Isopentenyl diphosphate isomerase deficiency in *Synechocystis* sp. strain PCC6803

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Abstract Isopentenyl diphosphate isomerase (IPP isomerase) in many organisms and in plastids is central to isoprenoid synthesis and involves the conversion between IPP and dimethylallyl diphosphate (DMAPP). It is shown that *Synechocystis* PCC6803 is deficient in IPP isomerase activity, consistent with the absence in its genome of an obvious homologue for the enzyme. Incorporation of [1-^{14}C]IPP in cell extracts, primarily into C_{20}, occurs only upon priming with DMAPP in *Synechocystis* PCC6803 and in *Synechococcus* PCC7942. Isoprenoid synthesis in these cyanobacteria does not appear to involve interconversion of IPP and DMAPP, raising the possibility that they are not within the plastid evolutionary lineage.

Key words: Dimethylallyl diphosphate; Isoprenoid biosynthesis; *Escherichia coli*; *Haematococcus*; *Synechococcus* PCC7942; *Synechocystis* PCC6803

1. Introduction

Isoprenoids in photosynthetic organisms, animals, fungi and bacteria play varied and vital roles in photosynthesis, electron transport, signaling and other cell processes. Two C_5 compounds, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), serve as the essential precursors for isoprenoid biosynthesis. IPP isomerase catalyzes the reversible isomerization of IPP to DMAPP, with the equilibrium of the reaction leaning very much in favor of DMAPP [1–3]. It is a soluble enzyme and occurs in plastids as well as in the cytosol of plants. Increased activity of IPP isomerase has been correlated with the increased biosynthesis of carotenoids in developing plastids of maize [4] and has been regarded as rate limiting in the production of carotenoid synthesis in differentiating chloroplasts. IPP isomerase has been purified from chromoplasts of peppers [5] and of daffodils [1].

In bacteria, fungi, mammals, green algae and plants IPP isomerase is encoded by one or more genes with relatively high DNA and predicted amino acid sequence similarity [6]. Characteristically, the IPP isomerases have conserved cysteine and glutamate active site residues [7]. In several plants, two nuclear encoded genes have been identified and one gene product might, therefore, be targeted to the cytoplasm and the other to the chloroplast. Expression in two separate compartments is consistent with the known locations of the two distinct pathways of isoprenoid synthesis in plants. The cytosolic IPP isomerase therefore occurs in conjunction with the mevalonate pathway and the other with the deoxyxylulose-5-phosphate (DOXP) pathway [8], an alternate pathway for isoprenoid synthesis [9,10] in the chloroplasts.

Cyanobacteria are the probable antecedents of chloroplasts [11] and, as in plant plastids, they synthesize isoprenoids via the DOXP pathway [8,10,12]. The photosynthetic apparatus of cyanobacteria is highly similar to that of plants and requires an active isoprenoid pathway for the formation of chlorophyll by phytolation, for carotenoid biosynthesis and photoprotection and for the synthesis of quinones that are essential to electron transport [10,13].

Given the importance of IPP isomerase plastids and the high conservation of amino acid sequence for the enzyme in most organisms [6], it is surprising that the fully sequenced genome of *Synechocystis* PCC6803 lacks an obvious homologue for this enzyme [14]. Two possible explanations are: (a) there is a gene for IPP isomerase in *Synechocystis* PCC6803 but the encoded polypeptide does not share essential features of the known IPP isomerases or (b) isoprenoid synthesis in this cyanobacterium does not require the interconversion of IPP and DMAPP. To distinguish between these two, we analyzed IPP isomerase activity in *Synechocystis* PCC6803 and in *Synechococcus* PCC7942, whose genome has not yet been sequenced. For comparison, we determined the IPP isomerase activity in organisms known to contain this enzyme including *Escherichia coli* [15] with one IPP isomerase gene and the green alga *Haematococcus pluvialis* which has two IPP isomerase genes [16].

2. Materials and methods

2.1. Cell culture and fractionation

Cyanobacteria were cultured at 30°C in continuous light (20 μmol/m^2/s). The culture medium was BG-11 for both *Synechococcus* PCC7942 and *Synechocystis* PCC6803 and was supplemented with 5 mM potassium-TES (*N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, pH 7.5). *H. pluvialis* was grown in a mineral medium as in Sun et al. [16] under continuous light (30 μmol/m^2/s, 22°C). Cultures of *E. coli* (TOP10, from Invitrogen) were grown in LB medium (37°C) with shaking. All organisms were harvested by centrifugation in the log phase of growth and cell pellets were rinsed with 100 mM Tris–HCl (pH 7.5).

