Purification and reactivation of recombinant *Synechococcus* phytoene desaturase from an overexpressing strain of *Escherichia coli*

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The *Synechococcus* phytoene desaturase has been isolated from an overexpressing strain of *Escherichia coli*. The plasmid pPDS*del*35 mediated the overexpression of the full-length polypeptide directly. The recombinant protein comprised 5% of the total cellular protein and was found predominantly in the inclusion body fraction. Urea was used to solubilize the recombinant protein from the inclusion fraction and the protein was subsequently purified to homogeneity on a DEAE-cellulose column. The purification scheme yielded 4.0 mg of homogeneous desaturase protein after a 20-fold purification, recovering 40% of the original protein from a 100 ml suspension culture of *E. coli*. The recombinant desaturase had an apparent molecular mass of 53 kDa on SDS/PAGE and crossreacted with an antiserum raised against the expressed protein. Desaturase activity was restored upon the removal of urea. The enzyme catalysed the conversion of phytoene to ζ-carotene via phytofluene. These products of the desaturase reaction existed predominantly in a *cis* configuration. Lipid replenishment enhanced activity. NAD^+ and $NADP^+$ were observed to be involved, whilst FAD was an ineffective electron acceptor.

INTRODUCTION

Carotenoid formation can be divided conveniently into several distinct phases: (1) the early reactions from mevalonic acid (MVA) to geranylgeranyl pyrophosphate (GGPP), which are common to many isoprenoids; (2) phytoene formation; (3) desaturation steps; (4) cyclization; and (5) later modifications such as hydroxylation [1]. Desaturation of the colourless C_{40} phytoene molecule proceeds via a series of sequential dehydrogenation reactions. At each desaturation step a new double bond is introduced in a symmetrical manner, extending the phytoene chromophore by two conjugated double bonds. The enzyme responsible for this conversion is phytoene desaturase. This enzyme is the principal target site for bleaching herbicides (see [2] for a review).

The phytoene desaturase from the cyanobacterium *Synechococcus* PCC 7942 has been used extensively in the study of such inhibitors [3]. As in other carotenogenic organisms, the phytoene desaturase (pds) of *Synechococcus* and related species is membrane-bound, being associated with the thylakoid membranes [4] and requiring the action of detergents for its release [5]. Biochemical characterization *in vitro* has indicated that the reaction involves oxidized nicotinamide nucleotides and that the enzyme is sensitive to feedback regulation by other carotenes [6]. It has been observed also that phytoene desaturase catalyses the rate-limiting step in the pathway [3].

The gene encoding phytoene desaturase and *Synechococcus* has been isolated and characterized [7] and shows close sequence similarity to the higher-plant enzyme [8,9], but poor sequence similarity to those isolated from *Neurospora crassa* [10], *Rhodobacter capsulatus* [11,12] as well as *Erwinia uredovora* [13] and *Erwinia herbicola* [14].

Functional complementation studies with various phytoene desaturase genes has illustrated the diversity of product formation that exists between different species [15]. The reaction catalysed by *Synechococcus* pds appears to be very similar to that of the higher-plant enzyme, where phytoene desaturation ends at the level of ζ-carotene (two additional double bonds) and proceeds via phytofluene (one additional double bond). In contrast, the *Erwinia* and *Neurospora* enzymes perform desaturation to lycopene (four additional double bonds).

This report describes the isolation of the *Synechococcus* pds from an *Escherichia coli* strain harbouring a construct responsible for overexpression of the recombinant protein. Reactivation of the desaturase enabled the elucidation of the cofactor and lipid requirements of the enzyme.

MATERIALS AND METHODS

Organisms and culture conditions

E. coli strain JM101, the host for the vector pPDS*del*35 was grown in Luria Bertani (LB) broth containing ampicillin (50 μg/ml) as described by Maniatis et al. [16]. The growth and maintenance of the C5 mutant strain of *Phycomyces blakesleeanus* has been described previously [17].

Vector construction and protein overexpression

The protocols concerning the construction of the plasmid pPDS*del*35 have been described previously [15]. The recombinant protein was overexpressed by inducing an overnight culture in LB containing 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h in accordance with Maniatis et al. [16].

