Feedback Inhibition of Deoxy-D-xylulose 5-phosphate Synthase Regulates the Methyl Erythritol 4-phosphate Pathway*

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Background: The methylerythritol phosphate (MEP) pathway is required for the biosynthesis of plastid derived isoprenoids from plants.

Results: Deoxyxylulose 5-phosphate synthase (DXS) was cloned from Populus trichocarpa and metabolic regulation was tested.

Conclusion: Both isopentenyl diphosphate and dimethylallyl diphosphate inhibit DXS by competing with thiamine diphosphate.

Significance: Prediction of isoprene emission from trees and bioengineering of MEP pathway will be aided by these results.

SUMMARY

The methylerythritol phosphate (MEP) pathway leads to the biosynthesis of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), the precursors for isoprene and higher isoprenoids. Isoprene has significant effects on atmospheric chemistry while other isoprenoids have diverse roles ranging from various biological processes to applications in commercial uses. Understanding the metabolic regulation of the MEP pathway is important considering the huge applications of this pathway. The deoxyxylulose 5-phosphate synthase (DXS) enzyme was cloned from Populus trichocarpa and the recombinant protein (PtDXS) was purified from E. coli. The steady-state kinetic parameters were measured by a coupled enzyme assay. An LC-MS/MS-based assay involving the direct quantification of the end product of the enzymatic reaction, 1-deoxy-D-xylulose 5-phosphate (DXP), was developed. The effect of different metabolites of the MEP pathway on PtDXS activity was tested. PtDXS was inhibited by IDP and DMADP. Both of these metabolites compete with thiamine diphosphate for binding with the enzyme. An atomic structural model of PtDXS in complex with thiamine diphosphate and Mg²⁺ was built by homology modeling and refined by molecular dynamics simulations. The refined structure was used to model the binding of IDP and DMADP and indicated that IDP and DMADP might bind with the enzyme in a manner very similar to the binding of thiamine diphosphate. The feedback inhibition of PtDXS by IDP and DMADP constitutes an important mechanism of metabolic regulation of the MEP pathway and indicates that TPP-dependent enzymes may often be affected by IDP and DMADP.

Isoprenoids, also known as terpenoids, constitute one of the largest groups of metabolites and are widely dispersed in all living organisms including both prokaryotes and eukaryotes (1). They represent the largest group of secondary metabolites having more than 35,000 members including sterols, carotenoids, dolichols, ubiquinones, plastoquinones, prenylated proteins, cytokinins, gibberellic acid, and abscisic acid (1,2). They are involved in various biological processes including photosynthesis, respiration, and regulation of growth and development (3). Isoprenoids also play significant roles in attracting pollinators and seed-dispersers, defense against
different biotic and abiotic stresses, intracellular signal transduction, vesicular transport within the cell, and construction of cellular and organelle membranes (1-5). In addition, some isoprenoids have commercial applications as flavors and fragrances, pigments, polymers, and drugs (6).

Despite the great diversity of structure and function, only two metabolic precursors, isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP) are used as the building blocks for all the isoprenoids (7,8). The well-known acetate/mevalonate (MVA) pathway was thought to be the only biochemical pathway for the synthesis of IDP (8,9). However, incorporation of $^{13}$C labeled precursors into polyterpenoids revealed that an alternative, mevalonate-independent pathway, known as the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway also leads to the biosynthesis of IDP in bacteria (8,10,11). Further studies showed that the MEP pathway is also present in green algae (12) and higher plants (13-18). Both the MVA and MEP pathways are present in plants, however, they are localized in the cytosol and the chloroplast respectively (4,14).

The MEP pathway starts with the synthesis of 1-deoxy-d-xylulose 5-phosphate (DXP) from glyceraldehyde 3-phosphate (GAP) and pyruvate. The reaction is catalyzed by the enzyme called 1-deoxy-d-xylulose 5-phosphate synthase (DXS), which requires a divalent cation, Mg$^{2+}$ or Mn$^{2+}$, and uses thiamine pyrophosphate (TPP) as a cofactor (19-24). In the next step, DXP is converted to the branched compound MEP, the first committed intermediate of this pathway, by 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR) using NADPH as a cofactor and a divalent cation, Mg$^{2+}$ or Mn$^{2+}$ (25). MEP is ultimately converted to a mixture of IDP and DMADP, precursors of all higher isoprenoids (26-29). The isomerization of DMADP and IDP is catalyzed by the enzyme isopentenyl diphosphate isomerase (IDI) (28).

The absence of MEP pathway in humans and its presence in different eubacteria, various apicomplexa parasites, and photosynthetic eukaryotes make it an attractive target for drug discovery and herbicides (7,20,30). Some isoprenoids also serve as important targets for biotechnological applications due to their nutritional and medicinal benefits (7,31,32).

Isoprene, the most abundantly produced isoprenoid in plants, significantly affects atmospheric chemistry (33). In this regard, a mechanistic model predicting isoprene emission from plants will be of great importance in atmospheric chemistry. A mechanistic model requires a deep understanding of the regulation of the MEP pathway.

Gene expression studies have demonstrated a potential regulatory role for DXS in the synthesis of DMADP/IDP (7) but metabolic regulation of MEP pathway is not yet understood completely. Wolfertz and coworkers used deuterium-labeled deoxyxylulose 5-phosphate (DOX-d2) to show that the carbon flux through the MEP pathway is under strong metabolic regulation (34,35). They suggested that a feedback inhibition of DXS enzyme by the metabolites of this pathway downstream of DXP, especially DMADP, plays a critical role in this regulation. However, no direct evidence has been shown so far that can explain the tight control of the carbon flux through the MEP pathway.

