Communication

Amino Acid Sequence and Molecular Weight of Native NADP Malate Dehydrogenase from the C₄ Plant Zea mays

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ABSTRACT

N-terminus amino acid analysis of purified corn (Zea mays) NADP malate dehydrogenase showed that the mature protein begins at serine-41 of the preprotein sequence and not threonine-58 as previously concluded; therefore, the transit peptide consists of 40 amino acids. The theoretical molecular weight of the mature subunit protein (392 amino acids) is 42,564, agreeing with an experimental value of about 43,000. The molecular weight of the native unactivated (dark form) and activated (light form) of NADP malate dehydrogenase, determined by analytical ultracentrifugation analysis, was about 84,000, indicating that both forms are dimers. However, conventional and high performance liquid chromatography gel filtration procedures indicated apparent molecular weights of about 110,000 to 120,000 for the unactivated native enzyme and about 143,000 to 150,000 for the active enzyme; in these cases, the molecular weight may be overestimated due to the effect of an unusual molecular conformation on the mobility of the enzyme.

NADP-MDH has an integral role in the photosynthetic process of NADP-malic enzyme-type C₄ plants, catalyzing the reduction of oxaloacetate to malate in mesophyll chloroplasts (6). Most studies have been conducted on the enzyme from Zea mays and there has been considerable disagreement about the mol wt of both the subunit of this enzyme and its native active and unactivated forms (4). More recent studies report subunit mol wts of between 42,000 and 43,000 (7, 10). Some workers find the native forms of both the active (reduced, light activated) and unactivated (oxidized, dark inactivated) forms of NADP-MDH (4) to be apparent dimers with a mol wt of about 88,000 (5, 10). However, other studies using gel filtration procedures have indicated higher mol wts (2) and that the activated enzyme has a higher apparent mol wt than the unactivated enzyme (11).

Recently, sequencing of a cDNA clone of the Z. mays NADP-MDH has indicated a preprotein consisting of 432 amino acids (14). Based on earlier N-terminal analysis of the native enzyme (3), Metzler et al. (14) concluded that the mature native protein consists of 375 amino acids with a mol wt of 40,934; hence, that the transit peptide would consist of 57 amino acids. The present study reports that the native Z. mays NADP-MDH is in fact 17 amino acids longer at the N-terminal end and, hence, contains 392 amino acids. Further information is also provided about the subunit mol wt and the origin and significance of the discrepancies between reported mol wts of the native forms of the enzyme.

MATERIALS AND METHODS

Materials

Corn (Zea mays) plants were grown in soil in a naturally illuminated glasshouse maintained between 20 and 30°C. Biochemicals were obtained from Sigma Chemical Co. or from Boehringer-Mannheim (Australia).

Purification of NADP-MDH

NADP-MDH was purified in its unactivated form from Z. mays leaves essentially as described previously (1). The purified enzyme was stored at −20°C either frozen in Tris-KCl buffer or in 50% (v/v) glycerol (equal volume of glycerol added). With our purification procedure, the apparent mol wt and N-terminus amino acid sequence of purified NADP-MDH were the same whether or not protease inhibitors were included in the extraction and purification medium as described by Kagawa and Bruno (10).

NADP-MDH in Extracts from Leaves or Mesophyll Chloroplasts

Active or unactivated NADP-MDH was prepared by extracting previously illuminated or darkened leaves, respectively, by blending with about 5 volumes of 25 mM Hepes-KOH buffer, pH 7.5, containing 0.5 mM EDTA. Five millimolar DTT was also included where active enzyme was extracted. The homogenate was filtered and then centrifuged at 10,000g for 5 min and the supernatant was fractionated by the addition of solid (NH₄)₂SO₄ to give the protein precipitating between 50 and 60% saturation. This precipitate was dissolved in the above buffer mixture prior to gel filtration as described in "Results and Discussion."

Mesophyll chloroplasts were prepared as described previously (8) but omitting the Percoll step, and then the stromal
contents were released by freezing and thawing twice in 25 mm Hepes-KOH, pH 7.5, containing 0.5 mm EDTA.

**Activation and Assay of NADP-MDH**

Unactivated NADP-MDH was activated by incubating the enzyme under nitrogen at 25°C with 50 mm Tricine-KOH buffer, pH 8.8, containing 0.5 mm EDTA, 0.003% Triton X-100, and 100 mm DTT until plateau activity was reached (generally 60–90 min).

**Mol Wt Determinations**

**SDS-PAGE Analysis**

Purified NADP-MDH was subject to electrophoresis on SDS-PAGE gels (18% acrylamide) as described by Laemmli (12).

