The relationship between carotenoid biosynthesis and the assembly of the light-harvesting LH2 complex in *Rhodobacter sphaeroides*

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Coloured carotenoids play some undefined role in the assembly of a functional light-harvesting 2 (LH2) complex in photosynthetic bacteria. We have used a series of transposon Tn5 insertion mutants disrupted at various stages of the carotenoid-biosynthetic pathway, together with an LH2 deletion/insertion mutant, to investigate this effect in *Rhodobacter sphaeroides*. Mutants were initially characterized by low-temperature absorbance spectroscopy and ultrastructural analysis; Northern-blot analysis demonstrated normal *pucBA* transcripts for LH2 polypeptides in all the carotenoid mutants. Analysis of translation of the *puc* transcript and investigation of the fate of any resulting

LH2 polypeptides by SDS/PAGE, Western-blot and pulse-chase experiments clearly demonstrated that, in the absence of coloured carotenoids, the LH2 α - and β -polypeptides are synthesized but are rapidly turned over and do not become stably integrated into the membrane. Complementation of mutants with lesions in the *crtB* and *crtI* genes, encoding phytoene synthase and phytoene desaturase respectively, with the cloned *R. sphaeroides crtI* gene, resulted in restoration of carotenoid biosynthesis and stable assembly of the LH2 complex in the *crtI* mutant but not in the *crtB* mutant, despite the presence of the CrtI protein.

INTRODUCTION

The photosynthetic apparatus in purple bacteria such as *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* consists of three main pigment-protein complexes. The light-harvesting (LH2) antenna complex is found in regions peripheral to the LH1-reaction centre 'core'. The antenna complexes harvest light and transfer excitation energy to the reaction centres where the primary photochemical events of photosynthesis take place. All three complexes contain bacteriochlorophylls together with carotenoids, which play a role in photoprotection; in addition, the carotenoids act as accessory light-harvesting pigments, absorbing light in the 450–570 nm region and transferring energy to the bacteriochlorophyll molecules (Cogdell and Frank, 1987). The proposed minimal structural unit for LH1 is 3 α :3 β :6 bacteriochlorophyll:6 carotenoid and for LH2, 3 α :3 β :9 bacteriochlorophyll:6 carotenoid (Hunter et al., 1989) or 2 α :2 β :6 bacteriochlorophyll:3 carotenoid (Kramer et al., 1984; Papiz et al., 1989).

The arrangement of the genes for photosynthesis was first described by Marrs (1981) for *R. capsulatus*, and a physical-genetic map of this region was reported by Zsebo and Hearst (1984). The carotenoid-biosynthesis genes (*crt*) within this region were subsequently sequenced in *R. capsulatus* by Armstrong et al. (1989). In *R. sphaeroides* the *crt* genes are clustered, as in *R. capsulatus*, and are flanked by *bch* genes encoding the enzymes of bacteriochlorophyll biosynthesis within a 45 kb region of photosynthesis-related genes; the boundaries of this region are the *puhA* gene and the *puf* operon (Coomber and Hunter, 1989). The genes encoding the reaction centre L- and M-subunits and the LH1 α - and β -polypeptides are located in the *puf* operon (Williams et al., 1983, 1984; Kiley et al., 1987). The *puhA* gene encodes the reaction centre H-subunit (Donohue et al., 1986; Williams et al., 1986). The *puc* operon contains the *pucBA* genes which encode the LH2 β - and α -polypeptides (Ashby et al., 1987; Kiley and Kaplan, 1987). An additional region downstream of

the *pucBA* genes has been shown to be essential for assembly of the LH2 complex (Lee et al., 1989). This region has been sequenced by Gibson et al. (1992), who have identified a 1.3 kb open reading frame designated *pucC* on the basis of its homology with the *R. capsulatus pucC* gene. The *puc* region in *R. sphaeroides* has been mapped 18 kb downstream of *puhA* (Suwanto and Kaplan, 1989) (Figure 1a).

