Redox Regulation of Arabidopsis 3-Deoxy-D-arabino-
Heptulosonate 7-Phosphate Synthase

Robert Entus, Michael Poling, and Klaus M. Herrmann*
Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

The cDNA for 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase of Arabidopsis encodes a polypeptide with an amino-terminal signal sequence for plastid import. A cDNA fragment encoding the processed form of the enzyme was expressed in Escherichia coli. The resulting protein was purified to electrophoretic homogeneity. The enzyme requires Mn²⁺ and reduced thioredoxin (TRX) for activity. Spinach (Spinacia oleracea) TRX f has an apparent dissociation constant for the enzyme of about 0.2 μM. The corresponding constant for TRX m is orders of magnitude higher. In the absence of TRX, dithiothreitol partially activates the enzyme. Upon alkylation of the enzyme with iodoacetamide, the dependence on a reducing agent is lost. These results indicate that the first enzyme in the shikimate pathway of Arabidopsis appears to be regulated by the ferredoxin/TRX redox control of the chloroplast.

The shikimate pathway is a series of enzyme-catalyzed reactions leading to chorismate, the precursor of Phe, Tyr, Trp, and numerous secondary metabolites derived from these aromatic amino acids. The first enzyme of this pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), yielding DAHP and inorganic phosphate. The pathway is found only in microorganisms and plants (Herrmann and Weaver, 1999).

In bacteria, carbon flow into the pathway is regulated by transcriptional control and by feedback inhibition of DAHP synthase, both mediated by the aromatic amino acids; in vivo, feedback inhibition is the major form of control (Ogino et al., 1982).

In plants, DAHP synthase activity is regulated somewhat differently, which may not be surprising, because there is only about 20% sequence identity between the bacterial and the plant enzyme. Plants frequently must provide additional chorismate for the synthesis of aromatic secondary metabolites, for example, when mechanically wounded or attacked by insects. Under those conditions, mRNA encoding DAHP synthase increases within hours followed by a rise in enzyme activity (Dyer et al., 1989). Detailed northern-blot analyses showed that the synthesis of all the enzymes in the shikimate pathway are transcriptionally regulated (Bischoff et al., 1996, 2001). However, despite extensive experimentation, feedback inhibition of plant DAHP synthase by the aromatic amino acids has never been observed.

1 This is journal paper no. 16,461 of the Purdue University Agricultural Experiment Station.
* Corresponding author; e-mail herrmann@purdue.edu; fax 765–494–7897.

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ondary metabolites derived from Phe, Tyr, and Trp is apparently redox regulated by the Fd/TRX system.

RESULTS

Purification of Arabidopsis DAHP Synthase

The Arabidopsis gene DAHP synthase isoenzyme 1 (DHS1; Keith et al., 1991) encodes a DAHP synthase precursor that contains a putative amino-terminal sequence characteristic for plastid import (Gavel and von Heijne, 1990). The sequence is removed during the import of the polypeptide into isolated chloroplasts (Zhao, 1992). We cloned a partial cDNA sequence encoding the mature form of the enzyme into a pET vector and transformed Escherichia coli for heterologous expression of the plant protein. Induction of the resulting bacteria by isopropyl β-d-thiogalactoside yielded cell extracts, in which up to 5% of the soluble protein is DAHP synthase. In transformed E. coli strains grown in Luria broth, the bacterial DAHP synthase is repressed, and only the plant enzyme is detected. Moreover, bacterial and plant DAHP synthases are distinguishable, because the former enzyme is feedback inhibited by aromatic amino acids, and the two types of enzyme do not cross-react with heterologous antibodies raised against either the bacterial or the plant enzyme, nor do they copurify.

