The Dihydrolipoamide S-Acetyltransferase Subunit of the Mitochondrial Pyruvate Dehydrogenase Complex from Maize Contains a Single Lipoyl Domain*

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The dihydrolipoamide S-acetyltransferase (E2) subunit of the maize mitochondrial pyruvate dehydrogenase complex (PDC) was postulated to contain a single lipoyl domain based upon molecular mass and N-terminal protein sequence (Thelen, J. J., Miernyk, J. A., and Randall, D. D. (1998) Plant Physiol. 116, 1443–1450). This sequence was used to identify a cDNA from a maize expressed sequence tag data base. The deduced amino acid sequence of the full-length cDNA was greater than 30% identical to other E2s and contained a single lipoyl domain. Mature maize E2 was expressed in Escherichia coli and purified to a specific activity of 191 units mg⁻¹. The purified recombinant protein had a native mass of approximately 2.7 MDa and assembled into a 29-nm pentagonal dodecahedron as visualized by electron microscopy.

Immunoblot analysis of mitochondrial proteins from various plants, using a monoclonal antibody against the maize E2, revealed 50–54-kDa cross-reacting polypeptides in all samples. A larger protein (76 kDa) was also recognized in an enriched pea mitochondrial PDC preparation, indicating two distinct E2s. The presence of a single lipoyl-domain E2 in Arabidopsis thaliana was confirmed by identifying a gene encoding a hypothetical protein with 62% amino acid identity to the maize homologue. These data suggest that all plant mitochondrial PDCs contain an E2 with a single lipoyl domain. Additionally, A. thaliana and other dicots possess a second E2, which contains two lipoyl domains and is only 33% identical at the amino acid level to the smaller isoform. The reason two distinct E2s exist in dicotyledon plants is uncertain, although the variability between these isoforms, particularly within the subunit-binding domain, suggests different roles in assembly and/or function of the plant mitochondrial PDC.

The pyruvate dehydrogenase complex (PDC) is a multienzyme complex that catalyzes the oxidative decarboxylation of pyruvate to yield acetyl-CoA, carbon dioxide, and NADH (1). In eukaryotic cells, PDC is located in the mitochondrial matrix. A second PDC isoform in plants has been localized to the plastidial stroma (2, 3). Both PDC isoforms probably provide acetyl-CoA for fatty acid biosynthesis, but a primary role of the mitochondrial isoform is to link glycolytic and tricarboxylic acid metabolism (reviewed in Ref. 4).

A minimal PDC is composed of multiple copies of three enzymatic components: pyruvate dehydrogenase (E1, EC 1.2.4.1), dihydrolipoamide S-acetyltransferase (E2, EC 2.3.1.12), and lipoamide dehydrogenase (E3, EC 1.8.1.4) (reviewed in Ref. 5). The E1 component catalyzes the decarboxylation of pyruvate via a thiamin pyrophosphate-hydroxycarboxylate intermediate and the reductive acetylation of the lipoate covalently bound to a lysine of E2. The E2 component then catalyzes the transfer of the acetyl group to CoA for the formation of acetyl-CoA. Finally, the reduced lipoamide is oxidized by the E3 component to yield NADH. These three reactions are facilitated by the swinging action of the E2 lipoyl domain, enabling active site coupling between the E2-bound E1 and E3 components (6). In addition to the three major catalytic components, mitochondrial PDCs possess a pyruvate dehydrogenase kinase and phosphopruvate dehydrogenase phosphatase that regulate the phosphorylation status of PDC (7–9).

The polypeptides that constitute PDC are held together by noncovalent interactions. The E2 subunit forms the core to which all other PDC subunits bind (10). In Gram-negative bacteria such as Escherichia coli, the oligomeric core contains eight E2 trimers arranged as a cube with octahedral symmetry (11), while in Gram-positive bacteria (12, 13) and eukaryotes (14, 15) the core contains 20 E2 trimers arranged as a pentagonal dodecahedron with icosahedral symmetry. Amino acid sequence alignments comparing E2s from divergent organisms revealed that they are multi-domain proteins (see Ref. 16 and references therein). Starting at the N terminus, an E2 polypeptide contains 1–3 lipoyl subdomains (~100 residues in length), which are linked together by variable, flexible hinge regions of 20–30 residues. The inner lipoyl domain is adjoined to the E1-binding domain (~40 residues) by another flexible hinge motif. Finally, a short, hypervariable region of 10–40 residues connects the E1-binding domain with the catalytic/assembly domain of 230 residues. Tightly associated with the E2 core of...
most eukaryotes is a lower abundance polypeptide termed protein X (17–19). The primary amino acid sequences of the human (20) and yeast (21) protein X subunits indicate that they are related to E2 subunits, quite possibly diverging from the same ancestor to yield a functionally distinct protein. The human and \textit{Saccharomyces cerevisiae} protein X subunits both have functional lipoyl domains, and the human (but not the \textit{S. cerevisiae}) homologue appears to have a functional acetyltransferase subdomain. Protein X from \textit{Ascaris suum}, in contrast, does not contain a lipoyl domain (22). A characteristic common to all protein X subunits is that they can bind the E3 component with higher affinity than the E2 core, suggesting a possible function for this protein and prompting it being renamed the E3-binding protein (17–19, 22). While it is clear that E3-binding proteins are present in animal and fungal PDCs, evidence for an E3-binding protein in plants is indirect and has not yet been confirmed (23).