It has been known for a long time that in *Rhodobacter* mutants which are unable to synthesize coloured carotenoids, there is a corresponding loss of the absorption peaks corresponding to LH2 (Griffiths and Stanier, 1956; Marrs, 1978). The resulting reduction in the size of the antenna complex of these mutants could be seen as an adaptive response which allows the bacteria to limit the size of the photosynthetic antenna when photoprotective carotenoid pigments are absent (Bartley and Scolnik, 1989); however, the mechanism for this response is not yet fully understood. Zsebo and Hearst (1984) and Giuliano et al. (1988) observed that, in *R. capsulatus*, mutations in the early stages of carotenoid biosynthesis affect the formation of the LH2 complex with a corresponding loss of absorption from LH2. However, Dörge et al. (1987) observed variable levels of LH2 in *R. capsulatus* mutants lacking coloured carotenoids, and conclude that coloured carotenoids are not obligatory for the assembly of the LH2 complex. As the *crtI* (phytoene desaturase) gene encodes the enzyme that mediates the conversion of phytoene into coloured carotenoids (Giuliano et al., 1986), it is an obvious target for studies of the relationship between carotenoid biosynthesis and LH2 assembly. Bartley and Scolnik (1989) have also shown that a little LH2 is formed in interposon mutants of the *crtI* gene of *R. capsulatus*, and they suggest a correlation between the levels of CrtI and the formation of the LH2 complex. Using a biochemical approach, Zurdo et al. (1993) employed a selective procedure of carotenoid extraction to investigate the structural role of the carotenoid in the LH2 complex of *R. capsulatus*, and they suggest that the carotenoid is required to stabilize the structure of the LH2 complex.

Abbreviations used: LH1 and LH2, light-harvesting complexes 1 and 2.

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Figure 1 Map of the carotenoid genes and the carotenoid-biosynthetic pathway in *R. sphaeroides*

(a) The 45 kb photosynthetic cluster and *puc* operon adapted from Coomber et al. (1990) and containing information from McGlynn and Hunter (1992). Shaded regions represent the positions of genes as mapped by insertion mutagenesis and subsequent sequencing studies. (b) Restriction map of the carotenoid genes and positions of the Tn5 insertions. Restriction sites are abbreviated as follows: a, *AccI*; b, *BamHI*; c, *SacI*; d, *HindIII*; h, *HincII*; l, *Sall*; m, *SmaI*; p, *PstI*; s, *SphI*. (c) The carotenoid-biosynthetic pathway.

In this report we have demonstrated the level at which carotenoids affect the assembly of the LH2 complex. A series of insertion mutants have been used, and were characterized by a variety of methods; the results demonstrate that the formation of the LH2 complex requires the presence of coloured carotenoids; disruption of the *crtI* gene is solely responsible for the lack of LH2 assembly, which has attendant effects on membrane morphology.

MATERIALS AND METHODS

Chemicals, enzymes and isotopes

The antibiotics kanamycin, streptomycin and tetracycline, *M*, markers for SDS/PAGE, phenol, glyoxal and 2-mercaptoethanol were obtained from Sigma. Acrylamide (30%, w/v) and restriction enzymes were from Northumbria Biologicals Ltd. ECL

detection reagents, Hybond-N and Hybond-C membranes, L-[³⁵S]methionine and [α -³²P]dCTP were purchased from Amersham International plc. (Amersham, Bucks., U.K.). Amplified alkaline phosphate immunoblot assay kit, Tween 20 and gelatin were from Bio-Rad Laboratories. The RNA ladder was supplied by Gibco-BRL. All other chemicals were purchased from Fisons Scientific equipment unless otherwise stated. All reagents were molecular-biology grade.

Media and antibiotics

R. sphaeroides strains were grown in M22 + medium (Hunter and Turner, 1988); liquid cultures were supplemented with 0.1% casamino acids. These were grown at 34 °C, semi-aerobically in the dark or photosynthetically in 30 ml screw-cap bottles at a light intensity of 10 W/m². Antibiotics were used at the following concentrations (μ g/ml): 20 (kanamycin), 5 (streptomycin) and 1 (tetracycline).

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1.

Absorption spectroscopy

All strains were grown under semi-aerobic conditions in the dark. Cells were disrupted in a French pressure cell at 124 200 kPa, and cell-free intracytoplasmic membrane fractions were isolated by passage through a discontinuous (15/40% w/w) sucrose gradient by a method adapted from Fraley et al. (1977). The intracytoplasmic membrane fraction, which bands just above the 15/40% interface, was collected and diluted with 80% glycerol. Low-temperature absorption spectra were obtained on a Guided Wave model 260 fibre-optic spectrophotometer (Guided Wave Inc.) using an Oxford Instruments liquid-nitrogen cryostat.

Electron microscopy

Cell samples were fixed overnight in 3% glutaraldehyde/0.1 M potassium phosphate (pH 7.4). After being washed in 0.1 M potassium phosphate (pH 7.4), the material was fixed for a second time in aq. 2% osmium tetroxide for 4 h and then progressively dehydrated using 75% ethanol, 95% ethanol, 100% ethanol, ethanol/propylene oxide (1:1, v/v) and 100% propylene oxide. The sample was then infiltrated with Spurr resin (Agar Scientific) and polymerized at 60 °C for 17 h. Thin sections, cut with a microtome, were stained with saturated alcoholic uranyl acetate and Reynolds lead citrate and examined in a Philips CM10 transmission electron microscope.

