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CHLOROPLAST BIOGENESIS: MOLECULAR STRUCTURE OF SHORT WAVELENGTH CHLOROPHYLL *a* (E432 F662)*

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Key Word Index—*Anacystis nidulans*; Cyanophyceae; Chlorophyll *a*; tetrapyrrole; novel chlorophyll; chemical structure.

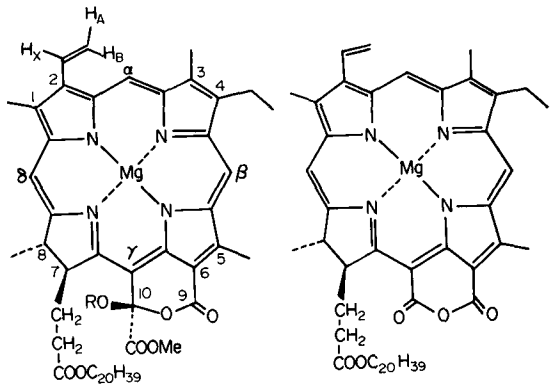
Abstract—^{1}H NMR and FAB mass spectrometric analyses are compatible with a monovinyl 10-hydroxylactone chlorophyll *a* [i.e. 13^{1}A-homo-13^{1}A-oxa-13^2-hydroxy-] structure for a chlorophyll (E432 F662) purified from cultures of *Anacystis nidulans*, a blue-green algae that lacks chlorophyll *b*. Its biosynthesis in greening cucumber cotyledons has been reported earlier.

INTRODUCTION

We have proposed and documented that the chlorophyll (Chl)‡ biosynthetic pathway of green plants is a multi-branched pathway, which is capable of giving rise to several different chemical species of Chl *a* and *b*. [1–5]. The chemical structures of two of these novel Chls, namely 2,4-divinyl Chl *a* (DV Chl *a*) [i.e. 4-vinyl-4-desethyl Chl *a*] and DV Chl *b* have already been confirmed by derivatization and by NMR analysis [6–8]. Divinyl Chl *a* appears to be the major Chl constituent in the phytoplankton of tropical oceanic waters [9].

During low temperature spectrofluorometric analysis of the Chl pool of greening tissues, we have observed the biosynthesis of small amounts of a novel Chl, Chl (E432 F662), which exhibits Soret excitation and fluorescence emission maxima at 431–432 and 662–663 nm respectively in ether at −196° [4]. This Chl exhibits a strongly blue-shifted fluorescence maxima in comparison to MV Chl *a* (E447 F674) which, under the same analytical conditions, exhibits Soret excitation and red fluorescence emission maxima at 446–447 and 674–675 nm respectively [4]. Likewise, Chl (E432 F662) exhibits blue-shifted Soret and red electronic absorption maxima in ether at 20° (416–417 and 651–652 nm respectively) in comparison to standard MV Chl *a* (428 nm and 660 nm respectively) [4]. Similar electronic absorbance properties have been attributed in the past to artifacts of MV Chl *a* such as Mg-purpurin 7-lactone-methyl-ether-methylphytyl ester (**1a**) and Mg-unstable chlorin-methylphytyl ester (**1b**) [10–12]. Such Chls have a lactone ring between the 6 and γ position of the macrocycle, instead of a cyclopentanone ring (Fig. 1). The assignment of the aforementioned chemical structures is based mainly on chemical derivatization coupled to electronic absorbance analysis [10–13] and on limited infrared spectroscopic analysis [10].

We have observed that photosystem II particles [14] and pigment-protein complexes of green photosynthetic membranes, corresponding to photosystem II reaction centres [2], as well as algal cultures of *Anacystis nidulans* [4], a blue-green algae that lacks Chl *b*, were all enriched in Chl (E432 F662). FAB mass spectrometric analysis of purified *Anacystis* Chl (E432 F662) and ^{1}H NMR analysis of synthetic Chl (E432 F662) are consistent with a 2-vinyl-4-ethyl-10-hydroxylactone Chl *a* (**1b**) structure for this naturally occurring Chl.



1

1a R = Me
1b R = H

2

Fig. 1. Chemical structures of monovinyl (MV) 10-methoxylactone Chl *a* (**1a**), of 10-hydroxylactone Chl *a* (**1b**), and of MV Mg-purprin 18 (**2**).

*Part 58 in the series 'Chloroplast Biogenesis'. For part 57 see Tripathy, B. C. and Rebeiz, C. A. (1987) in *Progress in Photosynthesis Research* Vol. 4. (Biggns, J., ed.) p. 8439.

