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Purification and Characterization of Chloroplastic NADP-Isocitrate Dehydrogenase from Mixotrophic Tobacco Cells

Comparison with the Cytosolic Isoenzyme

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Green, mixotrophic tobacco (*Nicotiana tabacum*) cell cultures in the exponential growth phase were found to have two clearly distinguishable NADP-isocitrate dehydrogenase (ICDH; EC 1.1.1.42) isoenzymes. Their elution behavior during anion-exchange column chromatography was similar to that described previously for the cytosolic (ICDH1) and chloroplastic (ICDH2) enzymes from pea (*Pisum sativum*) leaves. ICDH2 was absent in etiolated tobacco cell suspensions and appeared during the greening process. Both isoforms were purified to apparent electrophoretic homogeneity by ammonium sulfate fractionation and anion-exchange and affinity chromatography. The isoenzymes were separated on a DEAE-Sephacel column, but the most effective step was a Matrex Red-A column, which enabled an overall purification of 833- and 1328-fold for ICDH1 and ICDH2, respectively. Polyclonal antibodies were raised against each isoform. The ICDH2-specific antibody was used to localize tobacco leaf ICDH2 in situ by an immunogold labeling technique. The enzyme was found largely, if not exclusively, in the chloroplasts of green leaves. ICDH1 and ICDH2 were shown to have apparent native molecular weights of 117,000 and 136,000, respectively, and to consist of identical, 48.5-kD subunits. Similar apparent K_m values for NADP, D(+)isocitrate, and Mg^{2+} were found for the two enzymes when assayed with Mg^{2+} as the metal cofactor.

NADP-ICDH (EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitrate to yield 2-oxoglutarate. The enzyme is widely distributed in living organisms. It has been purified to homogeneity, and its properties have been investigated, from bacteria (Barrera and Jurtshuk, 1970; Reeves et al., 1972; Fukunaga et al., 1992), cyanobacteria (Muro-Pastor and Florencio, 1992), fungi (Meixner-Monori et al., 1986), and mammals (Farrell, 1980), as well as from a few plant tissues (Omran and Dennis, 1971; Curry and Ting, 1976; Henson et al., 1986; Ni et al., 1987; Chen et al., 1988). In higher plants, NADP-ICDH activity has been detected in all tissues and organs where it was assayed. Although the activity is usually cytosolic (Henson et al., 1986), it has also been detected in chloroplasts (Elias and Givan, 1977; Randall and Givan, 1981; Chen et al., 1989) and mitochondria (Curry and Ting, 1976; Rasmusson and Möller, 1990). To date, only the cytosolic higher-plant isoenzyme has been purified to homogeneity. It has been reported that the cytosolic (ICDH1) and chloroplastic (ICDH2) isoenzymes from pea leaves exhibit distinct elution profiles during column chromatography on DEAE-cellulose; the chloroplastic enzyme eluted at an ionic strength of 120 mM KCl compared with 60 mM for the cytosolic enzyme. ICDH1 represents approximately 95% of the total pea leaf NADP-ICDH activity (Chen et al., 1989). The very low activity of the chloroplastic isoform in pea leaves renders its purification impractical from this plant tissue.

Recently, we have observed that extracts from green tobacco (*Nicotiana tabacum*) cell-suspension cultures show, in addition to the well-characterized peak of ICDH1 activity, a second peak of activity eluting at about the same ionic strength as that of the ICDH2 from pea chloroplasts (Chen et al., 1989). This latter activity peak was absent in etiolated tobacco cells and appeared during the greening process. The increased ratio of ICDH2:ICDH1 activity in green tobacco suspension cultures and the light-induced ICDH2 activity suggest that this isoenzyme could have an important physiological role in such cells.

Taking advantage of this relatively high NADP-ICDH2 activity, in this paper we describe the purification and comparative characterization of the cytosolic and chloroplastic NADP-ICDH isoenzymes from cultured tobacco cells. The findings represent not only the first purification of a plastidic NADP-ICDH from a higher plant, but also a detailed comparison of the properties of two NADP-ICDH isoenzymes from the same plant species.

MATERIALS AND METHODS

Materials

NADP (sodium salt) was obtained from Boehringer (Mannheim, Germany). DEAE-Sephacel and Protein A-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Matrex Red-A was from Amicon Corp. (Danvers, MA). Complete Freund coadjuvant was obtained from Difco

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Abbreviations: FPLC, fast-protein liquid chromatography; NADP-ICDH, NADP-isocitrate dehydrogenase.

Laboratories (Detroit, MI). DL-Isocitrate, NADP, and horseradish peroxidase-conjugated sheep anti-rabbit antibodies were purchased from Sigma.

