Cloning and Analysis of the C4 Photosynthetic NAD-dependent Malic Enzyme of Amaranth Mitochondria*

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(Received for publication, July 15, 1993, and in revised form, August 27, 1993)

In some C4 plant species, a mitochondrial NAD-dependent malic enzyme (EC 1.1.1.39) (NAD-ME) catalyzes the decarboxylation of 4 carbon malate in the bundle sheath cells, releasing CO₂ for the Calvin cycle of photosynthesis. In amaranth, a dicotyledonous NAD-ME-type C4 plant, the photosynthetic NAD-ME purified as two subunits of 65 and 60 kDa, designated α and β, respectively. Antiserum raised against the α subunit reacted only with the 65-kDa protein in immunoblot analysis. Immuno-gold electron microscopy using the α subunit antiserum demonstrated that this protein was localized specifically to the mitochondrial matrix of bundle sheath cells. The complete nucleotide sequence of a 2300-base pair α subunit cDNA clone showed that this gene encodes a protein that contains all of the motifs required for a complete and functional malic enzyme. The α subunit has significant similarity along its entire length to other known NAD- and NADP-dependent malic enzymes from plants, animals, and bacteria. The findings presented here provide new insights about the C4 photosynthetic NAD-ME and its evolutionary relationship to other forms of malic enzyme present in eukaryotic and prokaryotic organisms.

Malic enzymes (ME)¹ are present in all organisms and catalyze the decarboxylation of L-malate to produce pyruvate and CO₂ (1, 2). Three classes of this enzyme have been identified. NAD-dependent malic enzyme (EC 1.1.1.40) (NADP-ME) preferentially utilizes NADP, has the ability to decarboxylate oxaloacetate (OAA), and has been found in cytosol, chloroplast, and mitochondria. Another class of malic enzyme (EC 1.1.1.38) preferentially utilizes NAD, has the ability to decarboxylate OAA, and has been found in bacteria and in insects. The third class is an NAD-dependent malic enzyme (NAD-ME) (EC 1.1.1.39), which preferentially utilizes NAD, is unable to decarboxylate OAA, and is specifically localized to the mitochondrial matrix (3). While cDNA clones for malic enzymes of the first two classes have been isolated and characterized by sequence analysis, the EC 1.1.1.39 form has not previously been characterized at the molecular level.

The mitochondrial NAD-ME (EC 1.1.1.39) has been found in many plant species (1-4). In most plants it appears to be involved in the conversion of C4 acids into acetyl-CoA, which can then be oxidized in the tricarboxylic acid cycle (5, 6). However, in some plant species that utilize the highly efficient C4 pathway of photosynthesis, the photosynthetic NAD-ME plays a key role in photosynthetic carbon fixation (5, 6). In these C4 plant species, classified as NAD-ME-type C4 plants (7), the NAD-ME is present in very abundant amounts and is found primarily in only one type of specialized photosynthetic leaf cell.

C4 plant species possess a specialized Kranz-type leaf anatomy, which consists of two photosynthetic cell types, the bundle sheath and mesophyll cells (8, 9). In C4 leaves, each vascular center is surrounded by one or two layers of bundle sheath cells, with one or more layers of mesophyll cells surrounding each ring of bundle sheath cells. The arrangement of these two cell types helps to isolate the bundle sheath cells from the leaf surface, greatly reducing their exposure to atmospheric O₂. In C4 plants, carbon fixation initially occurs in the mesophyll cells, where atmospheric CO₂ is combined with phosphoenolpyruvate by the enzyme phosphoenolpyruvate carboxylase. This reaction produces 4-carbon acids, which are then transported from the mesophyll cells to the neighboring bundle sheath cells. The 4-carbon acids are decarboxylated in bundle sheath cell mitochondria by the photosynthetic NAD-ME, thereby producing 3-carbon pyruvate and releasing CO₂ for refixation by ribulose 1,5-bisphosphate carboxylase. The C4 pathway thus acts as a "CO₂ pump" to concentrate CO₂ in the bundle sheath cells in the vicinity of ribulose 1,5-bisphosphate carboxylase for incorporation into the Calvin cycle of photosynthesis and to eliminate the metabolically wasteful oxygenation reaction of ribulose 1,5-bisphosphate carboxylase (8, 9).

