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Metabolism of plastid terpenoids: in vitro inhibition of phytoene synthesis by phenethyl pyrophosphate derivatives

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The inhibition of partially purified phytoene synthetase activity from *Capsicum annuum* chromoplasts was investigated using aminophenethyl pyrophosphate and azidophenethyl pyrophosphate. These compounds were effective inhibitors of phytoene synthesis and kinetic analysis showed that they were competitive with respect to the substrate isopentenyl pyrophosphate. These data were strengthened by the ability of azidophenethyl pyrophosphate to photoinactivate irreversibly the activity of the enzyme complex. These results suggest that the primary targets of these analogs are at the level of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthetase.

Plastid; Carotenoid; Phytoene synthesis; Phytoene synthetase; Inhibitor

1. INTRODUCTION

In higher plants, the plastid compartment is a site of synthesis of specific terpenoids (carotenoids, chlorophylls, plastoquinones, tocopherols, kaurene...) which play important roles in plant development. For this reason a large number of chemicals have been screened for the ability to inhibit their formation at various control points. There is little apparent structure or biochemical relationship among these chemicals. Moreover, none of them inhibited the synthesis of phytoene, the first C40 carotenoid formed in higher plants through a series of reactions catalyzed by an enzyme complex (phytoene synthetase) involving isopentenyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthetase, geranylgeranyl pyrophosphate-geranylgeranyl transferase and phytoene synthase [1].

Using a partially purified phytoene synthetase and isopentenyl pyrophosphate as a precursor, we have previously reported the inhibition of phytoene synthesis by different allylic substrates as well as inorganic pyrophosphate [2]. In these studies the concentrations required for significant inhibition were approx. 5–50-times higher than the K_m value for isopentenyl pyrophosphate. In continuing this work, our interest in this approach was stimulated by the ability of azidophenethyl pyrophosphate to site-affinity label and inhibit farnesyl pyrophosphate synthetase from avian liver which apparently shows a broad specificity [3] compared to geranylgeranyl pyrophosphate synthetase [4].

We have therefore synthesized aminophenethyl pyrophosphate and its azido derivative and tested their efficiency as inhibitors of phytoene synthesis, using *Capsicum* chromoplast stroma and a partially purified phytoene synthetase enzyme complex.

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2. MATERIALS AND METHODS

2.1. *Inhibitors*

o-Aminophenethanol was purchased from Aldrich. The azido derivative was prepared as described [5]. The corresponding pyrophosphate derivatives were synthesized as reported earlier [6].

2.2. *Enzyme systems*

Chromoplast stroma was prepared from *Capsicum annuum* fruits [6]. The procedure for partial purification of phytoene synthetase was as follows: chromoplast stroma isolated from 5 kg of fruit was subjected to precipitation by sequential addition of 50% (w/w) solution of polyethyleneglycol 6000 in 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM dithiothreitol (1 ml of polyethyleneglycol per 4 ml of stroma). The homogenate was equilibrated for 30 min before each centrifugation at 100000 × *g*. Using this method, 4 successive precipitations were performed and the last precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.6, containing 30% glycerol (buffer A). Fractions of this preparation were applied to a DEAE-Sephacel column (1.5 × 15 cm) equilibrated with buffer A and elution was accomplished by a step gradient of 0, 0.1, 0.2, 0.3, 0.4, 0.5 M KCl in buffer A.

2.3. *Assay of phytoene synthetase*

Phytoene synthetase was assayed by following the incorporation of isopentenyl pyrophosphate into phytoene. The assay mixture (1 ml final volume) contained: 2 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM dithiothreitol, 1 mM ATP, 0.2 M sorbitol, 400 μg Tween-80, 50 mM Tris-HCl buffer, pH 7.6, 0.1 μCi of [1-^{14}C]isopentenyl pyrophosphate (10 mCi/mmol), 1 mg stromal protein or 300 μg of partially purified phytoene synthetase and a definite amount of aminophenethyl pyrophosphate or azidophenethyl pyrophosphate. The reaction was allowed to proceed at 25°C for 4 h except for the determination of initial rates where linearity was observed for 90 min. Kinetic parameters were evaluated by fitting the initial rate data to the Michaelis-Menten equation using a curve fitting program. Phytoene was purified as described [6]. The identity of phytoene was further verified by high-performance liquid chromatography using a μ-Bondapak C18 column (Waters) and two different elution procedures (system I: methanol/cyclohexane/hexane, 80:10:10, v/v, detection at 215 nm; system II: acetonitrile/ethyl acetate/chloroform, 70:20:10, v/v, detection at 280 nm). Alternatively the reaction products were spotted on silica gel plates developed with benzene/ethyl acetate, 90:10, v/v. To test for dimethylallyl pyrophosphate, geranyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate formation, the reaction mixture was treated with acid phosphatase [7]. At completion, the lipid fraction was subjected to high-performance liquid chromatography using system I. For a more precise assay of dimethylallyl pyrophosphate formation, 4 ml of dichloromethane were added after acid phosphatase hydrolysis, then free terpenols in the lipid were esterified in the presence of naphthoic acid and carrier amounts of unlabelled isopentenol and dimethylallyl alcohol [8]. The purified ester fraction was analyzed by high-performance liquid chromatography using the above described column and a mobile phase of acetonitrile/H_2O, 60:40, v/v. Elution of naphthoic acid esters was detected at 240 nm. The incorporated radioactivity was determined by liquid scintillation.

