ADENOSINE 5'-TRIPHOSPHATE STIMULATION OF THE ACTIVITY OF A PARTIALLY PURIFIED PHYTOENE SYNTHETASE COMPLEX[1]

Bernard Maudinas,[2] Michael L. Bucholtz, Constantin Papastephanou,[3] Sarvagya S. Katiyar, Anita V. Briedis and John W. Porter

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

Received July 10,1975

SUMMARY

An enzyme complex capable of converting isopentenyl pyrophosphate to phytoene has been isolated from an acetone powder of tomato fruit plastids and partially purified. This complex, approximately 200,000 Daltons in molecular weight, is devoid of squalene synthetase activity. Mn^{++} is required for enzyme activity, but no other cofactors have been identified as being essential. However, a 6 to 7-fold stimulation in activity is effected by ATP (1.3 mM). Since there is no evidence that ATP participates directly in the synthesis of phytoene from isopentenyl pyrophosphate, it is suggested that this nucleotide may be an allosteric effector of the reaction.

A method for the preparation of an acetone powder from tomato fruit plastids was developed by Jungalwala and Porter (1). An extract of this powder was then found to be capable of converting isopentenyl pyrophosphate to phytoene. Subsequent papers from this laboratory have reported the incorporation of [^{14}C]terpenyl pyrophosphates into phytoene and more unsaturated carotenes by this preparation (2, 3). Some cofactor requirements for the crude enzyme system synthesizing phytoene have also been reported (1).

The present report describes the partial purification of the enzyme complex that converts isopentenyl pyrophosphate to phytoene. This purification is achieved by ammonium sulfate precipitation and gel filtration. Attempts to

---

[1]This investigation was supported in part by grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service, AM 01383, and the National Science Foundation, GB25275.

[2]On leave from Centre National de la Recherche Scientifique, Faculté de Pharmacie, ER 159, BP 403 Nancy 54000 - France.

[3]Present address: The Squibb Institute, Analytical Research, New Brunswick, New Jersey 08903.

further purify the enzyme system by a charge separation step are under investigation. The enzyme activity for phytoene synthesis is eluted as a symmetrical peak from a gel filtration column. This activity is stimulated up to 7-fold by 1.3 mM adenosine 5'-triphosphate. Other nucleotides either have no effect or are only partially stimulating.

EXPERIMENTAL PROCEDURE

Commercial semi-ripened tomato fruits were purchased locally and acetone powders of the plastids were prepared by the method of Jungalwala and Porter (1). All operations were carried out at 0 to 4° unless otherwise indicated. Six grams of an acetone powder were then homogenized three times with 25 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM dithiothreitol and 1% Tween 80. Following centrifugation at 10,000 g for 20 minutes, the supernatant solution was fractionated with ammonium sulfate between 20 and 60% of saturation. The pellet was resuspended in 20 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 30% glycerol and 2 mM dithiothreitol and then dialyzed three hours against the same buffer. This material was applied to a Biogel A-1.5 m column (2.5 x 45 cm) which was previously equilibrated with the above buffer. The active fractions eluted from the column were pooled and concentrated to a volume of 20 ml with an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass. 02173) and a PM-10 membrane. This concentrated protein fraction was used for all of the experiments described hereafter.

[1-^{14}C]Isopentenyl pyrophosphate, 60 μCi/μmole, was obtained from Amersham Searle. This compound was diluted with nonradioactive isopentenyl pyrophosphate to a specific radioactivity of 2.15 μCi/μmole.

The incubation mixture for the phytoene synthetase assays contained 10 μmoles boric acid, pH 8.2, 1 mg Tween 80, 1 μmole $MnCl_2$, 7.5 μmoles $MgCl_2$, 2 μmoles $NADP^+$, 5 μmoles dithiothreitol, 20 nmoles and 95,000 dpm isopentenyl pyrophosphate, 400 nmoles ATP, and 0.2 ml of the protein fraction (approximately 1 mg protein) in a total volume of 0.6 ml. Protein was determined by the method of Lowry *et al.* (5). The incubation was carried out under white light from fluorescent bulbs and a nitrogen atmosphere for three hours at 24°. The reaction was stopped by the addition of 2 ml of absolute ethanol, and the incubation mixture was then extracted with 3 x 3 ml aliquots of petroleum ether (B_p 40-60°). Authentic carrier phytoene, isolated from car-

rot oil by the method of Jungalwala and Porter (4), was added to the petroleum ether extract and the latter was dried over anhydrous sodium sulfate.

