The derivation of the oxygen atoms of the 13^1-oxo and 3-acetyl groups of bacteriochlorophyll *a* from water in *Rhodobacter sphaeroides* cells adapting from respiratory to photosynthetic conditions: evidence for an anaerobic pathway for the formation of *iso*cyclic ring E

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Received 5 July 1995

Abstract **Using mass spectrometry, we have demonstrated ^{18}O-labelling of both the 13^1-oxo and 3-acetyl groups of newly-formed bacteriochlorophyll *a* synthesized by *Rhodobacter sphaeroides* cells during adaptation from respiratory to photosynthetic conditions in the presence of $H_2{}^{18}O$. This derivation of the 13^1-oxo group of bacteriochlorophyll *a* from water provides a stark contrast with that of chlorophylls in higher plants where ring E formation is an aerobic process in which the 13^1-oxo group arises from molecular oxygen via an oxygenase activity. The formation of the 3-acetyl group of bacteriochlorophyll *a*, however, is consistent with the enzymic hydration of the 3-vinyl group of a derivative of chlorophyll *a*.**

Key words: Biosynthesis; Bacteriochlorophyll *a*; *Iso*cyclic ring E; Anaerobic cyclization; ^{18}O-labelling; Mass spectrometry

1. Introduction

Bacteriochlorophyll (BChl) *a*, an antenna and reaction centre chlorophyll of many photosynthetic bacteria, like all known functional chlorophylls, possesses a 13^1-oxo group arising from the introduction of an oxygen atom into the 13-propionic acid methylester side chain of protoporphyrin-IX-mono-methylester during *iso*cyclic ring E formation [1,2]. Several biosynthetic steps later a second carbonyl oxygen is introduced into some chlorophylls; for instance, into the 3-acetyl side chain of BChl *a* [3] and into the 7-formyl group of chlorophyll (Chl) *b* [4–6].

In this paper, we investigate the origin of the 13^1-oxo and 3-acetyl oxygens of BChl *a* in the facultative-photosynthetic purple bacterium, *Rhodobacter sphaeroides*. In the purple bacteria, including *Rb. sphaeroides*, BChl *a* formation is mainly regulated by oxygen tension and light. Respiring cells can form BChl *a* under mildly aerobic conditions in the dark but synthesis is more prolific during adaptation from a respiratory life mode in the dark to a photosynthetic existence in the light under microaerophilic or anaerobic conditions [7]: the maximum oxygen tension at which pigment synthesis can occur varies for individual species. This adaptation is usually performed in non-degassed media under microaerobic conditions in filled and sealed bottles rather than under strict anaerobiosis. Thus, a question arises about the origin of the 13^1-oxo and 3-acetyl groups (see Fig. 1): are they derived aerobically from molecular oxygen or from water by a potentially anaerobic mechanism?

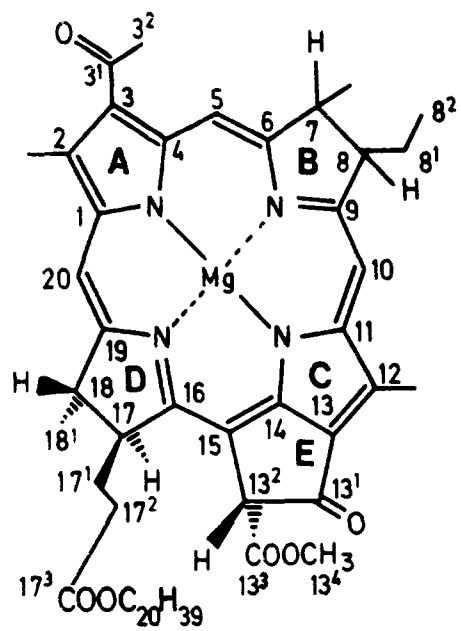

Fig. 1. Structure of BChl *a* showing the carbonyl oxygens discussed in this paper at C-3^1 and C-13^1. The carbon numbering system and ring labelling is that approved by IUPAC-IUB [19].

