Carotenoid Biosynthesis in Photosynthetic Bacteria

GENETIC CHARACTERIZATION OF THE RHODOBACTER CAPSULATUS CrtI PROTEIN

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Carotenoids are photoprotective pigments present in many photosynthetic and nonphotosynthetic organisms. The desaturation of phytoene into phytol is a step in the biosynthetic pathway that in the photosynthetic bacterium Rhodobacter capsulatus is mediated by the product of the crtl gene. Here we report the sequence of this gene and the identification of CrtI as a membrane protein of approximate M, 60,000. Mutant strains with 5-fold lower or 10-fold higher levels of CrtI with respect to wild type have only small differences in their carotenoid content, indicating that the cellular concentration of CrtI is not a limiting factor in carotenoid biosynthesis. However, a correlation was found between the levels of CrtI and the formation of a photosynthetic antenna system.

Materials and Methods

Growth Conditions—R. capsulatus cells were grown under photosynthetic conditions in ammonia-free minimal medium (RCVBNF) supplemented with threonine (10 mM) or ammonia as described (10).

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Matings were done as described (9). The wild-type strain was SB1003, the crtl point mutation used was BPY69, and the interposon insertion strains were described previously (8).

DNA Sequencing—Sequence was determined on both strands of pUBLUSCRIPT (Promega, Madison, WI) derivatives by the chain termination method (11) using both commercially available and custom-made primers. For sequence analysis we used the Wisconsin sequence analysis package (12).

Plasmid Construction—For expression of a CrtI-derived peptide in Escherichia coli we cloned the 1834-bp Ndel-BamHI fragment from CrtI (Fig. 1) into a T7 translation vector (13). The resulting plasmid (pT701) was transformed into E. coli BL21 (DE3) and expression was induced as described (13). For expression in R. capsulatus a 2400-bp NruI-BgII fragment carrying crtl and a segment of crtB (8) was cloned between the NdeI (converted to a blunt end with T4 DNA polymerase) and the BamHI site of pNF3 (10).

Antibody Production and Immunoblots—Sections of NaDodSO4-polyacrylamide gel electrophoresis gels containing the polypeptide expressed from pT701 were cut, frozen, and ground to a powder in liquid nitrogen. Antibodies were raised in New Zealand White rabbits by Hazelton Research Products, Denver, PA, and purified according to Olimstead (14). For immunoblots we used the Protoblot AP kit from Promega (Madison, WI) according to the manufacturer's instructions. The intensity of the reactive bands was visually compared with standards obtained with purified CrtI polyepitides expressed in E. coli.

Pigment Characterization—Bacteriochlorophyll was determined from acetone-methanol (7:2) extracts according to Cohen-Bazire et al. (15), and carotenoid according to Scolnik et al. (16).

Results

Nucleotide Sequence of the crtl Gene—Based on insertion mutagenesis, nuclease S1 protection analysis, and complementation of point mutations, we had determined previously (8) that crtl starts downstream of the NruI site (bp 6) and continues for about 1.4 kilobase pairs. The nucleotide sequence of crtl and the deduced peptide sequence of the only open reading frame that conforms to a compiled codon usage for R. capsulatus (data not shown) is shown in Fig. 1. There are three in-frame ATG codons in the first 150 bp that could serve as start codons. Based on R. capsulatus codon usage and on homology to the equivalent gene from Neurospora crassa,2 we locate the putative translation start at the ATG at bp 88. This ATG codon is preceded by a Shine-Dalgarno element that overlaps on homology to the equivalent gene from Neurospora crassa,2 we locate the putative translation start at the ATG at bp 88. This ATG codon is preceded by a Shine-Dalgarno element that overlaps

1 The abbreviations used are: bp, base pair(s); LH-I and LH-II, light-harvesting antenna I and II, respectively; NaDodSO4, sodium dodecyl sulfate.
2 G. E. Bartley and P. A. Scolnik, manuscript in preparation.

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**FIG. 1.** Genetic physical map, nucleotide sequence, and deduced polypeptide sequence of the *crtI* gene. A, physical map shows above the line the restriction sites at which interposon insertions were constructed and the location of initiation (ATG) and termination (TGA) codons. The names of the interposon mutant strains and their color phenotype are shown below the line. The color phenotype of wild-type and *crtZ* mutants is, respectively, red and blue-green. B, nucleotide sequence of *crtI* numbered from bp 1 and deduced peptide sequence in the one-letter code. Bars on top of the nucleotide sequence indicate relevant restriction sites: *NruI* (TCGCGA, bp 148 was changed by site-specific mutagenesis to CATATG (*NdeI*). Stretches of peptides likely to form transmembrane domains (18) are underlined.
R. capsulatus CrtI Protein

The occurrence of His residues in hydrophobic areas of CrtI compared with the putative bacteriochlorophyll-binding antenna sequence. His residues are numbered on the right.