Isolation and purification of the recombinant *Synechococcus* phytoene desaturase

IPTG-induced cells were harvested by centrifugation at 6000 *g* for 5 min at 4 °C. Pelleted cells were resuspended in 30 mM Tris/HCl buffer, pH 8.0, containing 20% (w/v) sucrose, 1 mM EDTA and lysozyme (1 mg/ml), then placed on ice for 10 min. After centrifugation at 12000 *g* at 4 °C for 15 min, the super-

Abbreviations used: DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria broth; PBS, phosphate-buffered saline; pds, phytoene desaturase.

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natant (periplasmic fraction) was discarded. The cells were resuspended in 0.1 M Tris/HCl, pH 8.0, containing 1 mM EDTA, and broken open by freeze–thawing (repeated three times). The viscosity of the resulting suspension was reduced by incubation with DNAase (10 μg/ml) for 30 min. Centrifugation at 12000 *g* for 10 min at 4 °C gave the cytoplasmic fraction (supernatant) and pelleted membranes. The membrane fraction was solubilized by resuspension in 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, 1.0% (w/v) Triton X-100 and 10 mM EDTA, then maintained on ice for 15 min prior to centrifugation at 12000 *g* at 4 °C for 15 min. The insoluble material (inclusion bodies) that remained was washed with distilled water and either stored at −20 °C or used immediately. The inclusion material containing the recombinant phytoene desaturase was solubilized with 8 M urea buffered in 25 mM Tris/HCl, pH 8.0, containing 1 mM dithiothreitol (DTT). Following a rapid incubation period (5 min) the solution was centrifuged at 12000 *g* at 4 °C for 5 min to remove any remaining insoluble debris. The urea solubilized inclusion material (about 3 ml) containing the recombinant phytoene desaturase was applied onto a DEAE-cellulose column (typically 1.5 cm × 6 cm). Unadsorbed proteins were washed from the column with running buffer (25 mM Tris/HCl, pH 8.0, containing 1 mM DTT, 1 mM EDTA and 8 M urea). Proteins bound to the column were eluted with a linear NaCl gradient (0–300 mM). A flow rate of 1 ml min^{-1} was used throughout chromatography and the eluent was monitored at 280 nm with a u.v. detector. Fractions of 4 ml were collected; those containing phytoene desaturase were pooled and stored at −20 °C in aliquots of 0.5 ml.

Reactivation of phytoene desaturase

A gel-filtration column (PD10, Pharmacia) was used to remove urea from the DEAE-purified preparation. Approximately 0.5 ml (about 300 μg protein) of the sample was exchanged into 0.4 M Tris/HCl buffer, pH 8.0, containing 5 mM DTT. During buffer exchange the protein content was diluted about twofold. DTT was added to the enzyme solution from a 1 M stock solution until a final concentration of 10 mM DTT was reached and the sample was maintained on ice for 30 min prior to assay. Lipids (purchased from Sigma) were prepared in chloroform as stock solutions (1 mg/ml). Soybean phospholipids were partially purified prior to use by washing in acetone for 24 h and the insoluble fraction that remained was suspended in diethyl ether for further use. Lipid suspensions were prepared by adding buffer (50 mM Tris/HCl, pH 8.0, containing 1 mM DTT) to a dried lipid residue and the mixture was sonicated for 20 min. An aliquot of this suspension was added to the enzyme solution to give a final concentration about 500 μg per incubation.

SDS/PAGE

SDS/PAGE was performed in accordance with the method of Laemmli [18]. The molecular mass of the recombinant protein on SDS/PAGE was determined by comparison with the relative mobilities of the standard proteins myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa).

Production of antiserum

Membrane fractions from the overexpressing *E. coli* strain were prepared and electrophoresed under denaturing conditions on a 10% polyacrylamide gel. The gel was stained for protein with Coomassie Blue, the heavily stained band corresponding to the recombinant pds was removed and protein recovered from the gel by electroelution. The electroeluted sample was further purified on a 10% gel containing 4 M urea and the protein from the single band that resulted was electroeluted again.

About 100 μg of the electroeluted antigen was emulsified with complete Freund's adjuvant and used to immunize a rabbit. Booster injections were made at 3 and 5 weeks after the initial immunization with 80 μg of the antigen. After 2 weeks from the last immunization, the rabbit was bled and serum collected by centrifugation of the blood at 3000 *g* for 15 min.

