Involvement of NADPH in the cyclization reaction of carotenoid biosynthesis

D. Hornero-Méndez[1], G. Britton*

School of Biological Sciences, University of Liverpool, Life Sciences Building, Crown Street, Liverpool L69 7ZB, UK

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Abstract Cyclic carotenoids, e.g. β-carotene, are formed by cyclization of an acyclic precursor, lycopene. The gene, *crtY*, which encodes lycopene β-cyclase, has a partial sequence characteristic of a pyridine nucleotide binding domain, and NAD(P)H has been reported to be an absolute requirement for the cyclization reaction in vitro. By complementary incubations with lycopene as substrate and with (4*R*)-[4-^{2}H]NADPH in ^1H_2O or with unlabelled NADPH in ^2H_2O in the presence of the purified enzyme, it has now been shown that the hydrogen atom introduced at C(2) in the cyclization comes from water and not from NADPH. The previously proposed mechanism involving the initiation of cyclization by H^+ attack at C(2) of the folded acyclic end group of the precursor is thus confirmed. No hydrogen is transferred from NADPH, which is therefore not involved directly in the cyclization reaction, but must play an indirect role, e.g. as an allosteric activator.

Key words: Carotenoid biosynthesis; Lycopene cyclase; Cyclization; NADPH

1. Introduction

Carotenoids are C_{40} tetraterpenoids that are major pigments in higher plants, and also occur commonly in fungi and bacteria. In photosynthetic organisms carotenoids play vital roles in light harvesting and in photoprotection through their ability to quench excited chlorophyll and prevent photooxidation. Mutants which cannot biosynthesize carotenoids or plants that have been treated with herbicides that block carotenoid biosynthesis cannot survive or have low viability [1].

Carotenoids also accumulate in chromoplasts of non-photosynthetic plant tissues, such as fruit and flowers, giving rise to a range of vivid colors from yellow to red, as a means of attracting animals, especially insects and birds, for pollen and seed dissemination. Some carotenoids, especially β-carotene, are important in human nutrition as the main dietary source of vitamin A. Dietary carotenoids have also been associated with many actions beneficial to human health, including reduced risk of cancer. Such effects are commonly attributed to an antioxidant action [2–5].

The pathway of carotenoid biosynthesis is an important branch of the isoprenoid pathway, and therefore many of the genes and enzymes for the early steps are common to the biosynthesis of all isoprenoid compounds and are well conserved. The first step exclusive to carotenoid biosynthesis is the condensation of two molecules of the C_{20} geranylgeranyl diphosphate to form the first C_{40} carotenoid precursor, phytoene. Four sequential desaturations then give phytofluene, ζ-carotene, neurosporene and lycopene (all acyclic compounds). Lycopene can subsequently undergo cyclization at one end or both ends to give monocyclic (e.g. γ-carotene, δ-carotene) and dicyclic (e.g. α-carotene, β-carotene) products, respectively. The introduction of oxygen functions (e.g. hydroxy, epoxide, keto groups) and other structural modifications of end groups, including esterification, then follow as the final steps of biosynthesis. The apparent complexity of the carotenoid pathway results in a great number of possible structures; about 700 different carotenoids isolated from natural sources have been described (for complete comprehensive reviews of carotenoid biosynthesis see [6–8]).

The general pathway and reaction sequences in carotenoid biosynthesis have been established over the past 40 years. Labelling with stable isotopes was used to determine the mechanism and stereochemistry of some important reactions, including the cyclization reaction that forms the β-ring in carotenoids such as β-carotene and its 3,3′-dihydroxy derivative zeaxanthin [9–11]. This work confirmed that, during the cyclization, a hydrogen atom from the medium is introduced stereospecifically at C(2) and this has been accepted as proof that the cyclization is initiated by a proton attack at C(2) (Fig. 1).

For many years it has been assumed that the cyclization reaction of carotenoid biosynthesis is similar, in principle, to the cyclization processes in the biosynthesis of other isoprenoid compounds, and involves the folding of the flexible acyclic end group of the precursor into the required shape by the cyclase enzyme and then a concerted electrophilic attack at C(2) and closure of the C(1)–C(6) bond to form the ring. Formally this would give a transient C(5) carbocation which is presumably stabilized by the enzyme. Finally, H^+ would be eliminated to give the neutral, stable, cyclic end group. If the H^+ lost is from the C(6) position this would generate the β end group, whereas alternative loss of H^+ from C(4) or from the C(18) methyl group would give the ε or γ end group, respectively (Fig. 1).