In this paper, we describe the cloning, and characterization of the recombinant DXS (PtDXS) protein from Populus trichocarpa. We also report here a rapid and convenient high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) based assay for PtDXS. This assay was used to study the activity of PtDXS in presence of different metabolites of the MEP pathway. Our results show that IDP and DMADP, the last metabolites of the MEP pathway significantly inhibit PtDXS by competing with TPP. Computational analysis shows that both of these metabolites bind with the enzyme in a manner similar to that of TPP. The inhibition of PtDXS by IDP and DMADP constitutes an important regulatory mechanism of the MEP pathway where the very last metabolite of the pathway regulates the activity of the very first enzyme of the pathway.

**EXPERIMENTAL PROCEDURES**

**Cloning.** The cDNA encoding the mature PtDXS protein from Populus trichocarpa was amplified by PCR using the primers 5’-GAA TTC CAT ATG GCA TCA CTA TCA GAA AGA GGA GAG-3’ and 5’-CG GGA TCC TTA TGA TGA CAT AAT CTC CAG AGC-3’. The PCR product was digested with the restriction
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enzyme BamHI to completion followed by a partial digestion with the restriction NdeI, as the coding DNA of PrDXS contained an NdeI site. The partially digested PCR product was ligated with a lab-made vector pET17b-HR digested with the same two restriction enzymes. The ligation mixture was transformed into the E. coli strain DH5α. The clones that contained the complete cDNA were selected by agarose gel electrophoresis of the isolated DNA constructs. The resultant overexpression plasmid construct was designated as pET17bHR/PrDXS for the production of PrDXS with a TEV protease-cleavable six-His tag at the N-terminus. An overexpression plasmid construct for the production of PrDXS with a TEV protease-cleavable ten-His tag at the N-terminus was engineered by PCR-based site-directed mutagenesis using pET17bHR/PrDXS as the template. The mutagenesis primers were 5'-G CAT CAC CAT CAC CAT CAC CAT CAC CAC CAT AGC GGT ACC GAG AAC CTG TAC TTC CAG GGT ACC GCT ATG GTG ATG GTG ATG GTG ATG GTG ATG GTG ATG C-3' and 5'-GAA GTA CAG GGT ACC GCT ATG GTG ATG GTG ATG GTG ATG GTG ATG C-3'. The resulting construct was designated as pET17b10HR/PrDXS.

An overexpression plasmid construct for the production of PrDXS with a TEV protease-cleavable six-His tag at both the N- and the C-terminus was engineered in two steps. First, the overexpression construct pET17bHR/PrDXS was digested with BamHI and EcoRI and ligated with a synthetic duplex DNA consisting of the oligos 5'-GA TCC GAG ATT ATG TCA TCA TCA GGA TCC GAG AAC CTC TAC TTC CAG GGT ACC CAC CAT CAC CAT CAC CAC TAA-3' and 5'-AA TT TTA GTG GTG GTG GTG GTG GTG GTG ACC CTG GAA GTA CAG GTT CTC GTC CTC G-3'. The clones with the inserted DNA were selected by EcoRI digestion, as the insertion destroyed the EcoRI site. Then the stop codon at the end of the PrDXS-coding sequence was converted to Ser by PCR-based site-directed mutagenesis using the primers 5'-CTG GAG ATT ATG TCA TCA TCA GGA TCC GAG AAC CTC TAC-3' and 5'-GTA CAG GTT CTC GGA TCC TGA TGA TGA CAT AAT CTC CAG -3'. The resultant DNA construct was designated as pET17bHR3'HR/PrDXS. The N-terminal His tag and TEV protease site was removed by PCR-based site-directed mutagenesis using pET17bHR3'HR/PrDXS as the template. The two primers for the mutagenesis were 5'-CT TTA AGA AGG AGA TAT ACC ATG GCA TCA CTA TCA GAA AGA GGA GAG-3' and 5'-CTC TCC TCT TCT TGA TAG TGA TGC CAT GGT ATA TCT CCT TCT TAA AG -3'. The resultant DNA construct was designated as pET17b3'HR/PrDXS. For all the plasmid constructs, the presence of the correct PrDXS coding sequence, and the absence of any undesired mutation, was confirmed by DNA sequencing.

Overexpression and Purification. The E. coli strain BL21(DE3)pLysS was used to overexpress the various forms of PrDXS. A liter of LB medium containing 100 µg/ml of ampicillin and 20 µg/ml of chloramphenicol was inoculated with colonies of fresh transformants of an overexpression construct and incubated at 37 °C with vigorous shaking (225 rpm) until OD600 reached 1. The culture was then cooled with ice to room temperature, induced with 0.5 mM IPTG (final concentration), and further incubated with vigorous shaking at room temperature for ~18 h. The E. coli cells were harvested by centrifugation and resuspended in 10 mL cold buffer A (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH8.0) per gram of bacterial paste. MgCl2 was added to a final concentration of 20 mM followed by the addition of DNase I and EDTA-free inhibitors. The cells were lysed with a French press. The cell debris was removed by centrifugation at ~27,000 x g for 20 min. Ammonium sulfate was added to the supernatant in small quantities to 45% saturation under gentle stirring. After 30 min, the suspension was centrifuged at ~27,000 x g for 20 min. The pellet was redissolved in the same volume of cold buffer A. The solution was centrifuged and the ammonium sulfate precipitation was repeated with the supernatant. The pellet of the second ammonium sulfate precipitation was redissolved in cold buffer A and the solution was centrifuged. The supernatant was dialyzed against 2 L of the same buffer for 3 h. The dialyzed protein solution was mixed with Ni-NTA resin with gentle shaking for 1 h and loaded onto a column. The column was washed with 10 mM imidazole in buffer A until OD280 of the effluent < 0.05 and eluted with a 10-250 mM linear imidazole gradient in buffer A. Fractions containing PrDXS were identified by SDS-PAGE and pooled. PrDXS was precipitated with ammonium sulfate (45% saturation) and the
pellet redissolved in a minimal volume of a cold buffer containing 50 mM Tris-HCl, 10% glycerol, and 1 mM DTT (pH 7.5). The protein solution was dialyzed against 1 L of the same buffer and centrifuged at ~27,000 x g for 20 min. The supernatant was dispensed into microtubes, frozen in liquid nitrogen, and stored at -80 °C. All protein purification procedures were carried out at 4 °C unless specified otherwise.