![Diagram of CrtI sequence](image)

**FIG. 2.** Occurrence of His residues in hydrophobic areas of CrtI compared with the putative bacteriochlorophyll-binding antenna sequence. His residues are numbered on the right.

A plasmid pT701 containing a segment of CrtI under control of the phage T7 promoter (T7P) and NaDodSO4-polyacrylamide gel electrophoresis of extracts from E. coli cells carrying pT701 stained with Coomassie Blue. 10 μg each of soluble (lane 1) or insoluble (lane 2) fractions were run in a 10% gel. Location of the CrtI-derived polypeptide is indicated. B, plasmid pGBN3.2 containing CrtI under control of the nif promoter (nifP). Black boxes and thin lines indicate, respectively, R. capsulatus and vector DNA. Below, immunoblot of R. capsulatus membrane fractions (10 μg/each) run in a 10% NaDodSO4-polyacrylamide gel electrophoresis gel, blotted onto nitrocellulose, and probed with the anti-CrtI antibody. Lane 1, SB1003; lane 2, SB203N; lanes 3 and 4, BPY69 (pGBN3.2). For lanes 1, 2, and 4 the nifP was activated by adding ammonia to the medium (10).

![Diagram of antigen expression](image)

**FIG. 3.** Overexpression of CrtI in E. coli and R. capsulatus.

A, plasmid pT701 containing a segment of CrtI under control of the phage T7 promoter (T7P) and NaDodSO4-polyacrylamide gel electrophoresis of extracts from E. coli cells carrying pT701 stained with Coomassie Blue. 10 μg each of soluble (lane 1) or insoluble (lane 2) fractions were run in a 10% gel. Location of the CrtI-derived polypeptide is indicated. B, plasmid pGBN3.2 containing CrtI under control of the nif promoter (nifP). Black boxes and thin lines indicate, respectively, R. capsulatus and vector DNA. Below, immunoblot of R. capsulatus membrane fractions (10 μg/each) run in a 10% NaDodSO4-polyacrylamide gel electrophoresis gel, blotted onto nitrocellulose, and probed with the anti-CrtI antibody. Lane 1, SB1003; lane 2, SB203N; lanes 3 and 4, BPY69 (pGBN3.2). For lanes 1, 2, and 4 the nifP was activated by adding ammonia to the medium (10).

![Table of pigment composition](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carotenoids</th>
<th>Bchl</th>
<th>Bchl/Carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1003</td>
<td>2.6 ± 0.10</td>
<td>10.4 ± 0.12</td>
<td>4.0 ± 0.22</td>
</tr>
<tr>
<td>BPY69 (pGBN3.2)</td>
<td>2.7 ± 0.02</td>
<td>17.7 ± 0.21</td>
<td>6.6 ± 0.12</td>
</tr>
<tr>
<td>SB203N</td>
<td>1.4 ± 0.12</td>
<td>6.0 ± 0.07</td>
<td>4.3 ± 0.43</td>
</tr>
</tbody>
</table>

The coding region.

It has been postulated that the structural polypeptides of the R. capsulatus antenna system interact with bacteriochlorophyll through His residues located in transmembrane α-helical domains (19, 20). The common motif for these residues is Ala-X-X-X-His (19). Conserved His, but not Ala, residues were observed in antenna polypeptides of different photosynthetic bacteria (20, 22). There are four His residues in hydrophobic areas of CrtI. Three of them contain either Ala or Gly, which is also a small side chain residue, at position -4 respect to His (Fig. 2). Using site-specific mutagenesis, Bylina et al. (21) have shown that the replacement of -4 Ala to Gly in a LH-I structural polypeptide does not alter the formation of this antenna complex.

Overexpression of a Segment of CRTI in E. coli and Immunochemical Detection of CrtI in R. capsulatus—To obtain an anti-CrtI antibody we cloned a 1084-bp segment of CRTI into a T7 expression vector (Fig. 3). Induction of expression resulted in the synthesis of a 41-kDa insoluble protein (Fig. 3). An antibody generated against this protein recognizes a 60-kDa membrane protein in the wild-type strain SB1003 (Fig. 3). This is consistent with the information derived from the nucleotide sequence. The protein remained bound to membranes washed with 1 M NaCl, and the apparent size was not affected by different heat treatments of the sample before electrophoresis or by including 8 M urea in the gel (data not shown).

CrtI was found only in low levels in SB203N (Fig. 3), and, as expected, it is absent in the interposon mutants SB203E and SB218 (data not shown).

Complementation of CRTI Mutants and Overexpression of CRTI in R. capsulatus—To further characterize the role of the CrtI protein we constructed pGBN 3.2, a plasmid in which the crtl gene is under the control of a promoter for nitrogen fixation (nif). We mobilized this plasmid by conjugation into the R. capsulatus mutant BPY69, a blue-green strain which contains a crtl 69 mutation (9). The use of a point mutation for complementation analyses of CrtI is necessary because interposon insertions in this gene are polar on the adjacent crtB gene (8). BPY69 (pGBN3.2) merodiploids are red even when the nif promoter is not induced (data not shown) and accumulate little CrtI (Fig. 3), but induction of the nif promoter led to a 10-fold increase in CrtI respect to wild type (Fig. 3).

