

Anaerobic carotenoid biosynthesis in *Rhodobacter sphaeroides* 2.4.1: H₂O is a source of oxygen for the 1-methoxy group of spheroidene but not for the 2-oxo group of spheroidenone

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Abstract Anaerobic biosynthesis of carotenoids in the purple facultative photosynthetic bacterium *Rhodobacter sphaeroides* was studied using mass spectrometry. We have demonstrated that ¹⁸O from H₂¹⁸O was incorporated into the 1-methoxy group of spheroidene and spheroidenone, the two major carotenoids produced by this bacterium during photosynthetic growth. Neither water nor CO₂ was shown to provide an oxygen atom for the 2-oxo group of spheroidenone in *R. sphaeroides* 2.4.1 grown photosynthetically in the absence of molecular oxygen. Possible mechanisms for the anaerobic biosynthesis of spheroidenone in *R. sphaeroides* are discussed.

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Key words: Carotenoid biosynthesis; Anaerobic oxidation; *Rhodobacter sphaeroides*; ¹⁸O Labeling; Mass spectrometry

1. Introduction

Spheroidene (SE, 1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro-ψ,ψ-carotene) and spheroidenone (SO, 1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro-ψ,ψ-caroten-2-one) are two major carotenoids (Crt) accumulated in variable amounts by the photosynthetic proteobacterium *Rhodobacter sphaeroides* 2.4.1 grown anaerobically (photosynthetically) or aerobically (chemoheterotrophically). SE, the penultimate product of the carotenoid biosynthesis pathway, is predominantly accumulated during photosynthetic growth at low light intensity. SO is the end product of this pathway, and is predominant under aerobic growth conditions or during photosynthetic growth at high light illumination.

The Crt biosynthetic pathway in *R. sphaeroides* and in the closely related *Rhodobacter capsulatus* has been described earlier [1,2] (Fig. 1). Most of the intermediate Crt products have been isolated and characterized [3]. However, there are few data on the detailed mechanisms of the enzymatic reactions involved in the final steps of this pathway. It was proposed by several workers that an incorporation of the 1-methoxy group in the Crt molecule proceeded via hydration of the C1-C2 double bond of neurosporene followed by methylation of the resulting 1-hydroxyneurosporene by the *S*-adenosylmethionine-dependent enzyme [3–6]. Nicotine was shown to inhibit the formation of SE and hydroxyspheroidene by photo-

synthetically grown *R. sphaeroides*, leading to the accumulation of neurosporene [7]. ²H was demonstrated to incorporate into the C2 position of SE accumulated by *R. sphaeroides* in the presence of deuterium oxide, suggesting the hydration mechanism for the introduction of the C1 tertiary hydroxyl group into the carotenoid molecule [8]. However, no direct experimental evidence supporting this proposed role of water as the source of oxygen for the 1-methoxy group of xanthophylls produced during anoxygenic, photosynthetic growth of *R. sphaeroides* is available.

Transformation of SE into SO, which is the final step of Crt biosynthesis in *R. sphaeroides*, involves the introduction of the 2-oxo function into the Crt molecule. Molecular oxygen was shown to be the source of oxygen in this reaction during aerobic biosynthesis of SO [9] and conversion of SE to SO was shown to be catalyzed by cell-free extracts of *R. sphaeroides* [10]. It was proposed that the enzyme was a mixed-function oxidase (monooxygenase). This reaction was long thought to be strictly dependent on the presence of molecular oxygen [5,9,11]. However, no data regarding the mechanism of the anaerobic biosynthesis of SO by *R. sphaeroides* under photosynthetic conditions were available. We have recently demonstrated that the SE-to-SO conversion is a redox-dependent process, and that SO can constitute a majority of the total Crt accumulated by *R. sphaeroides* during photosynthetic growth under high light illumination [12], or by *CcoP* and *RdxB* mutants of *R. sphaeroides*, during photosynthetic, diazotrophic growth (O'Gara and Kaplan, submitted). Therefore it was of interest to study the possible source(s) of oxygen for the conversion of SE to SO in the absence of molecular oxygen.

