The cyanobacterium *Acaryochloris marina* was cultured in the presence of either H$_2^{18}$O or O$_2^{18}$, and the newly synthesized chlorophylls (Chl $a$ and Chl $d$) were isolated using high performance liquid chromatography and analyzed by mass spectrometry. In the presence of H$_2^{18}$O, newly synthesized Chl $a$ and Chl $d$, both incorporated up to four isotopic $^{18}$O atoms. Time course H$_2^{18}$O labeling experiments showed incorporation of isotopic $^{18}$O atoms originating from H$_2^{18}$O into Chl $a$, with over 90% of Chl $a$ $^{18}$O-labeled at 48 h. The incorporation of isotopic $^{18}$O atoms into Chl $d$ upon incubation in H$_2^{18}$O was slower compared with Chl $a$ with $\sim$50% $^{18}$O-labeled Chl $d$ at 115 h. The rapid turnover of newly synthesized Chl $a$ suggested that Chl $a$ is the direct biosynthetic precursor of Chl $d$. In the presence of O$_2^{18}$ gas, one isotopic $^{18}$O atom was incorporated into Chl $a$ with approximately the same kinetic incorporation rate and 90% $^{18}$O-labeled Chl $d$ at 48 h. The incorporation of two isotopic $^{18}$O atoms derived from molecular oxygen ($^{18}$O$_2$) was observed in the extracted Chl $d$, and the percentage of double isotopic $^{18}$O-labeled Chl $d$ increased in parallel with the decrease of non-isotopic-labeled Chl $d$. This clearly indicated that the oxygen atom in the C31-formyl group of Chl $d$ is derived from dioxygen via an oxygenase-type reaction mechanism.

Until recently, all oxygenic photosynthetic organisms had been found to contain chlorophyll (Chl) $a$ as their major photopigment (1). However, a novel cyanobacterium, *Acaryochloris marina* (*Acaryochloris*) that contains Chl $d$ (< 95%) as its major photopigment (2), challenged the Chl $a$-centralized requirement in oxygenic photosynthesis. Chlorophyll $d$ only differs from Chl $a$ through one substitution at the C31 position: the vinyl group of Chl $a$ is replaced by a formyl group in Chl $d$ (Fig. 1). This substitution results in the following unique characteristics of Chl $d$: 1) its Q$_y$ absorption peaks (in vivo) lie between 690 and 740 nm (Fig. 1) where other oxygenic Chls (i.e. Chl $a$, $b$, or $c$) do not absorb (3) and 2) it is the only Chl found so far that can substitute for Chl $a$ in charge separation in the reaction centers of oxygenic photosynthetic organisms (4–6). However, the biosynthetic mechanism responsible for the formation of the C31-formyl group of Chl $d$ has not been determined.

The known Chl biosynthetic pathway contains at least 17 enzymatic steps from the precursor δ-aminolevulinic acid to Chl $a$. Eight molecules of δ-aminolevulinic acid are condensed together to form four monopyrroles that condense to a linear tetrapyrrole, which in turn is cyclized to uroporphyrinogen III (7). Metal-free protoporphyrin IX is formed after a number of decarboxylation and oxidation reactions. Protochlorophyllide is synthesized from protoporphyrin IX through magnesium insertion, methylation, and oxidative cyclization reactions forming a fifth ring (8). Reductions of ring D as well as of the vinyl group on ring B result in chlorophyllide $a$, which is converted to Chl $a$ by an esterification of phytol. All of the intermediate products up to the step of chlorophyllide are common in the synthesis of all (bacterio-) chlorophylls (7, 8). However, the enzymes carrying out most of the oxidation and reduction reactions are different in anaerobic and aerobic environments because they either are oxygen-sensitive or require different chemistry to catalyze the oxidations in the absence of oxygen (8, 9). Genes homologous to the cyanobacterial genes encoding enzymes for each of the reaction steps up to Chl $a$ are present in the genome of *Acaryochloris* (10).