Plant Material

Culture and subculture of tobacco (*Nicotiana tabacum* L. cv Xanthi) cells were performed as described previously (Nato et al., 1977). Cells were grown in a modified Murashige and Skoog (1962) medium supplemented with 30 g/L Suc, under a 16-h photoperiod at a PPFD of 180 μmol m^{-2} s^{-1} at 28°C as described elsewhere (Nato et al., 1983). Tobacco seedlings were grown in vermiculite in a greenhouse with 12 h of supplemental light (150 μmol m^{-2} s^{-1}) at 25°C. Mature leaves were harvested after 3 months of growth.

Greening Procedure

Green tobacco cells were first etiolated by three consecutive cultures of 2 weeks each in the dark, as described by Brangeon and Nato (1981). The nongreen cells were then transferred to fresh culture medium and greened by exposure to continuous light (180 μmol m^{-2} s^{-1}) for 6 d before harvest.

Purification of the NADP-ICDH Isoenzymes

Mature leaves from 3-month-old tobacco plants were excised and homogenized in a blender. Cultured tobacco cells were collected on cheesecloth (50-μm mesh diameter), washed with distilled water, and disrupted using a French press (120 bar). The ice-cold extraction medium (1:3, g fresh weight/mL) was composed of 100 mM potassium phosphate, pH 7.5, 14 mM 2-mercaptoethanol, 1% (w/v) PVP. The brei was filtered through two layers of cheesecloth and the filtrate was centrifuged at 9700*g* for 30 min. The supernatant fraction was precipitated at 45 to 80% saturation $(NH_4)_2SO_4$. The pellet was collected by centrifugation, dissolved in 1 volume of buffer A (10 mM potassium phosphate, pH 7.5, 5% [v/v] glycerol, 14 mM 2-mercaptoethanol, 1 mM $MgCl_2$), and dialyzed overnight at 4°C against 5 L of buffer A.

The desalted sample was applied to a DEAE-Sephacel column (1.8 × 15 cm) equilibrated with buffer A and washed until the eluate was protein free. A 500-mL linear gradient of 0 to 0.4 M KCl in buffer A was used to elute NADP-ICDH activity. The flow rate was kept at 25 mL h^{-1} during this chromatography. Fractions containing the two peaks of NADP-ICDH activity were combined separately and dialyzed overnight against buffer A.

The desalted enzyme solution was placed onto a Matrex Red-A affinity column (1.8 × 4 cm) equilibrated with buffer A. After washing, NADP-ICDH activity was eluted with a linear gradient of 0 to 1 mM NADP and 0 to 25 mM DL-isocitrate in buffer A at a flow rate of 18 mL h^{-1}. Fractions containing enzyme activity were dialyzed against buffer A and concentrated by ultrafiltration (Amicon YM30 membrane). The purified NADP-ICDH isoenzymes were stable after storage at −20°C for 2 months in a modified buffer A containing 20% (v/v) glycerol.

Enzyme and Protein Assays

NADP-ICDH activity was measured spectrophotometrically by following the reduction of NADP at 340 nm and 30°C in 100 mM potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$, 2.5 mM DL-isocitrate, and 0.25 mM NADP. The reaction was initiated by the addition of isocitrate. One unit of activity is defined as the production of 1 μmol NADPH min^{-1}.

Protein concentration was determined spectrophotometrically according to Bradford (1976) with BSA as the standard.

Gel Filtration

The molecular mass of each purified native enzyme was estimated using an FPLC Superose 6 (HR 10/30) gel-filtration column equilibrated with 50 mM Hepes, pH 7.5, 200 mM KCl. The molecular mass standards used to calibrate the column were thyroglobulin (669 kD), γ-globulin (158 kD), BSA (66 kD), ovalbumin (45 kD), myoglobin (17 kD), and vitamin B_{12} (1.4 kD).

Kinetic Properties

Kinetic analyses of both the cytosolic and chloroplastic NADP-ICDH isoenzymes were carried out at 30°C and pH 7.5. Apparent K_m values for each substrate were obtained at saturating concentrations of the other two substrates, using Mg^{2+} as the bivalent cation. To avoid the lag phase, which is probably due to formation of the Mg^{2+}-isocitrate complex (Maloney and Dennis, 1977), the reaction was started by addition of enzyme. Michaelis constants were calculated from Lineweaver-Burk double-reciprocal plots.

PAGE

SDS-PAGE was carried out by the procedure of Laemmli (1970). Gels (10% polyacrylamide) were run at 100 V for 2 h at room temperature and subsequently stained with 0.25% (w/v) Coomassie brilliant blue R-250.

Nondenaturing PAGE was performed at 4°C using a 4% stacking gel and a 7% separating gel at 60 V for 4 h. NADP-ICDH activity was visualized by staining with phenazine methosulfate and nitroblue tetrazolium (Markert and Möller, 1959).

