Communication

Enzymatic Synthesis of Isoprene from Dimethylallyl Diphosphate in Aspen Leaf Extracts

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ABSTRACT

Aspen (Populus tremuloides Michx.) leaf extracts contain a newly discovered enzyme activity that catalyzes the magnesium ion-dependent elimination of diphosphate from dimethylallyl diphosphate with rearrangement to form isoprene (2-methyl-1,3-butadiene). This isoprene synthase activity has been partially purified. The nonenzymatic reaction of dimethylallyl diphosphate to isoprene, known to be acid catalyzed, may be insignificant at physiological pH. In contrast, the enzymatic reaction may be responsible for the majority of light-dependent isoprene production by isoprene-emitting plants.

Isoprene (2-methyl-1,3-butadiene) is the major nonmethane hydrocarbon emitted from plants to the atmosphere, and plays an important role in atmospheric chemistry (see references in Monson and Fall [13]). In light of these facts, there is interest in understanding the processes that lead to isoprene biosynthesis and emission. Even though isoprene emission from plants was discovered over 30 years ago (16), surprisingly little is known about isoprene biogenesis. Studies describing incorporation of isotopically labeled metabolites into isoprene have been reported and biosynthetic pathways proposed (9, 16, 18), but as yet no clear evidence exists for the exact metabolic steps leading to isoprene synthesis. Considering that isoprene is perhaps the simplest member of the isoprenoid family, it seems likely that isoprene could be derived from 5-carbon intermediates common to the central mevalonic acid pathway of isoprenoid biosynthesis (7), a view expressed years ago (10) but still not proven.

No enzyme has yet been found to catalyze isoprene formation. Mead and coworkers (5) investigated the production of isoprene in rat liver extracts, and concluded that isoprene is formed from the acid-catalyzed solvolysis of DMAPP (20). In this scheme, ionization of diphosphate from DMAPP would yield a transient carbocation that would give rise to isoprene as well as solvolysis products, 3-methyl-2-buten-1-ol and 2-methyl-3-buten-2-ol. Sanadze (16) has also suggested that isoprene is formed nonenzymatically in plant chloroplasts. In the light, the thylakoid lumen is acidified to approximately pH 5 by proton pumping (1), and if DMAPP is contained in this compartment, the acid-catalyzed reactions described above could generate isoprene. Evidence for the presence of DMAPP in the thylakoid lumen is lacking.

We have been working to investigate the biochemical and physiological basis for the light-dependent formation of isoprene in plants (8, 13, 14), and as an extension of this work initiated experiments to directly test for the enzymatic production of isoprene from DMAPP. This report describes the detection of a new enzyme activity in leaf extracts that catalyzes the formation of isoprene.

MATERIALS AND METHODS

Plant Material and Reagents

Quaking aspen trees (Populus tremuloides Michx.) were obtained from a local nursery and maintained in a greenhouse as described elsewhere (13).

DMAPP was synthesized and purified by the method described by Davisson et al. (4). All reagents for the synthesis of DMAPP were supplied by Aldrich (Milwaukee, WI). Verification of the structure and purity of the DMAPP was by TLC and 1H- and 31P-NMR using a 300 MHz Varian VXR-300S instrument; this preparation was free of detectable isopentenyl diphosphate. Polyclar AT was supplied by Serva Biochemicals (Westbury, NY). DEAE-Sephacel was obtained from Pharmacia (Piscataway, NJ). All other chemicals were reagent grade.

Partial Purification of Isoprene Synthase

The partial purification of isoprene synthase activity was performed at 0 to 4°C as follows. Fully expanded aspen leaves of various ages (5 g) were homogenized with a mortar and pestle using a modified procedure suggested by Gegenheimer (6). The PEB contained 50 mM Tris-HCl, 20 mM MgCl2, 5% glycerol, pH 8.0. For the homogenization, we used 50 mL PEB and added 10% (w/v) Polyclar AT, 20 mM DTT, 1 mM PMSF, and 1 mM benzamidine-HCl. Polyclar AT and DTT were found to be essential for preserving isoprene synthase activity. After filtration through a layer of Miracloth (Calbiochem, LaJolla, CA) and centrifugation (20 min at 12,000g and 40 min at 40,000g), the supernatant contained isoprene...
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synthase activity, which was precipitated between 40 and 55% saturation with (NH₄)₂SO₄. The precipitate was collected by centrifugation (10 min at 12,000 g), and resuspended in 1/10 volume PEB containing 1 mM DTT, yielding a preparation that was stable at -70°C for at least 1 month. After dialysis against PEB containing 1 mM DTT, this preparation was applied to a column of DEAE-Sephacel (15 mL bed volume) equilibrated in the same buffer. Isoprene synthase activity was eluted with PEB containing 1 mM DTT and increasing concentrations of NaCl. The active fractions were concentrated by ultrafiltration (Ultrafree-PFL filter units, Millipore, Bedford, MA) to a final concentration of 1 to 2 mg protein mL⁻¹. This concentrated protein was flash frozen in liquid N₂ and stored at -70°C.

