

Novel Activity of *Rhodobacter sphaeroides* Spheroidene Monooxygenase CrtA Expressed in *Escherichia coli*[∇]

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The spheroidene monooxygenase CrtA of *Rhodobacter sphaeroides* introduces a keto group and/or hydroxy group at the ends of nonnative substrates in *Escherichia coli*, resulting in the production of novel oxocarotenoids. The heme-containing CrtA is not a P450 enzyme but a new type of oxygenase.

Carotenoids, which are produced by many microorganisms and plants, belong to a specific class of natural pigments. These structurally diverse pigments have different biological functions, such as coloration, photo protection, and light harvesting, and they also act as precursors for many hormones (2, 13). Carotenoids are commercially used as food colorants, animal feed supplements, cosmetic and pharmaceutical compounds, and more recently, as nutraceutical agents (20). Interestingly, recent studies have shown that carotenoids with more complex structures tend to have higher biological activity than those with simple structures (1, 5, 24). The increasing industrial importance of carotenoids has led to metabolic engineering of biosynthetic pathways, with the aim of generating new carotenoid structures (16, 19, 20), as well as developing microbial

processes for large-scale production of a range of carotenoids (6, 7, 13, 15, 21, 23, 26).

Rhodobacter sphaeroides monooxygenase CrtA is one of the scientifically interesting enzymes among the carotenoid enzymes frequently used to metabolically engineer the carotenoid pathways in heterologous producers (16, 19). In the original host *Rhodobacter* strains, the monooxygenase CrtA is known to catalyze the asymmetrical introduction of one keto group at the C-2 position of spheroidene (3, 4) and two keto groups at the C-2 and C-2' positions of spirilloxanthin (11). However, our recent studies (16, 19) showed that CrtA was able to introduce one or two keto groups into acyclic nonnative substrate structures, such as ζ-carotene and neurosporene, indicating that the substrate specificity of CrtA in *Escherichia coli*

TABLE 1. Properties of compounds identified in this study

Label	Suggested structure	Mass (m/z)	Observed fragment(s)	UV/visible light spectra (nm)
N1	1-OH-neurosporene-2-one	570		406, 425, 450
N2	1'-OH-neurosporene-2-one	572	554 (M-18), 536 (M-16), 480 (M-92)	456, 480
N3	1-OH-neurosporene-2'-one	572	480 (M-92), 435 (M-137), 417 (M-18-137)	453, 479
N4	Neurosporene-2',2-dione	570		405, 425, 450
L1	1'-OH-lycopene-2-one	570	552 (M-18), 534 (M-18-18)	435, 460, 489
L2	1-OH-lycopene-2-one	568		447, 472, 507
L3	Lycopene-2',2-dione	568		435, 460, 489
D1	1-OH-didehydrolycopene-2-one	566	548 (M-18), 532 (M-18-16), 520 (M-18-28), 508 (M-58), 498 (M-69)	482, 507
D2	Didehydrolycopene-2',2-dione	566	548 (M-18), 532 (M-34)	480, 501
D3	1-OH-didehydrolycopene-2',2-dione	582	565 (M-18)	482, 508
D4	1',1-(OH) ₂ -didehydrolycopene-2',2-dione	598	571 (M-27), 556 (M-42), 539 (M-59), 519 (M-79), 502 (M-96)	484, 514
T1	Tetradhydrolycopene-2-one	548		ND ^a
T2	1-OH-tetradhydrolycopene-2-one	564	548 (M-16), 546 (M-18), 528 (M-18-18), 518 (M-18-28), 508 (M-56), 506 (M-58)	492, 506,
T3	1',1-(OH) ₂ -tetradhydrolycopene-2-one	582	566 (M-18), 546 (M-18-18), 524 (M-58), 508 (M-16-58), 506 (M-18-58)	503, 515
T4	1-OH-tetradhydrolycopene-2',2-dione	580	563 (M-18)	503, 513

^a ND, not determined due to overlapping spectra.