The photosynthetic NAD-ME may represent a unique and specialized form of malic enzyme. Alternatively, this could be a basic metabolic enzyme common to all plant species, which has had its regulation or patterns of gene expression altered in C4 plant species to allow it to function in the photosynthetic pathway. Therefore, it was of interest to isolate and characterize the photosynthetic malic enzyme from a plant that utilizes the NAD-ME-type C4 pathway and compare it with other known malic enzymes. Several other malic enzymes from a variety of organisms have been characterized in terms of enzymatic activity, structure, and, in some cases, nucleotide sequence. These include two nonphotosynthetic forms of NAD-dependent malic enzyme (EC 1.1.1.39 or EC 1.1.1.38), as well as several types of NADP-ME (EC 1.1.1.40). The NAD-ME (EC 1.1.1.39) found in animals exists as a tetramer.

* This work was supported by United States Department of Agriculture Grants 91-37306-6523 and 93-01572 (to J. O. B.). The costs of publication of this paper were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U01162.

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¹ The abbreviations used are: ME, malic enzymes; NAD-ME, NAD-dependent malic enzyme(s); NADP-ME, NADP-dependent malic enzyme(s); α, large subunit of NAD-ME; β, small subunit of NAD-ME, OAA, oxaloacetate; PAGE, polyacrylamide gel electrophoresis.
composed of identical sized subunits (3). This same homotetramer arrangement has been determined for NADP-ME (EC 1.1.1.40) present in plants and in animals (10, 11) and for the NAD-ME (EC 1.1.1.38) from bacteria (12). In contrast, previous studies of NAD-ME from plant mitochondria indicate a unique structure consisting of two distinct subunits, designated α and β, which exist primarily in octomeric form (2, 4, 13). Both subunits appear to be required for enzymatic activity (2, 3).

In this study, we have characterized the photosynthetic NAD-ME from mitochondria of amaranth (Amaranthus hypochondriacus), a dicotyledonous C4 grain plant (14). Our results indicate that the amaranth enzyme, like other plant mitochondrial malic enzymes, is composed of two distinct subunits of 65 and 60 kDa. Antiserum produced against the larger of these two co-purifying proteins, the NAD-ME α protein, was used in immunogold electron microscopy to determine the cellular localization of this protein and to isolate an NAD-ME α protein cDNA clone. Our findings indicate that the bundle sheath cell-specific α protein contains all of the motifs required for a complete and functional malic enzyme, with a nucleotide and amino acid sequence comparable along its entire length to other known malic enzymes. The data presented here represent the first molecular characterization of an EC 1.1.1.39 malic enzyme and provide valuable insights into the relationships between the diverse forms of malic enzyme present in eukaryotic and prokaryotic organisms.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—Seeds of A. hypochondriacus var. 1023 were germinated and plants were grown in a Conviron growth chamber at 24 °C with 14 h/day illumination at an approximate intensity of 170–200 μmol m⁻² s⁻¹. Leaves were harvested from the plants at various time points as required for purification and analysis.

NAD-ME Purification and Antiserum Production—NAD-ME was purified from mitochondria isolated from mature amaranth leaves according to the methods of Wedding and Black (13). The purified NAD-ME preparation was separated by SDS-PAGE and contained two major protein bands of 65 and 60 kDa (Fig. 1A). These bands were present in approximately equal amounts and corresponded in size with the two subunits of plant mitochondrial NAD-ME as previously described (3). The two bands, designated α for the larger 65-kDa protein and β for the smaller 60-kDa protein, were separately cut out of the gel and injected into rabbits for production of antiserum. Therefore, the smaller β protein does not contain regions capable of cross-reacting with antiserum raised against the α protein.

Microsequencing of Purified α Protein—The gel-purified α protein was sequenced using a Beckman/Porton protein sequencer by the Center for Advanced Molecular Biology and Immunology (CAMBI) protein sequencing facility of SUNY-Buffalo.

Western Analysis—Total proteins were extracted from leaves 10 mm or greater in length. These proteins were then separated using SDS-PAGE and blotted to nitrocellulose filters. The filters were reacted with antisera raised against the α protein and probed with 32P-labeled protein A as previously described (15).