RNA preparation and Northern-blot analysis

RNA was prepared from 30 ml photosynthetic cultures, grown for 1–3 days, at 10 W/m², until the absorbance at 680 nm reached 0.3–1.0. Cells were resuspended in 150 μ l of solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl, pH 8.0), and 550 μ l of extraction buffer [4 M guanidium isothiocyanate, 50 mM Tris/HCl pH, 7.5, 25 mM EDTA, 8% (w/v) 2-mercaptoethanol] was added. Cells were sonicated for 3 $\times$ 5 s using a Soniprep sonicator, and 70 μ l of 3 M potassium acetate was added. The RNA was extracted with phenol/chloroform (1:1, v/v) and pelleted in a Beckman L2 JA20 rotor at 17 000 g for 1 h at 4 °C. The pellet was washed in 70% ethanol, vacuum-

desiccated for 20 min and redissolved in 80 μ l of water. Each RNA sample (20 μ g) was denatured by glyoxalation and electrophoresed through a 1.2 % agarose gel at 100 V for 2.5 h at room temperature, and Northern-blot analysis was performed as described by Thurston et al. (1988). DNA probes were excised from pSUP202-derived plasmids constructed by Coomber et al. (1990) and labelled with [α - 32 P]dCTP by the method of Feinberg and Vogelstein (1983).

SDS/PAGE, Western-blot and pulse-chase analysis

SDS/PAGE and Western blots were performed as described by Sambrook et al. (1989). Cultures were grown under semi-aerobic conditions in the dark, and cells were disrupted in a French pressure cell at 124200 kPa. Membrane, upper pigmented band and supernatant fractions were separated on a linear (5–35 %, w/w) sucrose gradient with a 60 % (w/w) sucrose cushion. Western blots were probed with LH2 antibodies using the Bio-Rad alkaline phosphate immunoblot assay system. Antibodies raised against the β -polypeptide of LH2 were kindly donated by Professor R. Cogdell (University of Glasgow). Pulse-chase techniques were adapted from Stiehle et al. (1990).

RESULTS

Five Tn5 insertion mutants, constructed by Coomber et al. (1990), were used in this study (see Table 1). They were identified initially on the basis of the colour of photosynthetic colonies, and the carotenoid content was analysed by either h.p.l.c. or t.l.c.; finally the position of the Tn5 insertion was physically mapped using restriction endonucleases (Coomber et al., 1990). The mutants used in this study were selected on the basis of their identification by Coomber et al. (1990) and were intended to represent the various stages in the carotenoid-biosynthetic pathway. (Figure 1c). All 25 carotenoid Tn5 insertion mutants have now been sequenced from the point of insertion (H. P. Lang, R. J. Cogdell and C. N. Hunter, unpublished work), and two of the mutants used in this study were found to be wrongly identified in Coomber et al. (1990). TC72, a blue-green mutant, initially identified as *crtB*, has been mapped to *crtI* and accumulates phytoene, although its correct identity was established before

selection for this study; TC19, initially identified as *crtD/E*, is in fact a *bchl*⁻ mutant (Figure 1b). The other three mutants used in this study had been correctly identified. The second blue-green strain, TC70, is a *crtB* mutant and contains no carotenoids. TC40 and TC18 are both green mutants; TC40 has a Tn5 insertion in *crtC* and accumulates neurosporene, and TC18 is a *crtD* mutant and accumulates neurosporene and hydroxyneurosporene. The sixth mutant is a deletion/insertion mutant, where the genomic copy of *pucBA* has been replaced by a streptomycin-resistance gene (Jones et al., 1992); this strain therefore lacks the LH2 complex, but retains the LH1 and reaction centre complex and possesses wild-type carotenoids.

Analysis of mutants by absorption spectroscopy at 77 K

All of the mutants and the wild-type strain were grown semi-aerobically in the dark, and photosynthetic membranes were isolated on a discontinuous sucrose-density gradient. Low-temperature (77 K) absorption spectra were recorded for membranes isolated from each strain.

The distinctive wild-type absorption spectrum contains three easily distinguishable peaks in the near i.r. region; the peaks at 800 and 855 nm are attributable to the LH2 complex, and the absorbance at approximately 883 nm, which appears as a

Table 1 Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source/reference
<i>R. sphaeroides</i> NCIB 8253	Wild-type	R. A. Niederman (Rutgers University)
DBC Ω	Genomic deletion of <i>pucBA</i> , insertion of streptomycin-resistance cassette	Jones et al. (1992)
TC70	Tn5 insertion into <i>crtB</i>	Coomber et al. (1990)
TC72	Tn5 insertion into <i>crtI</i>	Coomber et al. (1990)
TC40	Tn5 insertion into <i>crtC</i>	Coomber et al. (1990)
TC18	Tn5 insertion into <i>crtD</i>	Coomber et al. (1990)
TC19	Tn5 insertion into <i>bchA</i>	Coomber et al. (1990)
Plasmids pHLKPD1	PCR-amplified <i>crtI</i> gene from <i>R. sphaeroides</i> cloned into pRKSK1	H. P. Lang, R. J. Cogdell, A. T. Gardiner and C. N. Hunter (unpublished work)