In Arabidopsis, DHS1 is preferentially expressed upon demand for increased carbon flow into the shikimate pathway (Keith et al., 1991; Zhao, 1992). Therefore, we have analyzed the encoded protein in some detail. From extracts of transformed E. coli cells, using standard techniques, we purified Arabidopsis DAHP synthase to apparent electrophoretic homogeneity. Figure 1 shows an SDS polyacrylamide gel of fractions from such a purification. The final product was judged to be better than 95% pure plant DAHP synthase and was not contaminated by the bacterial ortholog, because it was not inhibited by any of the three aromatic amino acids and had a strict requirement for a reducing environment. This preparation was used for all further experiments.

Arabidopsis DAHP Synthase Is a Metallo Enzyme

Like all other known DAHP synthases, the enzyme from Arabidopsis requires a metal ion for activity. The most effective cation is divalent Mn, which has an apparent dissociation constant of less than 10 μM (Fig. 2). Mg\(^{2+}\) can replace Mn\(^{2+}\), but millimolar concentrations are required. We also tested Co\(^{2+}\), Fe\(^{2+}\), and Ca\(^{2+}\). These ions failed to satisfy the metal requirement.

Arabidopsis DAHP Synthase Requires Reduced TRX for Enzyme Activity

To obtain active DAHP synthase enzyme preparations from extracts of either Arabidopsis plants or E. coli expressing Arabidopsis DHS1, extraction buffers must contain reducing agents. The use of extraction buffers without reducing agents or the removal of the reducing agent after extraction yields enzymatically inactive proteins. We prepared pure Arabidopsis DAHP synthase in buffers containing reducing agent,
removed the agent by buffer exchange, and added it back in increasing concentrations.

Figure 3 shows the activation of Arabidopsis DAHP synthase by reduced TRX. We used dithiothreitol (DTT) to reduce TRX. DTT alone partially activates the enzyme as well (Fig. 3B). However, the dissociation constant for TRX to DAHP synthase is 50- to several 100-fold smaller than the corresponding constant of DTT to the enzyme, and maximal enzyme activity is only obtained with reduced TRX (Fig. 3). Plant TRX is a small protein containing two Cys residues that are readily oxidized by air and, in chloroplasts, reduced by Fd/TRX reductase. Spinach chloroplasts contain two isoforms, TRX\(_f\) and TRX\(_m\) (Wolosiuk et al., 1979), originally identified as activators for Fru-1,6-bisphosphate phosphatase and malate dehydrogenase, respectively. Spinach TRX\(_f\) and TRX\(_m\) were obtained in pure form from Dr. Peter Schürmann. Recombinant spinach TRX\(_f\) (Aguilar et al., 1992) activates Arabidopsis DAHP synthase with an apparent dissociation constant of \(K_D = 0.2\) \(\mu\)M (Fig. 3C). This value is close to the one reported for the interaction of TRX\(_f\) with Fru-1,6-bisphosphatase (Aguilar et al., 1992). Recombinant spinach TRX\(_m\) (Schürmann, 1995) at that concentration does not activate Arabidopsis DAHP synthase. Arabidopsis chloroplasts contain two TRX\(_f\) isoforms (Sato et al., 1997). We have not yet shown which of these is the preferred reducing agent for DAHP synthase.

Figure 3. Activation of Arabidopsis DAHP synthase by reducing agents. The relative enzyme activity is plotted as a function of the concentration of the reducing agent in the assay. Before all assays, the purified enzyme was passed over a gel-filtration column equilibrated with a buffer that contained no reducing agent. A, The indicated concentrations of Spirulina sp. TRX were preincubated with 10 mM DTT and enzyme for 15 min at 25°C. The reactions were started by addition of substrates as described in "Materials and Methods." B, Activation of the enzyme by DTT (squares) or \(\beta\)-mercaptoethanol (circles). C, Activation of the enzyme by recombinant spinach TRX\(_f\) (squares) or TRX\(_m\) (circles).

