Regulation of Serine Biosynthesis in Arabidopsis

CRUCIAL ROLE OF PLASTIDIC 3-PHOSPHOGLYCERATE DEHYDROGENASE IN NON-PHOTOSYNTHETIC TISSUES

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In plants, Ser is synthesized through a couple of pathways. 3-Phosphoglycerate dehydrogenase (PGDH), the first enzyme that is involved in the phosphorylated pathway of Ser biosynthesis, is responsible for the oxidation of 3-phosphoglycerate to phosphohydroxypyruvate. Here we report the first molecular cloning and characterization of PGDH from Arabidopsis thaliana. Sequence analysis of cDNA and a genomic clone revealed that the PGDH gene is composed of three exons, encoding a 623-amino acid polypeptide (66,453 Da). The deduced protein, containing three of the most conserved regions in the NAD-dependent 2-hydroxyacid dehydrogenase family, has 38–39% identity to its animal and bacterial counterparts. The presence of an N-terminal signal sequence for translocation into plastids was confirmed by particle-gun bombardment experiments using green fluorescent protein as a reporter protein for subcellular localization. Southern hybridization analysis and restriction fragment length polymorphism mapping indicated that PGDH is a single-copy gene that is mapped to the upper arm of chromosome 1. Northern hybridization analysis indicated preferential expression of PGDH mRNA in root tissues of light-grown plants, suggesting that the phosphorylated pathway of Ser biosynthesis plays an important role in supplying Ser to non-photosynthetic tissues. The recombinant enzyme overproduced in Escherichia coli displayed hyperbolic kinetics with respect to 3-phosphoglycerate and NAD*. 3-Phosphoglycerate dehydrogenase (PGDH); EC 1.1.1.95, the first enzyme in the Ser biosynthetic pathway from 3-phosphoglycerate (3-PGA), catalyzes the oxidation of 3-PGA to form phosphohydroxypyruvate by utilizing NAD” as a cofactor. Phosphohydroxypyruvate is subsequently transaminated by phosphoserine aminotransferase to yield phosphoserine. In the final step, dephosphorylation of phosphoserine to Ser is performed by phosphoserine phosphatase (Fig. 1). The molecular cloning and biochemical characterization of PGDH have been reported for a variety of bacterial (1–3) and animal (4–6) sources. In higher plants, the biochemical characterization of PGDH enzyme preparation has been carried out in pea (7) and spinach (8); however, no investigation on molecular cloning and characterization was reported.

Ser can be formed by more than two pathways in higher plants (9, 10). The photorespiratory pathway (11) of Ser biosynthesis via glyoxylate and Gly is the major route of Ser formation in photosynthetic tissues under light conditions. The glycine decarboxylase multienzyme complex (GDC), along with the enzyme serine hydroxymethyltransferase, is responsible for the respiratory conversion of Gly to Ser (12, 13). The cDNAs encoding the four component enzymes of GDC (14–17) and serine hydroxymethyltransferase (18, 19) from plants have been cloned and characterized. The other two Ser biosynthetic pathways from 3-PGA via either phosphorylated or non-phosphorylated intermediates are proposed to be important Ser sources in non-photosynthetic tissues or under dark conditions, depending on the type of tissues involved (20) (Fig. 1). For these pathways, 3-PGA is supplied by glycolysis or the pentose phosphate pathway.

The serA gene, which encodes PGDH, has been cloned and characterized in Escherichia coli (1, 21, 22). E. coli PGDH is a tetramer of identical subunits, each consisting of three domains for nucleotide binding, substrate binding, and regulatory function. PGDHs from pea (7), E. coli (23, 24), and Bacillus subtilis (25) are reported to be feedback-inhibited by Ser, whereas the enzymes from spinach (8) and animals (6, 26) do not exhibit similar feedback regulation. However, PGDH in rat livers is regulated at the transcriptional level (6). In this paper, we describe, for the first time, cDNA and genomic cloning, biochemical characterization, and expression of PGDH from a higher plant, Arabidopsis thaliana.