Figure 2 Low-temperature absorption spectra of photosynthetic membranes isolated from wild-type and mutant strains

Photosynthetic membranes were isolated on a discontinuous sucrose gradient, diluted in 80 % glycerol, and low-temperature (77 K) absorption spectra were recorded. A, DBC Ω ; B, TC70; C, TC72; D, TC40; E, TC18; F, wild-type.

Figure 3 Ultrastructural analysis of Tn5 insertion mutants

Ultrastructural analysis of insertion mutants by thin-section electron microscopy. (a) TC70, (b) TC72; (c) TC40; (d) TC18; (e) wild-type. Magnifications are as follows: (a) $\times 21\,000$; (b) $\times 34\,000$; (c) and (d) $\times 27\,500$; (e) $\times 18\,000$. All strains were grown semi-aerobically in the dark.

shoulder on the 855 nm peak, arises from the LH1 complex. The green mutants TC40, which accumulates neurosporene, and TC18, which accumulates methoxy- and hydroxy-neurosporene, both have wild-type levels of the LH2 complex. The 450–570 nm region contains absorbance peaks for carotenoids bound to photosynthetic complexes. The difference in carotenoid content of the strains used is reflected in the variety of absorbing species in Figure 2.

Absorption spectra of the blue-green mutants TC70 and TC72, which produce no coloured carotenoids, show that they lack the two peaks of the LH2 complex; the 800 nm peak attributable to

the reaction centre remains, and the LH1 complex dominates the spectrum, so the major peak in the near i.r. region is now at 883 nm compared with 855 nm for the wild-type, TC40 and TC18 strains. The absorbance maxima of TC70 and TC72 at 883 nm demonstrate that the LH1 bacteriochlorophylls are influenced by the type of carotenoid present, as described in Jones et al. (1992); the presence of coloured carotenoids shifts the bacteriochlorophyll peak to 890 nm. The visible region of the spectra contains no carotenoid contribution in strains TC70 and TC72, as the absorbance of phytoene lies outside the spectral range chosen here.

Ultrastructural analysis of mutants

In addition to obvious spectral differences, there is also a change in the intracytoplasmic membrane structure of LH2⁻ mutants. This has been reported by Lommen and Takemoto (1978) working on a carotenoidless LH2⁻ strain of *R. sphaeroides* and has been extended by Hunter et al. (1988) and Kiley et al. (1988) using LH2⁻ strains with normal carotenoids. Their studies indicated that, in the absence of LH2, the normal spherical membrane invaginations become tubular in shape. However, further studies by Golecki et al. (1989, 1991) showed that an LH2⁻ mutant of *R. sphaeroides* exhibited normal spherical membranes in addition to tubular membranes. Gibson et al. (1992) carried out ultrastructural analyses on genetically defined LH2⁻ strains, and they showed that it is possible to have differences in membrane morphology between different LH2⁻ strains depending on the type of mutation involved, which emphasizes the need to work with well-characterized strains. DBCΩ, which lacks both the LH2 α- and β-polypeptides, exhibits a tubular membrane structure (Gibson et al., 1992).

Figure 3 shows ultrastructural analysis, by thin-section electron microscopy, of the strains used in this study. Intracytoplasmic membranes from the wild-type strain have the normal spherical structure; however, TC70 and TC72, which lack both LH2 and coloured carotenoids, have some cells that display an additional distortion in the membrane structure, where the tubules appear to spiral round the cytoplasm. Cells that do not contain spiralling membranes appear almost identical with the *hemF* mutant described by Gibson et al. (1992) where, apart from a few rather large spherical structures, there is a lack of any significant intracytoplasmic membrane system. When coloured carotenoids are present and the LH2 complex is assembled, in TC40 and TC18, the membranes return to the wild-type structure. It is likely that this effect on membrane morphology is a consequence of the lack of LH2, which is produced in turn by the lesion in carotenoid biosynthesis.