Immunogold Labeling and Electron Microscopy—Leaf slices were fixed in 1% glutaraldehyde, 0.2% picric acid, 4% paraformaldehyde, and 0.1 M Sorensen’s phosphate buffer, pH 7.2, for 4 h at 4 °C. Fixed tissue was rinsed in phosphate buffer, and free aldehydes were quenched with 0.5 M ammonium chloride in phosphate buffer using a modification of the procedure of Berrymann and Bowdaw (16). Tissue was dehydrated in graded ethanol to 70% and embedded in LR white resin (London Resin Company). Resin was polymerized overnight at 50 °C.

Immunolocalizations were carried out on thin sections (100 nm) mounted on Formvar-coated nickel grids. Sections were first incubated with blocking buffer (2.5% normal goat serum, 0.5% bovine serum albumin, phosphate-buffered saline, 0.5 M NaCl, 0.5% Tween) for 15 min and transferred to a 1:50 dilution of primary antiserum in labeling buffer (blocking buffer minus goat serum). Effective incubations ranged from 10 min to 1 h, but a 30-min incubation gave optimal results. Grids were rinsed 3 x 20 min in rinse buffer (same as labeling buffer but with 0.1% Tween) and then incubated for 1 h in droplets of a 1:20 dilution of 2% secondary antibody (15 nm of gold-labeled goat anti-rabbit IgG (Amersham International) in labeling buffer. Labeled sections were again transferred to rinse buffer for a total of 1 h and finally stained with 2% uranyl acetate and lead citrate before examination with a Hitachi H500 electron microscope operating at 75 kV.

Isolation and Sequencing of an NAD-ME α Protein cDNA Clone—Antiserum raised against the α protein was used to screen a Lambda Zap cDNA expression library (14). A 2.3-kilobase full-length cDNA clone, designated pMe11, was isolated and is contained between the EcoRI-XhoI sites of pBluescript SK− (Stratagene, San Diego, CA). Both strands of pMe11 were sequenced using a Sequenase kit (U. S. Biochemical Corp.). The sequence was analyzed using the Genetics Computer Group Sequence Analysis software package (17).

RESULTS

Purification and Identification of NAD-ME Proteins—Previous studies of NAD-ME in plants indicate that the active form of this enzyme exists primarily as an octomer composed of two distinct subunits, α and β, both of which are required for enzymatic activity (3, 13). This arrangement is unique to plant mitochondrial NAD-ME, because chloroplastic NADP-ME from plants and NADP-ME and NAD-ME from animals are all tetramers composed of a single subunit (3). The NAD-ME from amaranth mitochondria purified as two polypeptides, one of approximately 65 kDa and one of approximately 60 kDa (Fig. 1A). These two proteins were present in approximately equal amounts and were similar in size to the α and β subunits of plant mitochondrial NAD-ME as previously described (3, 4). It is therefore likely that these co-purifying proteins represent the two subunits of the C4 photosynthetic NAD-ME in amaranth mitochondria. For this study we have focused on isolating and characterizing the larger protein present in the purified enzyme preparation, designated here as the amaranth NAD-ME α protein.

In immunoblot analysis with a total leaf soluble protein extract, antiserum raised against the α protein reacted specifically with the 65-kDa protein band (Fig. 1B) and showed little or no reaction with other proteins present in the extract. In particular, this antiserum showed no reaction with the smaller 60-kDa β protein that co-purified with the α protein, either with the total leaf protein extract as shown in Fig. 1B or with the purified enzyme preparation (data not shown). Therefore, the smaller β protein does not contain regions capable of cross-reacting with antiserum raised against the α protein.
larger α protein under these conditions. This finding is consistent with previous studies indicating that the large and small subunits of plant mitochondrial malic enzymes are separate and distinct proteins, each of which display independent mobilities in urea isoelectric focusing gels, possess unique primary sequences as determined by limited proteolysis analysis, and have significantly different amino acid contents (3). It is therefore likely that β is a completely separate protein from α and does not represent an isozyme, breakdown product, or a processed form of the larger protein.