Alkylated Arabidopsis DAHP Synthase Is Enzymatically Active without Reducing Agents

Arabidopsis DAHP synthase preparations stored in the absence of a reducing agent become permanently inactivated. When the enzyme is kept in the absence of a reducing agent for 5 h at 25°C and then assayed in the presence of DTT, only about 50% of the activity is recovered. The inactivation is faster at 37°C and much slower at 0°C. Thus, inactivation is...
time and temperature dependent. Figure 4 shows the data for the time-dependent inactivation at 25°C. Figure 4 also shows that the inactivation is prevented by treating the enzyme with iodoacetamide. Whereas the enzyme cannot be assayed in the presence of the alkylating agent, the enzyme is fully active after the removal of excess iodoacetamide. More importantly, the alkylated DAHP synthase is no longer dependent on a reducing agent for activity. Alkylation of native DAHP synthase requires DTT, a property previously observed for the redox-regulated NADP-malate dehydrogenase (Decottignies et al., 1988).

**DISCUSSION**

During photosynthesis in chloroplast thylakoid membranes, electrons flow from water, via photosystems II and I, to NADP⁺, generating oxygen, ATP, and NADPH + H⁺. ATP and the reducing equivalents are used to drive carbon fixation. The first water-soluble intermediate in the flow of these electrons is Fd. Reduced Fd can donate electrons not only to NADP⁺ but also to TRX, a small protein containing a prominent disulfide bridge that is reduced to two thiols in the process. It is this Fd/TRX oxidation/reduction reaction that imposes light control on a number of critical pathways in the chloroplast. Reduced TRX reduces disulfide bridges in several enzymes, thereby changing the catalytic properties of these proteins. Foremost among the light-regulated polypeptides are several enzymes in the Benson-Calvin cycle and one in the malate-oxaloacetate shuttle, NADP-malate dehydrogenase. These enzymes are inactive in the oxidized form and are readily activated by reduced TRX, which is available only during active electron flow through the photosystems, i.e. exposure to light.

In vitro, all of these regulated enzymes can be activated by reduced TRX and most of them by DTT alone as well. For some of these enzymes, the specific Cys residues involved in this regulatory process have been identified. For example, the TRX f-regulated Fru-1,6-bisphosphate phosphatase has been analyzed by site-directed mutagenesis (Jacquot et al., 1995; Rodriguez-Suarez et al., 1997). Seven conserved Cys residues of the rapeseed (Brassica napus) enzyme were changed to Ser residues. In three of the seven mutant enzymes, sensitivity to DTT was strongly reduced, suggesting the involvement of two cystine bridges in the redox regulation of this enzyme. Although the crystal structure of the oxidized pea Fru-1,6-bisphosphate phosphatase shows the location of only one disulfide bridge, the structure does provide an explanation for the apparently conflicting data of the mutagenesis study (Chiadmi et al., 1999). The TRX m-regulated NADP malate dehydrogenase structure provides a basis for the mechanism of the redox activation for this enzyme (Carr et al., 1999; Johansson et al., 1999).

We show here that the first enzyme of the shikimate pathway should be included in this group of redox-regulated chloroplast enzymes (Fig. 5), specifically into the TRX f-activated anabolic enzymes. Purified Arabidopsis DAHP synthase is activated by reduced TRX. When the reducing agent is removed from reduced enzyme, all enzymatic activity is lost. Furthermore, in the absence of reducing agent, the enzyme apparently undergoes a conformational change in a time- and temperature-dependent manner. For example, we cannot reactivate an enzyme preparation left at 37°C for 1 h without reducing agent.

When Arabidopsis DAHP synthase prepared in the presence of DTT is alkylated with iodoacetamide and, thereafter, the DTT and excess iodoacetamide are removed by buffer exchange, the resulting alkylated enzyme is fully active and no longer requires a reducing agent for enzyme activity. The alkylated enzyme is equally active in the absence or presence of the reducing agent, indicating that the Cys residues involved in the regulation by reduction are not directly required for catalysis.

Comparing the primary structures of redox-regulated enzymes with their unregulated orthologs from other cell compartments reveals additional Cys-containing peptide inserts in the regulated enzymes (Ruelling and Miginiac-Maslow, 1999). However, a consensus structure for such inserts cannot be deduced from the amino acid sequences. Redox-regulated plant DAHP synthases have about 170 more amino acid residues per chain than their prokaryotic homologs that do not require reducing agents for activity. Cys residues in these extra sequences may be involved in generating a redox-regulated enzyme.