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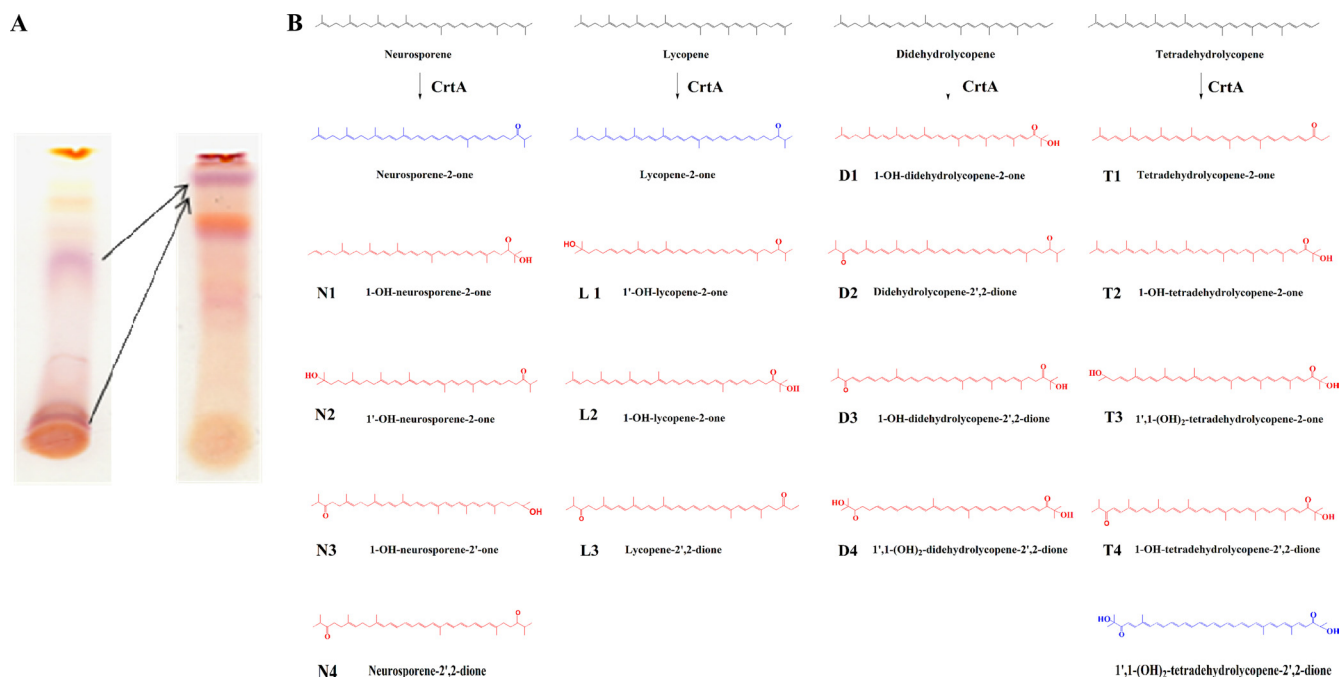


FIG. 1. TLC analysis of oxocarotenoids extracted from *E. coli*(pAC-CrtECrtBCrtI14, pUC-CrtA) (A), and the proposed structures of oxocarotenoids identified in this study (B). Carotenoids were visualized on TLC plates by using a low-polarity solvent system (hexane/acetone, 99:1 [vol/vol]) (left) and a high-polarity solvent system (hexane/acetone, 9:1 [vol/vol]) (right). The two arrows in panel A indicate the same spots seen on the TLC plates. Oxocarotenoid structures were proposed according to the assigned mass values and UV-visible spectra. In panel B, black and blue structures are those previously reported (19), and red structures were identified in this study.

was more promiscuous than expected. Although a few new oxocarotenoid structures have been reported, these provide only limited clues to understand the substrate specificity of CrtA. Therefore, additional structural information on unidentified oxocarotenoids, which are thought to be the derivatives of 3,4,3',4'-tetrahydrolycopene, 3,4-didehydrolycopene, lycopene, and neurosporene, and a spectroscopic analysis of the active site of CrtA should be carried out, because the data thus obtained will be useful in the elucidation of the catalytic mechanism of CrtA. In this study, therefore, we identified novel oxocarotenoids produced from the unexpected activity of CrtA heterologously expressed in *E. coli* and then carried out a spectroscopic analysis to better understand the enzymatic reaction of CrtA.