Immunogold Localization of NAD-ME α Protein — Antiserum raised against the α protein was used with immunogold electron microscopy to determine the cellular localization of this protein within a mature amaranth leaf (Fig. 2, A-C). Fig. 2A shows a leaf cross-section and reveals the Kranz anatomy characteristic of dicotyledonous plants that utilize the NAD-ME-type C4 pathway (7, 14, 18). Around the vascular center (vs) was a single layer of bundle sheath cells, with a layer of mesophyll cells surrounding the ring of bundle sheath cells. Fig. 2A also illustrates the relative positions of the various cell structures, including the vacuole, mitochondria, and chloroplasts, within the two photosynthetic cell types. The large vacuole present in bundle sheath and mesophyll cells (bv and mc, respectively) occupied much of the cell volume. The cytoplasm, mitochondria, and chloroplasts were compressed around the cell edges in mesophyll cells or in the centripetal portion of the cell (in toward the vascular center) in bundle sheath cells. The bundle sheath cell chloroplasts (bc) and mitochondria (bm) were larger and more numerous than those of mesophyll cells (mc and mm, respectively). In particular, the specialized C4 decarboxylating bundle sheath mitochondria (Fig. 2B) were 2–3 times larger than the mesophyll cell mitochondria (Fig. 2C). In bundle sheath cells, the mitochondria were clustered together with the chloroplasts against the cell edge adjacent to the vascular center.

The α protein antiserum reacted only with the mitochondria of bundle sheath cells and showed no reaction anywhere else within the leaf. The enlarged portion of a bundle sheath cell shown in Fig. 2B clearly shows specific reaction of the α protein antiserum only in the mitochondrial matrix. No reaction with this antiserum was observed in the cytoplasm, chloroplasts, or any other bundle sheath cell structure. The enlarged portion of a mesophyll cell shown in Fig. 2C shows that the α protein antiserum had no reaction with the mesophyll mitochondria or with any other organelles or structures in these cells.

The photosynthetic NAD-ME of amaranth leaves functions in the bundle sheath mitochondria to metabolize C4 acids to CO2 and pyruvate, and the released CO2 is then transported to the adjacent chloroplasts for incorporation into the Calvin cycle (6). The specific localization of NAD-ME α protein within the specialized bundle sheath mitochondria and the close association of mitochondria and chloroplasts within these cells are clearly reflective of the decarboxylation role of this enzyme in the NAD-ME-type C4 pathway.

Nucleotide Sequence of a cDNA Clone Encoding the NAD-
**ME α Protein**—The pMe1 cDNA has a length of 2283 nucleotides, followed by a 17-base pair poly(A) tail. This sequence contains an 1869-nucleotide open reading frame beginning with GCT at position 148, represent the coding region of the amaranth NAD-ME α subunit (20,21). Microsequencing of the gel-purified subunit isolated from amaranth mitochondria identified the first 14 amino acids of the mature protein (the sequenced amino acids are underlined in Fig. 3) and showed that the first 31 amino acids of the pMe1 open reading frame were not present at the N terminus. This indicates that cleavage occurred between the Phe and Ala residues (51.6%), of mitochondrial transit sequences, including enrichment for positively charged and hydroxylated amino acids (48.4%), and no Gly or Pro.

**Amino Acid Sequence of NAD-ME α Protein**—The precursor protein predicted from the pMe1 nucleotide sequence is 623 amino acids in length, with a calculated molecular mass of 69.7 kDa (Fig. 3). Two lines of evidence indicate that the first 31 amino acids encoded by the pMe1 open reading frame, highlighted in Fig. 3 with bold lettering, represent the mitochondrial transit sequence of the α protein. First, microsequencing of the gel-purified α subunit isolated from amaranth mitochondria identified the first 14 amino acids of the mature protein (the sequenced amino acids are underlined in Fig. 3) and showed that the first 31 amino acids of the pMe1 open reading frame were not present at the N terminus. This indicates that cleavage occurred between the Phe and Ala residues (51.6%), of mitochondrial transit sequences, including enrichment for positively charged and hydroxylated amino acids (48.4%), and no Gly or Pro present in the sequence (20,21).