Preparation of Antibodies

Antibodies raised against both denaturated ICDH1 and native ICDH2 from tobacco cell cultures were prepared as described by Vidal et al. (1980). The IgG fraction was precipitated with 33% saturation ammonium sulfate, redissolved in 50 mM potassium phosphate buffer, pH 7.5, 0.9% (w/v) NaCl, and purified by affinity chromatography on protein-A Sepharose. After washing the column with the above buffer, the IgG fraction was eluted with 0.2 M sodium citrate, pH 2.8.

Immunotitration of ICDH Activity

A given amount of ICDH1 or ICDH2 activity (0.3 unit) was incubated for 12 h at 4°C with increasing amounts of

antibody raised against either the ICDH1 or the ICDH2 isoform. Insoluble antigen-IgG complexes were pelleted by centrifugation, and the ICDH activity in the supernatant fraction was measured.

Immunoblotting

Protein samples were subjected to SDS-PAGE using a 4% stacking gel and a 10% separating gel. The separated proteins were electrotransferred to a nitrocellulose membrane using a Bio-Rad transfer apparatus at 200 mA for 4 h. The transfer buffer consisted of 25 mM Tris, pH 8.3, 192 mM Gly, 20% (v/v) methanol. The blocking of nonspecific binding sites was carried out by soaking the membrane in 25 mM Tris, pH 7.5, 150 mM NaCl containing 5% (w/v) nonfat powdered milk for 30 min at room temperature. The bands corresponding to the NADP-ICDH polypeptide were visualized by incubating the membrane with antiserum diluted 1:250 and horseradish peroxidase-conjugated sheep anti-rabbit IgG antibody (Tuckey and Holland, 1989), with 4-chloro-1-naphthol as substrate.

Immunolocalization of ICDH2

Preparation of Leaf Tissue for Light Microscopy

Tobacco leaves were cut into small pieces, fixed in 4% (v/v) paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.3, for 3 h at room temperature, and then dehydrated in a graded ethanol series terminating at 100% absolute ethanol. Samples were embedded in L.R. White resin, which was then polymerized at 60°C, and semithin sections (0.5–1.0 μm) were cut.

Immunogold Staining

The embedded leaf sections were washed twice for 10 min each in 100 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl (PBS) and 1 h in PBS containing 5% (w/v) BSA, and then incubated overnight at 4°C with the immunoaffinity-purified IgG fraction containing antibodies against ICDH2 (44 μg mL^{-1}) diluted with PBS and 0.1% (v/v) Triton X-100. After three washes in PBS containing 5% (w/v) BSA, the sections were incubated for 1 h at room temperature in biotinylated goat anti-rabbit IgG, washed three times in PBS, and then incubated for 2 h at room temperature in streptavidin conjugated to 5-nm gold particles diluted in PBS containing 0.1% (w/v) BSA. The samples were finally washed three times in PBS and then in distilled water. Immunogold-labeled sections were treated with 0.2 M sodium citrate, pH 3.5, the stain was intensified for 2 min at room temperature in silver, and the sections were then washed in distilled water. Slides were examined and photographed using bright-field optics or bright-field optics in conjunction with epipolarizing filters.

RESULTS

NADP-ICDH Isoenzymes from Tobacco Leaf and Cell Cultures

In previous studies, the leaf cytosolic and chloroplastic isoenzymes of NADP-ICDH have been characterized by their different elution patterns from an anion-exchange column (Chen et al., 1989). Figure 1A shows the elution profile of NADP-ICDH activity from a tobacco leaf 45 to 80% saturation $(NH_4)_2SO_4$ fraction; in this material ICDH1 represents 98% of the total activity, whereas the ICDH2 activity is extremely low. In contrast, two clearly separated peaks of NADP-ICDH activity can be seen in 45 to 80% saturation $(NH_4)_2SO_4$ fractions of green tobacco cells (Fig. 1B), the second peak eluting at an ionic strength (140 mM KCl) close to the value reported for the chloroplastic isoenzyme of pea leaves (Chen et al., 1989). In the green suspension cells, ICDH2 activity constituted 19% of the total activity. To investigate the possible physiological role(s) of these two isoenzymes we next compared the chromatographic isoenzyme pattern in tobacco suspension cultures grown under different conditions.

The 45 to 80% saturation $(NH_4)_2SO_4$ fractions from etiolated and green tobacco culture cells and from 6- (exponential-growth phase) and 25-d-old (stationary phase) tobacco cells cultured under mixotrophic conditions were obtained. Etiolated tobacco culture cells showed only a single peak of NADP-ICDH activity, which eluted at an ionic strength corresponding to cytosolic ICDH (Table I). Cells greened from etiolated material and S-phase and stationary growth-phase cells showed a very similar chromatographic profile, with an ICDH2 activity between 18 and 24% of the total ICDH activity.