Cys residues are also involved in the formation of the active site of DAHP synthase. The bacterial enzyme contains a CXXH motif involved in metal binding, a requirement for enzyme activity (Stephens and Bauerle, 1992). Even though the bacterial and plant

![Figure 5. Biosynthesis of aromatic compounds in chloroplasts. Electrons of photosystem I (P₇₀₀⁺)-reduced Fd can either reduce NADP⁺ or TRX. Reduced TRX activates DAHP synthase, the first enzyme of the shikimate pathway.](Image)
enzymes are only about 20% identical in their primary structures, the CXXH motif is found in both, and the plant enzyme also requires a metal for enzyme activity. Based on the NADP-malate dehydrogenase model (Ruelland and Migniac-Maslow, 1999), one can envision that the Arabidopsis DAHP synthase contains functionally different Cys residues, one that is subject to reduction/oxidation by theFd/TRX regulatory system and one in the metal binding portion of the active site. Our alkylation experiments indicate that the regulatory Cys residue is solvent exposed. The regulatory Cys residue can be alkylated without destroying enzyme activity, and the resulting alkylated enzyme is no longer dependent on a reducing agent for activity. We are in the process of addressing the function of individual Cys residues by site-directed mutagenesis.

That light may regulate DAHP synthase at the genetic (Henstrand et al., 1992) and enzyme level could suggest that light replaced the aromatic amino acids as regulators during evolution. If chloroplasts are of bacterial origin, one can assume that feedback-sensitive DAHP synthases were present in early plant cell organelles. Although today’s chloroplast DAHP synthase has lost the sensitivity to aromatic amino acids, the enzyme still retains a binding site for those amino acids, because the enzyme is slightly activated by Trp (Suzich et al., 1985). However, light may be the main regulator of the plant enzyme. This finding raises a number of interesting questions. For example, is wound repair faster in the light? Or even more basic, do plants synthesize aromatic amino acids only during the day, or are the other DAHP synthase isoenzymes active in the dark? The shikimate pathway is one of the major routes of carbon flow in green plants. It now becomes a challenge to demonstrate experimentally that, in vivo, light regulation of DAHP synthase links carbon flow directly to the generation of fixed carbon by the carbon reactions of photosynthesis through sharing reduced Fd as a trigger for pathway activity.

MATERIALS AND METHODS

Chemicals and Biochemicals

MnCl₂, MgCl₂, isopropyl cleotides were from the Purdue Laboratory for Macromolecular Structure. Vent polymerase was from New England Biolabs (Beverly, MA). Oligonucleotides were gifts from Dr. Peter Schuman and spinach (Spinacia oleracea) (1870 Plant Physiol. Vol. 129, 2002).

Expression of Arabidopsis DHS1 in E. coli

The open reading frame of the Arabidopsis DHS1 cDNA (Keith et al., 1991) encodes a DAHP synthase precursor with a putative signal sequence (Gavel and von Heijne, 1990) that is removed during plastid import. The actual cleavage site has been determined for potato (Solanum tuberosum) DAHP synthase (Zhao, 1992). From sequence alignments, we predicted that the Arabidopsis DAHP synthase cleavage site lies between Thr-48 and Ala-49. We used PCR to synthesize a DNA molecule encoding the mature form of Arabidopsis DAHP synthase beginning at Ala-49. The forward oligonucleotide was 5’-AACCTTCAATTCGCGCCGTCAG-3’, and the reverse oligonucleotide was 5’-ATTACGGTCGACACTAA-GACACACCTGG-3’. The resulting fragment was cloned into the E. coli expression vector pET23d at the Ncol and Sall sites using standard techniques (Ausubel et al., 1987). The correctness of the construct was verified by sequencing both strands of the DNA. Expression of the resulting E. coli BL21(DE3)/pET23dDHS1 yields a DAHP synthase polypeptide with two additional residues (Met-Gly) at the amino terminus. Eight milliliters of an overnight culture of this strain were inoculated into 1 L of Luria broth (10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl, and 1 mL of 1.0 N NaOH) containing 50 mg of ampicillin. The culture was grown at 37°C to an optical density of 0.6 at 600 nm. At that point, isopropyl β-d-thiogalactoside (final concentration 400 μM) was added, and incubation was continued for 4 h.