Western blotting

Following SDS/PAGE proteins were electrophoretically transferred from acrylamide gels to nitrocellulose (0.45 μm pore size, Schleicher and Schüll, Dassel, Germany). Transfer was performed in 25 mM Tris/HCl buffer, pH 8.0, containing 190 mM glycine and 20% (by vol.) methanol, for 60 min at a current of 0.75 A. Following transfer the nitrocellulose was treated with phosphate-buffered saline (PBS) (10 mM sodium phosphate, pH 7.0, with 0.14 M NaCl and 5% milk protein) for 1 h. The anti-pds antiserum was then added at a dilution of 1:1000 and incubated overnight. Prior to the addition of the secondary antibody, the nitrocellulose was washed three times with PBS. Goat anti-rabbit IgG labelled with peroxidase was used as the secondary antibody at a dilution of 1:10000 for an incubation period of 1 h. The blot was washed three times with PBS before colour development, which was performed in PBS with chloronaphthol (0.018%, w/v) and H_2O_2 (0.006%, v/v).

Enzyme assay

A coupled assay procedure using the *P. blakesleeanus* C5 mutant to produce [^{14}C]phytoene *in situ* was used to determine desaturase activity, as described previously [19,20]. The assay mixture (final volume 0.5 ml) contained 5 mM DTT, 1 μmol NADP, 5 μmol ATP, 6 μmol $MgCl_2$, 4 μmol $MnCl_2$, 150 μl of C5 cell extract and 0.25 μCi (3*R*)-[2-^{14}C]mevalonic acid lactone, converted to its Na^+ salt with 0.01 M NaOH before addition. The reactivated enzyme (approx. 20 μg of protein) was added to this assay mixture and incubated at 30 °C in dim light for 2 h. When determining cofactor requirements for desaturation, the [^{14}C]phytoene formed by the C5 cell extract was precipitated in association with protein, then resuspended in fresh incubation buffer (0.4 M Tris/HCl, pH 8.0, containing 5 mM DTT and the appropriate cofactors) [20]. The reactivated desaturase was added to this suspension and incubated for a further 2 h. Incubations were terminated by adding methanol (1.5 ml) and were stored routinely at −20 °C. The radioactive isoprenoids formed were extracted from the aqueous incubation mixture with 10% diethyl ether in light petroleum (boiling range 35–80 °C). Analysis of the reaction products was performed by h.p.l.c. A 3 μm reversed-phase Nucleosil C_{18} column (250 mm × 4.6 mm) was used to separate the carotenes of interest. The mobile phase consisted of acetonitrile/methanol/2-propanol (85:10:5, by vol.). Radioactivity was detected online with a radioactivity flow detector (Raytest Ramona LS). The reaction products were identified by chromatography with standard carotenes added before separation. The absorbance of the standard carotenes was recorded with a programmable Jasco u.v./visible photospectrometric detector model 8201. Conversion rates were calculated as the ratios of radioactivity accumulated in the product ζ-carotene and the sum of radioactivity in phytoene and ζ-carotene, and are expressed as percentages.

Protein determinations

Protein concentration was determined using the method of Lowry et al. [20a]. Protein concentrations were also estimated from SDS/PAGE by comparison with the staining intensity of a bovine serum albumin protein standard (2 μg, 5 μg and 10 μg), as reported previously by Sasiak and Rilling [21].

RESULTS

Expression of pds in *E. coli*

SDS/PAGE analysis of cellular proteins from IPTG-induced *E. coli* cells harbouring the plasmid PDS*del*35 indicated the presence of a prominently stained band corresponding to a molecular mass of 53 kDa (Figure 1, lane 2). This is consistent with the molecular mass predicted from the isolated *Anacystis pds* gene [7]. In cells containing the expression plasmid without the *pds* gene insert and also in uninduced cells the prominent band was absent. It was also observed that the intensity of the 53 kDa band increased with time of incubation with IPTG. Based on these criteria the heavily stained band migrating to a molecular mass of 53 kDa on SDS/PAGE was deduced to be the recombinant pds. Densitometric comparisons with stained protein standards suggested that the recombinant protein constituted about 5% of the total cellular protein.