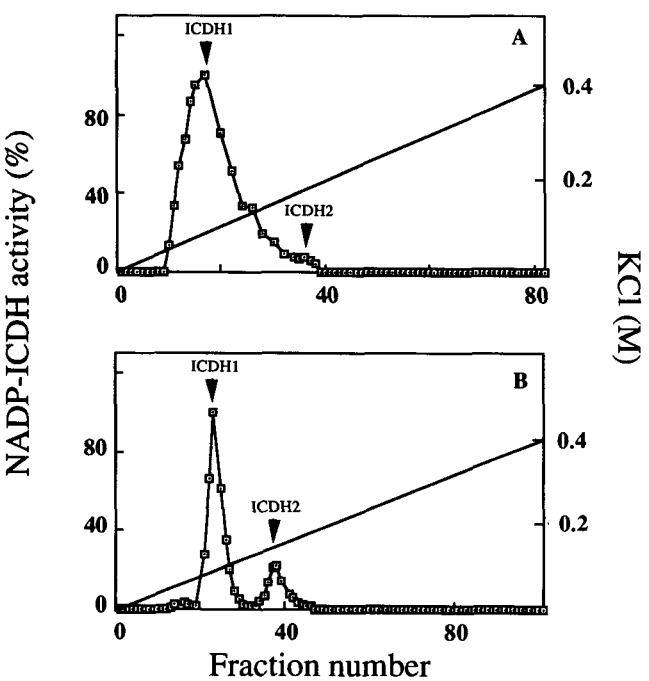

Figure 1. Elution pattern of NADP-ICDH activity during chromatography on DEAE-Sephacel. A, Dialyzed, 45 to 80% saturation $(NH_4)_2SO_4$ fraction obtained from green tobacco leaves. Maximal (100%) activity was 0.24 μmol min^{-1} mL^{-1}. The main peak corresponds to NADP-ICDH1, whereas ICDH2 appears as a minor shoulder of the main peak. B, Exponentially growing green tobacco cells. Maximal activity was 1.1 μmol min^{-1} mL^{-1}.

Table I. *Comparison of the NADP-ICDH isoenzyme pattern from 50 g of tobacco leaves and 9 to 11 g of tobacco cell suspensions*

The 45 to 80% saturation $(NH_4)_2SO_4$ fractions were prepared as described in "Materials and Methods." NADP-ICDH isoenzymes were separated on a DEAE-Sephacel column (see Fig. 1).

	Tobacco Leaves		Tobacco Cell Cultures							
	Green		Etiolated		Greened		S-phase (6 d)		Stationary phase (25 d)	
	ICDH1	ICDH2	ICDH1	ICDH2	ICDH1	ICDH2	ICDH1	ICDH2	ICDH1	ICDH2
ICDH activity (% of total)	98	2	100	0	82	18	81	19	76	24
Units/g fresh wt	0.11	0.002	0.7	0	0.58	0.13	1.36	0.32	0.21	0.068
ICDH2/ICDH1	0.018		0		0.22		0.23		0.32	

However, on a fresh weight basis, some significant differences were seen between the different tobacco cell-suspension cultures; exponentially growing cells showed 2- to 6.5-fold higher ICDH1 and ICDH2 activities per gram fresh weight than the other green cultures. In spite of the high ICDH2:ICDH1 ratio found in stationary-phase cells, their activity was relatively low (Table I).

The view that the ICDH2 isoenzyme may have some physiological role(s) is suggested by the observed induction of this isoenzyme during the greening of tobacco culture cells (Table I). This increased ICDH2 activity in green tobacco cells prompted us to further investigate the enzyme's properties and subcellular localization. For this purpose, exponentially growing tobacco cells were the most useful material for the purification of this isoenzyme because their ICDH2 activity was 160-fold higher than in green tobacco leaves and 2- to 5-fold higher than in greened or stationary-phase culture cells. Moreover, these S-phase cells make it possible to purify and compare both NADP-ICDH isoenzymes from the same plant material, which is important when investigating the possible physiological role(s) of each isoenzyme.

Purification of NADP-ICDH Isoenzymes from Tobacco Culture Cells

After solubilization of the 45 to 80% saturation $(NH_4)_2SO_4$ precipitate, extensive dialysis against buffer A was necessary to measure ICDH activity. Subsequent chromatography on DEAE-Sephacel produced two peaks of activity, the second one exhibiting 19% of the total NADP-ICDH activity (Table I). The respective total activities of each peak were used to calculate the relative contribution of each isoform to the total activity in both the crude and ammonium sulfate fractions, from which the respective purification factors were deduced (Table II). By far the most effective purification step was the elution from the Red-A affinity column by isocitrate plus NADP, which resulted in a 44- and 85-fold increase in specific activity of ICDH1 and ICDH2, respectively, and removed most of the contaminating proteins. If this final step was performed with a linear gradient of 0 to 0.4 M KCl, the ICDHs were not electrophoretically homogeneous. This three-step protocol resulted in an 833- and 1328-fold purification of the ICDH1 and ICDH2 isoforms, respectively, and yielded about 800 μg of ICDH1 and 670 μg of ICDH2 from 195 g of 6-d-old, S-phase cells.