By adapting respiring cells of *Rb. sphaeroides* to photosynthetic conditions in the presence of $H_2{}^{18}O$ and examining the BChl *a* formed by mass spectrometry, we show in this paper that the oxygen atoms of both the 13^1-oxo and 3-acetyl groups of BChl *a* are derived from water. These results contrast sharply not only with those of Nashrulhaq-Boyce et al. [1] and of Walker et al. [2] demonstrating the formation of the 13^1-oxo group in higher-plant Chls from O_2 but also with those of Schneegurt and Beale [4] and of Porra et al. [5,6] who showed

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Nomenclature and abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; Chl, chlorophyll; Chlide, chlorophyllide. The IUPAC-IUB numbering (see Fig. 1) and nomenclature systems [19] have been used together with [] bracket notation for substituents.

SSDI 0014-5793(95)00854-3

in algae and higher plants, respectively, that another carbonyl oxygen, the formyl group of Chl *b*, also arises from O_2; however, evidence that the formation of the 3-acetyl group may occur via hydration of the 3-vinyl group of chlorophyllide (Chlide) *a* (see section 4.3) was obtained by Jones [3].

2. Experimental

2.1. Chemicals and organism

$H_2{}^{18}O$ with 95.0% certified isotope enrichment was supplied by Novachem Pty Ltd (Melbourne, Australia). Argon (99.998%) was supplied by L'Air Liquide GMBH, Düsseldorf. $NaBH_4$ was obtained from Merck-Schuchardt (Darmstadt, Germany). DEAE-cellulose (DE52) was supplied by Whatman Laboratory Division and a suspension in methanol was prepared as previously described [8].

2.2. Organism and maintenance

Rhodobacter sphaeroides (NCIMB 8253) was supplied by the National Collection of Industrial and Marine Bacteria Ltd, Aberdeen, Scotland. Brown photosynthesizing cultures of *Rb. sphaeroides* were grown for 24 h before incandescent lamps (42.5 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 32°C in sealed 500 ml clear-glass, screw-top bottles filled to the top with a modified malate-glutamate medium [7,9]. At the end of this period the cell density was ≈1 mg dry wt./ml.

2.3. Preparation of dark-grown aerobic cells for adaptation experiments

Pale pink, respiring *Rb. sphaeroides* cells were grown at 32°C in the dark in 1 liter Erlenmeyer flasks containing 200 ml of malate-glutamate medium and shaken on a Brunswick Gyrarotary Shaker at 190–200 rpm for 24 h. At the end of this period the cell density was ≈1 mg dry wt./ml.

2.4. Adaptation of dark-grown Rb. sphaeroides *to photosynthetic conditions*

The aerobic, dark-grown cells (above) were washed with 0.02 M potassium phosphate buffer (pH 7) and then suspended (3 mg dry wt./ml) in an un-degassed adaptation medium [7] in which the substrate of BChl *a* formation was 2-oxoglutarate (10 mM). The suspension was incubated microaerobically in filled and sealed glass vessels and illuminated with white incandescent light (approximately 42.5 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 32°C for 12 h. During this period the pale pink cells became brown and no significant increase in turbidity was observed.

Adaptation experiments with $H_2{}^{18}O$ were conducted in 0.6 ml Pierce borosilicate reaction vials with 0.6 ml portions of adaptation mixture reconstituted with $H_2{}^{18}O$. Three vials were used for each experiment: one each for the preparation of BChl *a*, its 3-hydroxyethyl and 3-vinyl derivatives for mass spectrometry.

2.5. Extraction of BChl a

Cells were extracted twice with methanol (4 and 2 ml) in a Potter-Elvejhem homogenizor followed by centrifugation. The pigments in the pooled supernatants were transferred to diethylether, evaporated to dryness and applied in $CHCl_3$ to a DEAE-cellulose column to separate and purify the BChl *a* from other pigments (see below). The BChl *a* eluate was dried by Ar flushing and the sample further dried in toluene by Ar flushing.