Radioisotopes and stable isotopes were widely applied in the elucidation of the biosynthetic pathway of Chls, the magnesium branch of tetrapyrole biosynthesis (11–13). The oxygen atoms of Chl and BChl molecules have their origins in either molecular oxygen or water and thus are incorporated by an oxygenase or a hydratase reaction mechanism, respectively (9, 14). All four oxygen atoms of the C132- and C172-carboxyl groups arise from the precursor δ-aminolevulinic acid and thus originate from water during δ-aminolevulinic acid synthesis, which was confirmed by Porra *et al.* in 1995 and 1996 (15, 16). The fifth oxygen atom of the C132-oxo group is derived from molecular oxygen in most aerobic photosynthetic organisms and is thus an oxygenase-type reaction (9, 11, 15–17). Whereas this oxygen is derived from water using a hydratase reaction mechanism in anaerobic photosynthetic organisms, some exceptions to this generalization exist (9, 16, 18).

Chlorophyll $b$ contains six oxygen atoms: the same five oxygen atoms as in Chl $a$ plus an additional oxygen atom in the C7-formyl group. The oxygen atom of the C7-formyl group of Chl $b$ originates from dioxygen (O$_2$), i.e. an oxygenase-type
reaction mechanism (12, 19, 20). A single enzyme, chlorophyll a mono-oxygenase, facilitating this reaction was identified (21). The chlorophyll a mono-oxygenase converts the C71-methyl group of Chl a into a formyl group, thereby forming Chl b.

Chlorophyll d contains six oxygen atoms. They are the same five oxygen atoms as in Chl a plus an additional oxygen atom in the C31-formyl group. The formation of the C31-formyl group of Chl d must be catalyzed by a different enzyme than the chlorophyll a mono-oxygenase because it requires the breakage of a C=C double bond of the C31-vinyl group, converting it into a formyl group (Fig. 1). The determination of the origin of the C31-formyl group oxygen atom of Chl d will be a crucial step toward understanding the mechanisms of the biosynthetic pathway of Chl d.

There are two possible hypotheses for the origin of the formyl group oxygen atom in the Chl d molecule. Hypothesis A is that the formyl group oxygen atom was introduced into Chl d from dioxygen via an oxygenase-type reaction mechanism. Hypothesis B is that the formyl group oxygen of Chl d originated from water and was incorporated into Chl d via a hydratase-type reaction mechanism. In 1998, it was hypothesized that Chl d may represent an intermediate stage between BCHls and Chls, utilizing anaerobic or aerobic metabolic pathways (22). They based their hypothesis on the fact that the oxygen atom of the C31-acyl group of BCHl a is derived from water using a reductive mechanism, suitable to an anaerobic environment.

Until today, it was not known from where the six oxygen atoms of Chl d are derived, especially the oxygen atom of the C31-formyl group. Chlorophyll d is the major Chl in Acaryochloris with key roles in all photosynthetic reactions (23). Chlorophyll a is a minor photo pigment in Acaryochloris making up less than 3–5% of the total Chls (2). However, the functions and roles of Chl a in Acaryochloris have been debated since its discovery (5, 6, 24–27).

We report here the elucidation of the origins of all six oxygen atoms of Chl d utilizing the stable isotope 18O, in the form of 18O2 in the gaseous phase or H2 18O in the culture medium, as a molecular tracer. Our results suggest that Chl a is a direct precursor in the Chl d biosynthetic pathway and that the C31-formyl group of Chl d is formed via an oxidative reaction mechanism.

EXPERIMENTAL PROCEDURES

Culture Conditions and Growth Rate—The cells were incubated under continuous, fluorescent white light with an intensity of 30 μmol photons m−2 s−1, on a rotating flat bed shaker with ~100 rpm. Freeze-dried 1-ml aliquots of KES-sea water medium (ASW-KES) (2) were resuspended in 1 ml of H2 18O (98% 18O; Marshall Isotope) and inoculated with 100 μl of Acaryochloris (MBIC11017) culture with a Q transition (~710 nm) absorbance of 1.0. The experiments were terminated at 24, 48, 72, and 120 h, respectively, and the pigments were extracted from the cells as described below.