The purified forms of both NADP-ICDH isoenzymes were analyzed by nondenaturing PAGE and their positions were revealed by enzymic activity. Figure 2A documents that the two forms of ICDH differ in their R_F, with the ICDH2 protein having a greater electrophoretic mobility. This result, together with its elution position from an anion-exchange column (Fig. 1B), suggests that ICDH2 is more negatively charged than ICDH1. Following separation by SDS-PAGE, the final products of the purification procedure summarized in Table II appear as a single, Coomassie blue-stained polypeptide with a mol wt of 48,500 (Fig. 2B).

Table II. *Purification of the ICDH1 and ICDH2 isoenzymes from the same plant material*

Green tobacco suspension cells growing exponentially were extracted (195 g) as described in "Materials and Methods."

	Total Activity	Total Protein	Specific Activity	Yield	Purification
	units	*mg*	*units/mg*	%	*-fold*
ICDH1					
Crude extract	271	4620	0.06	100	1
$(NH_4)_2SO_4$ precipitate	284	1027	0.27	105	4.5
DEAE-Sephacel	267	235	1.14	99	19
Matrex Red-A	40	0.8	50	15	833
ICDH2					
Crude extract	64	4620	0.014	100	1
$(NH_4)_2SO_4$ precipitate	66	1027	0.064	103	4.6
DEAE-Sephacel	63	286	0.22	98	16
Matrex Red-A	12.5	0.67	18.6	20	1328

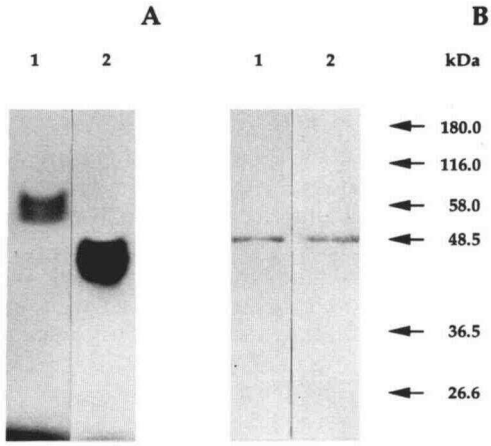

Figure 2. Native and SDS-PAGE analysis of the purified NADP-ICDH isoenzymes. A, Native gel stained for NADP-ICDH activity. B, SDS-polyacrylamide gel stained with Coomassie blue. Purified ICDH1 (lane 1) or ICDH2 (lane 2) was loaded in each well (2 μg).

Based on analysis by FPLC gel filtration, the native molecular mass of the NADP-ICDH isoforms was determined to be 117 kD for the cytosolic enzyme and 136 kD for ICDH2 (data not shown).

Kinetic Analysis

To be active, ICDH has an absolute requirement for a bivalent cation such as Mn^{2+} or Mg^{2+}. In this study, the kinetic constants of the two forms of tobacco culture cell ICDH were determined using Mg^{2+} as the cofactor.

The Mg^{2+} present in the purified enzyme preparations was eliminated by repeated concentration against buffer A lacking this cation. The saturation curves of both NADP-ICDH isoforms for their cofactor or substrates in the forward reaction were hyperbolic. The apparent K_m value for isocitrate was calculated using DL-isocitrate as the substrate; this material contained 50% D(+)isocitrate. The estimated K_m for D(+)isocitrate was 41 μM for the cytosolic enzyme and 28 μM for the chloroplastic isoform. The apparent K_m values for Mg^{2+} and NADP were 130 and 7.5 μM for the cytosolic enzyme and 120 and 7 μM for the chloroplastic ICDH, respectively.

Immunological Comparison of the Isoenzymes

Antiserum raised against ICDH2 markedly decreased the activity of the enzyme, but did not immunoprecipitate ICDH1 (Fig. 3B). As a consequence, this antiserum appears to be specific for ICDH2. On the other hand, the antibodies directed against ICDH1 inhibited the cytosolic enzyme, but some recognition of ICDH2 was also observed (Fig. 3A). These immunoassays suggest that the ICDH isoenzyme found in the cytosol and ICDH2 do not appear to share many common epitopes.

