THE USE OF DEUTERIUM FROM DEUTERIUM OXIDE AS A LABEL IN STUDIES OF BIOSYNTHETIC PATHWAYS

Carotenoid transformations in a *Flavobacterium* species

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1. Introduction

In studies of biosynthetic pathways extensive use has been made of inhibitors which prevent synthesis of the normal end-products and cause the accumulation of abnormal compounds that may be biosynthetic intermediates. In many cases the transformations that occur after removal of the inhibitor have also been examined. It may be very difficult to prove that the normal end-product is formed at the expense of the accumulated 'intermediates' rather than by new synthesis from small molecules. In some cases it has been possible to obtain good evidence that the apparent transformations are real by the use of radioisotopic labelling, or by using an inhibitor of an earlier biosynthetic stage to prevent new synthesis.

In studies of carotenoid biosynthesis (for reviews see [1–4]) diphenylamine and nicotine have been used very widely. In particular, nicotine inhibits the formation of cyclic carotenoids in many microorganisms, and causes the accumulation of acyclic pigments considered to be intermediates in the biosynthetic pathway. Detailed studies with a *Flavobacterium* species, R1519, have provided evidence for the transformation of lycopene (ψ,ψ-carotene), which accumulates in the presence of nicotine, into the normal main pigment zeaxanthin (β,β-carotene-3,3′-diol) when the inhibitor is removed. We now report a new method which, in appropriate cases, allows the direct and unequivocal demonstration of such transformations. This method has been used to demonstrate that the carotenoid transformations proposed for *Flavobacterium* R1519 do take place.

2. Methods

The culture of *Flavobacterium* R1519 (≡ 0147) was kindly provided by F. Hoffmann-La Roche and Co., Basel, Switzerland. Details of the culturing conditions and the procedures used for extracting and purifying the carotenoids have been given elsewhere [5,6].

The organism was cultured for 40 h at 19–21°C in medium containing nicotine (7.5 mM or 1.0 mM). The cells from 500 ml culture were harvested, washed successively with 0.1 M Tris–HCl buffer, pH 7.0, and deuterium oxide (99.6% D_2O) containing $MgSO_4$ (0.2%) and NaCl (4%), and then resuspended in culture medium (100 ml) prepared with deuterium oxide in place of ordinary water. After a further 48–72 h incubation the cells were harvested and the carotenoids extracted, purified and examined by mass spectrometry.

Mass spectra were determined by Mr G. Harriman or Mr M. Prescott, using an A.E.I. MS 12 instrument with the direct insertion probe, ion source temperature 200°C and ionizing voltage 70 eV.

3. Results and discussion

Nicotine inhibits the cyclization reaction of carotenoid biosynthesis.

Flavobacterium R1519 normally produces large amounts of the carotenoid zeaxanthin. In the presence of nicotine (7.5 mM), zeaxanthin synthesis is inhibited and lycopene becomes the main pigment.

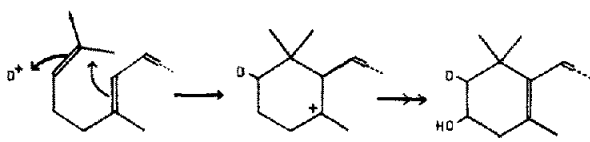

Fig.1. Introduction of deuterium at C-2 and C-2′ of zeaxanthin during cyclization in D_2O of an acyclic carotenoid precursor.

Previous work has indicated that, on removal of the nicotine, the accumulated lycopene is incorporated into zeaxanthin [6]. In the present work, *Flavobacterium* R1519 has been grown in the presence of nicotine so that lycopene accumulates, and the cells have then been washed free of nicotine and resuspended in deuterium oxide.

