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Meso-chlorination of chlorophyll *a* in the course of pigment extraction

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HPLC analysis has shown that the meso δ-carbon of chlorophyll (Chl) *a* undergoes chlorination to give δ-chloro Chl *a* (Cl-Chl *a*), possibly by an enzymatic reaction with chloride ion in the wet medium of crushed plant tissue. A sizeable amount, occasionally a 1% fraction, of the whole Chl *a* is chlorinated during tissue crushing/pigment extraction. The chlorination can be neatly or completely suppressed if plant materials are kneaded with a sufficient amount of a desiccant or with Cl^--scavenging silver ion before pigment extraction.

Chlorophyll chlorination; Pigment extraction; Photosynthesis; HPLC analysis

1. INTRODUCTION

A novel Chl *a* derivative, 10-hydroxy-δ-chloro-chlorophyll *a* (10-OH,Cl-Chl *a*: see fig.2 for carbon notation) [1,2], was recently isolated from spinach and cyanobacteria by Senger and co-workers [1,3,4]. They proposed that this pigment is a building block of the photosystem (PS) I reaction center (P700), since it reportedly occurred at a one to one molar ratio to P700 in extracts [5].

During our high-resolution HPLC analyses of pigment composition in plants, we sometimes detected a small and variable amount of a Chl-type pigment [6,7], which is entirely different from the well-known four Chls in higher plants: Chl *a*, Chl *b*, pheophytin (Pheo) *a* and Chl *a'* (C10-epimer of Chl *a*), where the latter two are apparently key components of PS II [8,9] and PS I [7] reaction centers, respectively. We previously speculated that this pigment was 10-OH, Cl-Chl *a* [6], because they were practically indistinguishable from each other with respect to visible spectroscopic properties.

We have reinvestigated this problem and show in the present communication that this pigment is δ-chlorinated but unhydroxylated Chl *a*, namely Cl-Chl *a*, which is not present in vivo but is easily produced in the wet medium of plant tissue, probably by an enzymatic reaction with chloride ion present in the tissue. We further confirmed that Cl-Chl *a* is oxidatively converted into 10-OH, Cl-Chl *a* during its handling under aerobic conditions.

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2. MATERIALS AND METHODS

Young leaves were harvested from a total of seven higher plants (*Brassica rapa, Hydrangea macrophylla, Spinacia oleracea, Cryptotaenia canadensis, Oenanthe stolonifera, Angelica keiskei* and *Fragaria chiloensis*) grown under sunlight. *Synechococcus* sp. (ATCC 27144) was grown in the BG-11 medium [10] without NaCl under white fluorescent tubes.

Pigment extraction/HPLC analysis was performed following our standard practice [7] unless otherwise noted. Briefly, the leaf tissue or the cyanobacteria (after separating the culture medium by centrifugation) was (or were) ground with a large amount of cold (-20°C) anhydrous Na_2HPO_4 as desiccant. Pigments were extracted with cold chloroform and the extract was loaded on a silica HPLC column cooled to 4°C. The pigments were eluted isocratically with hexane/2-propanol/

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methanol (100:0.8:0.4) at a flow rate of ~0.5–0.8 ml/min, and were monitored by absorbance detection (425 nm) and fluorescence detection (ex. 365 nm, em. 670 nm) in series.

The authentic sample of Cl-Chl *a* was prepared as follows. First, Pheo *a* was chlorinated by a modification of the hydrogen peroxide/hydrochloric acid method [2,11], and then Mg^{2+} was inserted inot Cl-Pheo *a* according to a method introduced by Isenring et al. [12]. The final products, Cl-Chl *a* and *a'*, were purified by precipitation with dioxane followed by chromatography on powdered sugar plates [13]. Cl-Chl *a/a'* was characterized thoroughly by proton NMR, UV/visible absorption, fluorescence and circular dichroism (CD) measurements. Further details of Cl-Chl *a/a'* preparation are to be published elsewhere.

The authentic 10-OH,Cl-Chl *a/a'* samples were generously supplied by Professor H. Senger, Biology/Botany Department of Philipps University, Marburg, FRG.

3. RESULTS AND DISCUSSION

An HPLC trace for a chloroform extract from *Brassica rapa* leaf tissue is displayed in fig.1. Besides the three major peaks of carotenes, Chl *a* and Chl *b*, two minor peaks of Pheo *a'* and Chl *a'* are clearly discerned. Here the Chl *a*/Pheo *a* and Chl *a*/Chl*a'* molar ratios are respectively 115 and 440, which are in line with a P680/P700 molar ratio of 1.91 based on the stoichiometries Pheo *a*/P680 = 2 and Chl *a'*/P700 = 1 [6,7]. In addition to these, a small peak (hereafter designated component X) is seen just after the Chl *a'* peak. These pigments, except for the xanthophylls eluted together at a retention time of around 180 min (not shown in fig.1), are all that can be detected by the present high-resolution HPLC. Component X is fluorescent to a much lesser extent than Chl *a/a'*, as judged from the absorbance/fluorescence intensity ratio on the HPLC trace.