The specificity of the antibodies for the NADP-ICDH isoenzymes was evaluated further by immunoblot analysis of both crude and partially purified extracts from tobacco leaves and tobacco cell suspensions. As shown in Figure 4,

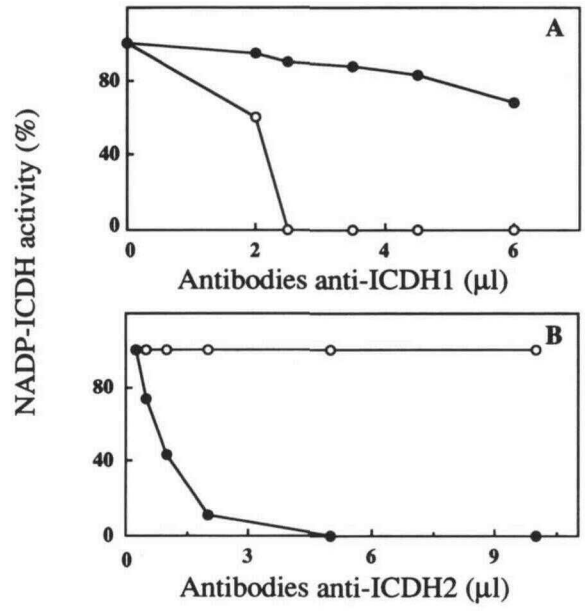

Figure 3. Immunotitration curves of the purified NADP-ICDH isoenzymes from tobacco cell cultures. O, NADP-ICDH1 activity; ●, NADP-ICDH2 activity.

no protein band was recognized by either antibody in a crude extract from green tobacco leaves; however, in a crude extract from green tobacco cell suspensions a weak, single band at 48.5 kD was recognized by both antisera. When partially purified samples of ICDH1 and ICDH2 from the anion-exchange step were subjected to immunoblot analysis, both ICDH1 and ICDH2 polypeptides were recognized by the antibodies raised against the cytosolic isoform (Fig. 4A), in

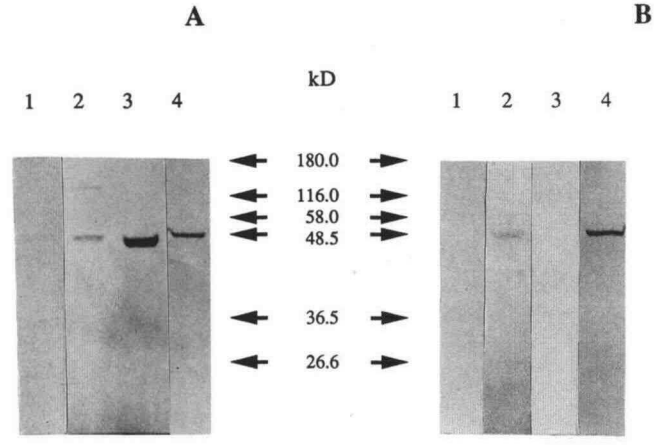

Figure 4. Western blot analysis of tobacco protein extracts. Proteins were separated by SDS-PAGE, electrotransferred onto a nitrocellulose sheet, and incubated with anti-ICDH1 (A) or anti-ICDH2 (B) antibodies. Lane 1, Crude tobacco leaf extract (30 μg of total protein); lane 2, green tobacco cell-suspension crude extract (30 μg); lane 3, partially purified NADP-ICDH1 from green tobacco cell suspensions (5 μg); lane 4, partially purified NADP-ICDH2 from green tobacco cell suspensions (5 μg).

agreement with the immunotitration assays (Fig. 3A). In contrast, antibodies raised against ICDH2 recognized a band only in the partially purified ICDH2 samples from tobacco cells (Fig. 4B) and leaves, failing to detect any band in an ICDH1-containing fraction from the DEAE-Sephacel column (Fig. 4B). In addition to confirming the immunotitration data, these western blot experiments also demonstrate that the ICDH2 antibodies appear to be more specific than those raised against the cytosolic enzyme.

In Situ Immunolocalization of ICDH2

The subcellular localization of tobacco leaf ICDH2 was examined using the monospecific antibody raised against the native, purified culture-cell protein. The use of an immunogold labeling technique coupled with an epipolarizing microscope showed the ordered areas where the antibodies were concentrated as brighter than the areas of more random distribution. This strategy indicated that ICDH2 is present largely, if not exclusively, in the chloroplasts of green tobacco leaves (Fig. 5A). The sparse but detectable gold labeling in the remainder of the cell could be due to either ICDH2 protein released from the chloroplasts during the cutting process prior to fixation or to nonspecific labeling. No particles were observed in control leaf sections treated with preimmune serum (Fig. 5B).