The mature α polypeptide, starting with Ala at position 32, is 592 amino acids in length with a calculated molecular mass of 65.1 kDa. This calculated size is consistent with the larger 65-kDa band detected in SDS-PAGE and labeled in immunoblot analysis (Fig. 1). Aligning the amino acid sequence of the amaranth NAD-ME α protein with malic enzymes of photosynthetic malic enzyme (ME) subunit cDNA. The leader peptide is shown in boldface. The underlined region represents amino acids determined by microsequencing.
maize, human, and *B. steaothermophilus* shows that the α protein has significant similarity along its entire length to the other enzymes. The alignment shown in Fig. 4 reveals five regions, which are boxed and designated I-V, that are highly conserved among the different malic enzymes. Region I is very highly conserved and corresponds to a possible NAD-binding region (11, 22). This region is most highly conserved between NADP-ME from maize and human, both of which utilize NADP preferentially. Region I is also present in the malic enzymes from amaranth and *B. steaothermophilus*, but it is more divergent in these organisms. This observation is consistent with the fact that the NAD-ME forms of malic enzyme can utilize NADP but less efficiently than NAD. Region II (Fig. 4) is also very highly conserved for all types of malic enzyme shown and represents a possible NAD-binding region (11).
Amaranth

C4 Photosynthetic Malic Enzyme of Amaranth Mitochondria

A. hypochondriacus extracts prepared from mature amaranth leaves (data not shown) indicate that the NAD-dependent malic enzymes possess several distinctive properties compared with other known malic enzymes. We performed an assay of enzyme activity using crude extracts of amaranth leaves, decarboxylation of C4 acids in bundle sheath cells is accomplished by a mitochondrial NAD-dependent malic enzyme. This C4 NAD-ME has been reported to be very abundant and bundle sheath cell-specific (5, 6, 25). Like the plant mitochondrial NAD-ME, it is composed of separate subunits, both of which are required for enzymatic activity (1-4, 13). Taken together with these other studies, our findings suggest that the amaranth NAD-ME, like mitochondrial NAD-ME in other plants, is composed of separate α and β subunits. This arrangement appears to be a unique property of plant mitochondrial malic enzymes, because all other known malic enzymes are composed of identical sized subunits (3).

DISCUSSION

Plant species that utilize the highly efficient C4 pathway of photosynthesis possess a specialized biochemical pathway that works in combination with a specialized Kranz-type leaf anatomy to concentrate CO2 in bundle sheath cells for fixation by ribulose 1,5-bisphosphate carboxylase and assimilation into the Calvin cycle, thereby increasing photosynthetic efficiency. C4 photosynthesis requires interactions between several enzymes, which are specifically compartmentalized to either the bundle sheath or mesophyll cells, and selective cell type-specific expression of genes encoding the various C4 enzymes. The various C4 enzymes, such as phosphoenolpyruvate carboxylase or pyruvate orthophosphate dikinase, are all present in C3 plant species as well as in C4 species (11, 23, 24). The difference is that, in C4 plants, specific enzymes that are required for the C4 pathway are all expressed at much higher levels than in C3 plants and are localized specifically to only one of the two photosynthetic cell types. In NAD-ME-type C4 dicotyledonous plant species such as amaranth, decarboxylation of C4 acids in bundle sheath cells is accomplished by a mitochondrial NAD-dependent malic enzyme. This C4 NAD-ME has been reported to be very abundant and bundle sheath cell-specific (5, 6, 25). Like the other C4 enzymes, the mitochondrial C4 NAD-ME has a less abundant, nonphotosynthetic counterpart present in C3 plant species (3, 4). Previous reports indicate that plant mitochondrial NAD-dependent malic enzymes possess several distinguishing characteristics, including an inability to decarboxylate OAA, a preference for NAD over NADP, and the use of either Mn2+ or Mg2+ as cofactors (1, 2, 4, 13). To determine how the enzymatic characteristics of the C4 NAD-ME from A. hypochondriacus compared with other known malic enzymes, we performed an assay of enzyme activity using crude extracts prepared from mature amaranth leaves (data not shown). We calculated a K_m for malate in the presence of Mn2+ of 0.77 ± 0.15 mM. This K_m for the photosynthetic NAD-ME from amaranth is similar to that reported for a NAD-ME from Crassula argentea (K_m = 0.59 ± 0.15 mM) and from Solanum tuberosum (0.76 ± 0.04 mM) (4, 13). Like these other plant mitochondrial malic enzymes, the NAD-ME in amaranth leaf extracts showed an inability to decarboxylate OAA, preferential utilization of NAD over NADP, and an ability to use both Mn2+ or Mg2+ as cofactors.