Water is considered to be a likely source of oxygen for the anaerobic biosynthesis of xanthophylls (O'Gara and Kaplan, submitted), and it has been recently demonstrated that in *R. sphaeroides* cells adapting from respiratory to photosynthetic conditions, the oxygen atoms of the 13¹-oxo and the 3-acetyl groups of bacteriochlorophyll *a* derive from water [14,15]. In the present study we describe the results of the mass spectrometric analysis of the ¹⁸O-labelled carotenoids produced by *R. sphaeroides* grown photosynthetically in the presence of H₂¹⁸O, and demonstrate that although water provides an oxygen atom for the 1-methoxy group of SE and SO, it does not for the 2-oxo group of SO, under anaerobic growth conditions.

2. Materials and methods

2.1. Bacterial strains and experimental conditions

Rhodobacter sphaeroides 2.4.1 (wild type) was used throughout.

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Cells were grown in Sistrom's minimal medium A [11] containing 0.4% succinate as the carbon source as described previously [16,17].

Experiments with H_2^{18}O were conducted in 1 ml Wheaton rubber-lined glass reaction vials. The total assay volume was 0.8 ml, including 0.7 ml of 70 atom% [^{18}O]water or 0.7 ml of unenriched water, and 0.08 ml of $10\times$ Sistrom's medium. Experiments were started by adding a suspension of photosynthetically grown cells (0.02 ml). Reaction vials were flushed with 98% N_2 -2% CO_2 for 1 h, and incubated at 28°C in front of the light source at 100 W/m^2 for 48–72 h. No residual O_2 was found to be present in the gassing mixture.

Experiments with C^{18}O_2 were conducted in 5 ml rubber-stoppered serum bottles. After 0.05 ml of photosynthetically grown cells (inoculum) was added to the medium (3.5 ml), the vials were flushed with oxygen-free nitrogen gas for at least 1 h, and the gas phase was replaced with 95 atom% [^{18}O] CO_2 , or with unenriched CO_2 . Cells were grown in front of the light at 100 W/m^2 for 48 h.

After incubation was completed, cells were pelleted by centrifugation, and the carotenoid pigments extracted and analyzed as described earlier [12]. The proportion of carotenoids introduced with the inoculum into the incubation medium did not exceed 2–3% of the total carotenoids isolated from the experimental samples.

2.2. HPLC separation of pigments

HPLC separation of pigments was performed on a Shimadzu SCL-10A system equipped with SPD-M10AV diode array detector. Pigment separation was achieved with a Spherisorb ODS2 (Rainin Instruments Company, Emerville, CA) reversed-phase column (25 by 0.46 cm), using a gradient elution system described previously [18].

2.3. Mass spectrometry

The carotenoid compounds were analyzed by FAB-MS. The mass spectra were acquired with a Kratos MS50 mass spectrometer equipped with a Mach 3 data system. The instrument was operated at 8 keV accelerating voltage. An Ion Tech B11NF saddle field gun was operated at 7 keV, and xenon was used. The probe tip was coated with a thin layer (1.2 μl) of the matrix (*m*-nitrobenzyl alcohol), to which was added 1.2 μl of sample dissolved in chloroform. The ^{18}O enrichment calculations were based on the intensity distribution of the ion cluster of the respective unlabelled carotenoid derivative.

2.4. Materials and reagents

Vitamins and HPLC solvents were obtained from Sigma. All other chemicals were of reagent-grade purity and were used without further purification. H_2^{18}O with 70.0% certified isotope enrichment was obtained from Cambridge Isotope Laboratories (Andover, MA). C^{18}O_2 with 95% isotope enrichment was supplied by Isotec Inc. (Miamisburg, OH). N_2 and CO_2 gases were purchased from Iweco Inc. (Houston, TX) and were 99.998% and 99.995% pure, respectively.