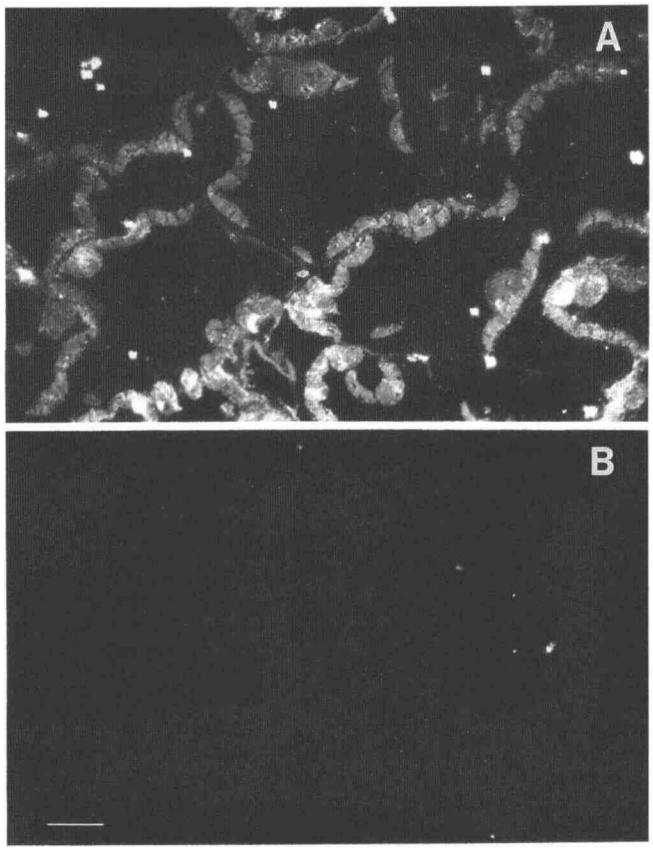

Figure 5. Low-magnification micrograph of a section through the mesophyll of a tobacco leaf. A, Incubated with monospecific antibodies against purified tobacco cell NADP-ICDH2. B, Preimmune control section. Antigen-antibody complexes are visualized as bright, refringent zones under epipolarized light. Bar = 5 μm.

DISCUSSION

A number of papers have been devoted to plant cytosolic NADP-ICDH. In contrast, in spite of the discovery of an ICDH activity in C_3 chloroplasts over 15 years ago (Elias and Givan, 1977), the properties of this enzyme have remained largely uninvestigated until the present study. This new progress in ICDH research has been achieved by the presence of a highly active plastidic enzyme in green, S-phase tobacco cells, which has made its purification feasible.

Indeed, an ammonium sulfate fraction from green tobacco cell cultures, in contrast to tobacco leaves, gave rise to two well-separated peaks of NADP-ICDH activity when subjected to DEAE-Sephacel column chromatography (Fig. 1), with the activity of the second peak being about 20% of the total ICDH activity. This second peak exhibits an elution pattern similar to that described previously for the pea chloroplast ICDH2 isoenzyme (Chen et al., 1989). Notably, this isoform is absent in etiolated tobacco culture cells but appears during the greening process. Moreover, it has the same relative electrophoretic mobility during native PAGE as the NADP-ICDH from isolated tobacco cell chloroplasts (data not shown). Taken together, these results suggest that this second peak of ICDH activity corresponds to a chloroplastic isoform of NADP-ICDH (ICDH2).

The purification procedure described in this report resulted in an 833- and a 1328-fold purification of the ICDH1 and ICDH2 isoenzymes, respectively, and yielded more than 500 μg of each purified protein from 195 g of exponential-phase cells (Table II). Following separation by SDS-PAGE (Fig. 2B), the final products appeared to be single, Coomassie blue-stained polypeptides with the same subunit mol wts of 48,500. However, the two isoforms differed in their relative electrophoretic mobilities on native polyacrylamide gels (Fig. 2A).

Antibodies raised against the purified cytosolic enzyme cross-reacted, at high concentrations, with the chloroplastic protein, but those raised against the latter protein did not immunoinhibit ICDH1 activity (Fig. 3). The observation that each isoform was, at best, poorly recognized by the antibodies raised against the other ICDH protein indicates that these isoenzymes share few similar epitopes.

Immunoblot analyses of crude extracts from tobacco leaves and cell suspensions showed that both antibodies were quite specific for ICDH (Fig. 4). Each partially purified ICDH from the DEAE-Sephacel column showed a single, 48.5-kD protein band with the antibodies (Fig. 4), suggesting that the antisera are monospecific for NADP-ICDH from this plant material. These results allowed us to examine the subcellular localization of the ICDH2 isoform in situ by immunogold labeling of semithin sections of tobacco leaves (Fig. 5). After incubating the sections with anti-ICDH2 serum, the chloroplasts appeared to be heavily gold labeled, whereas the labeling in the remainder of the mesophyll cell was very weak and randomly distributed. No gold labeling was detected in tobacco leaf sections treated with preimmune serum. This observation shows that the ICDH2 isoform is located largely,

if not exclusively, in the chloroplast. The density of the gold labeling suggests that the chloroplastic isoform is quite abundant, which seems to contradict the low activity of ICDH2 in whole leaf or chloroplast-stromal extracts. However, this finding can be partially explained by the 2.6-fold lower specific activity of the purified culture-cell ICDH2 when compared with ICDH1 (Table II). Along these same lines, anti-ICDH2 antibodies failed to recognize any protein band in a crude tobacco-leaf extract (Fig. 4B, lane 1). The different sensitivity of the detection techniques utilized in the western blot (horseradish peroxidase-conjugated secondary antibody) compared to the immunolocalization experiment (amplification with the biotin-streptavidin system, gold treatment, silver intensification) could help to explain this apparent discrepancy.