Previously, the purified maize mitochondrial PDC was shown to contain a single E2 subunit with a mass of 52 kDa (24). Alternatively, PDCs from pea (4) and potato (25) mitochondria contain two E2 proteins, 52 and 76 kDa in size. In both pea and potato, the smaller E2 appears to be the more abundant isoform (25). The presence of a 52-kDa E2 isoform in plants is enigmatic in that the cloning of the first plant mitochondrial E2, from \textit{Arabidopsis thaliana}, revealed two conserved lipoyl domains and a mature size of ~76 kDa on SDS-PAGE (26). These authors and others (23, 25) suggested that a 52-kDa protein, also detected immunologically, was therefore either a proteolytic derivative of the larger E2 or, more likely, a protein X homologue. Herein we report the molecular cloning of a cDNA encoding the 52-kDa E2 subunit of maize mitochondrial PDC and expression of the functional protein.

**MATERIALS AND METHODS**

**Plant Materials, Mitochondria Isolation, and Immunoblotting—Etiolated maize (\textit{Zea mays} L., B73; Illinois Seed Foundation, Urbana, IL) seedlings, peas (\textit{Psium sativum} L., cv. Little Marvel), \textit{A. thaliana} (Columbia), and \textit{Kalanchoe daigremontiana} seedlings were grown as described in Ref. 27. Cucumber (\textit{Cucurbitis sativus}) plants were grown in a growth chamber (10-h photoperiod, 18 °C) for ~20 days. Mitochondria were isolated from etiolated maize shoots and from the green leaves of \textit{Cucurbitis sativus} using the Basic Local Alignment Search Tool (BLAST; Ref. 32). The longer of the two cDNAs was retrieved and sequenced on both strands using AmpliTaq Gold with primers DDR 271 (5′-cagtctgct-3′) and DDR 272 (5′-cagtc-3′) and \textit{Hi-Bred} expressed sequence tag database using the N-terminal protein sequence (24) for the maize E2 subunit as the search query. Two cDNAs were identified from a 2–3-day root cDNA library using the Basic Local Alignment Search Tool (BLAST; Ref. 32). Automated sequencing was performed at the University of Missouri DNA core facility using an ABI model 377 sequencer. Nested deletions were generated using the Erase-A-Base procedure according to the manufacturer’s instructions (Promega, Madison, WI).

**Construction of Expression Plasmids—Primers DDB 277 (5′-agcttgaatgatgtgctgactagc-3′) and DDB 278 (5′-gatagtgctgactagcggcactc-3′) were used to amplify the open reading frame of the mature maize E2 protein corresponding to the region between 414 and 1721 bp (amino acids 103–542). Restriction sites were engineered into each primer at the 5′-end (lowercase letters) to facilitate subcloning of the PCR amplicon. The \textit{Ncol} (DDB 271) and \textit{SalI} (DDB 272) sites (underlined) were used to subclone the PCR product into pET28b expression vector (Novagen, Madison, WI). The resulting construct encodes for the mature maize E2 polypeptide with an extra Met-Val- at the N terminus.

Deletion constructs for mapping the monoclonal antibody epitope were generated by utilizing restriction sites within the cDNA to remove portions of the open reading frame. Since no stop codons were present upstream of the initiating Met, the entire cDNA was subcloned directly into an expression vector with the proper reading frame. Deletions of the first 400–500 nucleotides were created by digesting the plasmid with \textit{HindIII} or \textit{PstI} and then religating. Since both restriction sites are present within the cDNA and the multiple cloning region, the deletions were made to the 3′-end of the cDNA, keeping the reading frame intact. Colonies were screened by agarose gel electrophoresis, and proper size plasmids were confirmed by DNA sequencing. Expression constructs were transformed into BL21(DE3) cells for expression analysis. E. coli (DE3) cells were grown in 2 liters of LB medium supplemented with kanamycin (50 μg/ml) and incubated with shaking at 37 °C overnight. The cells were transferred to 0.5 liters of LB-kanamycin plus 1 μM lipoic acid and shaken at 37 °C until the \textit{A}_{600} reached 0.4 (2–4 h). The target gene was induced by isopropyl \textit{b}-D-thiogalactoside at a final concentration of 0.4 μM and continued shaking at 37 °C for another 4–8 h. The cells were harvested, resuspended in 60 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 0.2 mM NaCl, 10 mM 2-mercaptoethanol, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 1 μg/ml Triton X-100), and disrupted by ultrasonic treatment using three 20-s pulses at 50 watts, while keeping the cell lysis in an ice-water bath.