**HPLC Analysis**

Purified NADP-MDH (10–30 μg) or other proteins were separated on a Waters Protein-pak 300SW gel filtration column at 25°C. The elution buffer contained 50 mm Hepes-KOH, pH 7.5, and 200 mm KCl (recommended to prevent electrostatic binding of protein to the column matrix). Where active enzyme was chromatographed, 5 mm DTT was also included. The column was calibrated using the following mol wt marker enzymes: apoferritin (horse spleen, 443,000); β-amylase (sweet potato, 200,000); aldolase (rabbit muscle, 158,000); alcohol dehydrogenase (yeast, 150,000); malate dehydrogenase (pig heart, 70,000); ovalbumin (45,000); carbonic anhydrase (bovine erythrocytes, 29,000); chymotrypsinogen A (bovine pancreas, 25,000); Cyt c (horse heart, 12,500). Protein emergence was monitored using an in-line UV monitor set at 280 nm. Emergence of NADP-MDH was also monitored by assaying enzyme activity after 0.2-mL fractions were collected.

**Sephacryl S-300 Chromatography**

Active or unactivated NADP-MDH in the 50 to 60% saturated (NH₄)₂SO₄ fraction of leaf extracts or extracts from isolated chloroplasts (see above) were chromatographed on a 100-mL Pharmacia Sephacryl S-300 column at room temperature. The column was equilibrated and eluted with a medium containing 50 mm Hepes K⁺, pH 7.5, with or without 200 mm KCl as indicated, and also 5 mm DTT when active enzyme was chromatographed.

**Sedimentation Equilibrium Analysis**

Enzyme samples were dialyzed overnight at 4°C against 1000 volumes of 50 mm Hepes-KOH buffer, pH 7.5, containing 200 mm KCl and 0.2 mm EDTA. With active enzyme, 10 mm DTT was also included. The sedimentation equilibrium experiments were of the meniscus depletion type (16) and were performed in a Spincio model E analytical ultracentrifuge at 25°C at 24,000 or 29,000 rpm using double sector cells with dialyzed solution in one sector and its equilibrium dialysate in the other. Photographs at sedimentation equilibrium were taken and point average mol wts were evaluated by fitting successive groups of five points with a least squares quadratic method. The partial specific volume of the protein was calculated from its amino acid composition as described by Zamyatnin (17) to be 0.723 mL/g.

**Amino Acid Sequence Analysis**

N-terminal amino acid sequencing was performed on an Applied Biosystems model 477A peptide sequencer.

**RESULTS AND DISCUSSION**

**Amino Acid Sequence and Mol Wt of Mature NADP-MDH Subunit**

N-terminal sequencing of purified NADP-MDH (Fig. 1) indicated that the first N-terminal amino acid was Ser-41 of the preprotein derived from the cDNA sequence (14). The following 21 amino acids to Ala-62 were identical with the sequence published by Metzler et al. (14). Metzler et al. (14) had concluded that the N-terminus of the mature protein commences at Thr-58 based on the N-terminal sequence of Z. mays NADP-MDH reported by Decottignies et al. (3). Our purified enzyme had an additional 17 amino acids originally assumed by Metzler et al. (14) to be part of the transit sequence. It seems most likely that this N-terminal peptide must have been removed by proteolysis during purification of the enzyme analyzed by Decottignies et al. (3).

With the additional 17 amino acids to Ser-41, the mol wt of the mature subunit would be 42,564 instead of the 40,934 calculated by Metzler et al. (14). The subunit mol wt of the purified Z. mays enzyme determined by SDS-PAGE was approximately 43,000 (Fig. 2). This is similar to the values reported by Jenkins et al. (7) and Kagawa and Bruno (10), but substantially higher than values reported in earlier studies (2, 5).

**Mol Wt and Oligomeric Forms of Native NADP-MDH**

Previous estimates of the mol wt of the active and unactivated forms of native NADP-MDH have varied from about 60,000 to 150,000 (2, 5, 9–11). It has also been reported that the activated enzyme has a substantially higher mol wt than the unactivated enzyme determined by Sephadex gel filtration (11). The studies reported below suggest that both the active and unactivated forms of malate dehydrogenase occur as a

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Figure 1. N-terminal amino acid sequence of purified NADP-MDH from Z. mays. The numbering of amino acids is from the preprotein sequence predicted from cDNA analysis (14).
dimer and that anomalous results are obtained with this enzyme when the mol wt is determined by gel filtration procedures.

In a comprehensive study of the mol wt of native Z. mays NADP-MDH, Kagawa and Bruno (10) found a value of about 88,000 using both gradient PAGE and analytical ultracentrifugation. This value was lower than those deduced from earlier studies using gel filtration chromatography (2, 11). Therefore, we compared the apparent mol wt of both the active and unactivated form of the purified Z. mays enzyme determined by ultracentrifugation and gel filtration. A mol wt of about 84,000 was obtained for both forms using the ultracentrifugation procedure (see "Materials and Methods") and little or no protein appeared in other oligomeric forms. Samples of NADP-MDH prepared for ultracentrifuge analysis were also analyzed by HPLC gel filtration at the same time and under essentially identical protein concentration and buffer conditions. With this HPLC procedure, an apparent mol wt of about 118,000 was obtained for the unactivated enzyme and 143,000 for the active enzyme. The results of gel filtration analyses are described in more detail below.