**Coupled enzyme assay.** The steady-state kinetic constants of PtDXS were measured using a DXR-coupled assay. The assay components in a buffer containing 100 mM HEPES (pH 7.5) included pyruvate, GAP, 5 mM MgCl₂, 1 mM TPP, 50 mM NADPH, 4 µM recombinant *Acinetobacter baumannii* DXR (AbDXR, lab made), and 0.5 µM PtDXS. The reaction was initiated by the addition of GAP at room temperature. For measuring the $K_m$ of GAP at room temperature. For measuring the $K_m$ of pyruvate, GAP was fixed at 0.2 or 0.5 mM and pyruvate varied in the range of 0.05 - 1.2 mM. For measuring the $K_m$ of GAP, pyruvate was fixed at 2 or 5 mM and GAP varied in the range of 10 - 175 µM. The kinetic constants were evaluated by nonlinear least squares fitting of the data to the Michaelis-Menten equation using the program Origin (http://www.originlab.com/).

**Enzymatic synthesis of DXP and $^{13}$C₂-DXP.** DXP/$^{13}$C₂-DXP were synthesized enzymatically from pyruvate/2,3- $^{13}$C₂ pyruvate and GAP (produced in situ) using *E. coli* DXS (EcDXS) as described in (36) with some modifications. The reaction mixture was prepared by dissolving D-fructose 1,6-bisphosphate (406 mg, 25 mM) and pyruvate (or 2,3-$^{13}$C₂ pyruvate) (~220 mg, 50 mM) in ~39 mL of 50 mM Tris-HCl, pH = 7.5 with 1 mM DTT, 5 mM MgCl₂, and 0.5 mM TPP. The reaction mixture also contained recombinant *Staphylococcus aureus* fructose bisphosphate aldase (SaFBP aldolase) (2.4 µM, lab made), yeast triose phosphate isomerase (TPI) (0.04 µM, lab made), and EcDXS (1.5 µM, lab made). The reaction was then carried out at 37 °C for ~24 h. The enzymes were removed from the reaction mixture by ultrafiltration through YM10 (Millipore) membrane. The filtrate was then loaded on Dowex 1 x 8 column (40 mL, chloride form), which was equilibrated with water. The column was washed with 100 mL of water after collecting the flow-through. DXP was then eluted from the column with 100 mL of 1% NaCl solution. The fractions containing DXP were lyophilized to obtain solid DXP. The solid was then dissolved in ~3 mL of water. The solution was desalted with a Sephadex G10 column by eluting with water. The concentration of the pure DXP solution was obtained from NMR.

**Preparation of PtDXS assay mixture for LC-MS/MS based assay.** The activity of the purified PtDXS enzyme was studied using an LC-MS/MS-based assay. A mixture of dihydroxyacetone phosphate (DHAP) and TPI from rabbit muscle was used to maintain a constant supply of GAP in the reaction mixture. The ratio of equilibrium concentration of DHAP and GAP at the temperature of the reaction mixture (37 °C) was calculated to be 18:1. The assay mixture contained 40 mM Tris-HCl buffer at pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 100 µM TPP, 1 U/mL rabbit muscle TPI, and 0.25 µM PtDXS in a total volume of 100 µL. The reaction was initiated with a mixture of DHAP and pyruvate. The concentrations used to study the $K_m$ of DHAP and pyruvate were 0 - 197 µM of DHAP in presence of 5 mM pyruvate and 0 - 1 mM pyruvate in presence of 260 µM of DHAP respectively. The concentrations used to study the $K_m$ of TPP were 0 - 1 mM of TPP in presence of 260 µM of DHAP and 500 µM pyruvate. The reaction was incubated at 37 °C for 5 min. It was then quenched by freezing in liquid nitrogen followed by the addition of 400 µL of ice-cold acetoniitile keeping the frozen reaction mixture on dry ice. Then the reaction mixture was thawed on ice followed by the addition of 2 µM of $^{13}$C₂-DXP as an internal standard (IS) for the mass spectrometry. The assay mixture was then centrifuged at 28,000 x g for 10 min and the supernatant was stored at -80 °C until further analysis.

**Inhibition studies.** Different metabolites of the MEP pathway, namely 2-C-methyl-D-erythritol 4-phosphate (MEP), 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDPME), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP), 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP), IDP and DMADP were tested to study their effect on PtDXS activity. All the metabolites except IDP and DMADP were purchased from...
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Echelon Biosciences Inc. (Salt Lake City, UT, USA). IDP and DMADP were purchased from Isoprenoids, LC (Tampa, FL, USA). The assay mixture was prepared as mentioned before with 400 µM of the metabolites. The reaction was initiated with a mixture of DHAP (210 µM) and pyruvate (200 µM). Similarly, the assay mixtures for obtaining the Michaelis-Menten plot at different inhibitor concentrations were prepared as mentioned before at different TPP concentrations in the presence of 0 µM, 100 µM, and 1000 µM of IDP keeping the concentration of DHAP and pyruvate fixed at 210 µM and 200 µM respectively. The reaction was initiated with a mixture of DHAP and pyruvate. In order to calculate the $K_i$ of DMADP and IDP, the assay was done in presence of 25 µM TPP (~$K_m$ for TPP) and 0 - 3 mM of the metabolites. The reaction was initiated with a mixture of DHAP (210 µM) and pyruvate (200 µM). The assay was then carried out as described before.