Analysis of *puf* and *puc* transcripts

Transcription of the *puf* and *puc* operons in the mutants was examined in order to investigate at what level synthesis and/or assembly of the LH2 complex was affected by lesions in the carotenoid-biosynthesis genes. Figure 4 shows Northern-blot analysis of total RNA prepared from semi-aerobic cultures and probed with an *Xba*I–*Sal*I fragment of the *pufL* gene (Figure 4a) and a 2.5 kb *Bam*HI fragment including the *pucBA* genes (Figure 4b). The *puf* operon probe was used as a control, as spectroscopic analysis has shown that all the mutants contain LH1 and reaction centre complexes; in agreement with this, the Northern-blot analysis does indeed show that the *puf* transcript is present in all strains (Figure 4c). Figure 4(d) shows that the 0.55 kb *pucBA* transcript is also present in all strains, which indicates that the LH2 genes are transcribed in the mutants in which there is no LH2 complex formed.

LH2 polypeptide analysis

Crt strains were analysed for evidence of translation of the *pucBA* transcript and to investigate the fate of any resulting LH2 polypeptides. Semi-aerobically grown cells were disrupted in a French pressure cell, and the extract was fractionated on a continuous sucrose-density gradient. This procedure normally yields three fractions: the most rapidly sedimenting band contains mature photosynthetic membranes; the slowly sedimenting

Figure 4 Northern-blot analysis of *puf* and *puc* transcripts

(a) Restriction map of the *puf* operon showing the fragment used as a probe. (b) Restriction map of the *puc* operon showing the fragment used as a probe. (c) and (d) Autoradiographs of Northern blots of RNA prepared from photosynthetically grown cells, probed with (c) the *puf* probe and (d) the *puc* probe. Some 20 µg of RNA, denatured by glyoxalation, was loaded per lane and electrophoresed through a 1.2% agarose gel at 100 V for 2.5 h. Lane 1, TC70; lane 2, TC72; lane 3, TC40; lane 4, TC18; lane 5, TC19; lane 6, wild-type. The estimated sizes of the RNA species detected in the Northern blots are indicated.

upper pigmented band represents sites of preferential insertion of newly synthesized pigment–protein complexes (Niederman et al., 1979); finally, there is a supernatant fraction containing cytosolic components. Figure 5(a) shows SDS/PAGE analysis of the mature intracytoplasmic membrane fraction. The LH1 α- and β-polypeptides are clearly visible in all strains, whereas the LH2 α- and β-polypeptides can be seen only in the wild-type TC40, TC18 and TC19 strains and, as expected, are absent from the LH2 deletion strain control, DBCΩ; it is not entirely clear whether the LH2 polypeptides are absent from TC70 and TC72, as a faint band can be seen in both lanes which could be the LH2 polypeptides, but could equally well be another polypeptide that is normally masked by the LH2 band. In order to clarify the identification of the LH2 apoproteins, Western-blot analyses of membrane, supernatant and upper pigmented band fractions were probed with antibodies raised against the β-polypeptide of LH2 (Figures 5b, 5c and 5d). This confirms the identity of the LH2 polypeptides, and, in addition, Figures 5(c) and 5(d) (cytosolic and upper pigmented band fractions respectively) show that, if the LH2 α- and β-polypeptides are being synthesized in TC70 and TC72 at all, then they are not accumulating in other parts of the cell. A faint band can again be seen in the TC70 and TC72 lanes (Figure 5b); the identity of this band remains ambiguous, but it is worth noting that no such band is seen in the DBCΩ lane, in either the SDS/PAGE or Western-blot analyses, thus reducing the likelihood of it being a hidden band in the TC70 and TC72 lanes. Therefore we conclude that only very small amounts of LH2 are incorporated into membranes of strains TC70 and TC72. Transient accumulation of *pucBA*-

Figure 5 LH2 polypeptide analysis

All strains were grown semi-aerobically in the dark. Supernatant, upper pigmented band and photosynthetic membrane fractions were isolated on a linear 5–35% (w/w) sucrose gradient. All samples were run on 12–20% SDS/polyacrylamide vertical slab gels at a constant current of 12 mA overnight. (a) SDS/PAGE of photosynthetic membranes stained with Kenacid Blue. (b), (c) and (d) Western-blot analysis of SDS/PAGE of (b) photosynthetic membranes, (c) supernatant fractions and (d) upper pigmented band fractions probed with LH2 antibodies. Lane 1, TC70; lane 2, TC72; lane 3, TC40; lane 4, TC18; lane 5, TC19; lane 6, wild-type; lane 7, DBCΩ.

translation products could be occurring, and this requires analysis by pulse-chase methods.