2.6. Extraction of BChl a *as [3-(1-hydroxy)-ethyl]-BChl* a

BChl *a* was extracted as [3-(1-hydroxy)-ethyl]-BChl *a*. The pelleted cells were extracted twice with 4 and 2 ml of a fresh solution of $NaBH_4$ (3 mg/ml) in ethanol. The pigments, including the BChl derivative, were transferred from the pooled supernatants to diethylether and evaporated to dryness before purification on a DEAE-cellulose column (see below). After removing the eluant by Ar flushing, the BChl *a* derivative was again dried from toluene by Ar flushing to form a smear in a small tube.

2.7. Formation of [3-vinyl]-BChl a

After purification on a DEAE-cellulose column, a dry smear of 20–40 nmol of [3-(1-hydroxy)-ethyl]-BChl *a* (see above) was dehydrated to [3-vinyl]-BChl *a* by a modification of the method of Struck et al. [10] by heating in a small tube at 115°C for 5 h over a dessicant of P_2O_5 (cf. [11]) at a reduced pressure of approximately 0.25 mbars (25 Pa) and then purified by chromatography on DEAE-cellulose column (see below). The product was identified by its absorption spectrum and by chromatography against authentic markers on Merck RP-HPTLC plates (Type 13 725) developed in methanol [10].

2.8. Purification of BChl a, *[3-(1-hydroxy)-ethyl]-BChl* a *and [3-vinyl]-BChl* a

BChl *a*, [3-(1-hydroxy)-ethyl]-BChl *a* extracted from *Rb. sphaeroides* cells with methanol or ethanol containing $NaBH_4$, respectively, were purified on a DEAE-cellulose column using $CHCl_3$ to first elute the carotenoids and $CHCl_3$ containing 0.2% methanol to elute the slower-moving BChl *a*. $CHCl_3$ containing 0.4% methanol was used to elute the even slower-moving hydroxyethyl derivative. [3-vinyl]-BChl *a* was purified on DEAE-cellulose by eluting with $CHCl_3$ containing O.1% methanol as eluant [10].

2.9. Assay of BChl a

BChl *a* was assayed by absorption spectroscopy in quartz cuvettes (1 cm light path) in a Perkin-Elmer Lamda 2 UV-VIS spectrophotometer using an extinction coefficient (ε) of 42.0 $mM^{-1} \cdot cm^{-1}$ at 770 nm in methanol [12].

2.10. Mass spectrometry

BChl *a* and its derivatives were dissolved in methylene chloride and mass spectra recorded using liquid surface ionization technique in a *m*-nitrobenzyl alcohol matrix in a MAT9000 spectrometer (Finnigan MAT, Bremen) with a cesium gun (20 kV, ≈3 μA) and 1200-Da resolution. After a survey spectrum recorded in the exponential scan mode, 20–25 spectra of the molecular ion region in the linear scan mode were averaged. The ^{18}O enrichment calculation was based on the intensity distribution of the ion cluster of the respective unlabelled bacteriochlorophyll derivatives. This cluster was weighed according to the assumed distribution of higher masses due to ^{18}O enrichment and the resulting peak fit to the experimental spectrum.

3. Results

3.1. Growth of respiring cells of Rb. sphaeroides *capable of immediate adaptation to photosynthetic conditions*

In these studies of BChl *a* formation by adapting *Rb sphaeroides*, we found dark aerobic growth conditions (see section 2) that avoided long lag phases or additional dark treatments in 6% O_2 (cf. [7]). These cells contained between 1.5 to 3.5 nmol of BChl *a* per mg dry wt. and immediately commenced adaptation on exposure to light and microaerobic conditions; that is, in undegassed adaptation medium in filled and sealed glass containers. The BChl *a* concentration increased about 10-fold to about 30 nmol per mg dry wt. of cells over the 12 h adaptation period, which provided satisfactory conditions to detect ^{18}O-labelling of the newly-formed BChl *a* when the adaptation is performed in the presence of $H_2{}^{18}O$.