Recombinant *E. coli*(pAC-CrtECrtBCrtI, pUC-CrtA) and *E. coli*(pAC-CrtECrtBCrtI14, pUC-CrtA) (19) were cultivated for 24 to 36 h at 28°C in terrific broth (TB) medium supplemented with ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml). Carotenoids were isolated and analyzed using previously described methods (16, 19). For CrtA purification, *E. coli*(pUC-CrtA) was grown overnight on the TB medium and then harvested by centrifugation at 4,000 × *g* for 15 min at 4°C. The pelleted cells were resuspended in 50 mM sodium phosphate buffer, pH 7.9, and broken by sonication (18). The resulting homogenate was incubated with DNase (10 µg/ml) for 15 min on ice to reduce the viscosity of the suspension. After centrifugation of the treated homogenate for 30 min at 4,000 × *g*, the brown membrane pellet was resuspended in solubilization buffer A (50 mM sodium phosphate buffer, pH 7.9, 0.5% Triton X-100) and stirred on ice for 1 h. The suspension was

then centrifuged at 12,000 × *g* for 1 h at 4°C, and the supernatant was collected for further purification. The supernatant containing CrtA was then subjected to fast protein liquid chromatography (FPLC) using a column (16 × 45 cm; Pharmacia) filled with a cationic-exchanger SP resin. For gradient elution, buffer A (50 mM sodium phosphate buffer, 0.5% Triton X-100, pH 7.9) was mixed with increasing amounts of buffer B (50 mM sodium phosphate buffer, 0.5% Triton X-100, pH 7.9, 1 M NaCl). After a stepwise increase in the NaCl concentration, the protein was eluted, pooled, concentrated with a Centricon YM-10 filter (Millipore), and then desalted using a PD-10 desalting column (Amersham). This purified protein was used for *in vitro* characterization. All purification processes were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% gels. Proteins were stained with Coomassie brilliant blue, and protein concentrations were determined using the Bradford method (Bio-Rad protein assay kit). To obtain the crude P450-BM3 enzyme, *E. coli*(pUC-BM3) was cultured in TB medium at 30°C. After the cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4°C, the pelleted cells were resuspended in 50 mM sodium phosphate buffer, pH 7.9, and broken by sonication. The resulting homogenate was incubated with DNase (10 µg/ml) for 15 min on ice and centrifuged at 12,000 × *g* for 1 h at 4°C, following which the supernatant was collected for the spectroscopic experiment. Purified CrtA was reacted with imidazole and dithionite, and the absorption spectrum was measured. The purified CrtA in 0.1 M Tris (pH 7.9) buffer was equilibrated with nitrogen gas by gently bubbling for 5 min and then reacted by adding excess imidazole or dithionite (a few grains). Immediately after this