Cells were generally broken by sonication in 100 mM Tris–HCl (pH 7.5) in the presence of three protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride; 1.0 mM benzamidine–HCl and 5.0 mM ε-aminocaproic acid. The cyanobacterial cells, *Synechocystis* PCC6803 and *Synechococcus* PCC7942, were difficult to break unless treated with lysozyme (10 mg/ml, 60 min, 37°C and then washed with 100 mM Tris–HCl, pH 7.5) before breakage in a French pressure cell (20 000 psi, 4°C). The supernatant, after centrifugation for 1 h at 60 000 × *g*, was collected and used immediately or stored at −80°C for up to 3 weeks.

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2.2. Enzyme assay

IPP isomerase was assayed according to Spurgeon et al. [17] except that Tris–HCl was used instead of borate buffer. The incubation (37°C) in a total volume of 2 ml (100 mM Tris–HCl, 15 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM DTT, pH 7.5) began upon addition of [1-^{14}C]IPP (Amersham) to a final concentration of 8.5 μM IPP and 8.25×10^5 dpm/ml. The protein concentration of incubated samples ranged from 2.3 to 10.2 mg/ml and was determined by the bicinchoninic acid assay of Smith et al. [18] with bovine serum albumin as standard. Petroleum ether (b.p. 55–110°C) was used for the extraction of the allylic prenols after hydrolysis (37°C, 0.5 N HCl, 20 min). Phosphatase activity, distinguished by the radioactivity extracted into petroleum ether in the absence of acid hydrolysis, was subtracted from paired samples.

Iodoacetamide, a known inhibitor of IPP isomerase, at 5 mM decreased the [1-^{14}C]IPP incorporation of *H. pluvialis* extracts by virtually 100%. Inhibition of the IPP isomerase activity in cell extracts of *E. coli* with the empty vector (pBluescript SK-) was about 50 and 60% with 5 and 20 mM iodoacetamide, respectively. For *E. coli* containing additional copies of the IPP isomerase (pBluescript SK-pEcipi), inhibition was about 20 and 65% for the above respective iodoacetamide concentrations. The results are comparable to those previously published for *E. coli* [19], i.e. 41 and 77% at the same iodoacetamide concentrations.

2.3. Assay for terpenoids

Reversed-phase column chromatography was used to analyze the radioactive isoprenoids. A sample of 0.5 ml of the petroleum ether extract (see Section 2.2) was applied to a column (1×20 cm) of silica gel 6, RP-18 (EM Industries) and equilibrated with 85% acetonitrile/15% water (v/v). After elution with ca. 56 ml of the mobile phase (40 fractions of 1.4 ml each) at a flow rate of 8.4 ml/h, the mobile phase was changed to 100% acetonitrile. A 1 ml aliquot of each fraction was counted in 10 ml of Fisher Scientific Scinti-safe Econo 2 cocktail. The column was calibrated using the following alcohols as standards: geraniol (C_{10}), farnesol (C_{15}), nerolidol (C_{15}), geranylgeraniol (C_{20}) (all from Sigma) and linalool (C_{10}) (from Aldrich).

Petroleum ether-extracted products were also concentrated and analyzed by thin layer chromatography (TLC) on Whatman LKC 18 silica gel (60 Å) plates and developed with methanol/water (4:1 v/v). Radioactivity of the TLC plates was detected by exposure to X-ray film. Also, samples and standards on TLC plates were visibly monitored after exposure to iodine vapor. Standard alcohols, in adjacent lanes, were the same as those used for column chromatography.

2.4. Increasing the copy number of the IPP isomerase gene in E. coli

An *E. coli* genomic DNA fragment (essentially equivalent to GenBank U28375: 42316–43666) containing the entire open reading frame (ORF) for only the IPP isomerase gene (orf182; bases 42879–43424) was inserted in the *Eco*RI and *Sal*I sites of the high copy number plasmid vector pBluescript SK- (Stratagene). Cultures containing this plasmid, pEcipi, or the empty cloning vector were grown in LB medium containing 150 μg/ml ampicillin at 28°C with shaking and harvested in the late log phase.