The suspension was centrifuged at 10,000 × g for 20 min. The supernatant was transferred to a new centrifuge tube, and 50% polyethylene glycol 6000 was added dropwise to reach 10% final concentration and then placed at 4 °C for 16 h for complete precipitation of protein. The precipitated protein was collected by centrifugation at 10,000 × g for 20 min; resuspended in 2 ml of 20 mM TES-KOH, pH 7.4, 2 mM dithiothreitol; clarified at 10,000 × g for 20 min to remove insoluble protein; and then applied to a 10–50% linear glycerol gradient composed of 50 mM TES-KOH, pH 7.5, 14 mM 2-mercaptoethanol, 2 mM magnesium chloride. The proteins were resolved in the gradient by centrifugation in a SW28 rotor at 25,000 rpm for 16 h. The gradient was fractionated from the bottom, and 1-M fractions were collected. Fractions were assayed by immunoblot analysis with the monoclonal antibody. Peak fractions were pooled; dialyzed against 20 mM TES-KOH, pH 7.5, 30 mM KCl, 30 mM NaCl, for 16 h; and then concentrated using an ultrafiltration membrane (300-kDa cut-off; Amicon, Beverly, MA).

**Enzyme Assays—** The transfer of [1-14C]acetate to dihydrolipoamide from [1-14C]acetyl-CoA was assayed as described previously, with the following modifications (33). In a 1.5-ml Eppendorf tube, the following components were combined and allowed to incubate for the final concentration indicated: 50 mM TES-KOH, pH 7.3, 1.2 mM reduced dihydrolipoamide, 1 mM [1-14C]acetyl-CoA (~1 μCi). The assay was initiated with purified recombinant protein to reach a final volume of 500 μl and proceeded for 2 min at 25 °C. The [1-14C]acyldihydrolipoamide was extracted by adding 1 ml of benzene followed by vortexing and centrifugation to separate the phases. The upper benzene phase (0.5 ml) was removed and counted by liquid scintillation spectrometry. Controls without dihydrolipoamide, without recombinant protein, and using boiled protein (5 min) were performed.

The nonisotopic transfer of acetate to dihydrolipoamide was assayed using the following variations from a previously described procedure (33). The following components were added in order to a 1-ml quartz cuvette: 50 mM TES-KOH, pH 7.4, 1 mM acetylphosphate, 1 mM dihydrolipoamide, 10 μM CoASH, 20 mM cysteine, 1 unit of recombinant phosphotransacetylase (P-1329; Sigma), and recombinant E2. The formation of acetyldihydrolipoamide was measured by the \textit{A}_{340} for 1 min at 25 °C. Background rates, without E2 (~5% of the actual rate), were observed and stabilized after approximately 20 s. The extinction coefficient for the acetyldihydrolipoamide ester bond is 4400 M\(^{−1}\) cm\(^{−1}\). Rates were expressed as \textit{A}_{340}, due to the potential for formation of undetermined amounts of biacetylated dihydrolipoamide (34).

**Size Exclusion Chromatography—** The native size of purified recombinant maize E2 was determined using a Superose 6 column in conjunction with a fast protein liquid chromatography system (FPLC; Amersham Pharmacia Biotech). The exclusion size of this matrix is 2.8 MDa predicted mass) should not pass through the void volume. A 100-μl sample was applied to a column equilibrated with 20 mM TES-KOH, pH 7.4, 30 mM NaCl, 30 mM KCl, and eluted with the same buffer at 0.5 ml min\(^{−1}\). A calibration curve using high molecular weight standards was used to estimate the size of the protein complex.

**Electron Microscopy of E2—** Purified recombinant maize E2 eluted from the Superose 6 column was diluted to 0.2 mg/ml in the elution buffer.
buffer for negative staining. Approximately 100 μl of elution buffer was placed on a carbon-coated copper grid as a control. The diluted recombinant protein was also placed on a copper grid for 5 min. Unbound protein was removed from the copper grid, and bound protein was negatively stained with 1.3% (w/v) phosphotungstic acid, pH 7.4, for 5 min at 25 °C. The stain was then removed, and the grid placed in the column of a Hitachi H-600 for visualization.