2.4. *Photolysis of azidophenethyl pyrophosphate*

A stock solution of enzyme was transferred to 25 mM Hepes buffer, pH 7.6, containing 0.2 M NaCl, 1 mM 2-mercaptoethanol and 1 mM MgCl by filtration through a Sephadex G25 disposable column (V = 4 ml) equilibrated with the same buffer. This step prevents the non-enzymic reduction of the azide group which might occur in the presence of dithiothreitol [3,9]. Irradiation was accomplished in the presence of 100 μM of azidophenethyl pyrophosphate and 1 mg of enzyme proteins at 4°C in quartz tubes placed under a Mineralight lamp emitting at 254 nm. After each 1 min irradiation the enzyme solution was filtered through the Sephadex disposable column before determination of the activity. In control experiments, the treated enzyme was kept in the dark. The reaction mixture was filtered through the Sephadex G25 column before determination of enzymic activity as described above.

2.5. *Unspecific phosphatase assay*

Nitrophenylphosphate (1 mg) was added to the

basic reaction medium used for phytoene synthesis and after incubation for 1 h at 35°C, 4 ml of 0.5 N KOH were added before reading the absorbance at 405 nm.

3. RESULTS AND DISCUSSION

The potency of aminophenethyl pyrophosphate to act as inhibitor of phytoene synthesis was tested at 20 μM concentration using chromoplast stroma which synthesizes phytoene from isopentenyl pyrophosphate [6]. The reaction products in the total lipid extract from treated and untreated chromoplast stroma were absolutely identical (fig.1a). Isolated phytoene [6] was further unambiguously characterized by high-performance liquid chromatography in system I (retention time, 11.86 min) and system II (retention time, 7.18 min). Under these conditions, the reaction was only 30% inhibited. Since the reaction mixture was not subjected to hydrolysis after the incubation period, the appearance of free geranylgeraniol (fig.1a) was obviously due to interfering phosphatases or prenylhydrolases, which actively hydrolyzed nitrophenyl phosphate. These data allow us to conclude that the latter probably hydrolyzed aminophenethyl pyrophosphate thus vitiating its efficiency. This observation prompted us to use a partially purified phytoene synthetase as described in section 2. Phytoene synthetase emerged from the column at 0.25 M KCl. This partially purified enzyme complex remained undissociated and was devoid of contaminating unspecific phosphatases or prenylhydrolases. Incubation of phytoene synthetase was 20 μM of aminophenethyl pyrophosphate resulted in a strong loss (90% inhibition) of enzyme activity (fig.1b). Furthermore, this inactivation was not followed by accumulation of intermediates (geranyl pyrophosphate, farnesyl pyrophosphate or geranylgeranyl pyrophosphate) resulting from prenyl transfer reactions. In addition dimethylallyl pyrophosphate resulting from isopentenyl pyrophosphate isomerization was not detected.

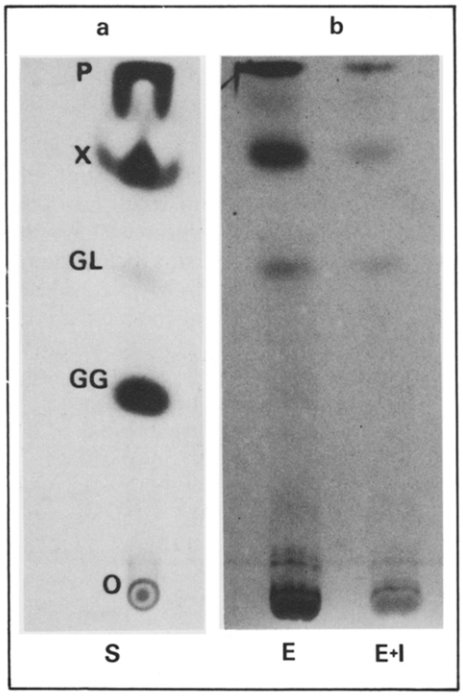

Fig.1. Radioautogram of total lipid extract obtained after incorporation of isopentenyl pyrophosphate. *Capsicum* chromoplast (S) or partially purified phytoene synthetase (E) was incubated with isopentenyl pyrophosphate in the absence of inhibitors. Inhibition experiments with the above enzyme sources (S or E), were performed in the presence of 20 μM aminophenethyl pyrophosphate (I). Since the profile of (S) was similar to that of (S + I), only (E + I) is shown. P, phytoene; X, unidentified prenyl derivative; GL, geranyllinalool; GG, geranylgeraniol; O, origin including prenyl pyrophosphate derivative.