All operations were carried out at 4°C. The cells were harvested by centrifugation, washed, and resuspended in buffer A (50 mM potassium phosphate, pH 7.4, containing 1 mM PEP, 1.5 mM Trp, and 0.2 mM DTT). All buffers contain PEP, which stabilizes the enzyme, and Trp, which slightly activates plant DAHP synthases (Suzich et al., 1985). The cells were broken in a French pressure cell. The resulting cell extract was clarified by centrifugation (20 min at 15,000 × g). The supernatant was loaded onto a cellulose phosphate column equilibrated with buffer B (25 mM potassium phosphate, pH 7.4, containing the buffer A supplements. Fractions containing enzyme activity were pooled and loaded onto a Sephacryl S-300HR column equilibrated with buffer A. Elution was with buffer A. The final preparation with a specific activity of 15 units mg⁻¹ protein is stable when stored at ~20°C.

Purification of Arabidopsis DHS1

DAHP Synthase Enzyme Assay and Protein Determination

DAHP synthase was assayed (Suzich et al., 1985) by measuring the absorbance of the periodate degradation product of DAHP complexed with thiobarbiturate. The unit of activity is defined as the amount of protein catalyzing the appearance of 1 μmol DAHP min⁻¹. Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

To analyze the metal ion requirements of the enzyme, all buffers and enzyme solutions were treated with Chelex 100 resin. The enzyme was passed through a Sephadex G25 column equilibrated with buffer B (25 mM EPPS, pH 8.4, containing 1 mM PEP, 1.5 mM Trp, and 0.2 mM DTT). The reaction mixture contained 50 mM EPPS, pH 8.6, 5 mM PEP, 2 mM E4P, 10 mM DTT, and suitably diluted enzyme in a total volume of 0.1 mL. The reaction was initiated by adding 50 μl of a solution containing various concentrations of metal ions. Incubation was at 37°C for 10 min. The reaction was stopped by the addition of 0.3 mL of 10% (w/v) trichloroacetic acid.

To analyze the enzyme for reducing agent requirement, the enzyme was passed through a Sephadex G25 column equilibrated with buffer C (25 mM EPPS, pH 8.4, containing 1 mM PEP and 1.5 mM Trp). The enzyme was then preincubated with various concentrations of TRX in 10 mM DTT or various concentrations of DTT alone (total volume of 50 μL), and the assay was started by the addition of 100 μL of reaction mixture containing 50 mM EPPS, pH 8.6, 5 mM PEP, 2 mM E4P, and 0.1 mM MnCl₂.

Chemical Modification of DHS1

The enzyme was treated with iodoacetamide using a procedure developed for arginyl-tRNA synthetase (Liu et al., 1999). DAHP synthase was incubated at room temperature for 30 min in 0.45 mM potassium phosphate, pH 7.4, containing 2 mM MnCl₂, 1.5 mM Trp, 0.2 mM DTT, and 20 mM iodoacetamide. After incubation, excess iodoacetamide was removed by...
buffer exchange into buffer C using a Sephadex G25 column at room temperature.

ACKNOWLEDGMENTS

We thank Dr. Peter Schürmann for generous gifts of recombinant TRX f and m, and Drs. Peter Goldsborough, Mark Hermodson, and Ronald Somerville for critical reading of the manuscript. This paper is dedicated to Dr. Nikolaus Amrhein for his unselfish support of our experimental efforts.

Received January 13, 2002; returned for revision March 22, 2002; accepted April 22, 2002.

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