**RESULTS**

The N-terminal protein sequence of the maize E2 (23) was used as a search query to identify two cDNAs from a maize EST data base. The two cDNAs were identical except for an extra 42 base pairs on the 5'-end of the cDNA reported herein. The maize E2 cDNA is 1981 base pairs in length containing a 542-amino acid open reading frame with an ATG codon starting at nucleotide 93 and an in-frame stop codon starting at nucleotide 1719. The corresponding polypeptide carries a 107-amino acid mitochondrial targeting peptide immediately N-terminal to the single predicted lipoyl domain. The mature protein starts at Ser108, based upon the similarity of the deduced sequence, -SSSADLPPHQEIGMPSLPT-, with the experimentally determined amino-terminal sequence, -SNXADLPPHQEIGMPXSPT- (24). The mature protein is 435 amino acids in length with a predicted mass of 46,704 and a pI value of 5.08.

The maize E2 polypeptide exhibits 37% overall amino acid identity to the yeast (35), 35% identity to the human (36), and 30 and 29% identity to the *A. thaliana* mitochondrial (26) and plastid (37) E2 sequences, respectively. In contrast, a hypothetical protein identified from the *A. thaliana* genome project exhibited 62% amino acid identity with the maize E2. Like the maize E2, this protein contains a single lipoyl domain downstream of a mitochondrial targeting sequence. For simplicity, we will refer to the hypothetical *A. thaliana* E2 as isoform 1, since it contains a single lipoyl domain, while the previously identified *A. thaliana* E2 will be referred to as isoform 2.

When the amino acid sequences of the maize and *A. thaliana* E2s are aligned with other known E2s, it is apparent that they contain the three conserved domains characteristic of this class of enzymes (Fig. 1). The lipoyl domain of the maize E2 is at least 55% similar to the inner lipoyl domain (Table I) but at most 50% similar to the outer lipoyl domain for other eukaryotic E2s. Likewise, the putative E1-binding domain of the maize E2 is similar to all other E2s except for *A. thaliana*2. The E1-binding domain for *A. thaliana*1, however, is nearly identical to the same region from the maize E2 (Fig. 1, Table I). Like the lipoyl and E1-binding domains, the catalytic domain of the maize E2 is highly conserved but more similar to *A. thaliana*1 than 2.

**Recombinant Expression of Maize E2**—Since the deduced amino acid sequence of the maize E2 is more related to a hypothetical *A. thaliana* protein than the previously identified *A. thaliana* E2, we deemed it necessary to characterize this recombinant protein. The mature maize E2 starting at amino acid Ser108 was expressed in the protease-deficient *E. coli* strain BL21. The expression construct encoded for two additional residues (MV-) at the N terminus of the recombinant protein. When expressed in this manner, the maize E2 represented at least 30% of the total *E. coli* protein (Fig. 2).
maize E2 was 80–90% soluble under all conditions employed and was easily isolated by sonication in the presence of non-ionic detergent. The native size of the recombinant E2 was noticeably large based on its ability to precipitate with ≥6% polyethylene glycol and the location of the protein peak upon density gradient centrifugation (30% glycerol, data not shown). The high molecular weight property of this recombinant protein facilitated its purification using a three-step procedure (Table II). Starting with 0.5 liter of culture broth, approximately 11 mg of purified protein were obtained with a 3-fold purification. The maize E2 was resistant to the few proteases in E. coli (lane 1, Fig. 2A). Furthermore, no internal initiation sites, resulting in truncated proteins, were observed by Coomassie Blue staining or immunoblotting with antibodies to the lipoyl or catalytic domains of plant E2s (data not shown). The purified E2 migrated primarily as a doublet at 52–53 kDa (Fig. 2A), considerably larger than the predicted mass of 46,934, which includes the N-terminal MV.

Identification of Monoclonal Antibody to Maize E2—A maize EST clone encoding a protein with a stretch of amino acids nearly identical to the N-terminal sequence of the maize E2 (24) was identified. This cDNA was judged to be full-length, based on the presence of two immunoreactive E2-like proteins from pea leaf mitochondria was first reported by Luethy et al. (4). The potential for two E2 isoforms in plants was also demonstrated by the purification of pea and potato mitochondrial PDCs. However, it was reported recently that purified maize mitochondrial PDC contains a single E2 (24).3 Additionally, the possibility of a gene-dilipoamide (Fig. 3). The activity observed in the isotopic transfer assay was unequivocally the result of an enzymatic activity, because boiling the recombinant E2 prior to assaying abolished 98% of the activity.

The purified recombinant E2 migrated as a distinct native species during Superose 6 chromatography. To obtain an estimate on the size of the recombinant protein, the standard curve had to be extrapolated, due to the lack of protein standards near the size of this complex. Based upon the standard curve in Fig. 4, the size of this protein was approximately 2.7 MDa. The recombinant protein from at least three different preparations was mostly associated with a sharp protein peak with some tailing (Fig. 4, inset). This elution profile is indicative of one predominant species.