Twenty-five ml of Acaryochloris MBIC11017 culture with an absorption maximum of 0.2 at 710 nm in ASW-KES medium (2) were grown in the presence of 50% 18O2 (97% 18O; Cambridge Isotopes) and 50% dinitrogen (N2) gas. One-ml cell culture aliquots were sampled for pigment extraction at 24, 48, 72, 96, 150, and 200 h, respectively. The growth rates of the cultures were monitored by recording the maximum absorption of each culture in vivo (at A710 nm) using a spectrophotometer (Shimadzu UV-2550) with a Taylor sphere attachment (JSR-240A; Shimadzu).

Pigment Extraction and HPLC Analysis and Fractionation—All of the pigment extraction processes were carried out quickly and under dim green light to minimize any potential photodamage. Acaryochloris cells were harvested by centrifugation at 16,000 × g in a microcentrifuge for 5 min. Pigments were extracted from the cell pellets using ice-cold 100% methanol for a maximum of 15 min on ice in dark. The methanolic pigment extracts were centrifuged and immediately injected into a Shimadzu VP series HPLC system using a reverse phase C18 column (Synergi Fusion, 250 × 4.6 mm; Phenomenex) at a flow rate of 1 ml/min. The column was equilibrated with solvent A and B (50%-50%). Solvent A was 80% acetonitrile and 20% 0.2 M

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ammonium acetate; solvent B was 100% methanol. The program was as follows: 6.5 min in mixed solvents A and B (50%: 50%); then a linear gradient of solvent B from 50 to 100% in 7 min followed by 23 min in 100% solvent B. Fractions of Chl d and a were collected at retention times of 19.7 and 21.2 min, respectively, for subsequent MALDI-MS analysis.

The ratios of pigments were calculated based on HPLC chromatogram peak areas using Shimadzu Class VP 6.14 software. Each peak was evaluated individually at its published maximum absorption wavelength and normalized to its extinction coefficient (in methanol) at that wavelength: molar extinction coefficient of Chl (in methanol) at that wavelength: molar extinction coefficient of Chl was 77.62 mol·cm⁻¹·mol⁻¹ at 696 nm; Chl a, ε = 68.72 mol·cm⁻¹·mol⁻¹ at 665 nm (28).

MALDI-MS Analysis—For increased signal intensity the collected fractions of Chl a and d were concentrated via vacuum centrifugation prior to mass spectrometry analysis. Concentrated pigments were mixed with the electron transfer matrix terthiopene (Sigma) dissolved in acetone at 10 mg/ml, and 2–5 μl of the mixture were spotted on a sample plate. Mass spectra were obtained by MALDI-MS on a Voyager-DE STR (Applied BioSystems), which was run in positive ion reflector mode at 22,000 V. The readings were obtained by averaging 250 single-shot spectra to achieve a good signal-to-noise ratio. The noise level/mass spectrum was averaged for each condition and regarded as the reading error.