EXPERIMENTAL PROCEDURES

Plant Materials—A. thaliana ecotype Columbia seeds were germinated and grown on germination medium (27) agar plates under 16/8-h light and dark cycles at 22 °C for 3 weeks. For the dark-treated seedlings that were used for Northern analyses, 2-week-old seedlings were wrapped in aluminum foil and subsequently grown for another week before RNA extraction was carried out.

Isolation of cDNA and Genomic Clones—cDNA library screening was carried out using a 32P-labeled synthetic 50-mer oligonucleotide probe (5'-GGTACGGAGCTTCTGAGGCTACCGCAGAAAGGCTCTGCTG-3') that was based on the deposited sequence of the cDNA insert of the Arabidopsis expressed sequence tag FAFH01 (GenBank™ accession number ATTS0347). Approximately 2.5 × 10⁶ plaques from the λgt11 cDNA library constructed from A. thaliana whole plants were screened. For genomic cloning, the cDNA clone CPDH-5 was used to screen ~4 × 10⁶ amplified plaques from the Arabidopsis genomic λEMBL3 SP6/7 library (CLONTECH).

Hybridization of the membranes (Hybond N+, Amersham Pharmacia Biotech) was carried out at 65 °C in 5× SSPE (0.9 M NaCl, 0.05 M

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sodium phosphate (pH 7.7), and 5 mM EDTA. 0.5% SDS, 5× Denhardt’s solution (28), and 25 μg/ml salmon sperm DNA. The final washing of membranes was conducted at 65 °C in 0.1× SSPE and 0.1% SDS for 10 min.

**DNA Subcloning and Sequencing**—The cDNA and genomic DNA inserts of the isolated clones were subcloned into appropriate cloning sites of pBluescript II SK+ (Stratagene). Sequencing of full-length cDNA was carried out on both strands using a series of overlapping exonuclease III-digested clones created with the Exo/Mung deletion kit (Stratagene). Autosequencing was conducted by the dideoxy chain termination method with Thermo Sequenase (Amersham Pharmacia Biotech) using a Shimadzu DNA sequencer (Model DSQ1000).

**Miscellaneous Techniques**—SDS-polyacrylamide gel electrophoresis, protein quantitation, and primer extension were carried out as described (28).

3-Phosphoglycerate Dehydrogenase from Arabidopsis

**FIG. 1. Main route of Ser biosynthesis in higher plants.** Arrow 1, ribulose-1,5-bisphosphate carboxylase/oxygenase; arrow 2, PGDH; arrow 3, phosphoserine aminotransferase; arrow 4, phosphoserine phosphatase; arrow 5, phosphoglycolate phosphatase; arrow 6, glycolate oxidase; arrow 7, amino-acid:glyoxylate aminotransferase; arrow 8, glycine decarboxylase-serine hydroxymethyltransferase complex; arrow 9, serine hydroxymethyltransferase; arrow 10, glycine-glyoxylate aminotransferase; arrow 11, Alanine-glyoxylate aminotransferase; arrow 12, serine:glyoxylate aminotransferase; arrow 13, glyceraldehydate dehydrogenase; arrow 14, glycine-glyoxylate reductase; arrow 15, 3-phosphoglycerate phosphatase; arrow 16, glyceraldehyde kinase, C, chloroplast/plastid localization; M, mitochondrion localization; P, peroxisome localization.
RESULTS