In recent years, the genes encoding the enzymes of carot-

*Corresponding author. Fax: (44)-151-7944349.
E-mail address: brittong@liv.ac.uk (G. Britton).

[1] Present address: Departamento de Biotecnología de Alimentos, Instituto de la Grasa (CSIC), Av. Padre García Tejero 4, 41012 Sevilla, Spain.

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enoid biosynthesis in a variety of organisms have been isolated and characterized [12–17]. Examination of the nucleotide base sequence for *crtY*, the gene encoding lycopene β-cyclase from several prokaryotic and eukaryotic sources, has revealed a partial sequence characteristic of a pyridine nucleotide binding site [18]. Because the cyclization reaction appears to be a simple rearrangement which does not involve any change in oxidation level, the involvement of $NAD(P)^+$ or NAD(P)H in the reaction would not be expected. Work with the isolated cyclase enzyme obtained by cloning the bacterial or algal gene in *Escherichia coli* has shown that, when lycopene is provided as a substrate, NADPH is an absolute requirement for cyclization [19]. This finding throws doubt on the long-accepted mechanism of cyclization or at least shows that the reaction is more complex than previously thought. There is thus an urgent need for reinvestigation of this mechanism, one of the fundamental steps of carotenoid biosynthesis. If NADPH directly participates in the cyclization as a hydrogen donor, this reaction would involve hydrogen transfer from NADPH to the carotenoid. Alternatively, the involvement may be indirect, and NADPH may bind to the cyclase enzyme as, for example, an allosteric activator.

2. Materials and methods

2.1. Bacterial strain and culture

E. coli strain JM101/pUC19, containing the cloned *crtY* gene from *Erwinia uredovora*, was a kind gift from Prof. G. Sandmann, University of Frankfurt, Germany. The cells were grown in 8×250 ml of LB medium containing ampicillin (100 μg/ml) at 28°C until the optical density (D_{600}) reached a value of 0.5. Then the recombinant protein was overexpressed by inducing the culture with 0.25 mM isopropyl β-D-thiogalactopyranoside overnight.

2.2. Extraction and purification of the enzyme

Cells from 10 culture flasks were pooled and harvested by centrifugation at 6000×*g* for 10 min at 4°C. The pellet was resuspended in 60 ml of 50 mM Tris/malate buffer, pH 6.5. Cells, in batches of 40 ml of suspension, were then disrupted by five successive passages through a French press at 30 MPa. The resulting suspension was incubated with 10 μg/ml DNase for 30 min at room temperature. Subsequent centrifugation at 105 000×*g* for 1 h at 4°C gave a supernatant which was used as the crude extract. Further purification was achieved by precipitating with 40% (w/v) ammonium sulfate over 30 min. The protein was collected by centrifugation at 6000×*g* for 10 min at 4°C, then resuspended in 10 ml of 50 mM Tris/malate buffer, pH 6.5, and incubated at 0°C for 20 min. The insoluble fraction was collected by centrifugation under the same conditions as above, and the pellet was resuspended in the same buffer containing 1 mg/ml soybean lipids (with 20% content of L-α-phosphatidylcholine). The resulting solution was used as the semipurified lycopene β-cyclase. The progress of the purification was monitored by SDS–PAGE [20].

2.3. Production of (4R)-[4-^{2}H]NADPH

The (4*R*)-[4-^{2}H]NADPH was prepared by the method of Jeong and Gready [21], by reduction of $NADP^+$ with an $NADP^+$-dependent alcohol dehydrogenase (EC 1.1.1.2) from *Thermoanaerobium brockii* (*Tb*ADH) and isopropanol-d_8 as substrate.

2.4. Substrate preparation

Lycopene was a kind gift from Hoffmann-La Roche (Switzerland). To ensure accessibility of the substrate to the enzyme in the aqueous buffered assay mixture, lycopene was mixed with soybean lipid suspension as follows: 1.3 ml of a 930 μM lycopene stock solution in chloroform were transferred to a roundcap test tube and evaporated under nitrogen, and 62.5 mg of soybean lipids were added. The mixture was dissolved in 5 ml chloroform and evaporated under nitrogen to give an intimate layered mixture of lipids and lycopene. A final solution containing 2.5 mg/ml soybean lipids and 48.36 μM lycopene was achieved by sonication of the above mixture with 25 ml of 50 mM Tris/malate buffer, pH 6.5. This suspension was used as substrate in the enzyme assay.