Pulse-chase analysis

Cells of the various *R. sphaeroides* strains were grown semi-aerobically under conditions that would be expected to favour the synthesis of pigment-protein complexes. They were given a 5 min pulse with L-[³⁵S]methionine, followed by a 3 h chase with unlabelled L-methionine. Samples were taken at 10–90 min intervals, the membranes extracted and analysed by SDS/PAGE; Western blots of the gels were autoradiographed (Figures 6a–d). In both the wild-type and TC40 membrane fractions, the intensity of the bands corresponding to the LH2 α- and β-polypeptides remains constant throughout the chase, indicating stable integration of these polypeptides into the photosynthetic membranes. With TC70 and TC72 membrane fractions, pulse-chase results did show detectable amounts of the LH2 polypeptides for the first 10 min, but the levels drop to barely detectable amounts by 30 min and are undetectable after 90 min (Figure 7). The positions of the LH2 subunits in the pulse-chase gels were verified by Western-blot analysis using the Amersham ECL detection kit (results not shown).

Complementation analysis

In order to verify that the effects of the lesions in carotenoid biosynthesis were exerted solely by lack of phytoene synthase

Figure 6 Pulse-chase analysis of LH2 polypeptides

SDS/PAGE (12–20% gels run as in Figure 5) was performed on photosynthetic membranes extracted from cells given a 5 min pulse with L-[³⁵S]methionine, followed by a 3 h chase with unlabelled L-methionine, blotted on to nitrocellulose and subjected to autoradiography. (a) Wild-type; (b) TC40; (c) TC70; (d) TC72. Lane 1, 0 min; lane 2, 10 min; lane 3, 30 min; lane 4, 90 min; lane 5, 180 min, after the chase. LH1 and LH2 α- and β-polypeptide bands and the reaction centre H-subunit (RC-H) bands are indicated.

Figure 7 Graph showing the stability of the LH2 α-polypeptide band in *crt* strains analysed by pulse labelling and autoradiography

The intensity of the LH2 α-polypeptide bands in Figure 6 was calculated on a Vilber Lourmat Bio-Profile densitometer using the Profile Intensity function, and expressed as a percentage of the intensity of the reaction centre H-subunit (RC-H) band in each track. ♦, Wild-type; ■, TC40; ▲, TC70; ●, TC72. Time points indicated are number of minutes after the chase.

and desaturase (encoded by *crtB* and *crtI* respectively) rather than any other effects on neighbouring genes, the *crtI* gene was amplified by PCR and cloned into a self-replicating non-integrative plasmid to give plasmid pHLKPD1 (H. P. Lang, R. J. Cogdell, A. T. Gardiner and C. N. Hunter, unpublished work). In this construct, transcription of inserted DNA is driven by the oxygen-regulated *puc* operon promoter (McGlynn and Hunter, 1992). Figures 8(a) and 8(b) show low-temperature

Figure 8 Complementation analysis of *crtB* and *crtI* mutants by transformation with the cloned *R. sphaeroides crtI* gene

The *R. sphaeroides crtI* gene was amplified by PCR and cloned into pRKSK1, to give pHLKPD1 (H. P. Lang, R. K. Cogdell and C. N. Hunter, unpublished work) and transformed into TC70 and TC72. Photosynthetic membranes were isolated on a discontinuous sucrose gradient, diluted in 80% glycerol and low-temperature (77 K) absorption spectra were recorded. (a) TC70 + pHLKPD1; (b) TC72 + pHLKPD1; (c) wild-type; (d) ultrastructural analysis of TC72 + pHLKPD1 by thin-section electron microscopy; magnification $\times 21\,000$. All strains were grown semi-aerobically in the dark.

spectra of membranes extracted from semi-aerobic cultures of both TC70 and TC72 transformed with this plasmid. TC70 is a *crtB* mutant and is not complemented by pHLKPD1; TC72 is a *crtI* mutant and, when it is complemented by pHLKPD1, not only are coloured carotenoids produced but the LH2 complex is restored and the morphology of the intracytoplasmic membranes returns to the spherical invaginations characteristic of the wild-type (Figure 8d). The level of the two LH2 absorbance peaks is significantly lower than in the wild-type with respect to the amount of LH1 absorbance at 875 nm.

We conclude from this experiment that the disruption of phytoene desaturase synthesis is solely responsible for the lack of stably assembled LH2 complex, and that this has attendant effects on membrane morphology.

DISCUSSION

The absence of the LH2 complex from the carotenoidless blue-green mutants TC70 and TC72 was established by absorption spectroscopy and Western blotting (Figures 2 and 5). As soon as the coloured carotenoid, neurosporene, is produced in TC40, the B800–850 absorption peaks of the LH2 complex reappear, so the assembly of the LH2 complex is clearly affected in some way by the function of the *crtI* gene. To establish at what level this effect takes place during the synthesis and assembly of the LH2 complex, it was first necessary to establish whether the *pucA* and *B* genes are transcribed. Northern-blot analysis of total RNA prepared from the carotenoid mutants shows that transcripts for the LH2 α - and β -polypeptides are indeed present in all the mutants analysed (Figure 4). This is in agreement with

Dörge et al. (1987) who also observed the presence of transcripts for the LH2 proteins in a *R. capsulatus* mutant defective in carotenoid synthesis.