Arabidopsis PGDH Is Closely Related to Its Counterparts from B. subtilis, Synechocystis sp., and Mammals—Phage plaques produced from an Arabidopsis whole plant cDNA library were screened with a synthetic 50-mer oligonucleotide probe based on the sequence of A. thaliana expressed sequence tag clone FAFH01, which shows high homology to PGDH from B. subtilis. Among the four positive clones selected for further studies, CPGDH-5, which contains the largest cDNA insert (2.2 kilobase pairs), was subcloned and sequenced. Sequence analysis revealed an open reading frame of 1881 nucleotides, encoding for 623 amino acid residues. The first ATG triplet, which is 14 nucleotides away from the 5'-end of CPGDH-5, is designated as the translational start point because the sequence around the Met codon (AGTCATGGC) matches well with the consensus sequence for plant gene initiation codons (AACAATGGC) (36). This is further supported by the primer extension result that mapped the transcriptional start point at 38 bp before the translational start site. A 3'-untranslated region of 265 nucleotides downstream of the translational stop codon (TAA) is present in the cDNA sequence. The AATAAA polyadenylation signal is located 129 nucleotides upstream of the poly(A) tail. The deduced amino acid sequence of Arabidopsis PGDH has been aligned with PGDHs from other organisms (Fig. 2). A phylogenetic tree (Fig. 3) indicates that Arabidopsis, B. subtilis, Synechocystis sp., rat, and human PGDHs form a family distinct from other bacterial and yeast PGDHs. The deduced 66,453-Da protein, containing three of the most conserved regions in the NAD-dependent 2-hydroxyacid dehydrogenase family, has 38–39% similarity to the amino acid sequences of PGDHs from other organisms (Fig. 2). The first pattern is based on a Gly-rich region that probably corresponds to the NAD-binding domain, Gly-X-Gly-X2-Gly-X17-Asp (37). Two other patterns contain a number of conserved charged residues, some of which may play a role in the catalytic mechanism. The 623 amino acid residues of Arabidopsis PGDH, sharing the three-dimensional structure of E. coli PGDH (22), is the longest sequence among all. It differs from the rest mainly due to its C-terminal domain and N-terminal extension.

PGDH Gene of Arabidopsis Is Mapped to Chromosome 1—The cDNA clone CPGDH-5 was used to screen the Arabidopsis genomic DNA library. Among various clones, CPGD-17 was selected for further studies. The 7.1-kilobase pair fragment that covers the PGDH structural gene and the 5'- and 3'-end FIG. 2. Alignment of deduced amino acid sequences of PGDHs from various organisms. ec, E. coli (1); bs, B. subtilis (2); sy, Synechocystis sp. (GenBank™ accession number S11998); hm, Homo sapiens (GenBank™ accession number AF006043); at, A. thaliana (this study). Black shading indicates identical amino acids, and gray shading indicates similar amino acids. Dots indicate gaps in the sequences for the best alignment. The lines above the sequences indicate the three most conserved regions in the NAD-dependent 2-hydroxyacid dehydrogenase family.

FIG. 3. Phylogenetic tree of PGDH proteins. This phylogenetic tree, constructed using the PHYLIP 3.57c program (J. Felsenstein), indicates that Arabidopsis, B. subtilis, Synechocystis sp., rat, and human PGDHs form a family distinct from other bacterial and yeast PGDHs. ec, E. coli; hi, H. influenza; sc, Saccharomyces cerevisiae; bs, B. subtilis; rn, Rattus norvegicus; sy, Synechocystis; hm, H. sapiens; at, A. thaliana.
The translational start codon (ATG) is indicated (TSP) (in boldface) and the translational initiation (ATG) and termination (TAA) codons are indicated by bars and arrows, respectively. The PGDH genomic region is shown with solid bars (coding regions) and open bars (untranslated regions) indicating the open reading frame. The transcriptional start site of the genomic clone was determined using primer extension analysis. A single major transcriptional start point (+1) was confirmed to be located 38 bp before the translational start codon (ATG) is double-underlined. The sequence of the cDNA clone CPGDH-5 is underlined.