**LC-MS/MS of the PtDXS assay mixture.** The assay mixture was analyzed by LC-MS/MS to separate the product DXP from the substrate DHAP. Liquid chromatography was performed using Merck SeQuant™ ZIC®-pHILIC (50 x 2.1 mm, 5 µm, 200 Å, polymeric beads PEEK) column (The Nest Group, Inc., MA, USA) fitted to two LC-20AD HPLC pumps and a SIL-HTc autosampler (Shimadzu, Kyoto, Japan) as described in (37). The rest of the instrumental setup for the mass spectrometer coupled to this chromatography system was done as described in (37). The assay mixture was filtered through Whatman™ syringeless filter device (Mini-Uniprep™ PTFE filter media) and 5 µL of the sample was injected into the column. The analyte was eluted with a binary gradient consisting of 50 mM ammonium acetate at pH 10 and acetonitrile (composition as shown in the inset of Fig. 1) at a flow rate of 0.15 mL/min. Mass spectrometry was performed as described in (37). Multiple-reaction-monitoring (MRM) mode was used to acquire the precursor/product ion pairs for DXP, $^{13}$C$_2$-DXP, and DHAP. The mass pairs used for scanning these compounds were 213/97, 215/97, and 169/97 for DXP, $^{13}$C$_2$-DXP, and DHAP respectively. The optimized declustering potential used for acquiring the mass spectra was -30V for DXP and $^{13}$C$_2$-DXP, and -15V for DHAP.

**Quantification and data analysis.** In order to obtain better quantification of the analytes, an IS was used in all the samples to allow a correction for the ionization efficiency. Standard DXP samples containing 2 µM of $^{13}$C$_2$-DXP as an IS were run to obtain a calibration curve. The calibration curve was used to quantify the amount of DXP produced in the assay samples. After correction for the dilution factor, the amount of DXP produced in the reaction mixture was used to calculate the specific activity of the PtDXS enzyme. The kinetic constants of PtDXS enzyme for different substrates and the IC$_{50}$ curves for IDP and DMADP were obtained by fitting the experimental data with non-linear regression using the program Origin (http://www.originlab.com/). The kinetic constants were evaluated using Michaelis-Menten equation. Calculation of the IC$_{50}$ values was done using a logistic equation and described as (38)

$$v = v_{\text{min}} + \frac{v_{\text{max}} - v_{\text{min}}}{1 + \left(\frac{[I]}{IC_{50}}\right)^H}$$

where, $v$ is the percent activity, $v_{\text{min}}$ is the minimum percent activity, $v_{\text{max}}$ is the maximum percent activity, $[I]$ is the concentration of the inhibitor, and $H$ is the Hill coefficient. The $K_i$ of the inhibitors were calculated using Cheng-Prusoff equation (39) as described below.

$$K_i = \frac{IC_{50}}{1 + \left(\frac{[S]}{K_m}\right)^H}$$

The IC$_{50}$ curves were obtained at $K_m$ concentration of TPP. At $[S] = K_m$, $K_i$ is calculated to be $\frac{IC_{50}}{2}$.

**Computational modeling.** A structural model of PtDXS was first built by homology modeling using the SWISS-MODEL server (http://swissmodel.expasy.org/) with the crystal structure of Deinococcus radiodurans DXS.
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(DrDXS; PDB ID: 2O1X) (40) as the template. The crystal structure of DrDXS, not that of *E. coli* DXS (EcDXS; PDB ID: 2O1S), was chosen as the template for the homology modeling because DrDXS has only one segment (residues 199–243) with no electron density whereas EcDXS has two segments (residues 183–238 and 292–317) with no electron density (40). Each monomer of the modeled homodimeric enzyme contains one Mg^{2+} ion and one coenzyme TPP. The Mg^{2+} ion is coordinated with four oxygen atoms, two from pyrophosphate of TPP and one each from Asp145 and Asn174. Two water molecules were placed near each Mg^{2+} ion based on the crystal structure of EcDXS so that the Mg^{2+} ion is coordinated with six oxygen atoms. The structural model was then refined by molecular dynamics using the AMBER program package (version 10) (41). The modeled homodimeric protein was solvated with ~32,900 TIP3P water molecules in a rectangular box with the edges at least 12 Å from the protein. The system was neutralized using 12 Na^+ ions. Glu377 was considered protonated as a hydrogen donor to form a hydrogen bond with N1 of the pyrimidine ring of TPP as in the crystal structures of EcDXS and DrDXS (40). Water molecules in the system were minimized first using a combination of steepest descent (15,000 steps) and conjugated gradient (5,000 steps) methods with protein and ligand heavy atoms restrained with a force constant of 100 kcal mol^{-1} Å^{-2}. The whole system was then minimized using a combination of steepest descent (5,000 steps) and conjugated gradient (5,000) methods without any positional restraint for any atoms except those interacting with the Mg^{2+} ions and forming the hydrogen bond between TPP and Glu377. The restraints for the Mg^{2+} coordination and the hydrogen bond between TPP and Glu377 were also enforced during the subsequent heating and equilibration steps. The minimized system was heated from 0 to 300 K in 500,000 steps in 1 ns at constant volume and equilibrated at 300K and constant pressure for 4 ns. The system was further simulated without any distance restraint for 1.5 ns. The minimization, heating and equilibration simulations were carried out using the Sander module in AMBER 10 (41) with the ff99SB force field.

The force field parameters for the coenzyme TPP were derived using the AMBER antechamber program (41). The PMEMD module in AMBER 10 (41) was used for the subsequent production simulation. The Particle-Mesh-Ewald method (42) was used to evaluate long-range electrostatic interactions. The nonbonded cutoff was 10 Å. All bonds to hydrogen atoms were constrained in the simulations with the SHAKE algorithm (43) permitting a time step of 2 fs. Temperature was controlled with Langevin dynamics. The data of the 1.5 ns simulation were analyzed using the PTRAJ module in the program AmberTools (41). The refined structural model of PrDXS in complex with TPP and Mg^{2+} was used to dock IDP and DMADP compounds. TPP was mutated to IDP and DMADP using the xleap program in AmberTools (41). The force field parameters for IDP and DMADP were derived using the same procedure as for the coenzyme TPP. The models of the inhibitor complexes were neutralized with Na^+ ions, solvated with explicit water molecules, and minimized using essentially the same procedure as for the TPP complex. All structural illustrations were drawn with the program PyMOL (Schrödinger, LLC; http://www.pymol.org/).