Visualization of Maize E2 by Electron Microscopy—The protein peak eluted from Superose 6 was visualized by negative staining electron microscopy using phosphotungstate. Initially, a protein sample of 2.5 mg ml⁻¹ was observed revealing numerous complexes. Dilution to 0.1–0.2 mg ml⁻¹ allowed optimal visualization for measurements. The E2 complexes were homogeneous with respect to size (29 nm ± 2.1) and best seen at ×50,000–60,000 (Fig. 5). None of the protein complexes were cubic in shape, and most of them appeared “six-sided.” The E2 complexes from animals and yeast, which are both pentagonal dodecahedrons, gave this same appearance (14, 15). Upon closer inspection, the three axes of symmetry characteristic of a pentagonal dodecahedron were observed (Fig. 5).

DISCUSSION

The presence of two immunoreactive E2-like proteins from pea leaf mitochondria was first reported by Luethy et al. (4). The potential for two E2 isoforms in plants was also demonstrated by the purification of pea and potato (25) mitochondrial PDCs. However, it was reported recently that purified maize mitochondrial PDC contains a single E2 (24). The two E2 isoforms from dicots were easily distinguishable by differences in molecular mass. In pea, the 76-kDa species appears to be much less abundant than the 52-kDa isoform.2 And in potato, both E2 species were shown to be functional, based upon their ability to be acetylated (25). This raised some speculation about the identity of the 52-kDa species. It is well known that the flexible nature of the lipoyl domain arms renders them susceptible to proteolytic degradation (39–41). Therefore, this was offered as a potential explanation for the presence of a 52-kDa species (23, 25, 26), i.e. a mono-lipoyl domain proteolytic product of the 76-kDa polypeptide. We were dubious of this explanation, because a 76-kDa E2 has never been observed in total protein extracts, purified mitochondria, or purified PDC from maize organs (24).3 Additionally, the possibility of a gene-encoded E2 with a single lipoyl domain is not new. The E2 subunits for S. cerevisiae (35), Bacillus stearothermophilus (42), and even A. thaliana plastid (37) PDCs all contain one lipoyl domain. In this report, we extend this list to include maize, A. thaliana, and other, if not all, plant mitochondrial E2s.

A maize EST clone encoding a protein with a stretch of amino acids nearly identical to the N-terminal sequence of the maize E2 (24) was identified. This cDNA was judged to be full-length, since only one Met was found in the first 150 amino acids, and

3 J. J. Thelen, N. R. David, and D. D. Randall, unpublished data.
translation starting at this Met resulted in a 107-amino acid targeting peptide that was predicted to sort exclusively to mitochondria using a subcellular sorting algorithm (PSORT). Also, transcription/translation of this cDNA in a reticulocyte lysate resulted in a 62-kDa precursor polypeptide (data not shown). Furthermore, the unusually large mitochondrial targeting peptide does not resemble a second lipoyl domain.

The mature protein contains the three domains characteristic of dihydrolipoamide S-acetyltransferases, the single N-terminal lipoyl domain plus E1-binding and catalytic domains. Based upon rather low similarity to E3-binding proteins (protein X) and the presence of the C-terminal -DHXXXDG- motif,

| Fractions                  | Total Protein | Specific Activity | Purification | Yield |
|---------------------------|---------------|-------------------|--------------|-------|
|                           | mg           | Volume (ml)       | Units/µg     | -fold | %    |
| Total                     | 164          | 63.0              | 10,630       | 64.8  | 1.00 | 100  |
| Clarified lysate           | 101          | 61.0              | 9460         | 93.7  | 1.45 | 88   |
| 0–10% PEG                 | 27.0         | 2.5               | 3960         | 147   | 2.27 | 37   |
| Density gradient           | 10.7         | 21.0              | 2040         | 191   | 2.95 | 19   |

**FIG. 3.** Dihydrolipoamide S-acetyltransferase activity assay of purified recombinant maize E2. Purified recombinant maize E2 (5 µg) was incubated with [1-14C]acetyl-CoA and dihydrolipoamide for 2 min and then extracted with benzene. The dihydrolipoamide partitions with the organic phase, carrying with it any transferred [1-14C]acetate. Controls without recombinant E2, without dihydrolipoamide, and using boiled E2 are shown. Values are expressed in dpm and are the mean of three experiments ± S.D.; without enzyme, 137 ± 11; without dihydrolipoamide, 87 ± 9; complete, 8328 ± 318; with boiled enzyme, 155 ± 16.

**FIG. 4.** Size exclusion chromatographic analysis of purified recombinant maize E2. Superose 6 chromatography was used to resolve high molecular mass standards (mean of three separate samples) for the standard curve. Blue dextran, 2000 kDa; thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa. The arrow indicates the position at which recombinant E2 eluted from at least three different preparations, and the inset shows a typical A280 elution profile.