It is not so easy to establish whether or not these transcripts are translated, as complexes with a modified structure could exhibit an increased susceptibility to endogenous proteolytic attack. It appears that an LH2 complex can only assemble in carotenoidless mutants if there is some compensating alteration in the structure (Davidson and Cogdell, 1981; Robert et al., 1984), but past studies have yielded conflicting results on the relationship between LH2 assembly and carotenoid biosynthesis. Both Zsebo and Hearst (1984) and Giuliano et al. (1988) concluded that, if biosynthesis of coloured carotenoids is blocked in *R. capsulatus*, the bacteriochlorophyll-binding LH2 α - and β -polypeptides are synthesized, but the non-bacteriochlorophyll-binding γ -subunit of the LH2 complex is absent. They suggest a pleiotrophic effect, where mutation of the carotenoid genes suppresses expression of the γ -subunit. However, Kiley et al. (1988) looked at carotenoidless mutants of *R. sphaeroides*, and, by means of SDS/PAGE and Western-blot analysis, found that the LH2 α - and β -polypeptides were absent. The results of SDS/PAGE and Western-blot analysis of the carotenoid mutants shown in Figure 5, although not conclusive, would appear to support Kiley et al. (1988) in respect of the lack of LH2 polypeptides; however, pulse-chase analysis (Figure 6) clearly demonstrates that, in TC70 and TC72, the LH2 α - and β -polypeptides are synthesized, but they are rapidly degraded and are not stably integrated into the membrane. TC40, which produces the coloured carotenoid, neurosporene, behaves like the wild-type. These results indicate that an active *crtI* gene is required for assembly and/or stable integration of LH2 in the photosynthetic membrane of *R. sphaeroides*. In any case, recent sequencing studies downstream of the *pucBA* genes of *R. sphaeroides* failed to identify a *pucE* gene encoding the LH2 γ -subunit (Gibson et al., 1992), despite its presence in the analogous position in *R. capsulatus* (Tichy et al., 1989). Thus it would appear that there are different requirements for stable assembly of LH2 complexes in these two species. This conclusion is supported by other results obtained for *R. capsulatus*. For example, both Dörge et al. (1987) and Bartley and Scolnik (1989) found that low levels of LH2 can be stably inserted into the photosynthetic membranes of *R. capsulatus* in the absence of coloured carotenoids, and they conclude that there is no direct connection between the presence of coloured carotenoids and the formation of LH2.

Another difference is revealed by the complementation studies in Figure 6. Bartley and Scolnik (1989) suggest that there is a correlation between levels of phytoene desaturase (CrtI) and the formation of LH2 in *R. capsulatus*, but this is clearly not the case in *R. sphaeroides*. Figures 8(a) and 8(b) show the absorption spectra of TC70 and TC72 mutants complemented by plasmid pHLKPD1, which contains the *R. sphaeroides crtI* gene under control of the *puc* promoter (H. P. Lang, R. K. Cogdell, A. T. Gardiner and C. N. Hunter, unpublished work). TC72 is a *crtI* mutant so it is complemented by pHLKPD1; it produces coloured carotenoids, and the B800–850 peaks are restored indicating stable assembly and integration of the LH2 complex. In contrast, TC70 is a *crtB* mutant and is not complemented by pHLKPD1; there is no restoration of the B800–850 peaks despite the presence of CrtI. This proves that it is the presence of coloured carotenoids and not the CrtI protein that is mediating LH2 assembly in *R. sphaeroides* and also negates the possibility of a pleiotrophic effect arising from Tn5 insertions into *crtB* and *crtI* in the mutants TC70 and TC72. If genes downstream of *crtIB* were essential for LH2 assembly and were linked transcrip-

tionally to *crtIB*, then addition of *crtI* on pHLKPD1 would not have restored LH2. These conclusions are supported by Zurdo et al. (1993) who used detergents to remove carotenoids selectively from LH2 in *R. sphaeroides*. They found that the spectroscopic changes that accompany carotenoid extraction are very similar to the spectral features of carotenoidless LH2⁻ mutants. They suggest that the carotenoid is involved in establishing and/or maintaining the native conformation of LH2 by stabilizing the wild-type conformation of the bacteriochlorophyll molecules in LH2.