**Calculation of 18O-labeled Chlorophyll Isotopomer Abundance—**Chlorophyll a (C₅₅H₇₂N₄O₅Mg) with an average molecular mass of 893.5 Da and a monoisotopic mass of 892.44 Da contains five oxygen atoms, and Chl d (C₆₃H₇₀N₄O₆Mg) with an average molecular mass of 895.5 Da and a monoisotopic mass of 894.5 Da has six oxygen atoms. Upon 18O incorporation zero (monoisotopic Chl mass, with 16O only) one, two, three, four, or all oxygen atoms (five for Chl a and six for Chl d) can be labeled with isotopic 18O. Therefore, the mass spectrum of Chl a (or Chl d) contains a mixture of 18O and 16O isotopes, which can be viewed as an overlay of all six (seven for Chl d) individual mass spectra, i.e. 18O, 16O, 18O₁, 16O₂, 18O₂, 16O₃, 18O₃, 18O₂, 16O₄, 18O₄, 16O₅, and 18O₅, 16O₆ group of molecules. An isotopic (18O₂, 16O₅ for unlabeled Chl a) mass distribution can be calculated, at 1 Da intervals, based on the naturally occurring carbon isotopes (Fig. 2). Thus the expected relative intensities (A₁, A₂, A₃… A₁₁) of the first six mass peaks (as M + 1, M + 2, M + 3, M + 4, and M + 5 ions) of Chl a are 100, 75, 42, 15.5, 3.7, and 0.5, respectively, and the first six mass peaks of Chl d are 100, 73.9, 41.3, 15.1, 3.7, and 0.4, respectively (Fig. 2). A 2-Da difference in mass (m/z) is expected from each isotopic 18O incorporation event in the newly synthesized Chls. The distribution of the measured intensities (I₁, I₂, I₃… I₁₁) of the mass peaks of Chl isolated from cells grown under isotopic 18O labeling conditions can be calculated by the following set of linear equations,

\[
A₁*C(18O₀) = I₁ \tag{1}
\]

\[
A₂*C(18O₂) + A₃*C(18O₄) = I₂ \tag{2}
\]

\[
A₄*C(18O₅) + A₅*C(18O₇) = I₃ \tag{3}
\]

\[
A₆*C(18O₈) + A₇*C(18O₁₀) + A₈*C(18O₁₂) = I₄ \tag{4}
\]

\[
A₉*C(18O₁₄) + A₁₀*C(18O₁₆) + A₁₁*C(18O₁₈) = I₅ \tag{5}
\]

\[
A₁₂*C(18O₂₀) + A₁₃*C(18O₂₂) + A₁₄*C(18O₂₄) + A₁₁*C(18O₂₆) \tag{6}
\]

where C(18O₀), C(18O₂), and C(18O₄) are relative concentrations (summing to 1) of the Chl isotopomers containing between zero and five 18O atoms (six for Chl d) and A₁, A₂, A₅… A₁₁ are the relative intensities of the natural abundance isotopomer peaks. I₁ corresponds to the monoisotopic mass of m/z = 892.5 for Chl a containing only monoisotopic 18O atoms (or m/z = 894.5 for Chl d). The sum of C(18O₀), C(18O₂), and C(18O₄) equals 100% (1.00), and each value represents the relative concentration of each isotopic 18O incorporation into the Chls in the sample, which can be determined for each mass spectrum.

**RESULTS**

**Pigment Analysis in the Presence of 18O (from Water or Dioxygen Gas)—**The samples were collected as described above. Because of the cost of H₂¹⁸O, we grew cells in 1-ml cultures. The results were obtained from a minimum of two individual experiments for each time point with at least one technical repeat. The level of Chl d was slightly higher in the presence of H₂¹⁸O, but no statistically significant difference between the two culture conditions was observed (Table 1).

**H₂¹⁸O Time Course Experiments—**The maximum mass of newly synthesized Chl a extracted from Acaryochloris cells...
grown in the presence of $H_2^{18}O$ in the culture medium for 48 h was 900.44 m/z. This was 8 Da heavier than the monoisotopic mass of Chl $a$ (892.44 m/z) because of the incorporation of four $^{18}O$ atoms derived from water (Fig. 3A). The extracted Chl $d$ from the same 48-h sample displayed a mass peak distribution pattern similar to the calculated monoisotopic/isotopic mass peak distribution of Chl $d$ in Fig. 2, i.e. no significant incorporation of $^{18}O$ in Chl $d$ originating from $H_2^{18}O$ could be observed at 48 h (Fig. 3B).