**FIG. 5.** Electron micrographs of negatively stained purified recombinant maize E2. Approximately 100 µl of a 0.2 mg/ml solution of recombinant E2 was applied to a carbon-coated copper grid. Adhered protein was negatively stained with 1.3% phosphotungstate for 5 min and visualized on a Hitachi H-600 model electron microscope. The upper panel shows that these complexes are homogeneous in size (~29 nm). The lower panels are magnified complexes demonstrating the three axes of symmetry that were observed.

**FIG. 6.** Mapping the epitope recognition domain for the monoclonal antibody to maize E2. A, model depicting the domain organization of the maize E2 subunit and relative location of deletion mutants. L, lipoyl domain; S, E1 subunit-binding domain; C, catalytic and assembly domain. B, immunoblot analysis of total bacterial cell extracts. Bacteria containing one of three constructs were grown to midlog phase. Lipoic acid (3 mM) and isopropyl-1-thio-β-D-galactopyranoside (0.3 mM) were added to the cultures and induced for 2 h. Exactly 1 ml of the cells was harvested and lysed in SDS-PAGE sample buffer. Approximately 2 µg of protein were resolved, blotted to nitrocellulose, and probed with the E2 monoclonal antibody. These data are representative of three different experiments.
important for catalytic activity of S-acetyltransferases (43), we concluded this was probably an E2. To test this, mature maize E2 was expressed without any fusion tags and purified from the heterologous host, *E. coli*. The putative maize E2 was highly induced, intact, and soluble under mild extraction conditions. The high degree of solubility, without the need for chaperone co-expression, probably indicates folding was unassisted. The ability to autoassemble seems to be a ubiquitous property of these enzymes (44-46). Once assembled, the E2 core was very stable to proteolysis and dissociation, facilitating purification. The nearly homogeneous E2 had a specific activity of 191 ΔA282 min⁻¹ mg⁻¹, considerably higher than the specific activity observed with purified recombinant human E2 (19.4 ΔA282 min⁻¹ mg⁻¹; Ref. 34). The purified maize E2 consisted of 52-, 53-, and 54-kDa polypeptides at a ratio of approximately 2:2:1, almost identical to the polypeptide pattern for native maize E2. The triplet pattern observed with native E2 is probably not due to different isoforms or proteolysis, since purified recombinant E2 gives this appearance and the N terminus of the 52- and 53-kDa E2 subunits are identical (24). One possible explanation is the lipoylation and/or acetylation status of E2.

The 52–54-kDa apparent size of maize E2 is much larger than the deduced size of 46,934. Anomalous migration during SDS-PAGE has been observed for E2 subunits from all organisms and was attributed to the flexible hinge regions (26, 36, 49–51). Some of the data presented here confirm this. The Δ240–542 maize E2 mutant, which contains the lipoyl and hinge region, appears 7 kDa larger upon SDS-PAGE than predicted. The migration shift during PAGE might be due to the unusual conformation of the hinge region resulting from the abundance of Pro (21%), Glu (21%), Ala (19%), and Lys (14%) residues within this 42-residue stretch.

The expressed maize E2 was not only active but also appeared to have assembled into the subcomplex core. This conclusion is based upon size exclusion chromatography showing it to be approximately 2.7 MDa, which is quite close to the predicted size of 2.82 MDa for a 60-mer pentagonal dodecahedron. Formation of this core is further supported by electron microscopy showing icosahedral symmetry of the maize E2. This report is the first example of a plant E2 core visualized by electron microscopy and establishes that the icosahedral symmetry of the plant mitochondrial E2 core is most likely identical to that for animal (14), fungal (15), and Gram-positive bacterial (12, 13) PDCs. Furthermore, the size of the maize E2 cores visualized by electron microscopy were about the same as those from other organisms. Whether the plastid E2 also obeys icosahedral symmetry is still unknown, but this question has interesting evolutionary implications.

Since the maize E2 contains a single lipoyl domain and is only 30% identical to the previously reported *A. thaliana* mitochondrial E2, which contains two lipoyl domains, we were interested to know if single lipoyl domain E2 homologues exist in other plants. The discovery of a hypothetical protein from *A. thaliana* that is 62% identical to the maize homologue (and contains a single lipoyl domain) suggested that two mitochondrial E2 isoforms exist in some plants. This finding raises some other questions. Do other plants also contain a smaller E2 homologue and two E2 isoforms? Which isoform is predominant? Can these isoforms co-assemble? Do these isoforms differ in function or assembly? While definitive answers to most of these questions await further investigation, some speculation is warranted.

The monoclonal antibody specific for the lipoyl domain of maize E2 provided a useful tool with which to address some of these questions. Probing plant mitochondrial protein isolated from four diverse plants revealed 50–54-kDa immunoreactive polypeptides in all of them (Fig. 7). A 66-kDa immunoreactive polypeptide from *A. thaliana* mitochondria is the position where *A. thaliana* plastid E2 (37) migrates, suggesting that these mitochondria were probably not as pure as those from the other plants. Indeed, these mitochondrial preparations from *A. thaliana* are contaminated with chloroplasts, judging by their green color. Highly purified pea mitochondrial PDC contains a 52-kDa E2 species as well as a faint immunoreactive species at 76 kDa, which is the position where the *A. thaliana* isoform 2 migrates (4, 26). Coomassie Blue staining also reveals this protein in purified preparations but at levels considerably (2-fold) less than the 52-kDa species. In contrast, a 76-kDa E2 isoform has never been observed in the monocot maize.

