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A novel regulatory mechanism based upon a dynamic core structure for the mitochondrial pyruvate dehydrogenase complex?

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ABSTRACT

The *Arabidopsis thaliana* genome includes three genes for mitochondrial dihydrolipoamide acetyltransferase, the E2-component of the mitochondrial pyruvate dehydrogenase complex (PDC). Two genes encode E2-proteins with a single lipoyl domain, while the third has a two-lipoyl domain structure. Transcripts for each E2 protein were expressed in all plant organs. Each recombinant AtmtE2 can individually form an icosahedral PDC core structure, and results from bimolecular fluorescence complementation assays are consistent with formation of hetero-core structures from all permutations of the AtmtE2 proteins. We propose a unique regulatory mechanism involving dynamic formation of hetero-core complexes that include both mono- and di-lipoyl forms of AtmtE2.

1. Introduction

The pyruvate dehydrogenase complex (PDC) is composed of multiple copies of three central components: E1, E2, and E3 (Bleile et al., 1981; Mooney et al., 2002; Zhou et al., 2001). Pyruvate dehydrogenase (E1; pyruvate:[dihydrolipoyllysine-residue acetyltransferase]-lipoyllysine 2-oxidoreductase (decarboxylating, acceptor-acetylating); EC 1.2.4.1) catalyzes the oxidative decarboxylation of pyruvate, using thiamine-pyrophosphate as a cofactor, followed by the reductive acetylation of lipoyl moieties covalently linked to dihydrolipoamide acetyltransferase (E2, enzyme-dihydrolipoyllysine:acetyl-CoA S-acetyltransferase; EC 2.3.1.12) which transfers the lipoyl-bound acetyl-group to CoASH, releasing acetyl-CoA as a product. Dihydrolipoyl dehydrogenase (E3, protein-N6-(dihydrolipoyl)lysine:NAD + oxidoreductase; EC 1.8.1.4) contains a FAD cofactor, and completes the reaction cycle by reoxidizing the lipoyl group of E2, using NAD^+ as an electron acceptor. Mitochondrial PDCs additionally include the regulatory components pyruvate dehydrogenase kinase (PDK, ATP:[pyruvate dehydrogenase (acetyl-transferring)] phosphotransferase; EC 2.7.11.2) and P-pyruvate dehydrogenase phosphatase (PDP, [pyruvate dehydrogenase (acetyl-transferring)]-phosphate phosphohydrolase; EC 3.1.3.43), that together control activity by reversible phosphorylation of E1α (Gey et al., 2008; Patel and Korotchkina, 2006; Strumilo, 2005; Tovar-Mendez et al., 2003).

The E2 protein sequences include three distinct domains. Depending upon the type of PDC, the N-terminus of E2 includes from one to three tandem lipoyl domains of approximately 100 residues. Lipoate protein ligase attaches the lipoic acid cofactor to a conserved Lys residue within each lipoyl domain (Reche and Perham, 1999). In mammals, the PDK and PDP regulatory components interact with the E2 lipoyl domains (Kato et al., 2005; Liu et al., 1995; Roche et al., 2003). The inner lipoyl domain is connected by a flexible linker to the subunit-binding domain (SBD), which is responsible for binding the E1 and/or E3 component(s) of the complex (Jung et al., 2003). The C-terminal region of E2 comprises the catalytic domain, and contains a conserved His residue necessary for CoA-binding. The C-terminal domain is also responsible for E2:E2 subunit association (Jung et al., 2002a,b). The E2 monomers form trimers, which can then self-assemble into the larger quaternary structural core of PDC (Yang et al., 1997). The E2 domains are connected by linker regions of approximately 20–30 residues, which allow structural flexibility of the core and attached components (Guest et al., 1985). This

Abbreviations: BiFC, bimolecular fluorescence complementation; E1, pyruvate dehydrogenase; E2, dihydrolipoyl acetyltransferase; E3, dihydrolipoyl dehydrogenase; E3BP, E3 binding protein; mt-, mitochondrial; MTS, mitochondrial targeting sequence; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; -pl, plastidial; SBD, subunit binding domain.

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flexibility, plus that of the lipoyl-groups themselves, contributes to the active-site coupling that is partially responsible for catalytic enhancement (Perham, 2000).