The molecular mass of the native NADP-ICDHs was 117 kD for ICDH1 and 136 kD for the chloroplastic isoenzyme. Taking into account the subunit mol wt of ICDH1, these values are indicative of a homodimeric ICDH1. In contrast, the mol wt of the native ICDH2 holoenzyme is compatible with a protein of two or three identical subunits. However, when using a gel-filtration column, the elution volume from which the apparent mol wt is calculated is theoretically related to the Stokes radius, not to size or mol wt (Scopes, 1982). Consequently, if the shapes of the unknown and standard proteins are dissimilar, the determination of the apparent mol wt from the elution volume can be in error. An anomalous native mol wt calculated from gel-filtration data has been described previously for NADP-ICDH from other sources (Kelly and Plaut, 1981; Jennings et al., 1990).

The two tobacco-cell isoenzymes did not differ significantly with respect to their kinetic properties. The apparent K_m values of both ICDH isoforms for isocitrate, NADP, and Mg^{2+} are very similar and in the same range as those already described for the chloroplastic enzyme from pea leaves (Randall and Givan, 1981) and other plant NADP-ICDHs (Chen and Gadal, 1990a).

The exact physiological role(s) of the different NADP-ICDH isoenzymes is, as yet, unknown. The oxidative decarboxylation of isocitrate is reported to be catalyzed by at least three distinct isoforms of NADP-ICDH in plant cells, located in the cytosol, chloroplast (Chen et al., 1989), and mitochondrion (Rasmusson and Möller, 1990). The subcellular localization of NADP-ICDH suggests that these forms may have different functions in plant metabolism. The cytosolic enzyme has been proposed to be involved in the supply of 2-oxoglutarate for chloroplastic glutamate synthase activity (Chen and Gadal, 1990b); in this pathway, citrate, either generated in the mitochondrion or stored in the vacuole, could be moved to the cytosol, where it could produce isocitrate via an active cytosolic aconitase (Brouquisse et al., 1987). Recently, this hypothesis has been supported (Hanning and Heldt, 1993) by measuring the flux through the tricarboxylic acid cycle in isolated spinach leaf mitochondria in the presence of metabolite concentrations similar to those occurring in the cytosol in vivo. The analysis of the products of malate oxidation under simulated photosynthetic conditions show that in illuminated spinach leaves the activity of the tricarboxylic acid cycle is very low, with citrate as the main product of mitochondrial malate oxidation. This result is in agreement with the suggested role of cytosolic NADP-ICDH.

The mitochondrial enzyme has been implicated in the provision of NADPH for glutathione reduction (Rasmusson and Möller, 1990). However, even less is known about the possible function(s) of the chloroplastic isoform.

Tobacco cell cultures growing exponentially are characterized by a doubling time of only 2.4 d. This active growth is supported by the utilization of exogenous Suc in the culture medium. There is now increasing evidence that photosynthetic metabolism is inhibited when carbohydrates accumulate in maize mesophyll protoplasts (Sheen, 1990), tobacco and *Arabidopsis* leaves (von Schaewen et al., 1990), and cell-suspension cultures and protoplasts of rape (Harter et al., 1993). If this trend holds true for our tobacco-cell system, such cultures growing on 3% Suc may not be capable of sufficient photosynthesis to provide the NADPH required for growth. The cytosolic pentose phosphate pathway has been proposed to provide NADPH (Lehninger, 1975). However, in plants this pathway does not seem sufficiently active to satisfy the requirements of the cell, since only 5 to 10% of the metabolism of Glc is catalyzed by this route (ap Rees, 1980). Thus, chloroplastic ICDH2 could be implicated in the production of reducing power in tobacco cells cultured on Suc. The experimental evidence for such a role, however, is not available, and, thus, this hypothesis is a speculative proposal.

The relative simplicity and effectiveness of the purification procedure described in this report should facilitate the elucidation of the possible function(s) of each NADP-ICDH isoform in tobacco cell cultures. In future studies, changes in composition of the culture medium will be performed to investigate the effect of different forms of exogenous nitrogen on the activities of the different NADP-ICDH isoforms.

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