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‡Abbreviations: MV: monovinyl; DV: divinyl; E: Soret excitation maximum in nm; F: red fluorescence emission maximum in nm.

RESULTS

Mass spectroscopic analysis of synthetic and natural Chl (E432 F662) *after demetalation*

It has been our experience that FAB mass spectroscopic analysis of chlorophylls is more successful with demetalated than with metalated tetrapyrroles [7]. The observed M_r of the molecular ions and of some of the fragments of the natural and synthetic Chl (E432 F662) corroborated the presence of a hydroxylactone ring in demetalated Chl.

Peaks at *m/z* 903 $[MH]^+$ [38% for the natural (n) and 47% for the synthetic (s) Chl respectively] and 902 $[M]^+$ (n 19%, s 23%) were consistent with monoprotonated and parent MV-10-hydroxylactone pheophytin *a* ($C_{55}H_{74}N_4O_7$) molecular ions respectively. Loss of one water molecule from the monoprotonated species or addition of one water molecule to it were suggested by peaks at *m/z* 885 $[MH-H_2O]^+$ (n 16%, s 24%) and 921 $[MH+H_2O]^+$ (n 28%, s 29%) respectively. This fragmentation pattern suggested very strongly that the initial protonation was at the hydroxyl group of the lactone ring and was accompanied either by elimination of the water unit thus formed, or by addition of the discharged water unit to the protonated molecular ion. The lability of the peripheral substituents on the hydroxylactone ring was evidenced by a fragment observed at *m/z* 860 $[MH+H_2O-HCO_2Me-H]^+$ (n = s = 24%). The generation of another fragment clearly observed in the natural sample, with *m/z* of 843 $[MH-HCO_2Me]^+$ (n 22%, s 9%) was compatible with the elimination of methyl formate to yield Mg-free-purpurin 18 (M = 843) (Fig. 1, **2**). The conversion of MV-10-hydroxylactone Chl *a*(**1b**) to Mg-purpurin 18 (**2**) has been proposed by others [11, 12] and is considered a strong evidence for the presence of C-10 hydroxy and C-10 carbomethoxy groups in a six-membered lactone ring. Other fragments compatible with the loss of various groups from the phytylpropionate residue at position 7 of demetalated MV-10-hydroxylactone Chl *a* were observed at *m/z* 565 $[MH-CH_2CO_2C_{20}H_{39}-H]^+$ (n 83%, s 25%), 551 $[MH-CH_2CH_2CO_2C_{20}H_{39}-H]^+$ (n 40%, s 28%), 547 $[MH-C_{20}H_{39}OH-HCO_2Me]^+$ (n 37%, s 33%) and 537 $[MH-CH_2CH_2CO_2C_{20}H_{39}-Me]^+$ (n 52%, s 47%).

^1H *NMR of synthetic Chl* (*E432 F662*)

Because of the relatively large sample size needed for meaningful NMR determinations, it was convenient to run the ^1H NMR analysis on synthetic Chl (E432 F662). This decision was justified since the chromatographic and electronic spectroscopic properties [4] as well as the mass spectroscopic properties (*vide supra*) of synthetic and natural Chl (E432 F662) were very similar. The following chemical shifts for synthetic Chl (E432 F662) were observed: ^1H NMR (360 MHz, $CDCl_3$, TMS): δ 9.63 (1H, *s*, H-β), 9.39 (1H, *s*, H-α), 8.38 (1H, *s*, H-δ), 8.00 (1H, *dd*, $J_{2B,2X}$ = 17.7 Hz, $J_{2A,2X}$ = 11.6 Hz, H_X-2), 6.19 (1H, *dd*, $J_{2B,2X}$ = 17.7 Hz, $J_{2A,2B}$ = 1.83 Hz, H_B-2) 6.01 (1H, *dd*, $J_{2A,2X}$ = 11.6 Hz, $J_{2A,2B}$ = 1.83 Hz, H_A-2), 3.75 (3H, *s*, H-10), 3.40 (3H, *s*, H-5), 3.31 (3H, *s*, H-1), 3.29 (3H, *s*, H-3), 1.74 (3H, *d*, *J* = 7.33 Hz, H-8).