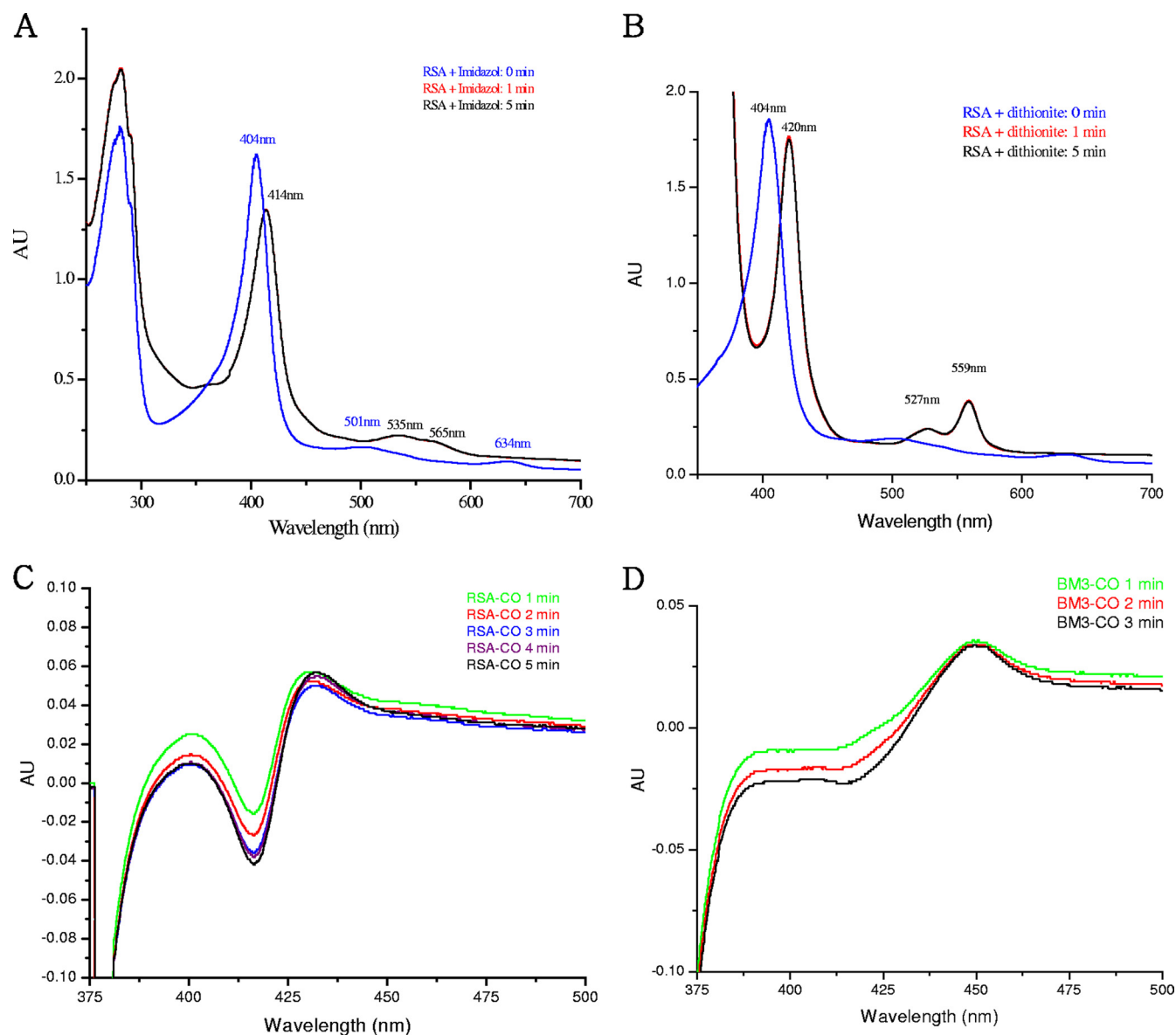


FIG. 2. (A to C) Absorption spectra of the purified CrtA (represented by RSA, for *R. sphaeroides* CrtA) reacted with imidazole (A), dithionite (B), and CO (C). (D) Absorption spectrum of P450-BM3 enzyme (represented by BM3) reacted with CO (used as a control). Absorption spectra were scanned at 1-min intervals. AU, absorbance unit.

step, the absorption spectra were obtained using a SPECTRAmax PLUS spectrophotometer (Molecular Devices). For the carbon monoxide (CO) difference absorption spectra, the purified CrtA and crude P450-BM3 were reduced by incubation with excess dithionite. The reduced CrtA and crude P450-BM3 were then treated with CO gas by gently bubbling for 1 min. Immediately after this step, the absorption spectra were collected using the SPECTRAmax PLUS spectrophotometer. Liquid chromatography-mass spectrometry (LC-MS), high-pressure liquid chromatography (HPLC), and thin-layer chromatography (TLC) analyses were performed under the same conditions as described in our previous study (19), and the structures of the carotenoids were determined using a combination of HPLC retention times, UV-visible absorption spectra, and mass fragmentation spectra (9, 10, 28).