The time course $^{18}O$ labeling profiles of Chl $a$ indicated that almost all extracted Chl $a$ at 48 h was newly synthesized, suggesting an almost complete turnover of Chl $a$ within 48 h (Fig. 4A). A trace amount of Chl $a$ containing five $^{18}O$ atoms was observed at 115 h of incubation time but below the noise level (data not shown), which may be due to the incorporation of accumulated $^{18}O_2$ released from $H_2^{18}O$ by photosynthesis.

On the other hand, Chl $d$ extracted from the same cells displayed a different $^{18}O$ incorporation rate profile over time. There was very little $^{18}O$ incorporation into Chl $d$ up to 65 h (>20%); the slight increase at 24 h being mostly due to an increased signal-to-noise ratio (Fig. 4B). Chlorophyll $d$ containing one or more isotopic $^{18}O$ atoms was only observed at $\sim$115 h (60% of total Chl $d$) (Fig. 4B). Comparison of the time course $^{18}O$ labeling profiles of Chl $a$ and Chl $d$ suggested that Chl $a$ is synthesized first, and Chl $d$ is produced from the newly synthesized (isotopic $^{18}O$ labeled) Chl $a$ after 65 h (Fig. 4). The fast incorporation rate of $^{18}O$ originating from $H_2^{18}O$ into Chl $a$ ($\sim$40% at 24 h; $\sim$90% at 48 h) is the result of a rapid turnover of Chl $a$ because of it being continuously synthesized into Chl $d$, which comprises $\sim$95% of the total Chls in Acaryochloris. The newly synthesized Chl $d$, with incorporated $^{18}O$, was only observable after it accumulated to a level detectable above the background of unlabeled Chl $d$ present at the start of the labeling experiment.

$^{18}O$ Gas Experiment — The mass spectrometry analysis of Chl $a$ isolated from cells that were grown in the presence of $^{18}O_2$ for 24 h had significant incorporation of one $^{18}O$ with a mass peak distribution of 40% monoisotopic mass (892.44 m/z) and 60% isotopic mass +2 Da (Fig. 5A). Even after 192 h of incubation time, there was only incorporation of one $^{18}O$ atom into Chl $a$, which is consistent with previous reports that only the C13$^{18}$-oxo group oxygen atom in Chl $a$ is derived from $O_2$ gas (10, 15). The time course incorporation profile of the $^{18}O_2$ labeling experiment showed a fast turnover of Chl $a$ similar to that in the $H_2^{18}O$ labeling experiment; thus after 48 h, almost all Chl $a$ is isotopic $^{18}O$-labeled originating from either $^{18}O_2$ or $H_2^{18}O$, respectively (Figs. 4A and 5A).

In the case of Chl $d$, two isotopic $^{18}O$ atoms are incorporated into Chl $d$ upon incubation of cells in the presence of $^{18}O_2$. The double isotopic $^{18}O$-labeled Chl $d$ followed a concomitant decrease in the amount of monoisotopic standard mass Chl $d$ (894.55 m/z) from 0 to 72 h in $^{18}O_2$ (Fig. 5B). Single isotopic $^{18}O$-labeled Chl $d$ was detected at 24 h before decreasing at 48 h (Fig. 5B). This isotopic $^{18}O$ labeling pattern followed inversely the $^{18}O$ incorporation into Chl $a$, suggesting that unlabeled Chl $a$ was the precursor of single isotopic $^{18}O$-labeled Chl $d$. The decrease in isotopic $^{18}O$ labeling at later time points is most likely due to dilution of the $^{18}O_2$ gas with $^{16}O_2$ gas released from $H_2^{18}O$ by photosynthesis in this time period.

Chlorophyll $d$ has six oxygen atoms including the additional oxygen atom of the C3$^1$-formyl group (Fig. 1). The $H_2^{18}O$ labeling experiment demonstrated that the four oxygen atoms of the two carboxyl groups at C13$^3$ and C17$^3$ positions of Chl $d$ originated from water, similar to Chl $a$, which would be expected if Chl $a$ is the precursor of Chl $d$. The incorporation of two $^{18}O$ atoms into newly synthesized Chl $d$ in the $^{18}O_2$ labeling experiment indicated that the C3$^1$-formyl group and the C13$^1$-oxo group oxygen atoms of Chl $d$ were derived through an oxygenase-type reaction mechanism.