2.5. In vitro enzyme assay

Incubation of lycopene cyclase with lycopene was carried out according to the method by Schnurr et al. [19] with some modifications. A final volume of 0.5 ml assay mixture contained 150 μl of the lipid–carotenoid suspension to give a final lycopene concentration of 14.51 μM. In addition, 5 mM NADPH or (4*R*)-[4-^{2}H]NADPH plus 25 μg of semipurified enzyme in 50 mM Tris/malate buffer, pH 6.5, was added. Incubation was carried out in the dark for 4 h at 30°C. The reaction was stopped by addition of 1 ml 10% (w/v) KOH. For experiments that involved the use of ^2H_2O, normal water was replaced by ^2H_2O in the preparation of all solutions that were used throughout the assay.

2.6. Carotenoid extraction and high-performance liquid chromatography (HPLC) analysis

After incubation the carotenoid pigments were extracted from the assay mixture with diethyl ether and the extract was evaporated under nitrogen and analyzed by HPLC on a Spherisorb ODS2 5 μm column (250×4.6 mm). A solvent linear gradient elution of 0 to 100% ethyl acetate in acetonitrile/H_2O (9/1, containing triethylamine 0.1% v/v) at 1 ml/min was used. The detection wavelength was 450 nm, and the UV/Vis spectrum of the eluate was recorded continuously on-line by use of a photodiode array detector (PDA, Waters 991).

2.7. Mass spectrometry (MS)

Analysis of the incubation products by LC–MS was performed at the Institute of Chemistry and Biochemistry of the University of Bern, Switzerland, as kindly arranged by Prof. H. Pfander.

2.8. Protein determination

Protein content of different fractions was measured by the method of Bradford [22]. Bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA) was used as standard.

2.9. Reagents and apparatus

$NADP^+$ and NADPH were purchased from Boehringer Mannheim, isopropanol-d_8 from Fluka, Tris buffer (Trizma HCl and base) and $NADP^+$-dependent alcohol dehydrogenase (EC 1.1.1.2) from *T. brockii* were from Sigma, and analytical grade NaCl was from BDH. CentriCell 20, a centrifugal ultrafilter, was obtained from Polysciences Inc., Warrington, PA, USA. For HPLC, analytical Spherisorb ODS2 5 μm (250×4.6 mm) and semipreparative Spherisorb ODS2 5 μm (250×10 mm) reverse-phase columns were purchased from Phase-Sep (Clwyd, UK). The HPLC system consisted of a Waters 600E pump delivery system and a Waters 991 PDA UV/Vis scanning detector.

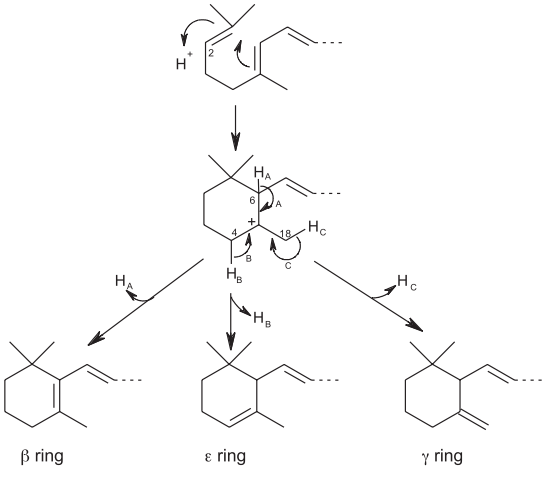

Fig. 1. Proposed mechanism for the biosynthetic formation of the β, γ and ε end groups of cyclic carotenoids [6].