Three additional novel oxocarotenoids (Table 1) were isolated from the crude extract of *E. coli*(pAC-CrtECrtBCrtI14, pUC-CrtA) (Fig. 1A) by using TLC, silica gel column chromatography, and preparative HPLC (19), and the new compounds were analyzed by HPLC and LC-MS. The purple compound T2 ($[M^+] = 564.4$) is proposed to be 1-OH-tetradecahydrolycopene-2-one, an oxocarotenoid derived from 3,4,3',4'-tetradecahydrolycopene. The deep-orange compound D1 ($[M^+] = 566.4$) is proposed to be 1-OH-didehydrolycopene-2-one, an oxocarotenoid derived from 3,4-didehydrolycopene. The purple compound T3 ($[M^+] = 582.4$) is proposed to be 1,1'-(OH)₂-tetradecahydrolycopene-2-one, an oxocarotenoid derived from 3,4,3',4'-tetradecahydrolycopene. The other minor compounds were also analyzed by LC-MS without further isolation. Based on the structural information, the new compounds are proposed to

be oxocarotenoids with a hydroxy and/or a keto group, derived from neurosporene, lycopene, 3,4-didehydrolycopene, or 3,4,3',4'-tetrahydrolycopene (Table 1). These novel oxocarotenoid structures suggest that CrtA can introduce a hydroxy group and a keto group at both ends of the acyclic carotenoid backbone, irrespective of the degree of saturation in the backbone (Fig. 1B). The hydroxylation reaction catalyzed by CrtA in heterologous *E. coli* was not entirely unexpected because CrtA was previously reported to form hydroxy intermediates during the ketolase reaction of CrtA in the wild-type *Rhodobacter capsulatus* host (11, 25). CrtA requires molecular oxygen and a reductant for its activity *in vivo* (29) and *in vitro* (11). Therefore, the heterologous environment of *E. coli* may force CrtA to incorporate molecular oxygen into the nonnative substrates and to accumulate reaction intermediates, such as hydroxy carotenoids, because of suboptimal enzymatic conditions.

CrtA is reported to be a heme monooxygenase that does not belong to the P450 family, although there is still no clear evidence of this hypothesis (11). Therefore, to better understand the structural properties of CrtA, CrtA was purified by solubilization of the enzyme from a membrane fraction by using Triton X-100, followed by fractionation on a cationic exchanger and spectroscopic analysis. First, the purified CrtA was reacted with imidazole or dithionite (8) and changes in the absorption spectra were determined. The addition of imidazole resulted in the movement of the Soret peak of CrtA from 404 to 414 nm. The two peaks at 501 and 634 nm changed to 535 and 565 nm, respectively (Fig. 2A). The addition of dithionite also resulted in the movement of the Soret peak from 404 to 420 nm, along with the distinguishable peaks at 527 and 559 nm (Fig. 2B). However, the addition of CO (12) after reduction of the heme with dithionite did not shift the Soret peak of CrtA (Fig. 2C), indicating that the distal pocket did not permit binding of CO to form the unique P450-type spectrum (30), as was seen in the case of P450-BM3 (Fig. 2D). The results together suggest that CrtA has a 5-coordinated heme at its active site and that it is not a P450 enzyme but a new type of oxygenase. Further structural investigation of CrtA, such as X-ray crystallography, could provide insights into the catalytic mechanism of CrtA. In conclusion, CrtA is a very unique oxygenase possessing interesting catalytic activity, which can be biotechnologically utilized to produce more diverse oxo chemical structures.

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