3.2. Adaptation experiments in the presence of $H_2{}^{18}O$

In this experiment, the cells were incubated in small 0.6 ml Pierce reaction vials (see section 2). After adaptation, about 50 nmol of BChl *a* was formed per tube representing about 27.6 nmol/mg dry wt. of cells.

BChl *a* was extracted directly from one aliquot of cells. A second aliquot was extracted with ethanol in the presence of sufficient $NaBH_4$ (3 mg/ml) to ensure reduction of the 3-acetyl to a hydroxyethyl group: this control tested for ^{16}O exchange between the potentially labile acetyl group and any unlabelled water present during adaptation or subsequent manipulations. The 3-acetyl group of BChl *a* requires higher concentrations of $NaBH_4$ for reduction than does the 7-formyl group of Chl *b* (cf.

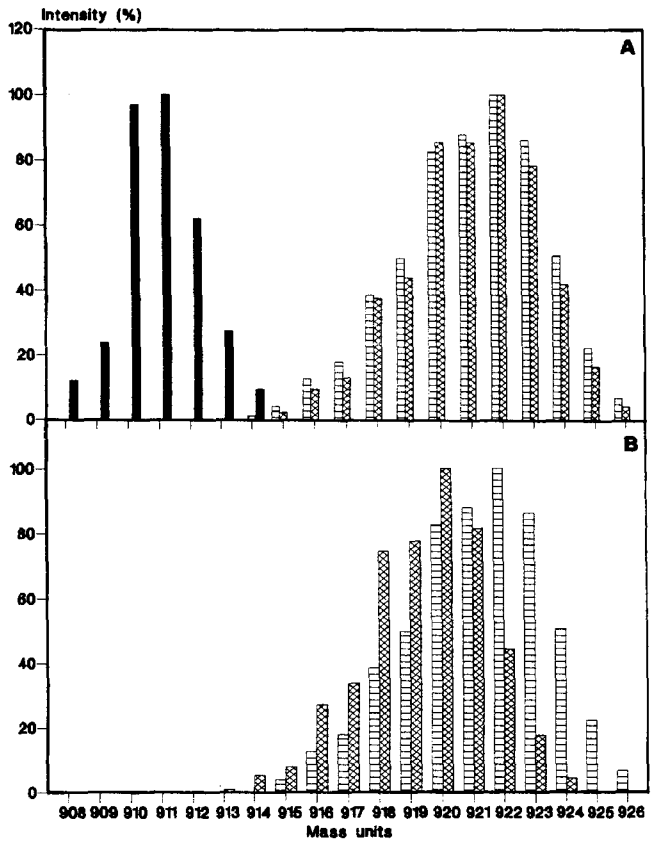

Fig. 2. Mass spectrometric analysis of ^{18}O-labelling of BChl *a* in adapting cells of *Rb. sphaeroides*. The observed mass spectrum pattern for ^{18}O-labelled BChl *a* is shown as horizontally hatched columns in both histograms A and B. The cross hatched columns represent the patterns calculated for equal 85% enrichment of all six oxygen atoms (histogram A) and of only five oxygen atoms (histogram B) of BChl *a*. As discussed in section 3.2., all spectra were calculated based on the experimental molecular ion cluster of unlabelled BChl *a* (solid columns, histogram A). The mass of BChl *a*, uncorrected for labelling by the natural abundance of heavy isotopes, is 910 mass units. The ordinate shows relative intensity of the peaks in relation to the most intense peak.