The extracted hydrocarbons were chromatographed on a Grade I alumina column (Baker aluminum oxide, suitable for chromatographic use). Increasing amounts of peroxide-free diethyl ether in petroleum ether were used to develop the column, as previously reported (4).

Thin layer chromatography was also performed on silica gel G (Sigma). Development of the chromatogram was effected with petroleum ether:diethyl ether (98:2). After development of the chromatographic plate, phytoene was visualized by exposure to iodine vapor. An R_f of 0.6 was obtained. Radioactivity associated with the C_{40} hydrocarbon obtained by each of the above methods was determined by analysis with a liquid scintillation spectrometer.

γ-^{32}P-Labeled adenosine triphosphate of high specific activity (1.1×10^8 cpm/nmole), a generous gift from Dr. Iku Nishigaki (Dept. of Pharmacology, University of Wisconsin, Madison) was used to ascertain whether phosphorylation of phytoene synthetase occurs. The purity of this compound was confirmed by high voltage electrophoresis.

RESULTS

The pattern of protein and enzyme activity on elution of the 20 to 60% ammonium sulfate fraction from a Biogel A-1.5 (200-400 mesh) column is shown in Fig. 1. Enzyme activity for phytoene synthesis was located by the assay reported in the Experimental section. The presence of phytoene was confirmed by alumina column and thin layer chromatography of the petroleum ether extract of the incubation mixture. A coincidence of radioactivity and mass of authentic phytoene was observed on elution from the alumina column. A coincidence of mass and radioactivity was also observed on thin layer chromatography. The behavior of the enzyme system for the synthesis of phytoene from isopentenyl pyrophosphate on Biogel filtration strongly suggests the existence of an enzyme complex for this reaction. This enzyme complex is devoid of squalene synthetase activity when assayed with farnesyl pyrophosphate by the method of Qureshi et al. (6). This complex has a molecular weight of approximately 200,000 Daltons when compared to the elution of standard globular proteins from Biogel.

Various possible cofactors have been examined for their ability to affect

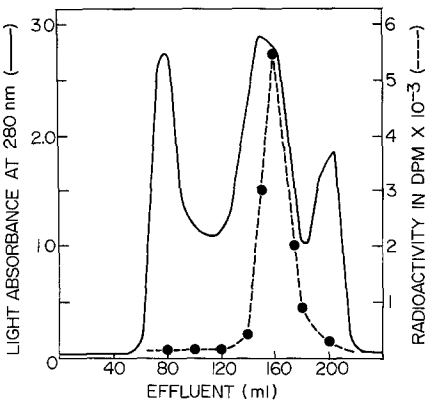

Fig. 1. Partial purification of phytoene synthetase by filtration on a column (2.5 × 45 cm) of Biogel A-1.5 m (200-400 mesh). Eluate fractions of 4 ml were collected. ——, Light absorption at 280 nm; -----, total radioactivity extracted by petroleum ether from an incubation mixture, expressed in dpm × 10^{-3}. Radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer. The product was characterized as reported in the Experimental section and 80% of the petroleum ether soluble radioactivity was phytoene.

---

the rate of conversion of isopentenyl pyrophosphate to phytoene by the crude enzyme system (1). The presence of divalent cations such as Mn^{++} has been shown to be a requirement for the synthesis of phytoene. The affect of ATP on the rate of phytoene formation has not previously been tested, however, mainly because there did not appear to be any reaction steps where this compound could be involved as a cofactor. When this compound was tested in the present study for its affect on the rate of synthesis of phytoene, a 6 to 7-fold increase was obtained, Fig. 2. At concentrations above 1 mM ATP is saturating for this reaction. At a concentration of 1.3 mM ATP there is a 7-fold stimulation over the activity of the control in the incorporation of isopentenyl pyrophosphate into phytoene.