**DISCUSSION**

**Chlorophyll Biosynthesis in Acaryochloris**—Chlorophyll $d$ has a formyl group at the C3$^1$ position of ring A in contrast to the C3$^1$-vinyl group of Chl $a$ (Fig. 1). Because of the similarity of the side chains of Chl $d$ and BChl $a$ at the C3 position of ring A (both contain an oxygen atom at the C3 of ring A) and the far-red light shifted absorption properties of Chl $d$, it was thought that Chl $d$ could be an evolutionary intermediate between BChl $a$ and Chl $a$ (4, 22). The oxygen atom of the C3-acetyl group of BChl $a$ is derived from water, and a similar reaction mechanism could be responsible for the incorporation of the oxygen atom into the C3$^1$-formyl group of Chl $d$ (15, 17). Con-

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**TABLE 1**

| Presence          | Ratio of Chl $a$ to Chl $d$ | Chl $d$ |
|-------------------|-------------------------------|---------|
| $H_2^{18}O$ (98%) | 0.6016 ± 0.018                | 94.19 ± 1.60 |
| $^{18}O_2$ (50%)  | 0.0594 ± 0.008                | 94.39 ± 0.69 |
versely, the oxygen atom of the C71-formyl group of Chl b had its origin from molecular oxygen (O2), which led to our initial hypotheses that either an oxygenase-type or a hydratase-type reaction mechanism is responsible for the incorporation of the C31-formyl group oxygen atom into Chl d (12).

Acaryochloris cells incubated in the H218O-containing culture medium displayed an increase from 20 to 60% in incorporation of isotopic 18O label into Chl d from 48 to 120 h. In the same time the unlabeled Chl d decreased from 80 to 40% (Fig. 4B). The lag phase (48 h) in the incorporation of 18O label from H218O into Chl d can be explained with the proposed model of Chl d biosynthesis in Acaryochloris if the cultural doubling time is ~48 h. Oxygen atoms originating from water are incorporated into the C131-oxo group and the C31-formyl group oxygen atoms of Chl d. After 48 h of incubation time, almost all Chl a of Acaryochloris was isotopic 18O-labeled because of the low Chl a/d ratio (0.03–0.05:1), resulting in a high turnover of Chl a as a precursor of Chl d (Fig. 4A). A doubling time of ~48 h for cells cultured under white light (30 μM photons m–2 s–1) is confirmed through reported Acaryochloris doubling times of 45–65 h under comparable light intensities (29).

The C131-oxo group and the C31-formyl group oxygen atoms of Chl d originate from molecular dioxygen (O2) and are incorporated into the Chl macrocycle in the last steps of the Chl biosynthetic pathway. Therefore, the incorporation of single isotopic 18O (at C131-oxo group) originating from 18O2 into Chl d occurred earlier in the time course experiments than the incorporation of 18O label originating from H218O. Double isotopic 18O labeled Chl d was increased from 14 to 29% in 24 h (from 24 to 48 h) equating to a doubling time of ~65 h (Fig. 5B).

The incorporation of isotopic 18O label into Chl a from 18O2 gas was not as high as from ASW-KES H218O medium. This may be due to the fact that the incorporation of isotopic 18O label was in competition with 16O2 gas produced by oxygenic photosynthesis that accumulated in the growth chamber. The calculated 18O2/16O2 gas ratio in the growth chamber after 5 days of incubation time was ~2.5:1, if it is assumed that the O2 evolution rate of slow growing Acaryochloris cells was ~100 μmol O2 h–1 (mg Chl)–1 at standard laboratory conditions and that the growth chamber had an initial concentration of 50% 18O2, 50% N2. This estimated 18O2/16O2 gas ratio was confirmed through gas chromatography mass spectrometry analysis of the gas mixture in the flask during the experiment (data not shown). Our results indicate that the incorporation of the oxygen atom into the C31-formyl group of Chl d is facilitated via
an oxygenase-type reaction mechanism and furthermore pinpoints Chl $a$ as the direct precursor in Chl $d$ biosynthesis.