In addition to sharing similar enzymatic characteristics, the amaranth C4 malic enzyme, like the other known plant mitochondrial malic enzymes, are composed of two separate proteins that were present in equal amounts. Antisera raised against the larger α protein showed no cross-reaction with the smaller β protein, suggesting that these two proteins are not antigenically related and most likely represent two completely separate polypeptides. The NAD-ME from other plants species, including the C3 plant S. tuberosum and the CAM plant C. argentea, have been shown to occur as a dimer, tetramer, or octamer composed of equal amounts of two separate and distinct subunits, both of which are required for enzymatic activity (1-4, 13). Taken together with these other studies, our findings suggest that the amaranth NAD-ME, like mitochondrial NAD-ME in other plants, is composed of separate α and β subunits. This arrangement appears to be a unique property of plant mitochondrial malic enzymes, because all other known malic enzymes are composed of identical sized subunits (3).

SDS-PAGE, immunoblot analysis, and immunogold electron microscopy demonstrated that the larger of the two subunits of the amaranth NAD-ME enzyme preparation, the α protein, is approximately 65 kDa in size and is localized specifically to the mitochondrial matrix of leaf bundle sheath cells. This would be expected for the C4 photosynthetic malic enzyme of amaranth. Microsequencing of the purified α polypeptide confirmed that this protein is encoded by the 1869 nucleotide open reading frame of pMe1, a clone that was isolated from an amaranth cDNA expression library using specific antisera raised against the α protein. Analysis of the nucleotide sequence of pMe1 identified this protein as a complete malic enzyme. As shown in Fig. 4, the amino acid sequence of the processed α polypeptide from amaranth shows significant similarity over its entire length to malic enzymes from a wide variety of organisms, including maize, human, and B. stearothermophilus.

How similar is the α protein component of the amaranth NAD-ME to other known forms of malic enzyme? Comparisons of several malic enzymes from various organisms indicate that the highest degree of similarity exists between the different types of malic enzymes that primarily utilize NADP (EC 1.1.1.40) (Table I). As expected within this group, a higher degree of similarity exists between more closely related organisms. For example, at the amino acid level the photosynthetic NADP-ME from maize chloroplasts (11) shares 75.9% identity with NADP-ME from ice plant (Mesembryanthemum crystallinum) (26), whereas cytoplasmic NADP-ME from mouse (27) shares 95.2% identity with cytoplasmic NADP-ME from rat (28). It is interesting to note that human NADP-ME shares more similarity with amaranth NAD-ME than does NADP-ME from maize, possibly due to the fact that both human and amaranth malic enzymes are localized and function in the mitochondria. The amaranth NAD-ME α subunit appears to have diverged from the NADP-ME group, showing between 40.1 and 41.4% identity. This emphasizes the fact that the plant mitochondrial malic enzymes in general, and the photosynthetic NAD-ME of amaranth in particular, may represent a distinct form of malic enzyme.

NADP-ME from a prokaryote, B. stearothermophilus (12), shows significant similarity to both NADP-ME from maize chloroplasts, human mitochondria, and mouse cytoplasm and with the NAD-ME (EC 1.1.1.38) from B. stearothermophilus. Comparisons are based on predicted polypeptide sequences, not including transit sequences. Numbers to the left of the slash marks indicate amino acid similarity, and numbers to the right indicate amino acid identity.