When purified inactive Z. mays NADP-MDH was chromatographed on Sephacryl S-300, it emerged between the marker enzymes alcohol dehydrogenase (Mr 150,000) and pig heart NAD malate dehydrogenase (Mr 70,000). The apparent mol wt was about 120,000 (results not shown). Similar behavior was observed with unactivated enzyme isolated from chloroplasts and with extracts from darkened leaves simply concentrated by isolating the protein fraction precipitating between 50 and 60% saturated (NH4)2SO4 (Fig. 3). This mobility corresponded to an apparent mol wt of approximately 110,000. When the active enzyme extracted from preilluminated leaves was concentrated with (NH4)2SO4 in the same way, it emerged with alcohol dehydrogenase, indicating an apparent mol wt of about 150,000 (Fig. 3). This agrees with a previous report suggesting that activation is associated with an apparent increase in mol wt as judged by mobility on Sephadex G-200 (11).

In an attempt to resolve the somewhat anomalous results obtained by conventional gel filtration chromatography, the apparent mol wt, and hence apparent oligomeric states, of active and inactive NADP-MDH was determined by an HPLC procedure using a gel filtration column. The unactivated enzyme stored at −20°C in 50% (v/v) glycerol emerged as a single peak with an apparent mol wt of about 118,000 (Fig. 4A). After storage in frozen aqueous solution at −20°C, a somewhat more complex picture was observed, although

Figure 2. Protein band pattern after SDS-PAGE of purified NADP-MDH in fractions from the activity peak eluted from Sephacryl S-300. The mol wt markers shown are BSA (67,000), ovalbumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), and Cyt c (12,000). Protein was detected by staining with Coomassie blue.

Figure 3. Chromatography of NADP-MDH in leaf or chloroplast extracts on Sephacryl S-300. The emergence patterns for the mol wt markers alcohol dehydrogenase (yeast, 150,000) and malate dehydrogenase (pig heart, 70,000) are also shown. A, Unactivated enzyme in chloroplast extracts (A). B, Unactivated enzyme in 50 to 60% saturated (NH4)2SO4 fraction of extracts from darkened leaves (A). C, Active enzyme in 50 to 60% saturated (NH4)2SO4 fraction of extracts from illuminated leaves (A). For other details, see "Materials and Methods."
the major protein peak and associated activity still emerged at a position corresponding to a mol wt of about 118,000 (Fig. 4B). Most of the minor peak in the higher mol wt position (about 150,000 or above) was associated with activatable enzyme, but the minor lower mol wt protein peak (approximately 80,000) was not. These minor peaks increased during storage at −20°C and following repeated thawing and refreezing. Repeated thawing and refreezing also caused increasing proportions of protein to appear at a mol wt position above 150,000 or at the excluded front peak. This protein could not be activated and was presumably denatured.

The chromatographic behavior of the unactivated enzyme during HPLC gel filtration was unaffected by varying the protein concentration over a 10-fold range or the pH from 6.5 to 7.8, or during chromatography on Sephacryl S-300 with and without 200 mM KCl (results not shown). In addition, NADP-MDH in chloroplast extracts emerged from HPLC columns at the same position as the purified enzyme, that is at a position corresponding to a mol wt of about 118,000 for the unactivated enzyme and 145,000 for the active enzyme (see below). These observations are not consistent with an interpretation that these apparently “intermediate” molecular forms of NADP-MDH seen by gel filtration analysis are due to rapid equilibration between dimer and tetramer forms during chromatography.

After purified unactivated NADP-MDH was activated by incubating the enzyme with 100 mM DTT to reduce S-S groups (see "Materials and Methods"), most of the protein and essentially all the activity emerged from the HPLC column at a position corresponding to a mol wt of about 143,000 (Fig. 4C). Most of the remaining protein appeared as a sharp peak at the exclusion front. This latter peak increased with storage, contained no active or activatable enzyme, and was presumably denatured protein.

In conclusion, the revised amino acid sequence of the native NADP-MDH deduced from the present study indicates a subunit mol wt for the Z. mays enzyme of 42,564, a value confirmed experimentally in the present study. Accordingly, our ultracentrifuge analysis indicating a mol wt of 84,000 for the active and unactivated native NADP-MDH is consistent with both forms existing as dimers. This agrees with the results of Kagawa and Bruno (10), but much higher apparent mol wts were obtained in both previous and current studies using gel filtration procedures. The simplest interpretation of these data is that results from ultracentrifugal analysis are correct and that NADP-MDH behaves anomalously during gel filtration, possibly the result of an unusual conformation for a globular protein. It is well established, but still not widely recognized, that the behavior of proteins during gel filtration chromatography is modified by their conformation, and is a function of Stokes radius rather than mol wt as such (13). Hence, accurate determination of the mol wt of a particular protein by gel filtration requires that the proteins used for calibrating the system be of the same conformational type (13, 15). For NADP-MDH, both the unactivated and active forms emerge from gel filtration columns at a position well ahead of that expected for the dimer form of the enzyme when standard globular marker proteins are used. This suggests that both forms have an irregular conformation. It is particularly interesting that this deviation from expected behavior becomes even more apparent with the activated form of the enzyme. Apparently, the reduction of the S-S group leading to activation of the enzyme results in a major conformational change.

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