Closer SDS/PAGE analysis suggested that the recombinant protein existed as a doublet. The less abundant (20%) form, which had a slightly higher molecular mass (by approx. 2 kDa) than that expected from the *pds* gene, is probably due to an additional start codon 23 amino acids upstream from the coding region responsible for the native polypeptide. Indiscriminate reading thus results in the expression of the two forms which subsequent immunological studies have shown to be related (data not shown).

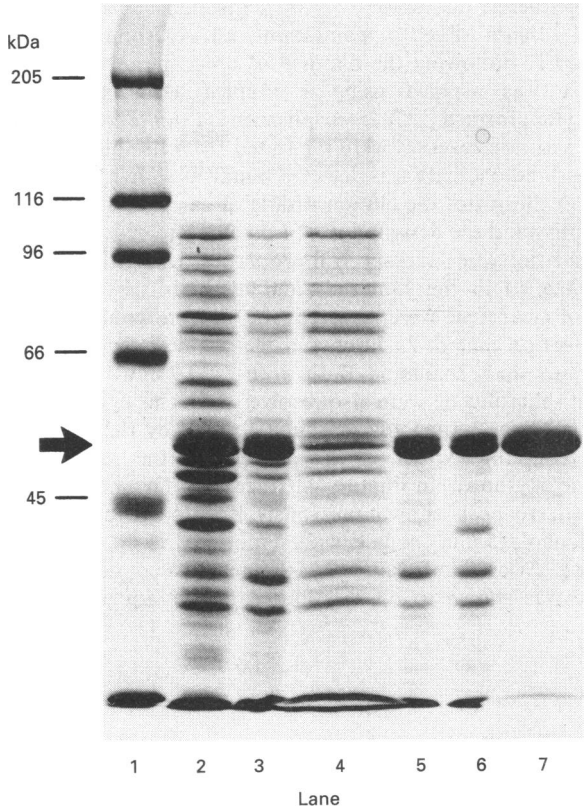

Figure 1 Purification of the *Synechococcus* recombinant phytoene desaturase monitored by SDS/PAGE

Fractions during the purification protocol were analysed by electrophoresis on a 10% polyacrylamide gel. Lane 1, molecular mass markers (kDa); lane 2, induced cells, 20 μg of protein; lane 3, isolated membranes, 15 μg of protein; lane 4, supernatant after detergent treatment of membranes, 15 μg of protein; lane 5, inclusion bodies, 10 μg of protein; lane 6, urea-solubilized inclusion bodies, 10 μg of protein; lane 7, purified recombinant desaturase from DEAE-cellulose column, 8 μg of protein. The migration of the recombinant pds is indicated by the arrow.

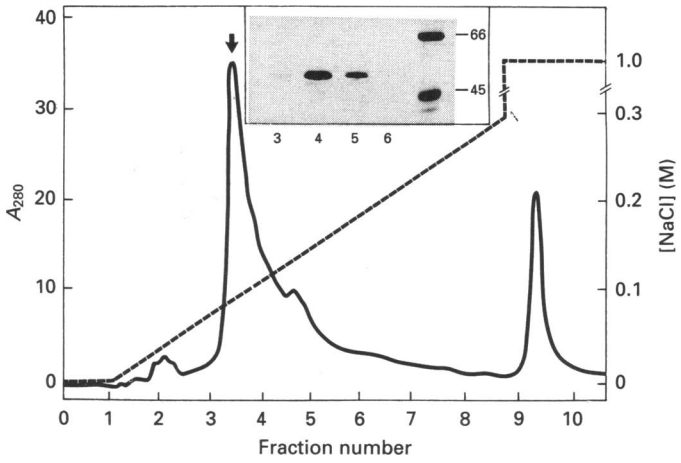

Figure 2 DEAE-cellulose ion-exchange chromatography of the urea-solubilized inclusion body material

The urea solubilizate (around 15 mg of protein) was applied onto the column and the bound proteins were eluted with a linear NaCl gradient (0–0.3 M), illustrated by the broken line (– – –). Fractions of 4–5 ml were collected. The u.v. absorbance (A_{280}) of protein eluted from the column is shown by the continuous line; the arrow indicates the peak of the recombinant phytoene desaturase protein. SDS/PAGE analysis of fractions 3, 4, 5 and 6 is shown in the insert. The equivalent of 15 μl of each fraction was applied to the gel.