RESULTS

Cloning, overexpression, and purification. To produce PrDXS in *E. coli*, the cDNA encoding the matured PrDXS was cloned into a home-made overexpression vector derived from the commercial vector pET17b from Novagen, under the control of a IPTG-inducible T7 promoter. A large quantity of PrDXS can be produced using this overexpression construct in the *E. coli* strain BL21(DE3)pLysS at 37 °C (not shown) but more soluble PrDXS protein could be obtained at room temperature (data not shown). The recombinant protein contained a six-histidine tag at its N-terminus to facilitate its purification using Ni-NTA resin but the His-tag did not help the purification, as the majority of the protein molecules did not bind to Ni-NTA resin. To address this issue, the His-tag was extended to 10 histidine residues, but the longer His-tag did not improve the binding. Then a six-histidine tag was engineered at the C-terminus of the protein and the N-terminal His-tag was removed as it did not help the purification. The C-terminal His-tag helped the purification of the protein. However, the purified protein could be easily degraded. Ammonium sulfate precipitation effectively removed protease contamination. Two
steps of ammonium sulfate precipitation before the Ni-NTA chromatography and one step of ammonium sulfate precipitation after the Ni-NTA chromatography yielded a stable protein preparation. The average yield for the purified protein was ~14 mg/L of *E. coli* culture.

**Steady-state kinetic analysis.** Steady-state kinetic parameters of the recombinant *PtDXS* were measured by a DXR-coupled enzyme assay. *AbDXR* was used as a coupling enzyme in the assay as this enzyme is stable. The assay was validated by varying the concentrations of *PtDXS* and *AbDXR*. *AbDXR* at 4 µM was deemed sufficient as the reaction rate doubled as the *PtDXS* concentration doubled and increasing the *AbDXR* concentration further did not increase the reaction. The kinetic data are summarized in Table 1. The *K_m* values for pyruvate and GAP (87.8 and 18.5 µM, respectively) were both higher than those of *Mycobacterium tuberculosis* DXS (40 and 6.1 µM, respectively) (44) but significantly smaller than those of *Rhodobacter capsulatus* DXS (440 and 68 µM, respectively) (45).

**Development of LC-MS/MS based assay for *PtDXS* enzyme.** The effect of different MEP pathway metabolites on the activity of *PtDXS* enzyme was studied by measuring the amount of DXP produced by the *in vitro* reaction of the enzyme in presence of those metabolites. The chromatogram of the assay mixture is shown in Fig. 1. Manual addition of substrates to the assay mixture followed by its quenching in liquid nitrogen involves a time lag. This leads to the production of small amount of DXP at 0 min. Study of the time course of the *PtDXS* enzymatic reaction showed that it was linear for the initial 10 minutes (data not shown). Samples were collected at 5 minutes to calculate the specific activity of the enzyme. Table 1 shows the kinetic constants of *PtDXS* enzyme for different substrates measured by this method.

**Effect of pH on *PtDXS* activity.** The activity of the *PtDXS* enzyme was monitored at different pH using bis-tris propane buffer. The useful pH range for this buffer is 6.3-9.5. The activity of the enzyme was found to be highly sensitive to the pH of the assay mixture (Fig. 2). The enzyme does not show substantial activity below pH 6.5 and above pH 8.5. The highest activity of this enzyme is obtained at pH 8.0. This is typical for a chloroplastic enzyme.

**Effect of different metabolites of the MEP pathway using LC-MS/MS method.** DMADP was selected for testing based on previous suggestions of its role in metabolic regulation of the MEP pathway (34,35). The rest of the intermediates of the MEP pathway were screened for potential effects on *PtDXS* activity (Fig. 3A). The intermediate 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol 2-phosphate (CDPMEP) could not be tested due to its instability. Fig. 3B shows the effect of MEP on *PtDXS* activity. Because the molecular weight of MEP is the same as the internal standard (13C2-DXP) used for the LC-MS/MS based assay, we could not normalize the data for MEP with respect to the internal standard. We compared the effect of MEP relative to the control instead of using absolute values since the ion suppression effect could not be eliminated in this case. ANOVA followed by Bonferroni posttest indicated that IDP alone showed statistically significant inhibition (p = 0.0036). HMBDP also showed some effect though not statistically significant in this experiment. Li and coworkers found that the physiological concentration of HMBDP under normal conditions is very low (~4.2 µM) (37). Therefore, we believe that HMBDP plays little or no role in feedback within the MEP pathway.

**Mechanism of inhibition.** The next goal was to study the mode of inhibition of IDP on *PtDXS* enzyme. The effect of each of the substrates on the inhibition of IDP on *PtDXS* enzyme was tested. In each case, the concentration of a particular substrate was doubled as compared to the control, keeping the concentration of the inhibitor (400 µM) same as that in the control. It was observed that the extent of inhibition was reduced in presence of higher concentration of TPP (Fig. 4). Use of higher concentration of pyruvate and DHAP did not affect the inhibition significantly. A two way ANOVA followed by Bonferroni posttest indicated that doubling the concentration of each metabolite had no effect on the rate in the absence of inhibitor and the inhibition by IDP is significantly reduced (p < 0.01) only in the presence of twice the amount of TPP. This
suggests that IDP acts as a competitive inhibitor of TPP. The inhibitory effect of IDP on PrDXS enzyme was tested by varying the concentration of IDP in presence of different concentration of TPP and fixed concentration of pyruvate and DHAP. The Michaelis-Menten plot of PrDXS activity at different TPP concentration showed that the activity was decreased in presence of higher concentration of IDP (Fig. 5). This also indicates that IDP acts as a competitive inhibitor of TPP. Regression of the experimental data points was done using the method of least squares. The Michaelis-Menten plot for the activity of the enzyme at different substrate concentration with varying inhibitor concentration could not be fitted well by assuming the equation for standard competitive inhibition kinetics. A better curve-fitting was obtained by incorporating a Hill coefficient in the rate equation (Fig. 5). The $H$ value obtained from the least squares regression was 0.68.