The inhibition of phytoene synthesis was closely paralleled by a decreased incorporation of isopentenyl pyrophosphate into the total extract (not shown). This observation prompted us to investigate whether or not the first enzyme in the biosynthetic pathway (isopentenyl pyrophosphate isomerase) was inhibited. Attempts to achieve this were made by addition of dimethylallyl pyrophosphate (50 μM) to the reaction mixture containing aminophenethyl pyrophosphate. Studies of these samples showed that phytoene synthesis was no longer inhibited. One could simply interpret this effect to mean that when the reaction catalyzed by isopentenyl pyrophosphate isomerase was artificially by-passed, the capacity to synthesize phytoene was restored and consequently this enzyme was the target for the inhibitor. However this explanation is unsatisfactory since one may wonder that the homoallylic site of geranylgeranyl pyrophosphate synthetase remain-

ed unaffected by the inhibitor which is structurally an analog of isopentenyl pyrophosphate. Considering the results from animal farnesyl pyrophosphate synthetase which have established that homoallylic substrates can occupy the allylic as well as the homoallylic site of this enzyme [10] and the reversibility of the reaction catalyzed by isopentenyl pyrophosphate isomerase, an overall explanation of our results could be that isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthetase had much more affinity for dimethylallyl pyrophosphate than for aminophenethyl pyrophosphate, thus eliminating the inhibitory effect.

In an attempt to get more insight into the mechanism involved, double reciprocal plots of the initial reaction velocities against isopentenyl pyrophosphate concentrations were made at 5 and 20 μM inhibitor concentrations. Although a multienzyme complex was used, the inhibition pattern obtained clearly showed that aminophenethyl pyrophosphate acted competitively (fig.2). Dixon plot (fig.2) of reciprocal phytoene synthesis versus the concentration of aminophenethyl pyrophosphate gave a K_i of 12 μM.

Given the fact that aminophenethyl pyrophosphate efficiently inhibits phytoene synthesis we prepared and used the azido derivative to photoinactivate the enzyme complex. In the absence of ultraviolet treatment, the inhibition pattern of azidophenethyl pyrophosphate was similar to that of aminophenethyl pyrophosphate. For photoinactivation treatment, the enzymic preparation was incubated with 100 μM azidophenethyl pyrophosphate before gel filtration. We noted under these conditions a 5 to 10% inhibition in the control preparation kept in the dark (fig.3). This may not be significant: if it is, it suggests that a small fraction of azidophenethyl pyrophosphate remains firmly bound to the enzyme after gel filtration instead of a specific inactivation due to the ultraviolet irradiation alone [11] which has not been observed in this study. In spite of this, the main observation after ultraviolet irradiation was the time-dependent inhibition of phytoene synthetase by azidophenethyl pyrophosphate (fig.3). Since this inhibition was irreversible, we conclude that azidophenethyl pyrophosphate was covalently linked to the active site(s) occupied by isopentenyl pyrophosphate and

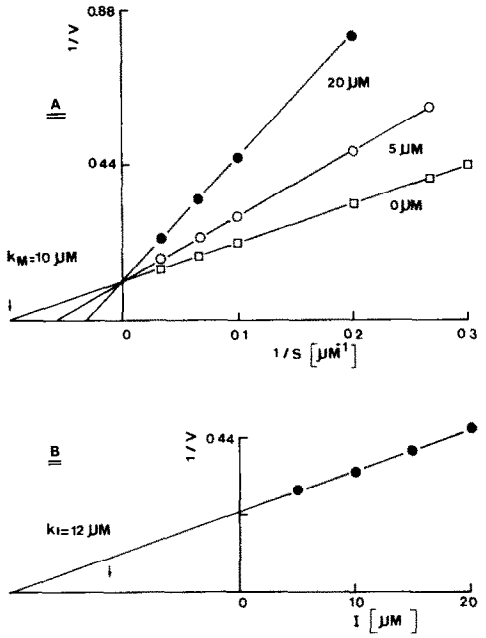

Fig.2. (A) Lineweaver-Burk plot of inhibition of phytoene synthetase activity by aminophenethyl pyrophosphate. Phytoene synthetase was determined over the indicated range of isopentenyl pyrophosphate concentrations (S). Initial velocity (V) is expressed as nanomoles of isopentenyl pyrophosphate incorporated per hour. The concentrations of inhibitor are indicated below the trace. (B) Dixon plot of aminophenethyl pyrophosphate inhibition of phytoene synthetase activity in the presence of 10 μM isopentenyl pyrophosphate and various concentrations of inhibitors (I). V is expressed as shown above.