Solubilization and purification of the recombinant phytoene desaturase

Analysis of subcellular fractions by SDS/PAGE indicated that the recombinant protein was present exclusively in the membrane fraction (Figure 1, lane 3). Treatment of the membrane fraction with a variety of detergents, including Triton X-100, Tween 40, cholic acid and zwitterionic agents, over the concentration range 0–3% (v/v) was ineffective in releasing the recombinant protein, although a slight solubilization (1% of the total recombinant protein) was apparent following treatment with 3% (w/v) Triton X-100. Despite its inability to solubilize the recombinant protein, detergent treatment did release numerous membrane-associated proteins (Figure 1, lane 4) aiding purification. Typically the insoluble material remaining after detergent treatment is termed the inclusion body material, and it is within this fraction that the recombinant protein was sequestrated as the principal component (Figure 1, lane 5). Further treatment with detergents failed to solubilize the recombinant pds from the inclusion bodies, as did the action of lipases and an alkaline environment (pH 10). Solubilization was achieved eventually with the denaturant urea at a concentration of 8 M (Figure 1, lane 6). However, the

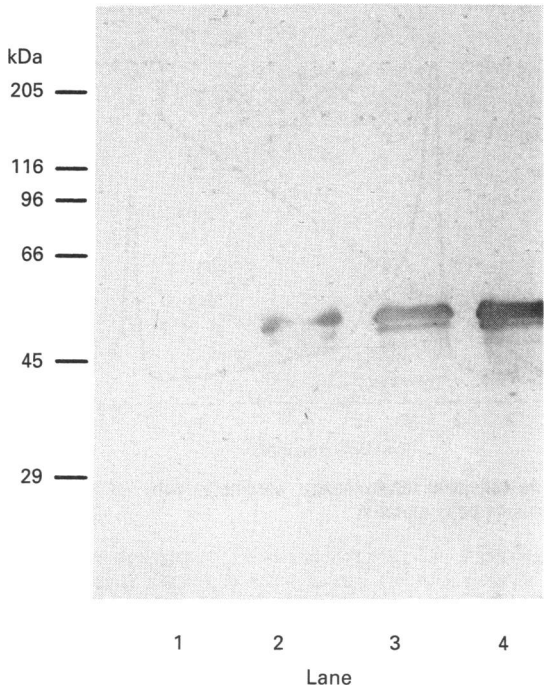

Figure 3 Western blot analysis with an antiserum raised against the recombinant protein

Lanes 1–4 contain the purified protein applied to a polyacrylamide gel (10%) at concentrations of 0.5, 1.5, 3.0 and 6.0 μg, respectively. The positions of the blotted molecular-mass markers (kDa) are indicated.

solubilization was relatively non-selective and did not result in significant purification.

The urea-solubilized inclusion body material was chromatographed on a DEAE-cellulose column, as detailed in the Materials and methods section. The profile of eluted protein (Figure 2) showed that virtually all the protein applied to the anion exchanger was bound; subsequent elution with NaCl gave a profile characterized by a major peak at a NaCl concentration of 100 mM. SDS/PAGE of the eluted fractions indicated that the recombinant protein was responsible for this peak (Figure 2, insert). The doublet nature of the recombinant protein observed on SDS/PAGE became more pronounced as salt elution progressed, suggesting that the higher-molecular-mass form was slightly more cationic in nature.

An antiserum raised against the recombinant protein was used to monitor purification by Western blotting. Crossreactivity with the purified preparation is shown in Figure 3. The antiserum detected approx. 1–10 μg of protein after a 1:1000 dilution. Preimmune serum showed no crossreactivity with the purified protein even when loaded at high concentrations (6–10 μg), demonstrating good specificity. This antiserum has been used further in the immunocharacterization of phytoene desaturase in cyanobacteria and higher plants (H. Linden, unpublished work).

The overall purification procedure (summarized in Table 1) resulted in 4.0 mg of homogeneous protein after a 20-fold enrichment with a 40% recovery, from an initial *E. coli* suspension culture of 100 ml. The absolute purity of the protein was judged by SDS/PAGE, which showed a single stained band, even after overloading (6–10 μg), corresponding to a dissociated molecular mass of 53 kDa (Figure 1, lane 7). The purified protein was susceptible to degradation, particularly after electroelution or prolonged or repeated freezing. These degradation products were also detected by the antiserum (Figure 3).