3. Results and discussion

Previous results showed that, if the accumulated acyclic precursor lycopene is allowed to cyclize in situ, in ^2H_2O medium, one ^2H atom is introduced stereospecifically at C(2) of each ring formed, and the β-carotene produced is the $^2H_2$ species. This result was considered to confirm the long-assumed mechanism of cyclization, according to which the initiating step is H attack at C(2) (Fig. 1). More recently, however, molecular genetics and work with the natural or cloned cyclase enzyme have indicated that the reaction may be more complex. The sequence of the lycopene cyclase gene from a number of sources shows the presence of a nucleotide (NADH/NADPH)-binding domain [15,23], and work with cell-free systems revealed that NADPH was an essential cofactor for the cyclase [19]. The possibility was raised therefore that the H atom introduced during cyclization is actually transferred from NADPH, not directly from water of the medium. Under the conditions that had been used to demonstrate the incorporation of deuterium during cyclization of endogenous lycopene in situ, rapid equilibration would have meant that the endogenous NADPH rapidly became deuterated, so the earlier work does not exclude the possibility that hydrogen was introduced from NADPH during cyclization. To clarify this unambiguously, the partially purified lycopene β-cyclase enzyme, isolated after overexpression in *E. coli*, has been used in complementary labelling experiments. In one, the cyclization of the exogenous substrate lycopene was allowed to proceed on ordinary water (H_2O) containing an excess of the deuterated (4*R*)-[4-^{2}H]NADPH; in the other, unlabelled NADPH was used but the incubations were carried out in ^2H_2O. After the incubations, the carotenoids were isolated and analyzed by HPLC and HPLC–MS.

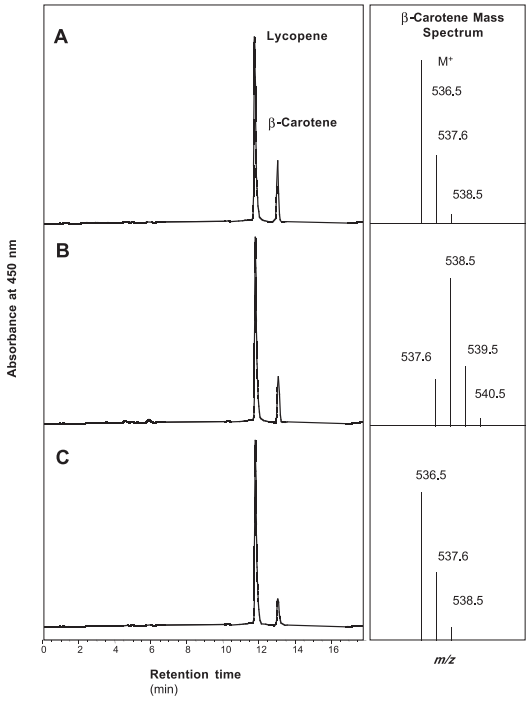

Fig. 2. HPLC–MS analysis of the carotene fraction obtained by incubation of lycopene β-cyclase with lycopene (A) with unlabelled NADPH in ^1H_2O (control), (B) with unlabelled NADPH in ^2H_2O, (C) with (4*R*)-[4-^{2}H]NADPH in ^1H_2O. Left: HPLC trace. Right: MS pattern of β-carotene. Incubation conditions as described in the text.

In control experiments without the use of ^2H labelling, it was confirmed that β-carotene was formed from lycopene to a significant extent (38%) in the presence of NADPH but not in its absence, in agreement with previous observations [19]. In incubations in ordinary water (H_2O) medium with deuterated NADPH, cyclization of lycopene also proceeded efficiently (34% conversion in 4 h), but the mass spectrum (Fig. 2) revealed that no deuterium was incorporated into the β-carotene product; only the normal molecular ion at *m/z* 536 was detected (allowing for the natural abundance of ^{13}C). In contrast to this, incubation with NADPH in ^2H_2O gave a β-carotene product in which the $^2H_2$ species (*m/z* 538) was dominant.

The remaining unconverted substrate lycopene contained no deuterium, showing the absence of deuterium exchange during the incubations. Some partial resolution of the unlabelled and deuterated β-carotene was observed during the HPLC–MS but, although the later fractions of the β-carotene were found to consist only of the $^2H_2$ form, no molecular species with more than two deuterium atoms were detectable, again consistent with the lack of simple exchange during the incubations.

These results prove that there is no transfer of hydrogen from NADPH during the cyclization. NADPH therefore plays an indirect rather than a direct role in the reaction. The mechanism of this activating role is unknown. The mechanism previously proposed for the cyclization, in which the reaction is initiated by addition of a proton from the water of the medium to C(2) of the folded acyclic precursor carotenoid, lycopene, is confirmed.

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