The activity of PrDXS enzyme was studied over a broad range of concentration of IDP and DMADP in presence of ~$K_m$ concentration of TPP (Fig. 6). The $K_i$ of IDP and DMADP for PrDXS enzyme were found to be 65.4 ± 4.3 µM and 81.3 ± 10.5 µM respectively. The IC$_{50}$ curves of IDP and DMADP were not easily modeled by standard competitive inhibition kinetics. We applied the logistic equation (as mentioned in the experimental procedure section) to the experimental data points using two different approaches. They involve the fitting using a fixed value of $H$ = 1 or a fixed value of $v_{min}$ = 0. A better fitting of the IC$_{50}$ curve was obtained using the second approach. This approach resulted in the $H$ values less than 1. The Hill coefficients of the inhibitor binding as obtained from the non-linear curve fitting using Origin (www.originlab.com) were $H$ = 0.69 ± 0.03 for IDP and $H$ = 0.61 ± 0.06 for DMADP. These $H$ values are consistent with both the inhibitors exhibiting negative cooperativity of binding for the enzyme, in other words, binding of one inhibitor to a dimer reduces the binding of a second inhibitor molecule to the other member of the dimer. One part of the IDP and DMADP molecules that they have in common with TPP is the diphosphate group. Therefore, the effect of pyrophosphate was tested (inset of Fig. 6). Sodium pyrophosphate did not show any effect on PrDXS activity even at a concentration of 1 mM. This suggests that the inhibitory effect of IDP and DMADP on PrDXS activity is not due to a nonspecific effect of diphosphate part of the molecules.

**Computational modeling.** To understand substrate and inhibitor binding, a three-dimensional atomic structure of PrDXS in complex with TPP and Mg$^{2+}$ was built by homology modeling based on the crystal structures of DrDXS and EcDXS (40) and refined by molecular dynamics simulations. The modeled structure was stable with an average root-mean-squares fluctuation (RMSF) of 0.75 Å (Fig. 7A). Significant fluctuations are located mainly in the region that was not observed in the crystal structure of DrDXS (residues 199–243), the template used for the homology modeling, and where PrDXS has an eight-residue insertion. The core structure of PrDXS aligns well with the crystal structures of EcDXS and DrDXS (Fig. 7B). The interactions between the enzyme and the coenzyme TPP and the metal ion Mg$^{2+}$ are illustrated in Fig. 8A and are essentially the same as in the crystal structures of EcDXS and DrDXS (40). The Mg$^{2+}$ ion is coordinated with Asp145 and Asn174 of the TPP-binding motif of GDGX25-30N (46), the pyrophosphate moiety of TPP, and two water molecules. The Mg$^{2+}$ coordination is stable during the production phase of the molecular dynamics simulation without any restraint. The coenzyme TPP is anchored at the active site mainly by its pyrophosphate and pyrimidine moieties (Fig. 8A) as in EcDXS and DrDXS. In addition to the interaction with the Mg$^{2+}$ ion, the pyrophosphate moiety of TPP is hydrogen bonded to the side chains of His73, Lys291, and His306 and the main-chain amides of Gly146 and Ala147. In addition to many van der Waals interactions, the pyrimidine ring of TPP is hydrogen bonded to the side chains of His73, Lys291, and His306 and the main-chain amides of Gly146 and Ala147. The protonation of Glu377 is crucial for the interaction with N1 of the pyrimidine ring. The interactions of IDP or DMADP with the enzyme are very similar to those of TPP (Fig. 8B). The binding of IDP or DMADP to the enzyme is mainly through the interactions of its pyrophosphate moiety, very similar to the binding of TPP (Fig. 8C) but several van der Waals interactions are also predicted.
DISCUSSION

To study potential feedback regulation in the MEP pathway required a kinetic study of DXS in presence of different MEP pathway metabolites. The most common assay for this enzyme involves the measurement of radioactivity incorporated into the product DXP from radiolabeled pyruvate (19-22, 47). However, this assay involves a laborious separation of the precursors from products. Another useful method to study DXS activity involves a coupled spectrophotometric assay exploiting the consumption of NADPH by DXR enzyme, which uses DXP as a substrate (48). We found some ambiguities in early results because of potential effects of tested metabolites on DXR. A fluorometric assay for DXS was developed using a fluorescent derivative of the product DXP (49). This assay suffered from the lack of selectivity. Another assay for DXS has been reported using HPLC based separation of derivatized DXP with a fluorophore using fluorescence detection (50). This assay still involved an additional step for derivatization of the product. Recently, an assay based on circular dichroism, has been reported for DXS (51). This assay appeared to be extremely important for studying the mechanistic behavior of DXS illustrating detailed insights about different TPP-bound intermediates involved in the DXS catalyzed reaction. Enzymatic synthesis of DXP from pyruvate and GAP by yeast transketolase has been successfully monitored by an HPLC-ESI-MS-MS based technique (52). Here we report another DXS assay in which DXP produced in the enzymatic reaction is measured by LC-MS/MS. This method is well suited for studying inhibitors of DXS activity.

Recombinant PtDXS enzyme from Populus trichocarpa exhibited a $K_m$ for pyruvate of 87.8 ± 3.2 µM and 119.2 ± 14.2 µM by coupled assay and LC-MS/MS-based assay respectively (Table 1). The $K_m$ for GAP obtained from the coupled assay (18.5 ± 0.7 µM) was higher than that obtained from the LC-MS/MS-based assay (5.9 ± 0.9 µM). This could be because of the consumption of GAP in the coupled assay and in situ production of GAP from DHAP and TPI in LC-MS/MS-based assay. The $k_{cat}$ values (~0.5 sec$^{-1}$) obtained from different measurements by the coupled assay were higher than the values obtained from the LC-MS/MS-based assay (~0.2 sec$^{-1}$) (Table 1). Use of a substantially lower concentration of TPP (100 µM) in LC-MS/MS-based assay compared to 1 mM TPP in the coupled assay may have caused this variation. The $k_{cat}$ value of ~0.5 sec$^{-1}$ for PtDXS is lower than the reported $k_{cat}$ value of ~1.9 sec$^{-1}$ from Rhodobacter capsulatus (45).