3. Results and discussion

3.1. Synechocystis PCC6803 and Synechococcus PCC7942 are deficient in IPP isomerase activity

In photosynthetic organisms IPP and DMAPP are precursors for the biosynthesis of compounds essential for photosynthesis: chlorophyll, carotenoids and quinones. Since IPP isomerase is required for the enzymatic conversion of IPP to DMAPP in many plants and algae, it was of interest to determine IPP isomerase activity in cyanobacteria, whose ancestors are the presumed progenitors of plant and algal chloroplasts [11] and yet appear to lack an IPP isomerase homologous to plant enzymes [14].

IPP isomerase activity was ascertained by quantifying the incorporation of [1-^{14}C]IPP into allylic diphosphates by soluble cell extracts. The assay is based on the acid lability of the allylic diphosphates formed by the prenyl synthases and on the solubility of the resulting prenyl alcohols in petroleum ether [20]. As seen in Fig. 1, IPP isomerase activity in *E. coli* and in the photosynthetic alga *H. pluvialis* is easily detectable. In both organisms IPP isomerase genes or cDNAs have been identified previously ([15,16], respectively). However, in the cyanobacterium *Synechocystis* PCC6083, which lacks an open reading frame that would predict a polypeptide similar in sequence to known IPP isomerases [14], and in *Synechococcus* PCC7942 the incorporation of [1-^{14}C]IPP into the allylic fraction was very low. The apparent lack of IPP isomerase activity in *Synechocystis* PCC6803 prevailed over a wide pH range (4–11.5) and was not affected by a range of divalent cations, by disulfide reducing agents or by surfactant treatment. The pellet fraction of these cyanobacteria was similarly without any apparent IPP isomerase activity.

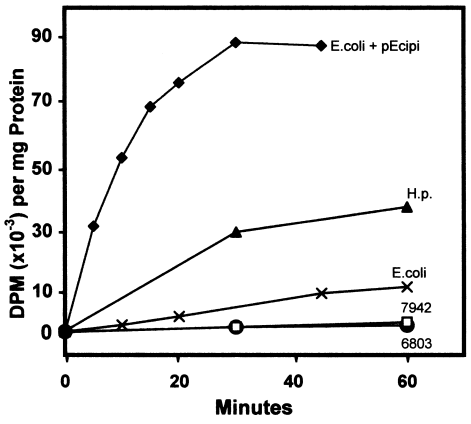

Fig. 1. Time course of IPP isomerase activity as indicated by incorporation of [1-^{14}C]IPP by soluble cell extracts. Organisms: (×) *E. coli* with empty plasmid vector, (♦) *E. coli*+vector containing a copy of the *E. coli* IPP isomerase gene, (▲) *H. pluvialis*, (□) *Synechococcus* PCC7942 and (●) *Synechocystis* PCC6803. After incubation in 100 mM Tris–HCl, 10 mM DTT, 15 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.5, 37°C, aliquots were extracted with petroleum ether and hydrolyzed in acid.

To confirm that the [1-^{14}C]IPP incorporation is directly related to IPP isomerase activity, a plasmid (pEcipi) with a copy of the *E. coli* IPP isomerase gene was introduced into *E. coli* (strain TOP10). Although *E. coli* contains an IPP isomerase-encoding gene (*ipi*) [15], the IPP isomerase activity in *E. coli* may be limiting for isoprenoid biosynthesis: we had previously reported that multicopy plasmid vectors containing genes or cDNAs encoding the *E. coli* or plant and algal IPP isomerases can more than double the accumulation of carotenoids (C_{40} isoprenoids) when introduced into strains of *E. coli* engineered to produce these pigments [6,21,22]. With the many additional *ipi* copies present in *E. coli* containing pEcipi, we find that the initial IPP isomerase activity is much greater, Fig. 1, than that in cultures with the empty vector plasmid (data not shown; dilution experiments indicate the activity to be as much as 80-fold). This result clearly demonstrates that [1-^{14}C]IPP incorporation and IPP isomerase activity are correlated.