Reactivation of desaturase activity

Previous complementation studies with the *Erwinia* gene cluster [15] have illustrated functional expression of the *pds* gene. However, *in vitro* activity was barely detectable in crude extracts and was lost during subcellular fractionation as well as purification, due to the presence of urea, which is a prerequisite for solubilization. For the purified preparation to regain activity the denaturant had to be removed to allow the protein to refold. This was done either by dialysis or by passage through a desalting-gel-filtration column (PD10), maintaining an environment containing DTT. Following the removal of urea, the recombinant desaturase was assayed using a coupled assay procedure [19,20,22] that formed [^{14}C]-15-*cis*-phytoene *in situ*. The products of the desaturase reaction were separated from its substrate by h.p.l.c. and radioactivity was detected online as shown in Figure 4. Trace A illustrates the elution profile of carotene standards; their identity and the designation of isomers were deduced from the relative polarities and spectral properties. Using the h.p.l.c. system detailed in the Materials and methods section, three isomers of ζ-carotene were resolved: the all-*trans* configuration had a retention time of 25.7 min and the two *cis* isomers eluted at 27.0 min and 28.0 min. Both *trans* (33.7 min)- and *cis* (35.0 min)-phytofluene were also resolved with this system. The separation of the radioactive products formed by the C5 *Phycomyces* coupling extract in the absence of the reactivated desaturase is shown in Figure 4 (trace B). The radioactive products have been shown previously [23] to correspond to prenyl alcohols (5 min), squalene (20.5 min) and 15-*cis*-phytoene (40.0 min). After incubation with the reactivated desaturase, phytoene was shown to be desaturated by the appearance of

Table 1 Purification of phytoene desaturase from *Synechococcus* expressed in *E. coli*

Fraction	Volume (ml)	Weight (mg)	Total protein* (mg)	Pds protein† (mg)	Specific pds content	Purification (fold)	Recovery (%)
Cells	100	1240	184	10	0.05	1	100
Membranes	10	216	80	8.3	0.104	2.0	83
Inclusion bodies	10	188	40	5.8	0.145	2.9	58
Solubilized inclusion bodies	10	–	22	5.5	0.25	5.0	55
DEAE fraction	4	–	4‡	4	1	20	40

*Estimated from SDS/PAGE using bovine serum albumin calibration (0–10 μg); †determined by modified Folin–Lowry for membrane proteins; ‡4 mg of homogeneous protein obtained from a total crude extract of 184 mg of protein with a 20-fold enrichement and a 40% yield.

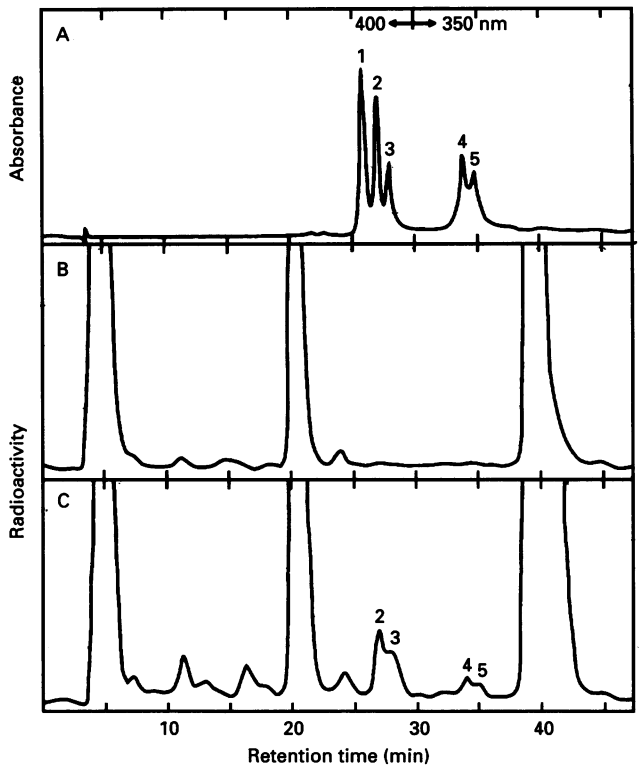

Figure 4 H.p.l.c. separation of radiolabelled carotenes formed by the reactivated phytoene desaturase after isolation