The *Acaryochloris* genome contains all of the enzyme-encoding genes that are required in the Chl $a$ biosynthetic pathway, which additionally supports our results (10). It was suggested that Chl $d$ could be produced by a member of the aromatic ring degradation protein superfamily, which also includes the potential chlorophyll $a$ mono-oxygenase enzyme (10). On the basis of the data presented here, it seems now ever more likely that a putative Chl $d$ synthase protein belongs to this family.

The Function of Chl $a$—Chlorophyll $a$ is a minor chlorophyll in *Acaryochloris* ($>5\%$), and its function has been debated since the discovery of this unique organism. Although it was soon established that Chl $a$ was not a prerequisite for functional photochemistry in the reaction centers of photosystem (PS) I of *Acaryochloris*, there are still disagreements regarding the role of Chl $a$ in the reaction centers of PS II (4–6, 24–27).

This work points out that Chl $d$ is synthesized from Chl $a$ in a reaction mechanism similar to the biosynthesis of Chl $b$ from Chl $a$. Although it is important to note that Chl $b$ is only an accessory pigment with Chl $a$ being the major photopigment in photosynthetic reactions. Furthermore, it suggests that the degradation of Chl $d$ could occur in analogy to the Chl $b$ degradation reaction as well, i.e. that Chl $d$ is broken down via Chl $a$ and pheophytin (Pheo) $a$. Pheophytin $a$ was previously identified as the primary electron acceptor of PS II in *Acaryochloris* (5, 6, 30, 31). Therefore, it is conceivable that the role of Chl $a$ in *Acaryochloris* is merely that of an intermediate product in the biosynthesis and degradation reactions of Chl $d$ without any potential involvement in the photochemistry. It also suggests Chl $a$ as a possible precursor for Pheo $a$ in *Acaryochloris*. This strengthens the role of Chl $a$ as the central Chl in oxygene photosynthesis with Chl $b$ and $d$ having evolved from it (32).

**Chlorophyll $d$ as the Major Photopigment in *Acaryochloris***—The red-shifted $Q_s$ absorption maximum (696 nm in methanol; Fig. 1) of Chl $d$ (over 30 nm red-shifted from Chl $a$) and its very high percentage of total Chls (95%) are the most striking characteristics of Chl $d$. Additionally, *Acaryochloris* exhibits the lowest Chl $a$ content of all aerobic photosynthetic organisms known to date. Containing Chl $d$ as the major photopigment, it would be expected to find Pheo $d$ in significant quantities if it is an intermediate product of major photopigment Chl $d$. Actually, no Pheo $d$ is detectable in *Acaryochloris*, which suggests that Pheo $d$ is not involved in photosynthetic reactions of *Acaryochloris* (33). Furthermore, it supports our hypothesis that Chl $d$ in *Acaryochloris* is broken down via Chl $a$.

Taken together these results sustain our hypothesis that Chl $d$ is biosynthesized from Chl $a$. However, it is uncertain yet whether a one-step reaction or a two-step reaction is responsible for the conversion of the C3'-vinyl group in Chl $a$ to the formyl group in Chl $d$.

The results of this study revealed the origins of all six oxygen atoms of Chl $d$. The revelation that the C3'-formyl group oxygen atom of Chl $d$ is incorporated via an oxygenase-type reaction mechanism suggests that genes encoding radical S-adenosylmethionine (SAM) superfamily enzymes should be excluded from the list of putative candidate genes for Chl $d$ biosynthesis (10). These findings are an important step toward the identification of the Chl $d$ synthase gene(s) in the future.

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