An experiment to examine the specificity of the ATP effect is reported in Table I. The monophosphate and diphosphate forms of adenosine had no effect on phytoene synthetase activity when compared to the control without nucleotides. The triphosphate nucleotides, CTP, GTP and UTP, produced only a 2.5-fold stimulation of phytoene synthetase activity. The addition of 1.6 mM EDTA to the phytoene synthetase incubation mixture inhibited, rather than

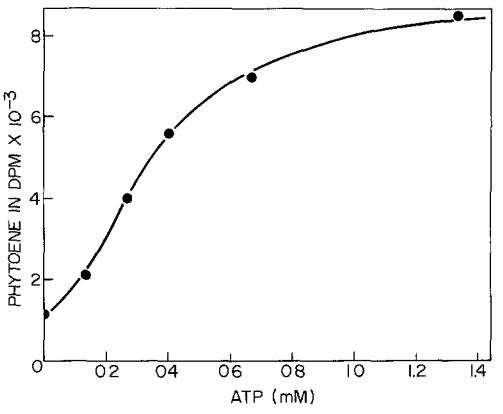

Fig. 2. Influence of adenosine 5'-triphosphate concentration on phytoene synthesis from isopentenyl pyrophosphate.

---

stimulated, phytoene formation. Hence, the ATP stimulation did not involve the removal of heavy metal ions by chelation.

In order to establish whether ATP is acting as an allosteric effector or a phosphorylating agent of the enzyme complex, 1.5 mg of enzyme protein were incubated in the presence of 400 nmoles of ATP (10^6 cpm [γ-^{32}P]ATP) for one hour at 23°C, in a total volume of 0.7 ml. Then the incubation mixture was loaded onto a Biogel A-0.5 m (200-400 mesh) column (1 x 24 cm), previously equilibrated under the conditions described in the Experimental section. Eluate fractions of 1 ml were collected and radioactivity was determined. The only radioactive fraction eluted from the Biogel A-0.5 column corresponded to the elution of the ATP molecule. No radioactivity was associated with the fractions that synthesize phytoene from isopentenyl pyrophosphate. This result rules out the role of ATP as a phosphorylating agent of the phytoene synthetase complex. Therefore it is most probable that ATP is acting as an allosteric effector of this enzyme complex.

DISCUSSION

This report demonstrates for the first time that the enzyme system, isolated from tomato fruit plastids, that synthesizes phytoene from isopentenyl pyrophosphate, behaves as a complex on Biogel filtration. Whether phytoene synthesizing enzymes of chloroplasts and of other species are also complexes remains to be ascertained.

TABLE I

INFLUENCE OF DIFFERENT NUCLEOTIDES ON PHYTOENE SYNTHESIS

Nucleotide	Phytoene
	dpm
None	1,102
cAMP	985
AMP	892
ADP	1,272
ATP	6,177
UTP	2,537
CTP	2,845
GTP	2,900

Each nucleotide was present at the same concentration (0.67 mM) in the incubation mixture described in the Experimental section.

Maximum phytoene synthetase activity by the tomato enzyme system is obtained when the incubation mixture contains adenosine 5'-triphosphate. The addition of ATP at a 1.3 mM concentration produced a 7-fold stimulation of phytoene synthesis. It was also demonstrated that AMP, ADP and cyclic AMP are not activators of this reaction. CTP, GTP and UTP produced only a 2.5-fold stimulation, as compared to the 7-fold stimulation by ATP. The exact role played by ATP in phytoene formation is not certain. However, our data indicate that it is acting as an allosteric effector and not as a phosphorylating agent of the phytoene synthetase complex.

REFERENCES

1. Jungalwala, F.B. and Porter, J.W. (1967) Arch. Biochem. Biophys. 119, 209-219.

2. Suzue, G. and Porter, J.W. (1969) Biochim. Biophys. Acta 176, 653-656.
3. Kushwaha, S.C., Suzue, G., Subbarayan, C. and Porter, J.W. (1970) J. Biol. Chem. 245, 4708-4717.
4. Jungalwala, F.B. and Porter, J.W. (1965) Arch. Biochem. Biophys. 110, 291-299.
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
6. Qureshi, A.A., Beytia, E. and Porter, J.W. (1973) J. Biol. Chem. 248, 1848-1855.