Trace A illustrates the elution profile of authentic marker carotenes required for identification; 1, *trans*-ζ-carotene; 2, *cis*-ζ-carotene; 3, *cis*-ζ-carotene; 4, *trans*-phytofluene; 5, *cis*-phytofluene. The corresponding radiolabelled carotenes resulting from the reactivated desaturase are shown in trace C, where identical retention times with the standards are numbered appropriately. In trace B the radiolabelled compounds resulting from the C5 coupling extract alone are shown as a control.

phytofluene (34.0 min) and ζ-carotene (26.0 min). Analysis with other h.p.l.c. systems, as described previously [22], showed that no desaturation beyond ζ-carotene occurred under the experimental conditions used. ζ-Carotene was the principal product of the recombinant phytoene desaturase reaction, constituting 80 %, with the intermediate phytofluene comprising the other 20 % of the total desaturase products. The ζ-carotene formed was exclusively *cis* in its configuration, although a mixture of *cis* isomers was observed. The relative abundances of the *cis* forms varied, but it was apparent that the *cis* isomer eluting directly after the *trans* configuration was always predominant. The positions of the *cis* bonds within the ζ-carotene molecule must await definitive designation by n.m.r. Of the phytofluene isomers, the *trans* configuration was reproducibly more abundant (77 %), with significantly lower levels of the *cis* form.

The conversion rate of phytoene into desaturase products was typically 10 % for the reactivated desaturase. This is less than that obtained with crude *Anacystis* membranes, but considerably higher than that found in crude extracts of the *E. coli* strain overexpressing pds. Further optimization of the purified desaturase activity was attempted by lipid replenishment (Table 2). With the exception of digalactosyl diacylglycerol, the presence of lipids enhanced activity, the most effective lipids being monogalactosyl diacylglycerol and phospholipid. Of the fatty acids, distearin (C_{18}) was responsible for the greatest increase in activity, whilst fatty acids possessing a chain length of C_{20} were less favourable.

Table 2 Effect of lipids on reactivation of phytoene desaturase

Conditions	Activity (%)*
Enzyme (pds)	100†
+ Monogalactosyl diacylglycerol	280
+ Digalactosyl diacylglycerol	0
+ Chaperone *GroE*‡	107
+ 1 % Tween 40	0
+ Cardiolipin	123
+ Phospholipid	212
+ Lipid mixture§	135
+ 1,3-Dipalmitin (C_{16})	147
+ Distearin (C_{18})	206
+ Diacharachidin (C_{20})	120
+ Tripalmitin (C_{16})	161
+ Tristearin (C_{18})	139
+ Triacharachidin (C_{20})	117

*Conversion rate of enzyme assayed as described in the Materials and methods section; †conversion rate of 10.2%, from 24709 d.p.m. of phytoene with a specific radioactivity of 140400 d.p.m./mg of protein; ‡the purified preparation of the recombinant protein was incubated with 100 μg of *GroE* and 3 μmol ATP for 20 min prior to assay; §lipid mixture contained monogalactosyl diacylglycerol, digalactosyl diacylglycerol, cardiolipin and phospholipid; each lipid was applied at a concentration of 120 μg.

Table 3 Cofactor requirement for desaturation

Incubation conditions	Activity (%)*
None	7.0
$NADP^+$, NAD^+, FAD	4.9
$NADP^+$	12.0†
NAD^+	10.4
FAD	5.6
NADPH	4.7

*Activity quantified as percentage conversion; †equivalent to a specific radioactivity of 68700 d.p.m./mg of protein from the conversion of 11413 d.p.m. of phytoene. All the cofactors were added to the final optimal concentration of 1 μmol.

Cofactor requirement for desaturation

Attempts were made to determine the cofactors involved in the desaturation of phytoene using a conventional coupled assay procedure. Unfortunately, these experiments were inconclusive, as endogenous cofactors present prevented elucidation. To circumvent this practical difficulty, the [^{14}C]phytoene formed in association with protein was precipitated [20] and then used as a source of substrate in the presence of a variety of cofactors. Table 3 shows the effects of nucleotide cofactors on desaturation. Some activity can be observed without their addition due to endogenous cofactors originating from the C5 extract. However, desaturation was enhanced by both NAD^+ and $NADP^+$. When used in a reduced form this enhancement disappeared. This was independent of the additional presence of FAD. Stimulation of desaturase activity was not significant in the presence of FAD. Neither the geometric isomers nor the ratio of desaturase products changed significantly under varying cofactor conditions.