3. Results

3.1. Incorporation of H_2^{18}O into spheroidene and spheroidenone during photosynthetic growth of *R. sphaeroides* 2.4.1

To study the formation of SE and SO during anaerobic (photosynthetic) growth of *R. sphaeroides* 2.4.1, two series of experiments were conducted: one with water with a relative abundance of isotopes found in nature (99.8% ^{16}O and 0.2% ^{18}O) and the second with 62% [^{18}O]water. Photosynthetically grown *R. sphaeroides* 2.4.1 cells were used as an inoculum, the reaction vials were flushed with N_2/CO_2 , and the cells were grown under high light illumination (100 W/m^2) to ensure an increased proportion of SO among the synthesized carotenoids. After the incubation was completed, cells were pelleted by centrifugation, and carotenoid pigments extracted and separated according to the procedure described in Section 2.

Fig. 2A represents the mass spectra in the region of the molecular ion cluster of SE isolated from cells grown in the presence of either H_2^{16}O (control) or H_2^{18}O (experimental). The peaks corresponding to the pigment isolated from the cells grown in the presence of H_2^{18}O (filled columns) were

displaced by two mass units toward higher molecular masses, when compared to the spectra of the control preparation of SE from the cells grown in the presence of H_2^{16}O . The peak pattern in the experimental mass spectrum was consistent with the pattern calculated for SE containing ^{18}O in the 1-methoxy group at 62% enrichment with the heavy oxygen isotope (Fig. 2B), thus indicating close to 100% incorporation of oxygen from water into the 1-methoxy group of SE.

Fig. 3A shows the mass spectra of SO isolated from photosynthetically grown *R. sphaeroides* 2.4.1 cells. For the control sample, obtained from the incubation with unenriched water, the major peak at m/z 582 corresponded to the molecular ion of SO. The spectrum obtained for the SO accumulated during the incubation in the presence of H_2^{18}O revealed a major peak at m/z 584, and only a minor peak at m/z 586, thus indicating that only one of the two oxygen atoms of SO originated from water. The mass spectrum pattern obtained for the labelled SO fits well with the pattern calculated for 62% enrichment of one of the oxygen atoms of SO (Fig. 3B, filled columns), but does not fit with the pattern calculated for the incorporation of [^{18}O] into both the 1-methoxy and 2-oxo groups of SO (Fig. 3B, open columns). Taking into account the 62% enrichment of the labelled water in the experimental sample, and the presence of some SO in cells used for the inoculum, this result corresponds to approximately 100% isotope uptake from H_2^{18}O into one (methoxy) oxygen of the newly formed SO. As no detectable (within 10% experimental error) incorporation of the label into the 2-oxo atom of SO could be registered, these results suggest that water is not a source of oxygen for the transformation of SE to SO.

3.2. Experiments with C^{18}O_2

The experiments described above clearly demonstrated that water provides an oxygen for the 1-methoxy group of SE and SO, but not for the 2-oxo group of SO, during photosynthetic growth of *R. sphaeroides*. Molecular oxygen is known to serve as a source of oxygen for the biosynthesis of the 2-oxo group of SO, under aerobic growth conditions [9]. However, all ex-

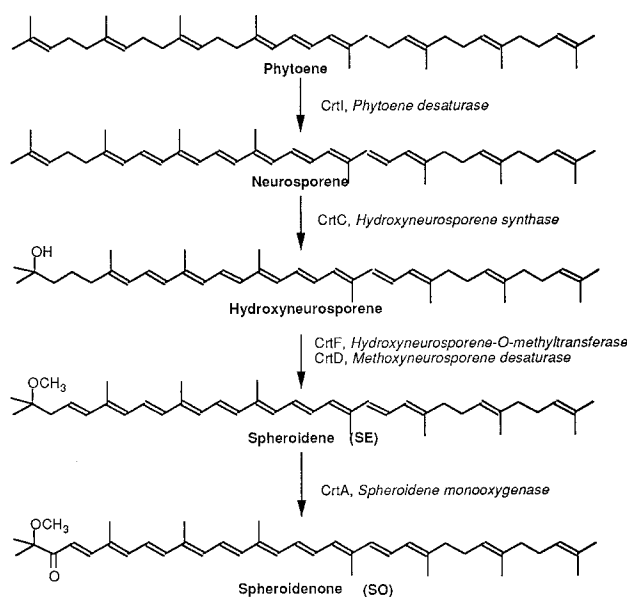


Fig. 1. The final steps and the respective enzymes of the proposed carotenoid biosynthesis pathway in *Rhodobacter* sp. [23].

