Communication

Dissociation of Prelycopersene Pyrophosphate Synthetase Complex from Phytoene Synthetase Complex of Tomato Fruit Plastids*

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MOHAMMAD A. ISLAM, STEPHEN A. LYRENE, EDWARD M. MILLER, JR., AND JOHN W. PORTER

From the Lipid Metabolism Laboratory, Veterans Administration Hospital and the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

The partially purified phytoene synthetase enzyme complex obtained from tomato fruit plastids dissociates into two or more subunit species on chromatography in low ionic strength buffer on DEAE-cellulose. One of these subunits, prelycopersene pyrophosphate synthetase, has a molecular weight of approximately 40,000, whereas the phytoene synthetase complex has a molecular weight of 200,000. The prelycopersene pyrophosphate synthetase catalyzes the conversion of isopentenyl pyrophosphate to geranylgeranyl and prelycopersene pyrophosphates. The identities of these subunits were established by thin layer chromatography in several solvent systems. The formation of both geranylgeranyl and prelycopersene pyrophosphates by this enzyme supports earlier results with cruder enzyme systems which suggested that these compounds are intermediates in the synthesis of phytoene.

Earlier studies from this laboratory (1–3) established the incorporation of radioactive isopentenyl, farnesyl, and geranylgeranyl pyrophosphates into phytoene by a cell-free enzyme system obtained from tomato fruit plastids. More recent studies (4) established that radioactive prelycopersene pyrophosphate and lycopersene pyrophosphates were converted to phytoene by the same enzyme system. The conversion of phytoene to more unsaturated acyclic and cyclic carotenes by a cell-free enzyme system obtained from tomato fruit plastids has also been demonstrated (5).

Early studies on the purification and properties of geranylgeranyl pyrophosphate and phytoene synthetases were carried out by Foldbrugge (6). These studies were then extended by Maudinas et al. (7) who partially purified phytoene synthetase by ammonium sulfate precipitation and gel chromatography on Bio-Gel A-1.5m. In an extension of this work Maudinas et al. (8) reported a 350-fold purification of phytoene synthetase complex which behaves as a complex with a molecular weight of approximately 40,000, and it catalyzes the conversion of isopentenyl pyrophosphate to geranylgeranyl and prelycopersene pyrophosphates. This enzyme appears to be comprised of only one protein inasmuch as it yields only one band on sodium dodecyl sulfate-gel electrophoresis.

EXPERIMENTAL PROCEDURES

Purification of Phytoene Synthetase—An acetone powder of plastids was prepared from commercial semiripened tomatoes according to the method of Jungalwala and Porter (1). The dry acetone powder was then extracted with phosphate buffer, the extract was centrifuged, and the soluble proteins were fractionated with ammonium sulfate (1). The fraction precipitating between 20 and 60% was collected and subjected to gel filtration on Bio-Gel A-1.5m as reported by Maudinas et al. (7). This preparation of the phytoene synthetase enzyme was used in the experiments reported herein unless otherwise stated.

Incubation System—The incubation system for the biosynthesis of phytoene and intermediates contained enzyme protein, 0.1 to 0.2 mg; borate buffer, pH 8.2, 10 μmol; Tween 80, 1 μg; MgCl₂, 1 μmol; MgCl₃, 7.5 μmol; NADP⁺, 2 μmol; dithiothreitol, 5 μmol; [1-¹⁴C]isopentenyl pyrophosphate, 21 nmol and 120,000 disintegrations per min in a total volume of 0.5 ml. The final pH of the incubation mixture was 7.1. Normally incubations were carried out for 2 to 4 h at 23° under a nitrogen atmosphere. Protein was estimated by the biuret method of Cornall et al. (9).

Assay for Phytoene and Acid Labiles—Incubations were terminated by the addition of 2 ml of absolute ethanol and the incubation mixture was then extracted three times with 3 ml of silica gel-purified petroleum ether. Phytoene in this extract was isolated by alumina column chromatography and then identified according to the method described by Jungalwala and Porter (10). Radioactivity in phytoene was determined in a Packard Tri-Carb liquid scintillation spectrometer. After removal of the nonsaponifiable compounds by extraction with petroleum ether (boiling point 40–60°), 100 μl of 12 N hydrochloric acid was added to the incubation mixture (final volume of 2.5 ml). This mixture was held at 37° in a water bath for 15 min. The acid-liberated terpenols were extracted with two 3-ml portions of petroleum ether, and the radioactivity of an aliquot of the extract was determined by liquid scintillation spectrometry.

RESULTS

Dissociation of the Phytoene Synthetase Complex—The phytoene synthetase complex effects at least one isomerization and four condensation reactions in the formation of terpenyl pyrophosphates and phytoene from isopentenyl pyrophosphate. This enzyme system, after partial purification by gel filtration, is stable for several weeks when stored at 20° in 0.1 M potassium phosphate buffer, pH 7.0, containing 30% glycerol and 2 mM dithiothreitol. However, when this complex is subjected to DEAE-cellulose chromatography in 0.02 M potassium phosphate buffer, pH 7.0, containing 2 mM dithiothreitol and 30% glycerol, it loses activity for the formation of phytoene. Enzyme activity for the formation of acid-libable compounds is retained (Fig. 1). The latter activity is eluted from the column with 0.2 M KCl.