3.2. Addition of DMAPP increases IPP incorporation

In Fig. 2 it is shown that the soluble cell extracts of the two cyanobacteria can actively incorporate [1-^{14}C]IPP when DMAPP is added initially ($t=0$) to the reaction mixture. By

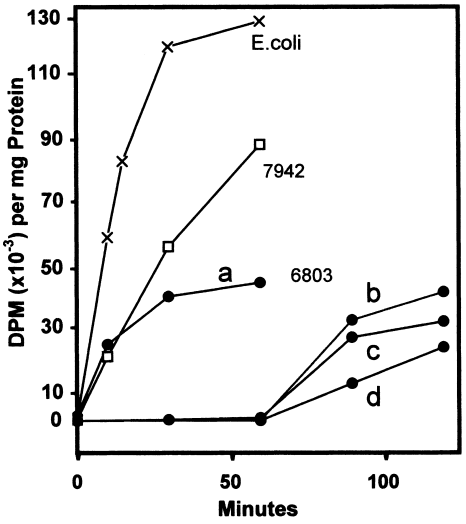

Fig. 2. Addition of DMAPP and [1-^{14}C]IPP at 0 or 60 min. Incorporation into allylic diphosphates upon addition of [1-^{14}C]IPP and DMAPP at $t=0$: (×) *E. coli*, (□) *Synechococcus* PCC7942. Selective additions of [1-^{14}C]IPP and DMAPP were made to cell extracts of (●) *Synechocystis* PCC6803 as follows: (a) DMAPP and [1-^{14}C]IPP added at $t=0$; (b) DMAPP added at $t=0$ and [1-^{14}C]IPP added at $t=60$ min; (c) DMAPP added at $t=60$ min and [1-^{14}C]IPP added at $t=0$; (d) DMAPP and [^{14}C]IPP both added at $t=60$ min. The reaction conditions and assay were the same as for Fig. 1.

about 30 min of incubation the rate of incorporation of [1-^{14}C]IPP is diminished. The extent of [1-^{14}C]IPP incorporation is substantial for both *Synechocystis* PCC 6803 and *Synechococcus* PCC7942, though both are exceeded by that of *E. coli* with the empty vector. We find no significant conversion of IPP to DMAPP or other allylic diphosphates in the cyanobacterial cell extracts lacking DMAPP. If the soluble extract is pre-incubated with DMAPP (for 60 min) before addition of [1-^{14}C]IPP, then we find that after another 60 min (Fig. 2b), the incorporation is nearly as rapid as when added initially (Fig. 2a). This suggests that the reaction components are reasonably stable. Conversely, if we pre-incubate with [1-^{14}C]IPP (60 min) and then add DMAPP (Fig. 2c), the incorporation of radioactivity is about the same. Finally, there is diminished incorporation (Fig. 2d), if both [1-^{14}C]IPP and DMAPP are not added until after the pre-incubation (60 min). The lower incorporation could result from a diminished IPP concentration from competing reactions such as the loss of one phosphate, or incorporation into products that are not recovered in the petroleum ether fraction after hydrolysis.

Incorporation of [1-^{14}C]IPP by *Synechococcus* PCC7942 extracts was similar to that of *Synechocystis* PCC6803 and our results indicate that (a) there is little or no conversion of IPP to DMAPP in these two cyanobacteria and (b) that DMAPP is a limiting substrate for isoprenoid synthesis in the cell-free extracts of the two cyanobacteria in these studies.

3.3. IPP incorporation into terpenoids

To ensure that the incorporation of [1-^{14}C]IPP was not spurious, petroleum ether extracts of acid-hydrolyzed incubation products were examined by reversed-phase chromatography, which separates isoprenoids of different carbon chain lengths. A major peak in the elution profile dependent on the addition of DMAPP (Fig. 3, fractions 46–56) was identified as C_{20} by the co-migration of the radioactivity with geranylgeraniol on this column and by TLC. The formation of such a product is consistent with the presence in the genome of two prenyltransferase-like ORFs [14] that correspond to putative geranylgeranyl diphosphate synthases. A smaller peak (fractions 25–30) similarly co-chromatographs with farnesol and, thus, is most probably a C_{15} compound. The other minor peaks were not identified. Incorporation of [1-^{14}C]IPP by the cell extract of *Synechococcus* PCC7942 gives essentially an identical elution profile under these conditions.