periments described here were conducted under anaerobic conditions, and precautions were taken to exclude oxygen from the incubation medium. Therefore it seems likely that neither molecular oxygen nor water could participate in the SE-to-SO conversion. It had been earlier demonstrated in this laboratory that the ratio between SE and SO is regulated by the redox poise existing in the cell [12; O'Gara and Kaplan, submitted]. It is also known that during photosynthetic growth of *R. sphaeroides* the cellular redox poise can be dependent upon the availability of CO₂, apparently through the RuBisCO-mediated pathway [19,20]. As CO₂ was one of the components of the gas mixture used to create anaerobic conditions for growth of the *R. sphaeroides* cells, one could consider the possibility that CO₂ was directly involved in anaerobic Crt biosynthesis, providing an oxygen atom for the 2-oxo group of SO. To explore this possibility, cells were grown photosynthetically in the presence of either C¹⁶O₂ or C¹⁸O₂. Photosynthetically grown cells of *R. sphaeroides* 2.4.1 were used as inoculum, and the incubation vials were flushed with a stream of N₂ to remove oxygen from the medium.

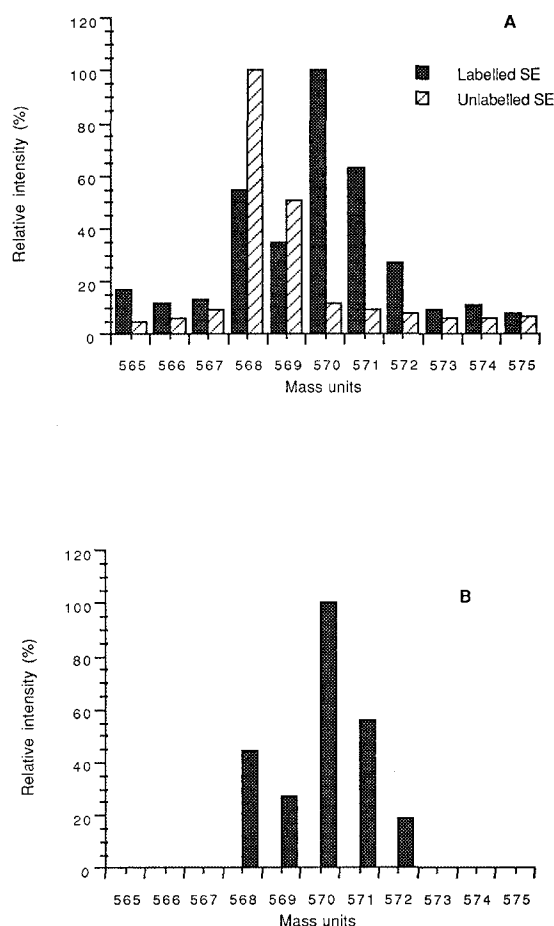


Fig. 2. A: Mass spectrometric analysis of ¹⁸O labeling of SE isolated from *R. sphaeroides* 2.4.1 grown photosynthetically in the presence of 62% [¹⁸O]-enriched water. The peak heights represent the relative intensity of the peaks in the mass spectrum in relation to the most intensive peak. B: Mass spectrum patterns calculated for 62% enrichment of the 1-methoxy group of SE. The spectrum was calculated based on the experimental molecular ion cluster of unlabelled SE (hatched columns, A) for 100% incorporation of ¹⁸O from 62% [¹⁸O]-enriched water.