A parallel situation has been observed in the photosystem of higher plants. It has been shown that photosystem II (PSII) requires carotenoids for stable accumulation (Plumley and Schmidt, 1987). Markgraf and Oelmüller (1991) used the herbicide Norflurazon to inhibit carotenogenesis in mustard seedlings (Norflurazon inhibits the CrtI or phytoene desaturase enzyme), and found that, although assembly and activity *in vivo* of photosystem I did not require carotenoids, photosystem II was not assembled. Herrin et al. (1992) examined mutants of *Chlamydomonas reinhardtii* blocked at specific steps of carotenoid or chlorophyll biosynthesis. They found that, in mutants completely blocked in carotenoid biosynthesis, the chlorophyll apoproteins of photosystems I and II, both core and light-harvesting components, do not accumulate, although the corresponding mRNAs accumulate to normal levels. Synthesis of the various apoproteins, as measured by pulse labelling *in vivo*, does occur in the carotenoid-biosynthesis mutants but to different degrees. They conclude that the light-harvesting complex apoproteins do not correctly assemble with chlorophylls unless carotenoids, specifically xanthophylls, are present and the unassembled polypeptides are rapidly degraded. In addition to the apoproteins, they found that chlorophyll is degraded in the carotenoid-synthesis mutants, presumably because the complexes do not properly assemble. This is in agreement with the results presented here and suggests that there is a general requirement for carotenoids for the biosynthesis of many photosynthetic complexes.

Figures 3(a) and 3(b) demonstrate that membrane morphology is also affected in carotenoidless strains of *R. sphaeroides*. The effect of the absence of the LH2 complex on the membranes of *R. sphaeroides* is well documented (Lommen and Takemoto, 1978; Kiley et al., 1988; Hunter et al., 1988; Golecki et al., 1989, 1991; Gibson et al., 1992). In general, the intracytoplasmic membranes of LH2⁻ strains form long tubes that appear to interfere with cell division and large irregularly shaped membranes, instead of the wild-type vesicular structures (Figures 3c, 3d and 3e). Hunter et al. (1988) and Kiley et al. (1988) both concluded that the LH2 complex is required to attain normal photosynthetic membrane morphology, and Hunter et al. (1988) suggested that in the absence of the LH2 complex, morphogenesis of the internal membranes is arrested at the tubular stage. However, Golecki et al. (1989, 1991) demonstrated intracytoplasmic membrane vesiculation in the absence of LH2 and in some mutants observed regular vesicular structures with 43 nm diameter and long tubes of 60–95 nm diameter in the same strain; they therefore exclude an important role of the LH2 complex in the formation of vesicular shaped intracytoplasmic membranes. None of these studies, though, were performed with genetically defined LH2⁻ mutants. Gibson et al. (1992) did use such mutants, and they also identified some mutant strains with both normal vesicular and abnormal tubular structures. They conclude that, in *R. sphaeroides*, membrane morphology may be affected by the presence of both the PucC protein and the LH2 complex. However, the situation is complicated by the dependence of LH2 expression on PucC, and gross alteration in

membrane morphology is most predictable when the whole *pucBAC* operon is disrupted.

The two carotenoidless mutants used in the present study, TC70 and TC72, both exhibit gross alteration in their intracytoplasmic membrane morphology (Figures 3a and 3b). Cells that do not contain spiralling membranes appear to lack any significant intracytoplasmic membrane system. Moreover, normal morphology is restored in transconjugant strains containing phytoene desaturase, with a consequent restoration of the LH2 complex (Figure 8d). The low levels of vesiculation seen in some LH2-deficient mutants appear, at least in those that have been genetically defined, to depend on the synthesis of small amounts of part or all of the LH2 polypeptides and depend largely on the nature of the mutation (Gibson et al., 1992). If Zurdo et al. (1993) are correct in their conclusions that carotenoids are involved in stabilizing the bacteriochlorophyll molecules in LH2, this would suggest a reason for the gross alteration in membrane morphology when carotenoids are absent. Spiralling membranes have only been observed in LH2⁻ mutants lacking coloured carotenoids and not in those containing coloured carotenoids. It is possible that this additional distortion is due to conformational changes in the LH1 and reaction centre complexes induced by the absence of carotenoids, and this is supported by the evidence of spectral shifts in LH2⁻ mutants containing coloured carotenoids (Figure 2; Jones et al., 1992). It is possible that small amounts of LH2 polypeptides are harmful in some way when carotenoids are absent; certainly the carotenoidless mutant R26 (Clayton and Smith, 1960) exhibits a tendency to revert to the strain R26.1 containing a modified LH2 complex (Davidson and Cogdell, 1981; Robert et al., 1984). The nature of this modification partially compensates for the carotenoidless condition as shown by our recent studies on site-directed mutants of LH2 expressed in carotenoid-deficient backgrounds (G. J. S. Fowler, H. P. Lang and C. N. Hunter, unpublished work).

We thank the Agricultural and Food Research Council (AFRC) for their support.

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