Our results for the two cyanobacteria (*Synechocystis* PCC 6803 and *Synechococcus* PCC7942) indicate that IPP isomerase activity is not detected using the conditions employed. Incorporation of IPP by extracts supplemented with DMAPP appears to be largely via prenyltransferase(s) and probably via geranylgeranyl diphosphate synthase, as has been shown in *Synechococcus elongatus* [23]. If IPP isomerase is absent, as can be inferred by the low incorporation of the radiolabeled IPP in the absence of DMAPP, then what is the source of DMAPP within cyanobacterial cells? Evidence for isoprenoid production in cyanobacteria via the DOXP pathway is strong and the known genes for this pathway are present in the genome of *Synechocystis* PCC6803. Labeling studies performed with precursors of the DOXP pathway on *Catharanthus roseus* cell cultures, while validating the expected incorporation into phytol and lutein, ruled out DMAPP as "the compulsory precursor of IPP" [24]. We agree with this assessment from our cyanobacterial findings and we also suggest the converse, i.e. that IPP is not the compulsory precursor of DMAPP. The possibility of independent synthesis of both DMAPP and IPP must be considered.

Interestingly, *E. coli* is a bacterium possessing the DOXP pathway. However, IPP isomerase is not essential for growth in this organism, because upon deletion of the *ipi* gene the bacterium grew at a rate comparable to wild type [15]. Nevertheless, additional copies of the IPP isomerase gene enhanced enzyme activity in cell-free *E. coli* extracts which underscores its role in enhancing carotenoid production in *E. coli* cells bio-

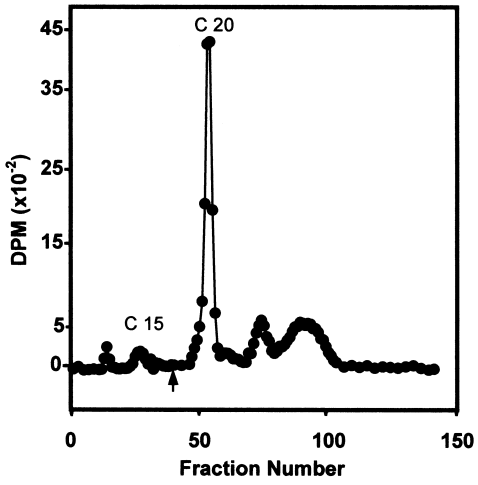

Fig. 3. Elution profile from reversed-phase chromatography of radiolabeled isoprenoids formed in a 60 min incubation of *Synechocystis* PCC6803 extract after extraction with petroleum ether and acid hydrolysis. The peaks labeled C_{20} and C_{15} co-chromatograph with geranylgeraniol and farnesol, respectively, on the same column and on TLC plates. The initial eluant was 85% acetonitrile–15% water which was changed to 100% acetonitrile at the arrow.

engineered with the carotenoid-enzyme genes [21,22]. However, when an *ipi* gene of yeast was inserted into *Synechocystis* PCC6803 by Lagarde et al. [25] the carotenoid production was not enhanced. Although mRNA for the yeast gene was detected, it is unknown why carotenoid production was not enhanced, or whether IPP isomerase activity is present in these transformed cells.

In chloroplasts, the DOXP pathway is assumed to lead to the production of a pool of IPP followed by IPP isomerase-mediated conversion to DMAPP and hence to isoprenoids of various lengths and functions. Thus, the deficiency of IPP isomerase activity in *Synechocystis* PCC6803 and *Synechococcus* PCC7942 raises some interesting questions regarding the assumption that cyanobacteria are the progenitors of chloroplasts. If chloroplasts are derived from cyanobacteria and if IPP isomerase is a central enzyme in the synthesis of critical photosynthetic components, our results would imply that either these two species of cyanobacteria are not part of the chloroplast lineage or chloroplasts gained IPP isomerase(s) secondarily. Thus, a search for homologous genes for this enzyme in other cyanobacteria will be of interest and the pathways for production of IPP and DMAPP in cyanobacteria and in chloroplasts may give insight into the evolution of chloroplasts from cyanobacteria.

Our results show that IPP incorporation, primarily into C_{20} compounds, in cell extracts of *Synechocystis* PCC6803 and *Synechococcus* PCC7942, is entirely dependent on added DMAPP. We suggest that IPP and DMAPP are synthesized separately and that IPP isomerase interconversion is not required in these cyanobacteria.

4. Note added in proof

Pertinent to this paper are the findings by Charon et al. (2000, Biochem. J. 345, 737–742) suggesting that in *E. coli* there are two different routes of synthesis to IPP and DMAPP.

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