We isolated component X from leaf tissue extracts by means of preparative-scale HPLC [14] for spectroscopic characterization. In fig.2 the absorption spectra of component X and Chl *a* in benzene are shown. Compared to Chl *a*, the peaks are slightly red-shifted and the blue/red absorbance ratio is noticeably larger in component X. The absorption spectrum of component X is clearly different from those of other Chl-type pigments, namely Chl *b*, Pheo *a* and Pheo *b* [14]. In benzene, the fluorescence peak wavelength of component X was approx. 3 nm longer, and the fluorescence quantum yield was roughly 5-fold lower, than Chl *a*.

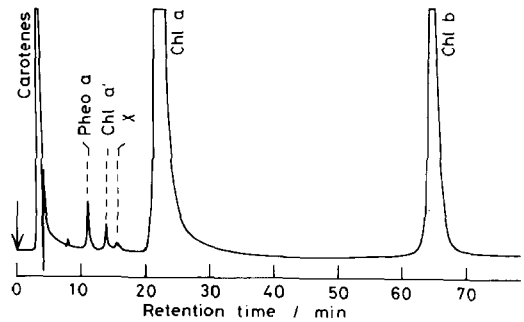

Fig.1. HPLC trace for a chloroform extract of *Brassica rapa* leaf tissue (absorbance detection at 425 nm).

Since these spectral features were essentially the same as those of 10-OH,Cl-Chl *a*, or Chl-RCl described by Senger and collaborators [1–5], we previously supposed that component X might be 10-OH,Cl-Chl *a* [6]. A significant change in the visible absorption spectrum in going from Chl *a* to 10-OH,Cl-Chl *a* is rationalized by invoking the (steric) perturbation on the π-conjugated system of the chlorin macrocycle, and the drastic drop of fluorescence yield could take place via an internal heavy atom effect of the Cl atom, and/or an increased steric hindrance introduced by it. Hydroxylation on carbon 10 (cf. fig.2), however, would exert little effect on these spectroscopic properties, because this site is out of the π-conjugated system. Further, the epimers (*a* and *a'*) cannot be distinguished from each other by absorption/fluorescence spectroscopy [14]. Hence there remain at least four possibilities as to the nature of component X; 10-OH,Cl-Chl *a* (or *a'*) or unhydroxylated Cl-Chl *a* (or *a'*).

A final idenfication of component X was done by thoroughly comparing it with the authentic Cl-Chl *a/a'* and 10-OH,Cl-Chl *a/a'* samples with respect to visible absorption, fluorescence (spectrum and quantum yield), CD and HPLC retention properties. All these measurements indicated conclusively that component X is identical to Cl-Chl *a*. As an example, the result from the HPLC investigation is given in fig.3. It was confirmed (not shown) that the 10-OH,Cl-Chl *a/a'* pair is eluted as two distinct peaks at around 45 min, namely just halfway between the Chl *a* and Chl *b* peaks (cf. fig.1).

We then examined the origin of Cl-Chl *a*. For this, the amount of Cl-Chl *a* versus the total

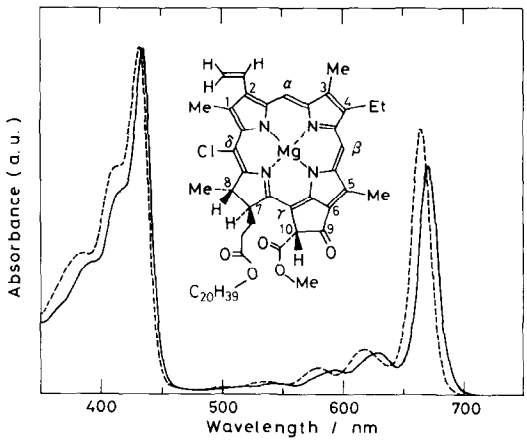

Fig.2. Absorption spectra of component X (——) and Chl *a* (----) in benzene. The Soret peaks are normalized to a common height. The inset shows the structural formula of δ-chlorinated Chl *a* (Cl-Chl *a*).

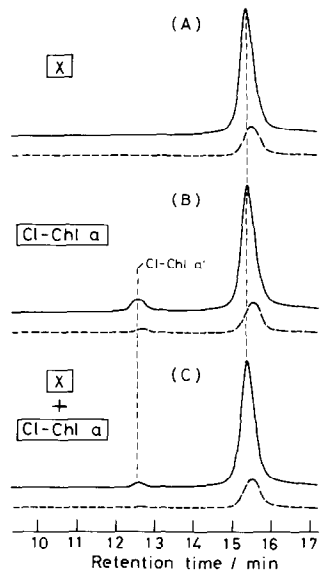

Fig.3. HPLC traces for (A) component X isolated from *Spinacia* leaf tissue, (B) authentic Cl-Chl *a* containing Cl-Chl *a'* as an impurity, and (C) a mixture of (A) and (B) at a molar ratio of ~1/1. The solid traces represent absorbance detection, and the broken traces (with slightly) longer retention time due to a time lag) fluorescence detection. The Cl-Chl *a* peaks are normalized to a common intensity.