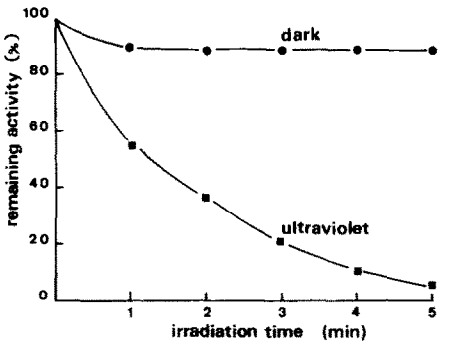

Fig.3. Time-dependent inhibition of phytoene synthetase irradiated in the presence of (100 μM) azidophenethyl pyrophosphate. Control preparations were kept in the dark while step ultraviolet irradiation was for 1 min up to a period of 5 min. After each time the enzymatic solution was filtered through a Sephadex G25 disposable column and incubated as described in section 2.

this reinforces the competitive mechanism stated above.

Considering the fact that phytoene synthetase is a multienzyme complex, plausible targets for these analogs could be at the level of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthetase (fig.4). Taking all the results together, we propose that these two enzymes were indeed the main targets for the following reasons: (i) a drastic reduction of isopentenyl pyrophosphate incorporation, in connection with an absence of accumulation of dimethylallyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate occurred when these inhibitors were used; (ii) addition of dimethylallyl pyrophosphate in the presence of these inhibitors restored the enzyme activity. Some recent results of our own have some bearing on this matter. We have observed recently that geranylgeranyl pyrophosphate synthetase can use isopentenyl pyrophosphate and dimethylallyl pyrophosphate to synthesize geranylgeranyl pyrophosphate (Dogbo and Camara, unpublished). Under these conditions, one may understand that an inhibition of isopentenyl pyrophosphate isomerase in connection to geranylgeranyl pyrophosphate synthetase

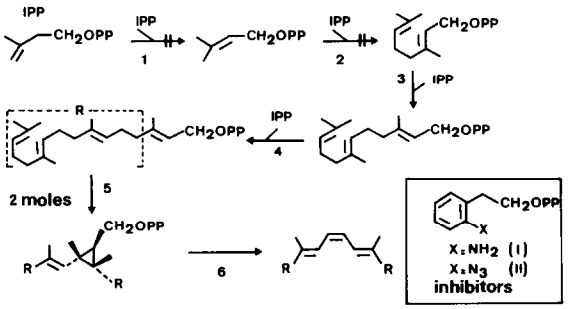

Fig.4. General and theoretical scheme of phytoene synthesis from isopentenyl pyrophosphate and suggested sites of inhibition by phenethyl pyrophosphate derivatives. The different steps are catalyzed by: (1) isopentenyl pyrophosphate isomerase; (2) geranyl pyrophosphate synthetase; (3) farnesyl pyrophosphate synthetase; (4) geranylgeranyl pyrophosphate synthetase; (5) geranylgeranyl pyrophosphate-geranylgeranyl transferase; (6) phytoene synthase. Structure of inhibitors: (I) aminophenethyl pyrophosphate; (II) azidophenethyl pyrophosphate. The enzymes involved in steps 2–4 are generally named prenyltransferase; in *Capsicum* chromoplast these steps are catalyzed by one dimeric protein.

leads to early abortion of chain elongation by prenyl transfer.

More precise information on these points as well as the use of these interesting inhibitors and related derivatives for site-affinity labelling of different plastid enzymes of terpenoid synthesis is underway.

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REFERENCES

[1] Porter, J.W. and Spurgeon, S.L. (1979) Pure Appl. Chem. 51, 609–622.
[2] Camara, B. (1985) Pure Appl. Chem. 57, 675–677.
[3] Brems, D.N. and Rilling, H.C. (1979) Biochemistry 18, 860–864.
[4] Shinka, T., Ogura, K. and Seto, S. (1975) Chem. Lett., 111–112.
[5] Rilling, H.C. (1985) Methods Enzymol. 110, 125–130.
[6] Camara, B. (1985) Methods Enzymol. 110, 244–253.
[7] Fuji, H., Koyama, T. and Ogura, K. (1982) Biochim. Biophys. Acta 712, 716–718.
[8] Yang, T.H., Zabriskie, T.M. and Poulter, D. (1985) Methods Enzymol. 111, 253–263.
[9] Staros, J.V., Bayley, H., Standring, D.N. and Knowles, J.R. (1978) Biochem. Biophys. Res. Commun. 80, 568–572.
[10] Rilling, H.C. (1979) Pure Appl. Chem. 51, 597–608.
[11] Ferrini, U. and Zito, R. (1963) J. Biol. Chem. 238, 3824–3825.