The chemical shifts of synthetic Chl (E432 F662) were quite comparable to those of MV-10-methoxylactone Chl *a* (**1a**) (^1H NMR, 100 MHz, THF-d_8, HMX) [15]. The latter differs from MV-10-hydroxylactone Chl *a* (**1b**), the proposed structure of Chl (E432 F662), by having a methoxy group instead of a hydroxy group at position 10 of the macrocyle (Fig. 1). The β, α and δ methine protons appeared at almost the same low field region for both Chls (*vide supra*, and [15]). The presence of only one vinyl group in Chl (E432 F662) was evidenced by the typical ABX spin–spin splitting pattern of a single vinyl group per molecule. A doublet of doublets centered at δ8.00 with J_{BX} (17.7) and J_{AX} (11.6) coupling constants was attributed to the resonance of the H_x proton. The two sets of doublet of doublets observed at δ6.19 and 6.01 were compatible with the resonance of vinylic H_B and H_A protons respectively.

The lack of ^1H resonance signals in the Chl (E432 F662) spectrum between δ6.21 and 6.38 is notable. These resonances are attributed either to a single C-10 proton (δ6.21) or to a hydroxy proton (δ6.38) respectively, at position 10 of the macrocycle [15]. It has been established that the proton of a hydroxy group at position 10 of the Chl macrocycle exchanges instantaneously with CH_3OD if the latter is present in the solvent while it takes several hours for the complete exchange of a single C-10 proton to take place [15]. Since the NMR spectrum of Chl (E432 F662] was recorded in $CDCl_3$ containing 3% CD_3OD, the C-10 single proton resonance around δ6.21 ppm would have been observed if that proton bound directly to C-10 as in MV and DV Chl *b* [8]. On the other hand, the absence of such a resonance in the δ6.21–6.38 region is fully compatible with the presence of a hydroxyl group instead of a single proton at position 10 of the Chl (E432 F662) macrocycle. The peripheral methyl proton resonances were observed in the same spectral region as in 10-methoxylactone Chl *a* [15].

Finally, the ^1H NMR spectrum indicated that the synthetic Chl (E432 F662) sample was contaminated with compounds absorbing below δ3. Furthermore, some weak signals that could not be assigned were observed at δ7, 6.9, 6.6, 5.4 and 5.2. These abberrations may be attributed to the formation of a certain amount of the C-10 Chl (E432 F662) epimer during synthesis. Such an epimer is an unfavourable conformational isomer in which the carbomethoxy group at C-10 and the phytyl propionic acid residue at C-7 are on the same side of the macrocycle. Altogether, in spite of these imperfections, the NMR analysis reinforced the FAB mass spectroscopic analysis and supported the assignment of a MV-10-hydroxylactone Chl *a* structure to Chl (E432 F662).

DISCUSSION

The putative Chl *a* lactones reported in [10–13] were derived chemically from MV Chl *a* by prolonged treatments involving methanol under basic conditions. It is acknowledged that under these conditions, the conversion of the cyclopentanone ring to a lactone ring proceeds rather readily via formation of a hydroperoxide at C-10 [13] or via formation of an enol double bond between C-9 and C-10 of the cyclopentanone ring [12]. The relative ease of conversion of the cyclopentanone to a lactone ring has raised the legitimate question of whether the occurrence of Chl lactones in biological systems is invariably a consequence of experimental artifacts or of catabolic processes [16]. The following observations argue against both of these possibilities: (i) the reported time course of MV-10-hydroxylactone Chl *a* biosynthesis in greening ethiolated cucumber cotyledons (Fig. 11 in [4]) does not

support a non-enzymatic origin for Chl *a* (E432 F662); (ii) Chl *a* (E432 F662) is also present in fresh *Anacystis* cultures air-shipped in dry ice and extracted immediately upon arrival (Fig. 10 in [4]). This in turn argues against its being a lyophylization artifact and finally (iii) when the Chl *a* pool of etiolated cucumber cotyledons formed by phototransforming the protochlorophyllide ester pool with a 2.5 msec light pulse, is extracted and purified in the same way as Chl *a* (E432 F662), it is found to consist mainly of MV Chl *a* (E447 F674) and smaller amounts of DV Chl *a* (E458 F674). It is devoid of any detectable Chl *a* (E432 F662) (Fig. 7 in [4]). This in turn is against it being an extraction and purification artifact.

EXPERIMENTAL

Plant materials. Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were germinated under a light (14 hr)/dark (10 hr) photoperiodic regime at 28° [17]. Lyophylized *Anacystis nidulans* (T × 20) [18], a gift of Professor J. Myers of the University of Texas at Austin, was stored in liquid N_2 until use. Dry ice-packed fresh *A. nidulans* (T × 20), also a gift of Prof. Myers, was air shipped to us and were extracted immediately upon arrival. *Preparation of MV Chl* a. Monovinyl Chl *a* was prepared from the green cotyledons of photoperiodically grown cucumber seedlings. Homogenization of the green tissue was followed by solvent-solvent extraction and by TLC purification on silica gel H and cellulose as described in [8].