The feeding experiment with labeled DOX-d$_2$ by Wolfertz and coworkers (35) indicated that a feedback regulation controls the carbon flux through the MEP pathway but did not provide evidence for a specific mechanism. Our results show that DMADP and IDP, the very last metabolites of the MEP pathway, inhibit PtDXS, the first enzyme of this pathway. The inhibitors compete with thiamine diphosphate for binding with the enzyme. The $K_i$ values for the inhibitor binding are 65.4 ± 4.3 µM for IDP and 81.3 ± 10.5 µM for DMADP. It is interesting that inhibitors can compete with TPP, which is generally thought to be an integral part of the enzyme. The results reported here indicate that IDP and DMADP have the potential to inhibit other TPP-dependent enzymes.

The absolute physiological concentrations of chloroplastic IDP and DMADP are not known. Non-aqueous fractionation to measure the chloroplastic DMADP pool of kudzu leaves has estimated a range of ~0.25 mM to ~3.5 mM (34). Measurement of chloroplastic DMADP pool by post illumination isoprene emission measurements estimated a concentration of ~43 µM in oak leaves (53, 54). Metabolic profiling studies by LC-MS/MS estimated the chloroplastic IDP/DMADP pool to be ~30 µM in poplar leaves (37). Considering the variability in the measurement of the metabolites by these methods, the physiological concentration for DMADP/IDP can certainly be assumed in the range of $K_i$ of these metabolites for PtDXS. Therefore, the inhibition of PtDXS by IDP and DMADP under physiological concentration constitutes a significant feedback regulation of the MEP pathway, which can play an important role in regulating the amount of carbon lost by plants as isoprene.

The apparent cooperativity of inhibitor binding causes PtDXS activity to be very sensitive to low concentrations of inhibitor but relatively less sensitive when the inhibitor concentration is
above the $K_i$. The crystal structure of DXS from *Escherichia coli* and *Deinococcus radiodurans* shows that the enzyme exists as dimer (40). Therefore, the inhibitor cooperativity would ensure some DXS activity even in the presence of high levels of DMADP and IDP. This could be important because DXP is also the substrate for thiamine and pyridoxol synthesis (21,22, 55). If DXS were too effectively shut off by IDP and DMADP it could interfere with thiamine and pyridoxol synthesis.

The observation by Wolfertz et al. (35) was that a very large reduction in DXS activity could be seen with little increase in the rate of isoprene synthesis. This work was done with Eucalyptus and the enzyme kinetics of Eucalyptus isoprene synthase has recently been published (56). Eucalyptus isoprene synthase has a $K_{m}^{DMADP}$ of 0.16 mM, $k_{cat}$ of 0.195 s$^{-1}$, and substrate inhibition ($K_{i} = 0.9$ mM). The inhibition of DXS by IDP and DMADP alone would not be sufficient to reduce DXS activity to such an extent that could explain the constant overall rate of isoprene emission. The complex kinetics of isoprene synthase also plays a significant role in this case. A combination of inhibition of DXS by IDP and DMADP and substrate inhibition of isoprene synthase by DMADP can satisfactorily account for the constant overall rate of isoprene emission using physiologically realistic assumptions (not shown).

Computational modeling shows that the binding of IDP/DMADP to PtDXS is mainly through the pyrophosphate moiety. Fig. 8A and 8B show that the oxygen atoms of the pyrophosphate moiety of both TPP and IDP have polar interactions with K291, H73, G146, and A147 residues of PtDXS. The pyrophosphate oxygen atoms from both TPP and IDP interact with N174 and D145 residues of PtDXS. The pyrophosphate oxygen atoms from both TPP and IDP interact with N174 and D145 residues of PtDXS through the Mg$^{2+}$ ion. However, the polar interaction of TPP through the pyrimidine N1 and N5 atoms with E377 and S116, nitrogen atom of NH$_2$ group of pyrimidine C4 with G114, and thiazolium sulfur atom with S178 residue of PtDXS is absent for the binding of IDP with the enzyme. The van der Waals interactions of the carbon chain of IDP/DMADP with Leu179, Ala352, Gly146, and Ala147 orients the pyrophosphate group in the appropriate position for binding with the enzyme. This interaction is important for binding of the molecule with the enzyme as pyrophosphate alone does not show any inhibitory effect (Fig. 6, inset).

Several studies indicated that DXS could have a role in the regulation of the MEP pathway. The regulation of gene transcription and translation by different developmental and environmental cues is most evident for DXS enzyme (57-59). Posttranscriptional regulation of DXS was observed by the level of the end product of the pathway (59). Higher or lower accumulation of isoprenoid end products in transgenic species having an over or under expressed DXS gene respectively was observed both in bacteria (60-62) and plants (63-65). DXS gene expression is strongly regulated with different developmental stages and strongly correlated with carotenoid accumulation in tomato fruits (58). DXS gene expression pattern under the influence of various exogenous elicitors in *Ginkgo biloba* is strongly correlated with ginkgolide accumulation (66). All of this evidence suggests a regulatory role for DXS in MEP pathway. Recently, a feed-forward activation of IspF enzyme (2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase) by MEP and a feedback inhibition of IspF-MEP complex by a downstream isoprenoid farnesyl diphasphate have been reported in *E. coli* (67). This explains a new regulatory mechanism that modulates the synthesis of one of the key intermediates of MEP pathway. It has also been shown that the steps using reducing power (steps involving DXR, HDS and HDR enzymes) act as strong regulatory points of the MEP pathway under different environmental conditions (37). This suggests the presence of a regulatory mechanism at the middle of the pathway. Our work shows the connection between the beginning and the end of the pathway. It suggests that the beginning of the pathway can control the flow of carbon through the pathway, coordinating with the signals provided by the end of the pathway.