Assay for Isoprene Production and pH Optimum

Isoprene production was routinely assayed in 4 mL glass vials sealed with Teflon-lined septa. Reactions containing enzyme, DMAPP, and Mg²⁺, as described in the text, were carried out at 28°C, and at appropriate times 1 to 2 cc samples of headspace were removed with a gas-tight syringe and analyzed by GC. The gas chromatograph used was a portable unit constructed in our laboratory. It was operated isothermally (85°C) with an n-Octane/Porasil C packed column (183 cm x 0.32 cm o.d.) with high-purity He or N₂ carrier gas at a flow rate of 30 cc min⁻¹. Samples were injected via a heated six-way valve (Valco Instrument Co., Houston, TX) and a 0.5 cc sample loop (Alltech Associates Inc., Deerfield, IL). Isoprene was detected using an RGD2 reduction gas detector (Trace Analytical, Menlo Park, CA) that is selective for gases that reduce mercuric oxide and can detect isoprene down to 0.06 pmol. For this work, 0.5 to 1 pmol isoprene were routinely detected. Calibration of the detection system was performed using an isoprene gas standard, and was monitored by daily injections of a propylene gas standard (Scott Specialty Gases, Longmont, CO).

To measure the pH optimum for isoprene synthase, and to establish the nonenzymatic conversion of DMAPP to isoprene, reactions rates were measured between pH 5.5 and 10.0.

Other Assays

Protein concentration was quantitated by the bicinchoninic acid procedure with serum albumin as a standard (19). Inorganic diphosphate was assayed using two different procedures (11, 15). Pi was assayed as described elsewhere (2).

RESULTS

As outlined in the introduction, we wanted to test the possibility that leaves contain an enzyme that catalyzes the conversion of DMAPP to isoprene. DMAPP was synthesized and used as a substrate for these reactions, and isoprene production was initially followed by GC-flame ionization detection. Many attempts at detecting an isoprene synthase activity in leaf extracts were unsuccessful. Two developments led to the detection of an isoprene synthase activity. First, we employed a more sensitive, selective detector for isoprene. Second, we used different leaf extraction techniques, including the addition of stronger antioxidants, that apparently stabilized the enzyme activity.

A reproducible conversion of DMAPP to isoprene was catalyzed by aspen leaf extracts, and the reaction was linear with time, dependent on the presence of Mg²⁺, and saturable with the substrate DMAPP, indicative of the presence of an enzyme. Reactions run without added leaf extract, with boiled leaf extract, or with protease-treated extract catalyzed a much slower nonenzymatic rate of isoprene synthesis.

The isoprene synthase activity was partially purified by a combination of (NH₄)₂SO₄ precipitation and ion exchange chromatography. Figure 1 shows the elution profile of the enzyme activity on a DEAE-Sephacel column. Using a stepwise NaCl gradient, isoprene synthase activity eluted as a single peak, between 0.2 and 0.3 M NaCl. This partially purified preparation of enzyme was used in all steps described below.

The isoprene synthase reaction was dependent on the presence of magnesium ion (Fig. 2), and maximal activity was found at 10 mM Mg²⁺. In contrast, Mg²⁺ had no apparent effect on the nonenzymatic conversion of DMAPP to isoprene at pH 5 to 8.

The pH dependence of the enzymatic and nonenzymatic reactions was investigated, using several buffers to span the pH range of 5.5 to 10. Isoprene synthase activity was maximal between pH 7 and 9 (Fig. 3). The non-enzymatic production of isoprene from DMAPP is also shown in Figure 3. Under these conditions, the nonenzymatic reaction accounted for only a fraction of the total isoprene produced. At lower pH (≤3), the nonenzymatic rate became much more significant (data not shown), indicative of acid-catalyzed solvolysis of DMAPP (20).