Extraction of Chl (E432 F662) from A. nidulans. 200 mg batches of lyophilized *A. nidulans* which had been stored in liquid N_2, or freshly shipped cultures packed in dry ice, were extracted twice by homogenization in a chilled mortar at 0–4°, with 20 ml Me_2CO: 0.1 M NH_4OH (9:1). This was followed by two extractions with 20 ml Tris–HCl, pH 8.0: Me_2CO: 0.1 M NH_4OH (5:18:2). The combined extracts were centrifuged and the Chls were extracted with hexane. Monovinyl Chl *a* (E447 F674) (R_f 0.73–0.79) was separated from Chl (E432 F662) (R_f 0.56–0.66) by TLC on silica gel H, developed in PhMe–EtOAc–EtOH (8:2:2) at 4° in darkness. There was no interference from Chl *b* since *Anacystis* does not form this Chl. In some cases, Chl (E432 F662) was further purified by TLC on cellulose MN 300 developed in petrol (60–80°)–PrOH (99:1) at room temp. In this solvent, MV Chl *a* and Chl (E432 F662) migrate with R_f values of 0.48 and 0.20 respectively. Chlorophyll (E432 F662) purified as just described, contained only trace amounts of MV Chl *a* (E447 F674).

Synthesis of Chl (E432 F662) from MV Chl a (*E447 F674*). Chlorophyll (E432 F662) was prepared from MV Chl *a* by adaptation of a procedure described in [10]. Microgram quantities of MV Chl *a* were dissolved in 2 ml C_5H_5N and the soln was stirred vigorously in air for 2 min. The soln was then placed in an ice bath and 0.1 ml of 10% KOH was added. After 5 min at 0–4° with occasional swirling, the soln was passed through an ice cold mixture of 5% KH_2PO_4–Et_2O (4:3). Most of the pigment passed into the Et_2O phase. The aq. phase was further extracted twice with 3 ml of Et_2O. The Et_2O extracts were combined and were washed twice with an equal vol. of 5% KH_2PO_4. This was followed by washing with an equal vol of H_2O. The washed Et_2O extract was concd under a stream of N_2 gas,and the unreacted MV Chl *a* was separated from Chl (E432 F662) by TLC on silica gel H and on cellulose, as was described for the Chl (E432 F662) of *Anacystis* (*vide supra*). Chlorophyll (E432 F662) was eluted in Et_2O.

Demetalation of Chl (E432 F662). Chlorophyll (E432 F662) was dissolved in 0.2 ml Et_2O to which was added 2 ml 4 M HCl. The mixture was placed in an ice bath and every few minutes was swirled gently. After 40 min, the reaction mixture was diluted with 5 ml Et_2O and was neutralized with $NaHCO_3$. Neutralization, drove the demetalated pigments into the Et_2O phase and the latter was washed with H_2O until neutral. Demetalated Chl (E432 F662) was purified by TLC on silica gel H developed in n-C_6H_{14}–Me_2CO (2:1) at room temp. At −196° in Et_2O, it exhibited a Soret excitation maximum at 405 nm and a red emission maximum at 670 nm. At 20°, It exhibited absorbance maxima similar to ones reported by others [19]. The absorbance at the Soret maximum divided by the absorbance at the indicated wavelengths are given in parenthesis: UV/VIS $\lambda_{max}^{Et_2O}$: 668 (2.40), 638 (19.2), 610 (19.1), 560 (57.9), 526 (14.6), 496 (9.59) and 398 (1.00). The formation of metal-free Chl (E432 F662) was accompanied by small amounts of degradation products with R_f of 0.54, 0.48, 0.43 and 0.30. Since the latter exhibited the same fluorescence properties as metal-free Chl (E432 F662), this compound could very well be a demetalated C-10-hydroxy Chl (E432 F662) epimer.

Spectroscopy. Absorbance and fluorescence spectra were recorded as described elsewhere [6]. ^{1}H NMR spectra were recorded on a 360 MHz FT instrument. The pigment was dissolved in $CDCl_3$ containing 3% CD_3OD (w/w). TMS was used as an int. standard. The FAB spectra were recorded on a VG ZAB-1F mass spectrometer. Samples were prepared in a mixed dithiothreitol and dithioeryritol matrix.

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