[5,6]) but the 13^1-oxo group of BChl *a* is stable to $NaBH_4$ [10]. Comparison of the mass spectra of BChl *a* and its hydroxyethyl derivative showed that the acetyl oxygen of BChl *a* is not prone to exchange.

To distinguish between ^{18}O-labelling at $C3^1$ and $C\text{-}13^1$, a third aliquot of cells was extracted with ethanol containing $NaBH_4$ and the hydroxyethyl derivative formed was dehydrated to [3-vinyl]-BChl *a* (see section 2). After purification by DEAE-cellulose, the [3-vinyl]-BChl *a* was free of the corresponding chlorin, Chl *a*, as judged by HPLC and absorption spectroscopy: this latter pigment is 2 mass units lighter than the corresponding bacteriochlorin and could therefore interfere in the mass spectrometric analysis of ^{18}O-isotope enrichment. The mass spectrum of the 3-vinyl-BChl *a* was consistent with ^{18}O-labelling of all five available oxygens. Indeed, BChl *a*, its 3-hydroxyethyl and 3-vinyl derivatives possess 6, 6 and 5 atoms of oxgen per molecule, respectively, and the analysis of the mass spectra of these pigments, obtained from cells adapted in the presence of $H_2{}^{18}O$, were consistent with approximately equal labelling at a level of 85% of all the available oxygens with ^{18}O.

The analysis of the results obtained for the labelling of BChl *a* is shown in Fig. 2. The complex pattern of the molecular ion cluster of unlabelled BChl *a* (Fig. 2A, solid columns) is derived from superimposing the M^+ ion series originating from the natural abundance ^{13}C content, with the $(M+1)^+$ and $(M-1)^+$ series arising from both protonation and deprotonation of the chlorophylls in the ionization chamber of the mass spectrometer [13,14]. Under the controlled experimental conditions used, the pattern of this cluster was constant for a given pigment and is not expected to change significantly on ^{18}O-labelling other than by the relevant increase in mass.

Fig. 2A not only shows the mass spectrum pattern obtained for the molecular ion region of unlabelled BChl *a* (solid columns) but also the fit of the observed pattern of ^{18}O-labelled BChl *a* (horizontally-hatched columns) with the pattern calculated for equal 85% enrichment of all six oxygen atoms of BChl *a* (cross-hatched columns): the error involved in the fit was less than 10% over the mass range of 914 to 926 mass units. Taking into account the 95% enrichment of the labelled water and the presence of about 10% unlabelled BChl *a* before adaptation commenced, these results correspond to approximately 100% isotope uptake from $H_2{}^{18}O$ into all six oxygens of the newly-formed BChl *a*. Fig. 2B shows the fit of the observed pattern of ^{18}O-labelled BChl *a* (horizontal hatching) with the pattern calculated for equal 85% labelling of only five oxygen atoms of BChl *a* (cross hatching): clearly the fit is less satisfactory than that shown in Fig. 2A.

3.3. Control experiments

Two control experiments were conducted. Firstly, fully-adapted cells containing unlabelled BChl *a* were incubated for 12 h in the dark at 32°C in adaptation medium reconstituted with of $H_2{}^{18}O$: when the mass spectra of BChl *a* and its 3-hydroxyethyl and 3-vinyl derivatives were analysed, no labelling with ^{18}O had occurred at any position. Secondly, a suspension of pink respiring cells was incubated in the light ($42.5\ \mu E \cdot m^{-2} \cdot s^{-1}$) for 12 h at 32°C under Ar (<0.002% O_2; see section 2) after a five-fold repetition of evacuation and flushing with Ar: under these strictly anaerobic conditions the BChl *a* content increased from 3.4 to 29.2 nmol/mg dry wt. of cells.