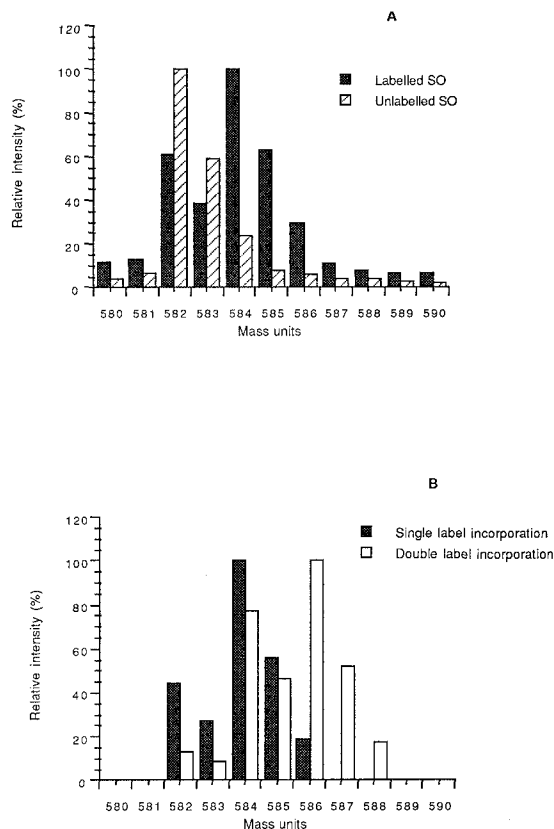


Fig. 3. A: Mass spectrometric analysis of ¹⁸O labeling of SO isolated from *R. sphaeroides* 2.4.1 grown in the presence of 62% [¹⁸O]-enriched water. The peak heights represent the relative intensity of the peaks in relation to the most intensive peak. B: Mass spectrum patterns calculated for 100% incorporation of [¹⁸O] from 62%-enriched water into the 1-methoxy group of SO (filled columns); and 1-methoxy and 2-oxo groups of SO (open columns). Calculations are based on the experimental molecular ion cluster of unlabeled SO (hatched columns, A).

After that reaction vials were sparged with CO₂ (control) or with CO₂ 95% enriched with [¹⁸O], as described in Section 2.

Fig. 4 represents the mass spectra obtained for SO isolated from control and experimental samples. As is evident from these spectra, no difference in the pattern of the molecular ion between the SO isolated from C¹⁶O₂ and C¹⁸O₂ cells could be detected. Similar results were obtained for SE isolated from the cells grown in the presence of either C¹⁶O₂ or C¹⁸O₂ (spectra not shown). These results indicate that CO₂ is not a significant source of oxygen for the anaerobic biosynthesis of SO in *R. sphaeroides*. However, one should not exclude the possible incorporation of oxygen from CO₂ into SO via the carbon assimilatory (RuBisCO-mediated) pathway. In that case, however, the ¹⁸O label would be highly diluted, which makes its detection by FAB-MS hardly possible.

4. Discussion

The labelling of the 1-methoxy group of SE and SO with ¹⁸O indicates that the conversion of neurosporene into hydroxyneurosporene proceeds via hydration of the C1-C2 double bond. It was suggested earlier [7,13] that water could provide oxygen for the 1-hydroxyneurosporene via a hydration reaction. Patel et al. [8] demonstrated that ²H from ²H₂O is incorporated into the C2 position of SE synthesized from

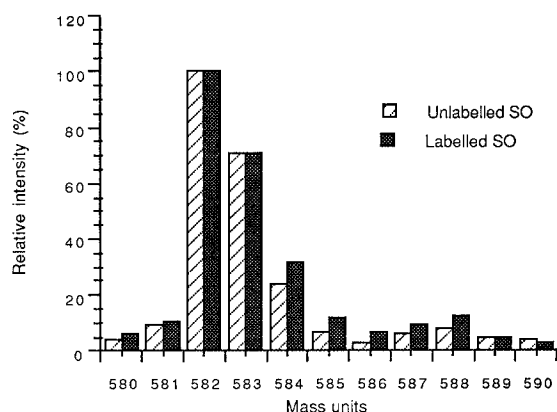


Fig. 4. Mass spectrometric analysis of SO isolated from *R. sphaeroides* 2.4.1 grown photosynthetically in the presence of CO_2 (hatched columns); and 95% ^{18}O -enriched CO_2 (filled columns). The peak heights represent the relative intensity of the peaks in relation to the most intensive peak.