The 60-amino acid leader sequence exhibits the general features of a transit peptide for transportation of protein to plastid. It starts with Met-Ala; is rich in hydroxylated amino acids, Ser and Thr (19/60); is rich in small hydrophobic amino acids, Ala and Val (13/60); is essentially deficient in acidic amino acids, Asp and Glu (1/60); and has a net positive charge (pI 11.5). Prediction by the PSORT program also suggested its localization in chloroplasts. The recombinant fusion protein CPGDH-GFP, containing the N-terminal 82-amino acids fused with GFP, could be detected in intact tissues after delivering the constructs into Arabidopsis leaves by particle-gun bombardment. The observed signals in the construct containing the predicted N-terminal transit peptide of PGDH from Arabidopsis were observed as green fluorescence that lighted up the chloroplasts (data not shown). They were similar to those exhibited by GFP fused with the transit peptide of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit polypeptide of Arabidopsis (34), which was already known to be sufficient for translocation of a passenger protein to chloroplasts (33). Despite a fainter degree of signals exhibited by CPGDH-GFP compared with the positive control, the fluorescent pattern observed for CPGDH-GFP was clearly distinct from those exhibited by mitochondrial and cytosolic proteins. These results confirmed that the N-terminal sequence of Arabidopsis PGDH is sufficient for translocation of passenger protein into chloroplasts, and thus, PGDH is a plastidic protein.

**Arabidopsis PGDH Can Functionally Complement E. coli serA**—The identity of the isolated cDNA clone CPGDH-5 was confirmed by successful complementation of the E. coli serine auxotroph y536 (HfrOR11 glu V42 SerA13 T3") (1) (Fig. 6). Mutant E. coli cells were transformed with the expression plasmid pPGDH-AB14, in which the expression of PGDH is regulated by the lacZ promoter. Transformants could grow on M9 minimal medium in the absence of Ser, whereas pTV118N-transformed E. coli y536 cells were not able to grow without supplementation of Ser, indicating the authenticity of CPGDH-5 encoding the functional PGDH. The shortened cDNA with 95 amino acids truncated at the N terminus was not able to complement the E. coli y536 mutant. This failure of complementation could be due to deletion of amino acid residues conserved among PGDHs from different organisms and thus necessary for functional expression.

**Fig. 5. Southern blot analysis of genomic DNA.** Genomic DNA was extracted from the leaves of 3-week-old seedlings. For every lane, ~5 µg of genomic DNA was digested with restriction enzyme, separated by electrophoresis through a 0.8% (w/v) agarose gel, transferred to a Hybond N+ membrane, and then hybridized with 32P-labeled CPGDH-5. The final washing was performed in 0.1× SSPE and 0.1% SDS at 65 °C for 10 min. kb, kilobase pairs.

**Fig. 4. Structure of PGDH gene.** A, partial restriction map of the PGDH genomic region. Open bars indicate 5'- and 3'-untranslated regions. Solid bars indicate the open reading frame. The transcriptional start point (TSP) and the translational initiation (ATG) and termination (TAA) codons are indicated by bars, arrows, and open bars, respectively. The sequence of the PGDH gene at the promoter region is shown with solid bars (coding regions) and open bars (untranslated regions) indicating the open reading frame. The transcriptional start site of the genomic clone was determined using primer extension analysis. A single major transcriptional start point (+1) was confirmed to be located 38 bp before the translational start codon (ATG) is double-underlined. The sequence of the cDNA clone CPGDH-5 is underlined.
Biochemical Properties of Recombinant Arabidopsis PGDH Produced in E. coli—Recombinant PGDH was overproduced in E. coli AD494 cells using a pET32a(+) vector system with a strong promoter. The recombinant protein was visualized on SDS-polyacrylamide gel as the expected 90-kDa protein in the insoluble fraction of crude extract as an inactive form. However, production of PGDH in the soluble fraction was too low to be visualized by SDS-polyacrylamide gel electrophoresis. Nevertheless, the soluble form of the protein exhibited PGDH activity of 0.14 ± 0.01 units/mg of protein in the physiological direction, catalyzing the oxidation of 3-PGA to phosphohydroxypyruvate, and of 10.95 ± 1.36 units/mg of protein in the opposite direction, reducing phosphohydroxypyruvate to 3-PGA. Enzyme activity was not detected in the cells transformed by the cDNA in the antisense orientation relative to the promoter. The construct of an insert with a 95-amino acid truncation at the N terminus could not be overexpressed in E. coli AD494 cells.