4. Discussion

4.1. ^{18}O-labelling of the 13^3- and 17^3-carboxyl oxygens by $H_2{}^{18}O$

The labelling of the 13^3- and 17^3-carboxyl oxygens is readily explained by non-enzymic exchange between $H_2{}^{18}O$ and intermediates of chlorophyll biosynthesis with free carboxylic acid groups before the formation of the 13^3-methyl and 17^3-phytyl esters: esterification renders these four carboxyl oxygens unsusceptible to exchange with water which is supported by the results of the first control experiment.

4.2. ^{18}O-labelling of the 13^1-oxo group oxygen by $H_2{}^{18}O$

The results of the first control experiment showed that direct exchange of ^{18}O between $H_2{}^{18}O$ and the 13^1-oxo group of BChl *a* does not occur, indicating that this group may be labelled enzymically. A possible enzymic mechanism for formation of the *iso*cyclic ring E and concommitant labelling of the 13^1-oxo group involves the dehydrogenation of the 13-propionic acid methylester side chain to yield 13-acrylic acid methylester which would produce a labelled 13^1-hydroxy-propionic acid

methylester derivative on hydration with $H_2{}^{18}O$. Dehydrogenation would form a labelled 13^1-oxo propionic acid methylester derivative and further dehydrogenation at C-13^2 and C-15 would result in formation of ring E. Labelling of the 13^1-oxo group by this non-O_2-dependent enzymic mechanism is further supported by the second control showing that BChl *a* formation can occur under strict anaerobiosis. Interestingly, Mg-protoporphyrin derivatives with methylesters of acrylic acid, hydroxypropionic acid and oxo-propionic acid at C-13 have been isolated from *Chlorella* mutants [15,16]; this is, perhaps, rather strange since *Chlorella* is an aerobic organism.

4.3. ^{18}O-labelling of the 3-acetyl oxygen by $H_2{}^{18}O$

The labelling of the 3-acetyl group is consistent with enzymic hydration with $H_2{}^{18}O$ of the 3-vinyl group of chlorophyllide (Chlide) *a*: this enzymic hydration step was proposed by Jones [3] after isolating [3-acetyl]-phaeophorbide *a*, a demetallated degradation product of Chlide *a*, from 8-hydroxyquinoline-treated cultures of *Rb. sphaeroides*. Dehydrogenation of the labelled hydration product, [3-(1-hydroxy)-ethyl]-Chlide *a*, would result in the formation of the corresponding labelled 3-acetyl derivative. Labelling by this non-O_2-dependent enzyme mechanism is supported by the results of the first control experiment which showed that ^{18}O cannot be exchanged directly from $H_2{}^{18}O$ into the 3-acetyl group of BChl *a* and by the second control experiment showing that BChl *a* can be formed anaerobically.

4.4. Concluding remarks

It is clear from the work of Nashrulhaq-Boyce *et al.* [1] and Walker et al. [2] that the 13^1-oxo group of Chls *a* and *b* in higher plants is derived from molecular oxygen and that the formation of *iso*cyclic ring E is therefore an aerobic process. This is in marked contrast, therefore, to the above labelling experiments with *Rb. sphaeroides* in which we show that the 13^1-oxo group oxygen of BChl *a* arises from water and that the formation of the *iso*cyclic ring is an O_2-independent process which can occur under strictly anaerobic conditions. Currently, we cannot entirely exclude the possibility that an aerobic process for 13^1-oxo group formation exists under some aerobic conditions.

Because BChl *a* biosynthesis can occur in *Rb. sphaeroides* under strictly anaerobic conditions, it demonstrates that electron acceptors, other than oxygen, exist in these cells *not only* for the oxidative decarboxylase which converts the 2- and 4-propionic acid side chains of coproporphyrinogen III to the vinyl groups of protoporphyrinogen IX but also for the oxidase which converts protoporphyringen IX to protoporphyrin IX: anaerobic forms of coproporphyrinogen III oxidative decarboxylase [17] and protoporphyrinogen IX oxidase [18] have been demonstrated in *Rb sphaeroides*.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (SFB 143, Projekt A9).

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