There are two types of PDC: I and II. The type I complexes, found in some Gram-negative bacteria, have a 24-mer E2 core with 8-fold symmetry (de Kok et al., 1998; Jones et al., 2000; Mattevi et al., 1992) to which the other components bind non-covalently. In eukaryotes and some Gram-positive bacteria, the E2 core is a 60-mer with icosahedral (5-3-2 fold) symmetry (Niu et al., 1988; Perham, 2000; Stoops et al., 1997; Zhou et al., 2001). Despite the seemingly substantial structural differences, the core structures are considered quasi-equivalent (Izard et al., 1999; Milne et al., 2006).

An additional protein component is present in the PDC of yeast, nematodes, and mammals, E3-binding protein (E3BP, also described as protein X). It has been estimated that the E3BP is present at 5–24 copies per complex (Behal et al., 1989; Hodgson et al., 1986; Jilka et al., 1986; Klingbeil et al., 1996). The E3BP primary sequence is similar to that of E2, and includes a lipoyl domain, but it cannot form the PDC core structure. Instead E3BP appears to sit inside the PDC E2 core, with its high affinity E3 binding domain exposed (Harris et al., 1997; Hiromasa et al., 2004; McCartney et al., 1997; Sanderson et al., 1996). The E3BP does not have acetyltransferase activity, although the lipoyl-group of yeast and mammalian E3BP can be reduced and acetylated (Behal et al., 1989; Rahmatullah and Roche, 1987).

Analysis of the *A. thaliana* genome using the maize mitochondrial PDC E2 sequence described by Thelen et al. (1999) as the query revealed multiple homologous sequences. The experiments described herein were undertaken to clarify potential functions of the proteins encoded by these genes, with emphasis on determining if one of them might encode a protein that functions as a PDC E3BP. Additionally, we consider the possibility of specialization of the multiple forms of AtmtE2.

2. Materials and methods

2.1. In silico analyses

The tBLASTn algorithm was used to search the non-redundant *A. thaliana* database of GenBank (http://www.ncbi.nlm.nih.gov/), using the amino acid sequence of the *Zea mays* mitochondrial PDC E2 (ZmmtE2, accession AF135014) as the search parameter. The full-length deduced amino acid sequences were aligned using the PHYLIP function of DNAMAN version 4.3 (Lynnon BioSoft, Vaudreuil, Quebec, Canada), and 1000 rounds of bootstrapping. The TreeView algorithm (version 1.6.1) was used for sequence alignments and comparison (Page, 1996). Three different programs were used for prediction of subcellular localization: TargetP (Emanuelsson et al., 2000), Predotar (Small et al., 2004), and PCLR (Schein et al., 2001). GENEVESTIGATOR was used to search and analyze microarray transcript-profiling data (Zimmermann et al., 2004).

2.2. Analyses of PDC E2 transcript abundance

Young seedlings, leaves, flowers, stems, siliques, and seeds were harvested from greenhouse-grown *A. thaliana* ecotype Col-0 plants. Samples were processed immediately for total RNA isolation, and the RNA was subsequently reverse-transcribed to produce DNA. Gene specific primers were made to a 500 bp region of each E2 sequence for use in the PCR (AtmtE2IA, DDR 328, GCGAATTCATGGCTTATGCGTCACGC ATCATT, DDR 355, TTGGCGAGATAGCCCTCTTCC; AtmtE2IB, DDR 363, AAGCCAAGCTCAGCTCCTTCAGAAG, DDR372, GGCGGATGTATTCATCTG TCC; AtmtE2II, DDR95, GCATCTAGAAGTCCTGCTGCAAAG, DDR99, GCAG TCGACAGATTTCTGGTCTGC). Conditions for thermal cycling were as described previously (Mooney et al., 1999). The RT-PCR products were separated by agarose gel electrophoresis and stained with EtBr, and the bands were quantified using 1-D gel imaging software (Eastman Kodak, New Haven, CT). Values were corrected for variation in sample loading using a 300 bp 18S rRNA internal standard (Ambion, Austin TX).