Isoprene produced from DMAPP by the partially purified isoprene synthase was verified by (a) cochromatography with authentic isoprene on capillary GC (flame ionization detec-
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buffer, pH enzyme would be partially eliminated by increasing concentrations of MgCl₂. Each 30 µL reaction contained 20 µg partially purified protein, 1.4 mM DMAPP, and 0 to 20 mM MgCl₂. All samples were incubated for 10 min and tested for isoprene.

Figure 2. Magnesium ion dependence of isoprene synthase activity. DEAE-purified enzyme was first dialyzed against 50 mM Tris-HCl buffer, pH 8.0. Magnesium-free enzyme was then assayed with increasing concentrations of MgCl₂. Each 30 µL reaction contained 20 µg partially purified protein, 1.4 mM DMAPP, and 0 to 20 mM MgCl₂. All samples were incubated for 10 min and tested for isoprene.

Figure 3. pH profile for enzymatic and nonenzymatic isoprene synthesis. To cover the range of pH 5.5 to 10.0, several buffers were employed: Mes, pH 5.5 to 6.8; Bis-Tris-propane (1,3-bis[tris(hydroxymethyl)methylamino]propane), pH 6.5 to 9.0; and CHES (2-[N-cyclohexylamino]ethanesulfonic acid), pH 9.0 to 10.0. Each 20 µL reaction contained 100 mM buffer, 20 mM MgCl₂, 7.1 mM DMAPP, and 10 µg DEAE-purified protein. Nonenzymatic samples were identical except that they contained no protein. All samples were incubated for 15 min and tested for isoprene.

Figure 4. Scheme for the isoprene synthase reaction and probable mechanism for acid-catalyzed conversion of DMAPP to isoprene. As discussed in the text, the enzymatic and acid-catalyzed reactions may proceed through the delocalized carbocation shown on the figure, and it is uncertain at this time whether inorganic diphosphate or Pi is formed in the isoprene synthase reaction.

This preparation. Thus, we cannot yet determine whether the isoprene synthase reaction releases diphosphate or Pi.

DISCUSSION

This is the first description of the enzymatic production of isoprene. Figure 4 shows the isoprene synthase reaction we propose, as well as a scheme for the acid-catalyzed formation of isoprene. In each case, elimination of diphosphate to give a delocalized carbocation intermediate followed by proton abstraction and rearrangement would yield isoprene. We attempted to detect the other expected product of these reactions, inorganic diphosphate, using two different assay methods. Diphosphate production was detected when DMAPP was acidified (pH 1), but not in enzymatic incubations. However, Pi production was detected in the latter samples. Upon addition of sodium diphosphate to the partially purified enzyme, Pi production was observed, indicating the presence of a diphosphatase in

It may be significant that the pH optimum for the enzymatic reaction is approximately pH 8. This may indicate that the enzyme is located in the chloroplast stroma, where the pH reaches 8 in the light (1). Mgaloblishvili et al. (12) have obtained evidence that isoprene emissions from poplar are located in the chloroplast. We do not have enough information to estimate the nonenzymatic rate of conversion of DMAPP to isoprene in a leaf, but it can be seen in Figure 3 that this rate is only a fraction of the enzymatic rate at pH 8.0. It may be that the majority of isoprene produced by aspen leaves is through isoprene synthase activity. Further work will be needed to ascertain the in vivo rates of the enzymatic and nonenzymatic reactions, and the relative contributions of each to leaf isoprene emissions.

Isoprene emission from leaves is dependent on light, and emission declines rapidly (e.g., in 2 min) to zero after a light-to-dark transition (14). This suggests that isoprene synthase activity is associated with a light-activated enzyme or that the availability of its substrate, DMAPP, is light dependent. Light modulation of chloroplast enzymes is well documented (reviewed in ref. 17). Alternatively, light-dependent isoprene
production could be explained by the model of Sanadze (16),
which proposes that proton pumping into the thylakoid lumen
could promote the acid decomposition of DMAPP, if this
prenyl-diphosphate is also transported into this chloroplast
compartment. As discussed by Sharkey et al. (18), this model
would not explain why some plants are isoprene emitters and
others are not.

We have also detected isoprene synthase activity in leaf
extracts of other isoprene-emitting plants, including plains
cottonwood (Populus deltoides var occidentalis Rydb.), cow-
pea (Vigna unguiculata L.), and velvet bean (Mucuna sp.)
data not shown), showing that the enzyme is not limited to
aspen leaves.

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