusted to 7.5, and NAD$^+$-dependent ICDH activity was measured.

DISCUSSION

The mitochondrial NAD$^+$-specific isoform of ICDH serves a major role in the tricarboxylic acid cycle (10). Despite its important physiological role, the instability of this enzyme has prevented detailed analysis of its molecular structure and kinetic capabilities. After the enzyme has been eluted from Matrix Gel Blue A, it can be stabilized in 40% glycerol, such that it can be stored for several days at 4°C. The enzyme cannot be frozen without significant changes in its kinetic characteristics. Similarly, when kept in 20% glycerol, exposure of the enzyme to salts needed for gel filtration and ion exchange chromatography result in substantial changes in the molecular species of the protein. All of these changes appear to be associated with the dissociation of the high molecular mass form of the protein into smaller polymeric aggregates. The native form of the enzyme in animal and yeast is a homooctamer. This would probably correspond to our 300 ± 50-kD peak. In crude preparations from pea mitochondria, the major forms of NAD$^+$ ICDH have apparent molecular masses of 1400 and 690 kD with about half of the activity in each peak. Although the 300-kD form is present in the crude matrix extract, it represents a relatively minor species. This suggests that in the fresh matrix extract the major forms appear as polymeric aggregates of either two or four octamers. Interestingly, as the purification proceeded,

Superose 6, and Mono Q chromatography (data not shown). The protein purified though Matrix Gel Blue A had an apparent isoelectric point of about pH 4.5 based on the major elution peak from a PBE-94 chromatofocusing column (Fig. 5). The shoulder at pH 5.3 is reproducible and may represent a different aggregation state of the enzyme or the association of ICDH with other matrix proteins. The unusual isoelectric point of ICDH combined with the large molecular mass of the crude protein should allow for its ready purification if the stability problem could be addressed.

we observed the loss of these high molecular mass forms and their replacement with enzymes that moved like octamers, tetramers, and dimers. Although we were unable to obtain homogeneity with the enzyme, we identified two bands at 45 and 47 kD on SDS-PAGE that consistently copurified with NAD$^+$-ICDH activity. Because the 47-kD band is the predominant form, we are tentatively identifying it as the NAD$^+$-ICDH monomer from pea mitochondria.

These characteristics are all very similar to the enzymes characterized from yeast and animal systems in which a subunit size of 39 to 43 kD is seen and the native enzyme appears to be a homooctamer. The one exception may be blowfly flight muscle mitochondria in which the most purified form of the enzyme showed subunits at 39 and 46 kD. The presence of various molecular mass forms under nondenaturing conditions has also been observed with other preparations. The bovine enzyme, for example, has been observed at 330, 670, and 1100 kD (14). In this case, the degree of enzyme association was closely related to the protein concentration. This may have been a factor in the dissociation of our enzyme. The bovine enzyme was sensitive to adenine nucleotide concentration, and this sensitivity was mediated through subunit association/dissociation. By contrast, the pea enzyme appears insensitive to AMP, ADP, or ATP (Table II).

Mitochondrial NAD$^+$-specific ICDH is allosteric with respect to isocitrate. This has been noted in most studies on the enzyme from a number of sources (5, 16, 17, 22). In an earlier study (7, 8), it was suggested that citrate is a potent effector of the pea mitochondrial NAD$^+$ ICDH. Although we were unable to reproduce this effect with fresh enzyme, we were able to demonstrate a citrate-dependent change in the kinetics of the enzyme that had been aged. In keeping with our observations that the oligomeric level of the enzyme changes during storage and manipulation and that the degree of allosterism for enzymes in general is related to oligomer status, it may be that change in kinetic properties is related

to citrate inducing reassociation of enzyme subunits. The physiological role, if any, of citrate in regulating the enzyme is not clear at this point and must be questioned given that it is only a property of the aged enzyme.

As with the other mitochondrial NAD⁺-linked dehydrogenases, ICDH is competitively inhibited by NADH. This inhibition is probably a key regulator of the flow of carbon through the tricarboxylic acid cycle. Surprisingly, however, NADPH also inhibits this enzyme. The specificity of this inhibition was supported by the observation that NADP⁺ did not act as a substrate for the enzyme or an inhibitor for the NAD⁺-dependent reaction. Because the inhibition by NADPH was noncompetitive, it appears that NADPH binds at a different site from NAD⁺/NADH. It is intriguing to postulate that the inhibition by NADPH might also be physiologically significant. Rasmusson and Moller (20) have noted that respiration by potato mitochondria oxidizing malate and isocitrate is stimulated by added NADP⁺ under conditions where external NADPH oxidation is inhibited. This suggests that electrons cycled through NADPH are entering the mitochondrial electron transport chain either directly through complex I or via the nicotinamide nucleotide trans-hydrogenase activity with the resulting NADH reoxidized by complex I. To control and balance the production of NADH and NADPH, the feedback inhibition of ICDH activity would need to be affected by both NADH and NADPH.

NAD⁺-dependent ICDH is often considered the rate-limiting step in tricarboxylic acid cycle activity by plant mitochondria (19, 24), largely based on its limited in vitro activity. The enzyme in vitro is affected by a number of potential regulators, including divalent cations, NADH, NADPH, pH, and possibly citrate. Given the limited pH gradient across plant mitochondrial membranes and the observation that the citrate affect is limited in freshly prepared enzyme, it would appear that the concentration of NADPH and the [NADH]/[NAD⁺] are the major regulators in situ.

NOTE ADDED IN PROOF

An antibody specific for the 47-kD protein band inhibited the NAD-ICDH activity in a crude matrix extract, verifying that this protein is the monomer of the enzyme.

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