| Amaranth Mitochondria | 62.6/40.5 | 66.2/41.4 | 63.7/40.1 | 50.2/26.8 |
|------------------------|-----------|-----------|-----------|-----------|
| EC 1.1.1.39            |           |           |           |           |
| Maize Chloroplasts     |           |           |           |           |
| EC 1.1.1.40            |           |           |           |           |
| Human Mitochondria     |           |           |           |           |
| EC 1.1.1.40            |           |           |           |           |
| Mouse Cytoplasm        |           |           |           |           |
| EC 1.1.1.40            |           |           |           |           |
| B. stearothermophilus EC 1.1.1.38 | 52.2/27.3 | 53.1/28.1 | 49.7/26.6 |
and the amaranth α protein but at a lower level (between 26.6 and 28.1% identity) than the similarity shared between the eukaryotic NADP-ME and the amaranth NAD-ME α subunit. These results indicate that the two classes of malic enzyme that have been characterized from eukaryotic cells are more closely related to each other than they are to the more divergent prokaryotic enzyme.

The multiple subunit structure and enzymatic characteristics of the specialized photosynthetic malic enzyme suggest that it is closely related to other plant mitochondrial NAD-dependent malic enzymes. It is very likely that the malic enzyme used in the C4 photosynthetic pathway of amaranth is evolutionarily derived from a metabolic NAD-ME, such as that found in plants that use the less specialized C3 photosynthetic pathway. A new C4 photosynthetic form of NAD-ME, showing increased levels of expression and bundle sheath cell specificity, might have been created through duplication of nuclear genes encoding the two subunits of an original metabolic NAD-ME. If this were the case, then it might be expected that amaranth leaves would contain both the abundant photosynthetic form of the enzyme in bundle sheath cells and a less abundant metabolic form that is not cell type-specific. Our antiserum did not detect any of the α subunit in mesophyll cells, either in immunogold electron microscopy (Fig. 1) or in immunofluorescent microscopy (data not shown). However, it is possible that very low levels, below the level of detection with our antibody, were present in these cells and represent a nonphotosynthetic form of this enzyme. Alternatively, the expression of previously existing NAD-ME genes may have been modified, due to alterations in promoter or other regulatory sequences, such that the original NAD-ME genes are now expressed as C4 genes. In maize, it has been found that the gene encoding the C4 photosynthetic form of pyruvate orthophosphate dikinase is derived from an overlapping gene encoding a nonphotosynthetic cytosolic form of this enzyme, and the same coding sequence is used for producing both forms of the enzyme (29). Genetic rearrangement has led to the occurrence of two separate upstream controlling regions that differentially regulate the expression of the overlapping pyruvate orthophosphate dikinase genes, such that an abundant C4 chloroplastic form and a less abundant cytosolic form of this enzyme are both produced from the same locus.

Because the α subunit appears to be a complete malic enzyme in itself, the role of the co-purifying β subunit is open to speculation. Previous studies (5, 4, 10), as well as this current investigation, indicate that the two subunits of plant mitochondrial malic enzymes are two completely separate proteins, with distinct physical and antigenic properties. Because both subunits of this type of malic enzyme appear to be required for enzymatic activity (3), it is possible that the small subunit could function as a regulatory protein. Sequence analysis of putative β protein cDNA clones is in progress and should allow us to determine the structure and function(s) of the small subunit of this enzyme.

The enzyme present in amaranth leaves represents a form of malic enzyme that has acquired a unique photosynthetic function. This current investigation has identified and characterized the α subunit as the malic enzyme component of this plant mitochondrial NAD-ME and has indicated that there are several unique properties of this enzyme that distinguish it from the previously characterized EC 1.1.1.40 and EC 1.1.1.38 malic enzymes. The sequence data presented here represents the first molecular characterization of the EC 1.1.1.39 form of malic enzyme from any organism. New insights into the evolution of the C4 pathway may be derived by comparing this photosynthetic form of NAD-ME with nonphotosynthetic forms of EC 1.1.1.39, when these sequences become available. In addition, understanding the mechanisms controlling the abundant and cell type-specific production of the photosynthetic NAD-ME in amaranth leaves will provide new information about the origin, development, and regulation of C4 photosynthetic capacity in higher plants.

Acknowledgments—We are grateful to Alan Siegel for excellent technical skill in preparing the samples for and performing the immunogold electron microscopy and to Jerry Koudelka for advice on performing the enzyme assays. We also thank Mary Bisson and Jeremy Brunn for helpful advice and discussion and Jim Stamos for preparing the illustrations.

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