In conclusion, PtDXS activity was monitored in presence of different MEP pathway metabolites. Only IDP and DMADP were found to have significant inhibitory effect on PtDXS activity. Both IDP and DMADP compete with TPP for binding with the enzyme. The inhibitors also exhibit negative cooperativity for binding
with the enzyme. Computational modeling shows that IDP/DMADP uses similar polar and non-polar contacts as TPP for binding with the enzyme. Inhibition of \( PtDXS \) by IDP and DMADP shows a potentially important metabolic regulation within the MEP pathway that plays significant role in controlling the carbon flow through this pathway.

Beyond its role in regulation of the MEP pathway, the competition among IDP, DMADP and TPP could affect nearly any other TPP-dependent reaction, depending on the relative affinities for these compounds.

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FOOTNOTES

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Table 1. Kinetic constants of the PtDXS enzyme measured by the DXR-coupled assay and LC-MS/MS based assay. Each number represents mean ± SE, n = 6 for DXR-coupled assay and mean ± SE, n = 3 for LC-MS/MS based assay. Kinetic constants were obtained from the non-linear curve fitting of the Michaelis-Menten plot in Origin.

| Substrate | DXR-coupled assay | LC-MS/MS based assay |
|-----------|-------------------|----------------------|
|           | $K_m$ (µM) | $k_{cat}$ (sec$^{-1}$) | $K_m$ (µM) | $k_{cat}$ (sec$^{-1}$) |
| Pyruvate  | 87.8 ± 3.2 | 0.53 ± 0.01 | 119.2 ± 14.2 | 0.16 ± 0.01 |
| GAP       | 18.5 ± 0.7 | 0.64 ± 0.01 | 5.9 ± 0.9 | 0.22 ± 0.02 |
| TPP       | – | – | 26.8 ± 4.3 | 0.55 ± 0.02 |
FIGURE 1. Chromatogram of PtDXS assay mixture at 0 min and 5 min. DHAP, DXP, and $^{13}$C$_2$-labeled DXP are represented by blue, red, and green color respectively. $^{13}$C$_2$-labeled DXP was used to quantify the amount of DXP produced. The inset shows the composition of the solvents used in the binary gradient for the elution of DXP and DHAP. Solvent A is 50 mM ammonium acetate, pH 10 and Solvent B is acetonitrile.

FIGURE 2. pH optimum for PtDXS enzyme. Specific activity of the PtDXS enzyme at different pH was monitored using LC-MS/MS based assay. Different pH of the assay mixture was maintained using bis-tris propane buffer. Each data point represents mean ± SD, n = 3. The enzyme is most active at pH 8.0.
FIGURE 3. Panel A. Effect of different metabolites of MEP pathway on \(Pt\)DXS activity based on LC-MS/MS based assay. Each bar represents mean \(\pm\) SD, \(n = 3\). The effect is most significant for IDP (\(p = 0.0036\)). Panel B. The effect of MEP on \(Pt\)DXS activity based on LC-MS/MS based assay without using any internal standard. MEP does not have any inhibitory effect on \(Pt\)DXS activity.
FIGURE 4. Effect of IDP on PtDXS activity in presence of increased amount of each of the substrates. The light and dark gray bars represent the enzymatic activity in absence and presence of IDP respectively. The different categories represent the activity in presence of twice the amount of a particular substrate (as designated below) compared to that present in the control. Each bar represents mean ± SD, n = 3. Inhibition by IDP is significantly less (p < 0.01) in presence of twice the amount of TPP.

FIGURE 5. Michaelis-Menten plot for PtDXS activity at different concentration of TPP and fixed concentration of pyruvate and DHAP in presence of varying concentration of IDP. Each data point represents mean ± SD, n = 3. Different symbols represent the experimental data points. The solid lines represent the regression of the experimental data points using the method of least squares. The black, pink and blue colors represent the PtDXS activity in presence of 0, 100, and 1000 μM of IDP respectively. PtDXS activity decreases with increasing concentration of IDP.
FIGURE 6. IC₅₀ curve of DMADP and IDP for the PtDXS enzyme in presence of $K_m$ concentration of TPP. Each data point represents mean ± SD, n = 3. The IC₅₀ curves were obtained from the non-linear curve fitting of the experimental data points using Origin. The solid and empty circles represent the experimental data points for IDP and DMADP respectively and the solid and dotted lines represent the fitted IC₅₀ curve for IDP and DMADP respectively. The $K_i$ values of IDP and DMADP were calculated to be ~65 μM and ~81 μM respectively. The inset shows the effect of sodium pyrophosphate on PtDXS activity. Each bar represents mean ± SD, n = 3. Sodium pyrophosphate did not show any inhibitory effect on PtDXS activity even at a concentration of 1 mM.
FIGURE 7. Panel A. Root-mean-square fluctuations of Cα atoms of the first subunit of PtDXS during 1.5-ns production phase of the molecular dynamics simulation. Panel B. Structural alignment of PtDXS (green) with EcDXS (yellow) and DrDXS (pink). The ligand binding site of PtDXS is shown in red contour.
FIGURE 8. Interactions of \textit{PtDXS} with the coenzyme TPP (A) and IDP (B). The Mg$^{2+}$ ion is shown as a gray sphere. Mg$^{2+}$ coordination and hydrogen bonds are shown in yellow dashed lines and van der Waals interactions in cyan dashed lines. Two Mg$^{2+}$-coordinated water molecules are also shown. (C) Simulated binding pose of IDP (Carbon in green) in the \textit{PtDXS} active site as compared to that of TPP. The Mg$^{2+}$ ion is shown as a gray sphere. Two Mg$^{2+}$-coordinated water molecules are also shown.