neurosporene by photosynthetically grown *R. sphaeroides*, thus supporting the operation of a C1-C2 hydration pathway. In this study we confirm these earlier observations and present more direct evidence that water provides oxygen for the 1-methoxy group in anaerobic Crt biosynthesis by *R. sphaeroides*.

Another question which was addressed in this study concerns the source of oxygen for the 2-oxo group of spheroidene under anaerobic conditions. For some strains of *Chromatium*, an obligately anaerobic bacterium, which produces the 4-keto carotenoid okenone, it was proposed that an introduction of the keto function in the absence of molecular oxygen might involve hydroxylation at C4 followed by oxidation of the secondary alcohol group to the 4-oxo group [5,21]. However, this proposed mechanism did not receive experimental support, as the corresponding intermediate carotenols have never been isolated [13]. It is clear from the results presented in this study that water is not a significant source of oxygen during anaerobic synthesis of the 2-oxo group, as virtually no incorporation of ^{18}O into the 2-oxo group of SO could be detected. The presence of molecular oxygen (which is known to be a source of oxygen for the 2-keto group during aerobic growth) in the medium could be excluded as the incubation vials were thoroughly degassed and flushed with an oxygen-free mixture of N_2/CO_2 prior to incubation. In addition, O'Gara and Kaplan (submitted) have shown that in cells of *R. sphaeroides* missing both Cyt *cbb*₃ terminal oxidase and RdxB, grown under diazotrophic growth conditions, the level of SO reaches 93% of the total carotenoid. Under these conditions there is no free oxygen in the medium. One can consider the possibility of the derivation of molecular oxygen from water as a result of photolysis. However, we did not detect any significant incorporation of the label into the *m/z* 586 peak, which suggests water was neither directly nor indirectly a major source of oxygen for SO synthesis.

We have also demonstrated that ^{18}O from C^{18}O_2 was not incorporated into newly formed SO during photoheterotrophic growth of *R. sphaeroides*. As neither water nor CO_2 was proved to be a source of oxygen for the anaerobic biosynthesis of SO, it seems likely that the oxygen atom for the 2-oxo group of SO is provided by some oxygen-containing species derived from cellular metabolites. Other results obtained

in this laboratory indicate that a *CrtA*[−] mutant accumulated SE, but not SO under both aerobic and anaerobic conditions (J. O'Gara and S. Kaplan, in preparation), thus suggesting that one and the same enzyme (CrtA) likely converts SE to SO under both types of growth conditions. Therefore, under conditions of high light illumination SE might act as a sink for the proposed harmful oxygen-containing species, and the transformation of the SE into SO conversion serves as an important mechanism of photoprotection. This proposed mechanism would correspond to photosynthetic anaerobic growth, and apparently resembles the suggested earlier photoprotection reaction by chemical interaction of carotenoids with singlet oxygen resulting in oxidized carotenoids, which takes place during growth under mixed conditions in the presence of both high light and low oxygen tension [21].

We have previously demonstrated that SO is more abundant in the B875 antenna complex, and SE is predominantly associated with the B800-850 complex synthesized by *R. sphaeroides* 2.4.1 during photosynthetic growth [12]. It is possible to speculate that the transformation of SE to SO may involve carotenoids physically bound to the structural polypeptides of the photosynthetic antenna complexes. In that case the harmful oxygen species evolved under conditions of high light illumination are quenched by the carotenoids which are located in close proximity to the endogenous photosensitizer. If this mechanism is operative, one should expect the CrtA enzyme catalyzing the introduction of the 2-oxo function, to be tightly associated with the photosynthetic antenna complex. Ref. not mentioned in text [22]

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