2.3. Promoter–reporter construction and analysis

The promoter region of each E2 (~1.5 kb 5′ of the ATG start codon) was amplified from *A. thaliana* leaf genomic DNA by PCR using primers specific for E2IA (DDR499; 5′-*tc*cctgcaggCTAACTCCAAGATTATCG-3′, DDR496; 5′-*cgg*gatcc**CAT**TGATGCACTATCGTT-3′), E2IB (DDR498; 5′--*tc*cctgcaggTCGAGATAATAGATAGAC-3′, DDR494; 5′-*cgg*gatcc**CAT**TGTT GTGCAATCGGA-3′) and E2II (DDR497; 5′-*tc*cctgcaggCAATAGTTAAA GAGTATT-3′, DDR492; 5′-*cgg*gatcc**CAT**GAGAGCACTTGGAGA-3′). Additional bases added to facilitate cloning are in italics, start codons are in bold, and engineered restriction endonuclease cleavage sites are lowercase. The CaMV 35S promoter region was excised from the pBI121 [(Jefferson et al., 1987), AF485783] plant transformation vector using SbfI and BamHI restriction sites, and replaced with an AtmtE2 promoter regions to drive *in planta* expression of β-glucuronidase (GUS). Constructs were verified by sequencing and then used to transform *Agrobacterium tumafaciens* strain GV3101. Bolting *A. thaliana* plants were transformed by floral dip (Clough and Bent, 1998) and grown to maturity, and seeds were collected. Seeds were plated on solid media containing 30 μg/mL kan to identify transformants. At least 15 transformants (for each construct) were transferred to soil and grown to maturity. The seeds of these plants were subjected to kan-selection, and survivors were used for histochemical staining with X-gluc using vacuum infiltration (Weigel and Glazebrook, 2002). Plants were stained in the dark at 37 °C for 8–18 h and then cleared by multiple washes in 70% ethanol. After de-staining, samples were viewed and photographed using a Leica MZFLIII stereo microscope equipped with an Optronics MagnaFire digital camera, at the University of Missouri Molecular Cytology Core Facility (http://www.biotech.missouri.edu/mcc/).

2.4. Construction of E. coli expression plasmids

Oligonucleotides were designed to amplify the complete open reading frame (ORF) for each of the AtmtE2 proteins. The primary sequences for AtmtE2IB (AF367302) and AtmtE2II [CAA86300] were verified relative to GenBank accessions from the *A. thaliana* genome project. However, our sequence for AtE2IA differed from the original accession. The corrected sequence of the full-length ORF is in GenBank as AY033001.

Oligonucleotides were designed to amplify each AtE2 ORF minus the mitochondrial targeting sequences (MTS). The position of the MTS cleavage site for each E2 was predicted based upon the N-terminal sequence of native ZmmtE2 (Thelen et al., 1999). The amplified products correspond to the region of the ORF between 307 and 1620 bp for E2IA and E2IB (DDR 403; 5′-ccatg*gtt*TCATCGGGTTCAGAT-3′ and DDR 367; 5′-*gggaac*tcgag**TTA**GAGTAACATAGATTTAGG-3′, and DDR 404; 5′-ccatg*gtt*TCATCCAGTTCAGATC-3′ and DDR 304; 5′-*ccg*gagctc**TTA**GAGC AACATAGATTCTGGG-3′, respectively), and 232–1914 bp for E2II (DDR 435; 5′-*cccgcg*catatgTCATCTACAGGACCCATATCA-3′ and DDR 260; 5′-*gggcccc*tcgag**TCA**CAGAAGAAGTCTTCG-3′). Additional bases used to facilitate cloning are in italics, stop codons are bold, and engineered restriction endonuclease cleavage sites are in lowercase letters. Primers for PCR were synthesized by Integrated DNA Technologies (Coralville, IA). Unless otherwise noted, all biochemicals were from the Sigma Chemical Co. (St. Louis, MO).

Each ORF was amplified using the QIAGEN One-step RT-PCR kit (QIAGEN, Valencia, CA). Template (total) RNA was extracted from flowering greenhouse-grown *A. thaliana* ecotype Col 1 leaves using the RNeasy Plant Mini Kit (QIAGEN). Approximately 5 ng of total leaf RNA was used per 15 μL RT-PCR reaction. Amplifications followed the manufacturer's instructions for reverse transcription and Taq activation, and the three-step cycling consisted of 94 °C, 30 s, 60 °C, 60 s, 72 °C, 90 s for 35 cycles, followed by a final extension at 72 °C for 10 min.

The RT-PCR products were purified by agarose gel electrophoresis and then ligated with pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated using a standard alkaline-lysis method, and samples