The cyclization reaction of carotenoid biosynthesis is generally considered to be a proton-initiated process [4]. Cyclization in deuterium oxide should therefore result in the introduction of a deuterium atom at C-2 of the cyclic carotenoid (fig.1). Zeaxanthin formed by cyclization of existing unlabelled lycopene in D_2O would therefore incorporate two deuterium atoms, at C-2 and C-2′. The high mass region of the mass spectrum of a *Flavobacterium* zeaxanthin sample produced under these conditions is illustrated in fig.2. The most abundant ion (*m/e* 570) was that of a dideuterio species of zeaxanthin (C_{40} H_{54} D_2O_2). No dideuterio species (*m/e* 538) was detected in the mass spectrum of lycopene present in the same extract, although the normal molecular ion (*m/e* 536) and more highly deuteriated species (average *m/e* 548) were present. The location of the

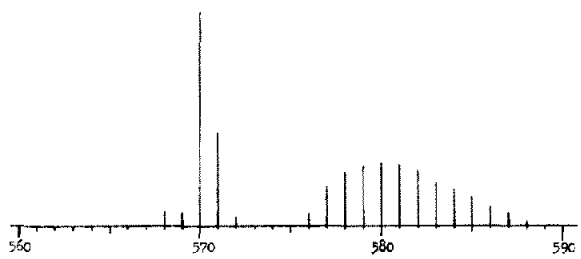

Fig.2. High mass region of the mass spectrum of deuteriozeaxanthin produced by cyclization in D_2O of lycopene accumulated by *Flavobacterium* R1519 in the presence of nicotine.

deuterium at C-2 and C-2′ in the zeaxanthin was established by ^1H NMR spectroscopy (W. J. S. Lockley, G. Britton and T. W. Goodwin, unpublished results). The dideuterio zeaxanthin could only have arisen by cyclization in D_2O of an existing, undeuteriated acyclic precursor (lycopene), thus proving that the lycopene that accumulates in the presence of nicotine does serve as a precursor for zeaxanthin formation after removal of the inhibitor.

Other important features of the zeaxanthin mass spectrum are:

(i) The normal zeaxanthin molecular ion at *m/e* 568 ($C_{40}H_{56}O_2$) was virtually absent. Only a very low level of zeaxanthin is present in nicotine-grown *Flavobacterium* cultures, and also no normal (undeuteriated) zeaxanthin had been produced after removal of the inhibitor.

(ii) The range of ions between *m/e* 576 and *m/e* 588, accompanied by *M*-deuteriotoluene fragmentations, indicated the presence of zeaxanthin species containing larger numbers of deuterium atoms (average D_{14}). This is the composition to be expected for zeaxanthin newly synthesized from glucose and D_2O, and provides evidence that carotenoid synthesis has occurred de novo from small molecules.

In the same experiment a small amount of the monocyclic carotenoid rubixanthin (β,ψ-caroten-3-ol) was also isolated, but no monodeuteriated species was detected in the mass spectrum. In a similar experiment in which *Flavobacterium* R1519 was grown in medium containing 1 mM nicotine, and was then washed and resuspended in D_2O medium, the mass spectra showed that monodeuterio zeaxanthin and β-cryptoxanthin (β,β-caroten-3-ol) were present in appreciable amounts and must have been formed by cyclization in D_2O of the rubixanthin which accumulated in the presence of the lower concentration of nicotine.

This method has therefore allowed us to demonstrate the direct conversions in vivo of the acyclic lycopene into zeaxanthin, and of the monocyclic rubixanthin into β-cryptoxanthin and zeaxanthin, thus proving the validity of the conclusions drawn previously [6] about alternative pathways of zeaxan-

thin biosynthesis in *Flavobacterium*. The absence of monodeuteriorubixanthin means that we have failed to detect any conversion of acyclic into monocyclic carotenoids on removal of the inhibitor, in agreement with the indications obtained from the previous radioisotope work [6].

A similar procedure should prove especially useful with photosynthetic bacteria, which will not readily incorporate radioactive substrates into their carotenoids. Nicotine inhibits (reversibly) the addition of water across the C-1,2 double-bond in the biosynthesis of the characteristic tertiary hydroxy- and methoxy carotenoids of photosynthetic bacteria. With the D_2O-labelling procedure it should be possible to demonstrate directly the carotenoid transformations that occur on removal of the nicotine, and hence establish the validity of the biosynthetic pathways that have been proposed for the carotenoids of photosynthetic bacteria. Related procedures may also prove useful for elucidating the biosynthetic pathways of other classes of natural products.

Acknowledgements

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References

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