DISCUSSION

This article describes the isolation of a phytoene desaturase originating from the cyanobacterium *Synechococcus*. To our knowledge, it is the first report of an isolated phytoene desaturase

derived from a photosynthetic source. Sequence similarity with the higher-plant genes from tomato [9] and soybean [8] suggests that the *Synechococcus* enzyme is an authentic model for the higher-plant protein and provides a valuable comparison with the recently isolated phytoene desaturase from the non-photosynthetic bacteria *Erwinia uredovora* [24].

Isolation was facilitated by overexpression of the full-length *pds* gene in *E. coli*. The recombinant protein constituted 5% of the total cellular protein and, like many other overexpressed systems, was localized in the inclusion body fraction, which is usually associated with inactivity, denaturation and insolubility of the protein. The release of the recombinant protein from the inclusion bodies required treatment with the denaturant urea. Despite the presence of urea, subsequent purification to homogeneity was achieved using a rapid and reproducible purification protocol, that enabled the attainment of large quantities of pure protein that could be reactivated upon the removal of urea. These findings mean that the system is suitable for monoclonal and polyclonal antibody production, as well as making crystallization and the study of other physical-chemical properties an interesting possibility.

The reactivated pds was able to desaturate phytoene to ζ-carotene via phytofluene, thus two double bonds are inserted into the phytoene molecule alternately to the left and right of the central chromophore at positions 11 and 11′, respectively. A *cis* geometric configuration of the desaturase products was found relatively exclusively. Such products of desaturation are characteristic of those found in higher plants and other photosynthetic organisms, but differ from the desaturase products found in fungi [10] and in non-photosynthetic bacteria [15,24], where phytoene desaturase is responsible for the desaturation of phytoene to lycopene via phytofluene, ζ-carotene and neurosporene, with geometric isomers predominantly in the *trans* configuration and with a non-enzymatic [25] isomerization step occurring early in the sequence. The activity of the reactivated desaturase quantified as the percentage conversion was less (P. D. Fraser, H. Linden and G. Sandmann, unpublished work) than that found in thylakoid membranes and lower than that expected from the levels of protein expressed. Unfortunately such findings probably reflect the loss of functional reactivation upon the removal of urea. Attempts were made to increase activity by including chaperone proteins, and although there were no significant effects of inclusion of chaperones in this study, investigations in this direction are in progress.

Significant stimulation in activity did occur as a result of lipid replenishment; monogalactosyl diacylglycerol, which is a major lipid component of cyanobacteria, was the most favourable lipid [26]. Of the fatty acids, the C_{16} and C_{18} moieties had the most beneficial effect on activity. Similar findings were observed previously [5] during the solubilization of carotenogenic enzymes from *Aphanocapsa*, where the C_{16} and C_{18} fatty acids were the principal lipids associated with the release of activity from thylakoid membranes.

The preferential involvement of the nicotinamide cofactors NAD^+ and $NADP^+$ indicates that the desaturation of phytoene to ζ-carotene in *Synechococcus* occurs via a hydride/proton transfer mechanism, as previously suggested from similar experimental data determined with crude membranes [6]. The cofactor requirements determined in this biochemical study correlate with those predicted from the gene sequence [7], and presumably the inhibitory effect of FAD in the presence of nicotinamides arises as a consequence of competition for the cofactor binding site. This situation is the reverse of that found with the previously isolated *Erwinia* phytoene desaturase, where FAD was the principal electron mediator [24].

Hopefully the isolation of *Synechococcus* pds will permit the properties of this enzyme to be further elucidated and will allow valuable comparison with other desaturases, both in their native environment and after overexpression. Although the complexity of assaying the enzyme does hinder rapid characterization, when compared with other more conventional enzymes, the photosynthetic origin of this enzyme and the previously illustrated sensitivity to bleaching herbicides should enable a comprehensive study of the action of these structurally diverse compounds upon phytoene desaturation to ζ-carotene, as well as the effects of those inhibitors shown to have a preferential inhibitory effect on ζ-carotene desaturation.

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