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The names "prelycopersene pyrophosphate" and "prephytoene pyrophosphate" are used interchangeably.
1524 Prelycopersene Pyrophosphate Synthetase

Forming Acid-Labile Compounds - Assays were carried out as described under "Experimental Procedures." DEAE-cellulose column. The enzyme, 7.2 mg of protein, was added to the column (9.5 x 1.3 cm) in 2.0 ml of 0.02 M potassium phosphate buffer, pH 7.0, containing 30% glycerol and 2 mM dithiothreitol. Protein was eluted from the column by stepwise addition of 0.2 M and 0.4 M KCl in 0.02 M potassium phosphate buffer, pH 7.0. Eluate fractions of 2.0 ml were collected and the protein elution profile was determined by recording the absorbance at 280 nm (0-O). Assays for the formation of phytoene (Δ-Δ) and acid labiles (O----O) were carried out as described under "Experimental Procedures."

Determination of Molecular Weight of the Enzyme System Forming Acid-Labile Compounds - Assays were carried out for the molecular weight of the enzyme system forming acid labiles in order to determine whether the loss of phytoene synthetase activity was due to selective inactivation of one activity of the complex or to dissociation of the enzyme system. The protein fractions eluted from the DEAE-cellulose column with 0.2 M KCl were pooled and concentrated in an Amicon concentrator equipped with a PM-10 filter. This protein fraction was subjected to Sephadex G-200 gel filtration and the elution volume of the acid-labile synthesizing enzyme was compared with those of other proteins of known molecular weight (Fig. 2). The molecular weight determined by this procedure was approximately 40,000, thus indicating that the phytoene synthetase complex had dissociated into two or more subunits on DEAE-cellulose chromatography. A molecular weight of approximately 40,000 was determined for the acid-labile synthesizing enzyme by gel filtration on a Sephadex G-200 column. A particle size of 40 to 120 μm and a column of 1.7 x 35.5 cm were used. Each standard protein, 2.0 mg, was dissolved in 0.2 ml of potassium phosphate buffer, 0.1 M, pH 7.0, containing 2 mM dithiothreitol and then applied to the column. The void volume of the column was determined with blue dextran. Fractions of 1.0 ml were collected and protein was determined in the eluate by recording the absorbance at 280 nm. The elution volume of the prelycopersene pyrophosphate (PLPP) synthetase was determined by assay for enzyme activity.

Identification of Acid-labile Products - Preliminary experiments indicated that at least two acid-labile compounds were synthesized by the above enzyme system. Extraction of these compounds from an incubation mixture was effected with 2 ml of 3-ml aliquots of H2O-washed n-butyl alcohol and one 6-ml aliquot of benzene. The combined extracts were concentrated under reduced pressure at a temperature of 35° or lower. The concentrated residue was chromatographed on a 0.1 m ammonium phosphate-buffered, pH 6.8, silica gel plate prepared according to the method of Rilling (11). Small sections of the silica gel chromatogram were scraped into counting vials and then assayed for radioactivity by liquid scintillation spectrometry. An Rf value of 0.4 to 0.5 was obtained. This Rf value is characteristic of both geranylgeranyl and prelycopersene pyrophosphates.

To identify the allylic pyrophosphates further, the compounds were hydrolyzed with HCl as reported under "Experimental Procedures." and the liberated terpenols were extracted with silica gel-purified petroleum ether (boiling point 40-60°C). The extract was concentrated under nitrogen and then subjected to thin layer chromatography in two systems. In one of these, chromatography was performed on Silica Gel G in a solvent system of benzene:ethyl acetate (80:20, v/v) (System IV of Qureshi et al. (12)). The radioactive terpenols had Rf values of 0.4 to 0.5 (geranylinalool) and 0.78 to 0.80 (prelycopersene alcohol). In the second system, reverse phase thin layer chromatography was carried out on Silica Gel G plates impregnated with paraffin oil (System VI of Qureshi et al. (12)). After development of the chromatogram in methanol:H2O (88:12, v/v), 1-cm sections of the gel were scraped from the chromatographic plate into counting vials. Radioactivity was determined by liquid scintillation spectrometry. In the reverse phase chromatographic system (Fig. 3) prelycopersene alcohol remained close to the origin (Rf of approximately 0.1),
whereas geranyl linalool migrated with an $R_f$ of approximately 0.7.

**DISCUSSION**

Data are presented in this paper which show that the enzyme system that synthesizes geranylgeranyl and prelycopersene pyrophosphates from isopentenyl pyrophosphate dissociates from the phytoene synthetase complex on DEAE-cellulose chromatography. The dissociated subunit has a molecular weight of approximately 40,000, whereas the phytoene synthetase complex has a molecular weight of 200,000. The dissociation of the phytoene synthetase complex is not affected by low ionic strength buffer inasmuch as treatment of the enzyme under the same conditions as used for chromatography, except that the enzyme is not passed through the DEAE-cellulose column, results in relatively little loss of phytoene synthetase activity. It is evident, therefore, that further investigations will be required to establish the mechanism of dissociation that is effected by DEAE-cellulose chromatography.

The identities of the products synthesized by the prelycopersene pyrophosphate synthetase portion of phytoene synthetase were determined by thin layer chromatography. The products obtained behaved on chromatograms as either geranylgeranyl and prelycopersene pyrophosphates or the alcohols derived from these compounds on acid treatment. The accumulation of geranylgeranyl pyrophosphate by the prelycopersene pyrophosphate synthetase was somewhat unexpected. It may be, though, that the geranylgeranyl pyrophosphate resulted from product inhibition. If so, further experimentation will be required to establish this point. The formation of both geranylgeranyl and prelycopersene pyrophosphates by the enzyme system derived from phytoene synthetase provides additional evidence in support of previous findings (2, 4, 12) that these compounds are intermediates in the synthesis of phytoene.

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