Double-reciprocal plots of the data for the initial rates demonstrated $K_m$ values of 0.35 and 0.12 mM for phosphohydroxypyruvate and NADH, respectively, at pH 7.1. The activity was inhibited by phosphohydroxypyruvate (−90 μM), as reported for the rat enzyme (6). This inhibition could be released by 100–400 mM KCl. $K_m$ values for 3-PGA and NAD$^+$ were 1.19 and 0.01 mM, respectively, at pH 9.0. Ser, Thr, Val, Gly, Trp, $O$-acetyl-L-Ser, and Cys (in the range of 5–50 mM) had no effect on the reaction rates in both orientations.

**Preferential PGDH Expression in Root Tissues**—The mRNA abundance of PGDH was examined in leaf and root tissues from both light-grown and dark-treated plants. The highest level of PGDH mRNA expression was observed in light-grown root tissues (Fig. 7A). It was 2–3-fold higher than the mRNA expression in dark-grown root and leaf tissues. A minor amount of mRNA expression (−1:15 of roots in light) was detected in the light-grown leaf tissues. The preferential expression of PGDH in root tissues of light-grown plants was in contrast with the expression pattern exhibited by H-protein (a component protein of GDC) (13) and serine hydroxymethyltransferase (18, 19), which accumulated primarily in the light-grown leaf tissues (Fig. 7, B and C). These RNA blot analyses suggested that the regulation of the PGDH gene is mainly exerted at the level of transcription or by stability of mRNA.

**DISCUSSION**

This is the first investigation on the molecular characterization of plant PGDH, a key enzyme committed to the entry step of the phosphorylated pathway of Ser biosynthesis. The isolated cDNA contains an open reading frame encoding the entire PGDH polypeptide of Arabidopsis. The deduced protein with a molecular mass of 66,453 Da, sharing the three-dimensional structure of the E. coli enzyme (22), is composed of three distinct domains: a nucleotide-binding domain, a substrate-binding domain, and a regulatory domain or a Ser-binding domain in each subunit of the tetrameric protein of E. coli PGDH. The main contact points between the subunits are at the level of the coenzyme-binding domains and the regulatory domains, indicating the importance of these zones for tetramerization. The deduced amino acid sequence has high similarity to eukaryotes (human and rat), but not yeast. Surprisingly, the nucleotide- and substrate-binding domains of B. subtilis PGDH exhibit more similarity to the eukaryotic enzymes than to other bacterial PGDH enzymes (E. coli and Haemophilus influenzae), whereas the yeast enzyme is closer to the latter. This suggests that there are two different types of PGDH that may have evolved at its origin before diverging to eukaryotes and prokaryotes. Three of the most common regions in the NAD-dependent 2-hydroxyacid dehydrogenase family are conserved in Arabidopsis PGDH.
Alignment of the *Arabidopsis* PGDH sequence with bacterial, yeast, and mammalian sequences reveals the presence of a presequence, presumably targeting the nuclear encoded protein to the chloroplasts/plastids. The essential common features of the chloroplast presequence are exhibited by the first 60 deduced amino acid residues at the N terminus. The exact cleavage site of the transit peptide was difficult to determine. Based on the multiple alignment results (Fig. 2) showing a low homology between various organisms at its first 80 amino acids, together with our complementation experiment and attempts to overexpress PGDH protein with its 95 amino acids truncated at the N terminus, the cleavage site is most probably located between 80 and 90 amino acids away from the N terminus. Even if the protein is actually processed after entering the chloroplasts, the kinetic properties may not change much. From our recent results with phosphoserine aminotransferase (38), the full-length proteins and the proteins with the transit peptide truncated exhibited essentially the same properties.

We have provided evidence for visualization of the targeting of the fusion protein of the N-terminal transit peptide and GFP to leaf chloroplasts. The weaker signals shown by CPGDH-GFP truncate at the N terminus, the cleavage site is most probably located between 80 and 90 amino acids away from the N terminus. Even if the protein is actually processed after entering the chloroplasts, the kinetic properties may not change much. From our recent results with phosphoserine aminotransferase (38), the full-length proteins and the proteins with the transit peptide truncated exhibited essentially the same properties.

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