These data clearly demonstrate the existence of two E2 isoforms from *A. thaliana* and pea mitochondria, which contain either one or two lipoyl domains. The mono-lipoyl domain E2 is probably present in all plant mitochondria, but the di-lipoyl domain E2 is not, since we have yet to detect one in the monocot, maize. It is possible that the di-lipoyl domain isoform is only present in dicots, while the mono-lipoyl domain E2 is the ubiquitous and predominant isoform. At this point it is unknown whether these isoforms can co-assemble, since co-migration during purification does not rule out the possibility of two homogeneous E2 cores of similar mass. Assuming these isoforms are capable of co-assembly, it is possible that they have different regulatory or binding properties. For instance, the low overall conservation within the E1-binding domain of *A. thaliana* isoform 2 suggests that it might not bind E1 but instead E3 and/or PDH kinase. This scenario could explain why the isoform 2-deficient maize PDC loses E3 (70–80%) and PDH kinase (>95%) during purification (7, 52). Expression of the two recombinant *A. thaliana* E2 isoforms will enable comparative analyses of catalytic and binding properties.

In conclusion, the 52-kDa polypeptide observed in purified maize mitochondrial PDC is not a proteolytic product or E3-binding protein, but rather a unique E2 subunit containing a single lipoyl domain. Identification of an *A. thaliana* homologue of the maize E2 demonstrates the existence of a mono-lipoyl domain E2 isoform in dicots and accounts for the 50–54-kDa immunoreactive E2-like proteins observed in *A. thaliana*, pea, potato, cucumber, and *K. daigremontianum* mitochondrial PDCs. Previous discoveries of mitochondrial E2s with two lipoyl domains (from dicots) suggest that two mitochondrial E2s...
isoforms exist in some plants and point toward a potentially unique organization of the E2 core in plants.