We analyzed the pigmentation composition of cells grown under nitrogen fixing conditions (Table I). Compared to wild type, the strain SB203N has about 54 and 60% of, respectively, carotenoids and bacteriochlorophyll. BPY69 (pGBN3.2) has about the same concentration of carotenoids but 70% higher bacteriochlorophyll than the wild type. The ratio of bacteriochlorophyll to carotenoids is similar in SB203N and the wild-type strain, but it is about 60% higher in BPY69 (pGBN3.2). We did not observe accumulation of photheo in SB203N (data not shown).

Formation of Antenna Complexes—R. capsulatus has two
photosynthetic antenna systems, LH-I and LH-II, both formed by structural proteins, bacteriochlorophyll, and carotenoids (20). Under the low-light phototrophic growth conditions used in this work, LH-II is dominant. The infrared absorption maxima are 875 nm for LH-I and 800 and 850 nm for LH-II (20). SB203E and SB218 do not form LH-II (8), a result consistent with previously published work showing that mutations in the early steps of carotenoid biosynthesis affect the formation of this antenna complex (23). We have now examined the spectrum of SB203N and found that little LH-II forms in this strain (Fig. 4). In contrast, BPY69 (pGBN3.2) forms more LH-II than the wild type. We used electron microscopy to examine cells of all three strains and found no significant differences in cell size or degree of development of the photosynthetic membranes (data not shown).

DISCUSSION

This investigation shows that the *R. capsulatus* CrtI protein is an intrinsic membrane protein of approximate *M*₅₀₀₀₀. The association with the membrane might be mediated by at least one of the three hydrophobic segments identified. Several of our results show that there is little correlation between the levels of CrtI and the concentration of carotenoids. First, SB203N contains approximately 5-fold less CrtI than SB1003 (Fig. 3), but accumulates about 54% of the carotenoids of the wild type. Second, when the plasmid pGBN3.2 was introduced into cells of the *crtI* mutant strain BPY69, the phenotype changed from blue-green to wild-type red, although the *nif* promoter was not induced, and no CrtI protein was detected in immunoblots (Fig. 3). Third, overproduction of CrtI with the *nif* expression vector (Fig. 2) did not result in an increase in carotenoid content with respect to wild type (Table 1). However, there is a significant increase in the bacteriochlorophyll/carotenoid ratio (Table 1).

Ultimately, characterization of the function of the individual proteins of the carotenoid biosynthetic pathway depends on our ability to reconstitute purified components into either natural or artificial membranes containing carotenoid precursors. At the present time, purification of these proteins in an active form cannot be easily achieved. The anti-CrtI antibody described here could be used to purify CrtI from *R. capsulatus* cells without having to rely on biosynthetically active systems. When the nucleotide sequence of other genes for carotenoid biosynthesis becomes available (28), the protocols described here can be used to characterize the remaining proteins of the pathway.

We have also investigated the formation of the photosynthetic antenna LH-II system in relation to the stoichiometry of CrtI. In photosynthetic bacteria there is a correlation between the presence of colored carotenoids and the formation of antenna systems (8, 23). The mechanism for this adaptive response, which allows the bacteria to limit the size of the photosynthetic antenna when photoprotective carotenoid pigments are absent, is not known. Carotenoids per se do not mediate the interaction, because an *R. capsulatus* mutant that lacks colored carotenoids can form low levels of LH-II (27). Our results with SB203N, which contains a significant amount of carotenoids but only low levels of LH-II, tend to support this lack of direct connection between the presence of colored carotenoids and the formation of LH-II. However, our observations on the formation of LH-II in the strains SB203N and BPY69 (pGBN3.2) (Fig. 3) suggest that there is a correlation between the levels of CrtI and the formation of LH-II complexes. In the case of overproduction of CrtI, the increased formation of LH-II is accompanied by an increase in the ratio of Bchl to carotenoids (Table 1). There are two possible explanations for these phenomena. First, it is possible that changes in the stoichiometry of CrtI give rise to unspecific interactions, for instance changes in the properties of the photosynthetic membrane, that alter pigment concentration and antenna formation. Second, it is possible that CrtI plays an accessory role in bacteriochlorophyll binding (24, 25, 26) and LH-II formation. The data currently available does not allow us to distinguish between these two possibilities.

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REFERENCES


**Fig. 4. Near-infrared absorption spectra of membranes from SB203N, SB1003, and BPY69 (pGBN3.2).** Absorption maxima of LH-I and LH-II are indicated on top. Samples were normalized for bacteriochlorophyll content.