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REFERENCES

1. Reed, L. J. (1974) Acc. Chem. Res. 7, 49–56
2. Williams, M., and Randall, D. D. (1979) Plant Physiol. 64, 1099–1103
3. Camp, P., J., and Randall, D. D. (1985) Plant Physiol. 77, 571–577
4. Luethy, M. H., Miernyk, J. A., David, N. R., and Randall, D. D. (1996) in Alpha-Keto Acid Dehydrogenase Complexes (Patel, M. S., Roche, T. E., and Harris, R. A., eds) pp. 71–92, Birkhauser Verlag, Basel, Switzerland
5. Patel, M. S., and Roche, T. E. (1996) FASEB J. 4, 3224–3233
6. Wallis, N. G., Allen, M. D., Broadhurst, R. W., Lessard, I. A. D., and Perham, R. N. (1996) J. Mol. Biol. 263, 463–474
7. Thelen, J. J., Muszynski, M. G., Miernyk, J. A., and Randall, D. D. (1998) J. Biol. Chem. 273, 26618–26623
8. Miernyk, J. A., Thelen, J. J., and Randall, D. D. (1998) in Plant Mitochondria: From Gene to Function, pp. 321–328, Backhuys Publishers, Margraf Verlag, Germany
9. Randall, D. D., Miernyk, J. A., David, N. R., Gemel, J., and Luethy, M. H. (1996) in Protein Phosphorylation in Plants (Shewry, P. R., and Halford, N. G., eds) pp. 87–103, Clarendon Press, Oxford
10. Reed, L. J., and Hackert, M. L. (1990) J. Biol. Chem. 265, 8971–8974
11. Stephens, P. E., Darlison, M. G., Lewis, H. M., and Guest, J. R. (1983) Eur. J. Biochem. 133, 481–489
12. Scoop, J. L., Westphal, A., Ben, J. A. E., Teixeira de Mattos, M. J., Nejssel, O. M., and de Kost, A. (1992) Eur. J. Biochem. 203, 245–250
13. Henderson, C. E., Perham, R. N., and Finch, J. T. (1979) Cell 17, 85–93
14. Wagenknecht, T., Grassmer, R. A., and Roche, T. E. (1991) J. Mol. Biol. 266, 2450–2455
15. Stoops, J. K., Cheng, R. H., Yazdi, M. A., Maeng, C. Y., Schroeter, J. P., Kluepfenberg, U., Kolodziej, S. J., Baker, T. S., and Reed, L. J. (1997) J. Biol. Chem. 272, 5757–5764
16. Perham, R. N. (1991) Biochemistry 30, 8501–8512
17. Gopalakrishnan, S., Rahmatullah, M., Roche, T. E. (1989) Biochem. Biophys. Res. Commun. 160, 715–721
18. Powers-Greenwood, S. L., Rahmatullah, M., Roche, T. E. (1989) J. Biol. Chem. 264, 3655–3657
19. Lawrence, J. E., Behal, R. H., and Reed, L. J. (1991) Biochemistry 30, 2834–2839
20. Harris, N. A., Bowker-Rinke, M. M., Wu, P., Jing, J., and Popov, K. M. (1997) J. Biol. Chem. 272, 19746–19751
21. Behal, R. H., Browning, K. S., Hall, T. B., and Reed, L. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8732–8736
22. Klingbeil, M. M., Walker, D. J., Arnette, R., Sidaway, E., Hayton, K., Komuniecki, P. R., and Komuniecki, R. (1996) J. Biol. Chem. 271, 5451–5457
23. Taylor, A. E., Cogdell, R. J., and Lindsay, J. G. (1992) Planta 188, 225–231
24. Thelen, J. J., Miernyk, J. A., and Randall, D. D. (1998) Plant Physiol. 116, 1443–1450
25. Miller, A. H., Knopp, C., Laver, C. J., and Hill, S. A. (1998) Biochem. J. 334, 571–576
26. Guan, Y., Rawsthorne, S., Scofield, G., Shaw, P., and Donnan, J. (1995) J. Biol. Chem. 270, 5412–5417
27. Thelen, J. J., Miernyk, J. A., and Randall, D. D. (1999) Plant Physiol. 119, 635–643
28. Hayes, M. K., Luethy, M. H., and Elthon, T. E. (1991) Plant Physiol. 97, 1381–1387
29. Fang, T. K., David, N. R., Miernyk, J. A., and Randall, D. D. (1987) Curr. Top. Plant Biochem. 6, 175
30. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
31. Elthon, T. E., Nichols, R. L., and McIntosh, L. (1989) Plant Physiol. 89, 1311–1317
32. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
33. Randall, D. D., and Miernyk, J. A. (1990) Methods Plant Biochem. 3, 175–192
34. Yang, D., Song, J., Wagenknecht, T., and Roche, T. E. (1997) J. Biol. Chem. 272, 6361–6369
35. Niu, X. D., Browning, K. S., Behal, R. H., and Reed, L. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7546–7550
36. Coppell, R. L., McNeilage, J., Surb, C. D., Van de Water, J., Spithill, T. W., Whittingham, S., and Gershwin, M. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7317–7321
37. Mooney, B. P., Miernyk, J. A., and Randall, D. D. (1999) Plant Physiol., 120, 443–451
38. Luethy, M. H., David, N. R., Elthon, T. E., Miernyk, J. A., and Randall, D. D. (1995) J. Plant. Physiol. 145, 443–449
39. Bleie, D. M., Hackert, M. L., Pettit, F. H., and Reed, L. J. (1981) J. Biol. Chem. 256, 514–519
40. Komuniecki, R., Rhee, R., Bhat, D., Duran, E., Sidaway, E., and Song, H. (1992) Arch. Biochem. Biophys. 296, 115–121
41. Kresse, G. B., and Ronft, H. (1980) Eur. J. Biochem. 112, 589–599
42. Borges, A., Hawkins, C. F., Packman, L. C., and Perham, R. N. (1990) Eur. J. Biochem. 194, 95–102
43. Guest, J. R. (1987) FEBS Microbiol. Lett. 44, 417–422
44. Behal, R. H., DeBuysere, M. S., Demeler, B., Hansen, J. C., and Olson, M. S. (1994) J. Biol. Chem. 269, 31372–31377
45. De Marcucci, O. L., DeBuysere, M. S., and Olson, M. S. (1995) Arch. Biochem. Biophys. 323, 169–176
46. Allen, M. D., and Perham, R. N. (1997) FEBS Lett. 413, 339–343
47. Russell, G. C., Machado, R. S., and Guest, J. R. (1992) Biochem. J. 287, 611–619
48. Quinn, J., Diamond, A. G., Masters, A. K., Brookfield, D. E., and Wallis, N. G., Yeaman, S. J. (1995) Biochem. J. 298, 81–85
49. Rubin, P. M., and Randall, D. D. (1977) Arch. Biochem. Biophys. 178, 342–349
50. Allen, A. G., and Perham, R. N. (1991) FEBS Lett. 287, 206–210
51. Machado, R. S., Clark, D. P., and Guest, J. R. (1992) FEBS Microbiol. Lett. 100, 243–246
52. Thelen, J. J., Miernyk, J. A., and Randall, D. D. (1998) in Plant Mitochondria: From Gene to Function Backhuys Publishers, pp. 417–422, Margraf Verlag, Germany