number of Chl *a* molecules in extracts would provide a primary clue. If Cl-Chl *a* is a key component in either PS I or PS II, the Chl *a*/Cl-Chl *a* molar ratio must be at a level of several hundred, nearly independently of the plant species [7]. If Cl-Chl *a* is, on the contrary, an artifact produced via δ-chlorination of Chl *a* during tissue crushing, the Chl *a*/Cl-Chl *a* molar ratio may exhibit a significant scatter from one measurement to another, because the reaction would proceed in a locally wet environment (or in the fragments of crushed tissue) embedded in the powdery desiccant, for which precise control of conditions is hardly attainable. In the latter case, the observed Chl *a*/Cl-Chl *a* molar ratio may also depend on the plant species due to a difference, from one species to another, in the chemical composition of cell contents.

The analytical results, displayed in fig.4 in the form of a correlation between the Chl *a*/Cl-Chl *a* and Chl *a*/Chl *a'* molar ratios in leaf extracts of six higher plants, strongly suggest that, whereas Chl *a'* is an ingredient of the photosynthetic apparatus [7], Cl-Chl *a* is an artifact produced in the course of pigment extraction.

This view has been substantiated by the following additional experiments. The *Fragaria chiloensis* leaf tissue, which otherwise gave Chl *a*/Cl-Chl *a* molar ratios of around 300 on repeated runs, was first kneaded with a small amount of $AgNO_3$ aqueous solution in such a manner that the Chl *a*/Ag^+ molar ratio was in the range 4 to 1000. The mixture was then submitted to the standard pigment extraction procedure mentioned in section 2. In these extracts from eleven independent runs, we were unable to detect Cl-Chl *a* by HPLC. This reflects to complete suppression of δ-chlorination of Chl *a* via efficient scavenging of the chloride ion by the precipitation reaction $Ag^+ + Cl^- \rightarrow AgCl$. One of the results is given as trace (A) in fig.5. The $AgNO_3$ treatment has caused, incidentally, somewhat heavy pheophytinization and epimerization of Chl *a* in addition to chlorination suppression: the relative contents of Pheo *a* and Chl *a'* in trace (A) are several-fold higher than those found in extracts prepared without the $AgNO_3$ treatment. This is an example of metal ion-induced molecular alterations of Chl derivatives (to be published), but the mechanistic elucidation thereof is beyond the scope of the present study.

The possibility that Cl-Chl *a* had been present in vivo but was dechlorinated by the action of added Ag^+, has been excluded by confirming the molecular integrity of the authentic Cl-Chl *a*

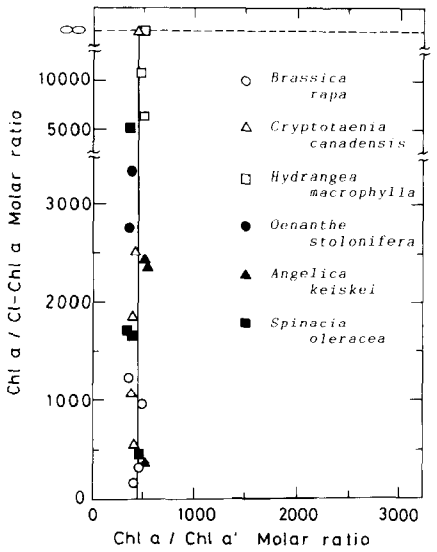

Fig.4. Chl *a*/Cl-Chl *a* and Chl *a*/Chl *a'* molar ratios in leaf extracts of six plants. The former value is based on the assumption that the sensitivity of the detector is the same for Chl *a* and Cl-Chl *a* at the detection wavelength (425 nm). The single point (□) at Chl *a*/Cl-Chl *a* = ∞ (i.e., Cl-Chl *a* not detected) for *Hydrangea macrophylla* represents more than ten independent measurements.

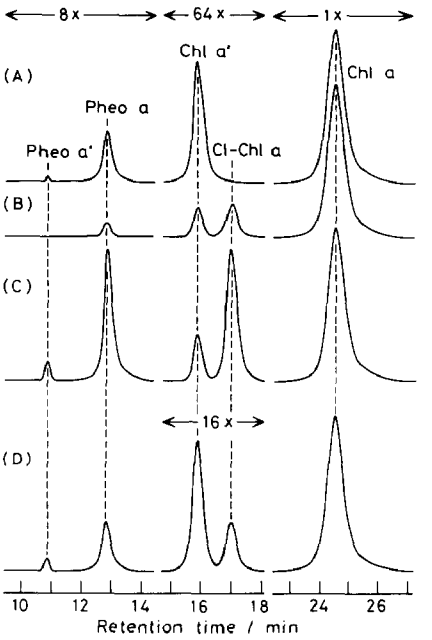

Fig.5. HPLC traces showing pigment compositions of (A) *Fragaria chiloensis* leaf tissue kneaded with $AgNO_3$ such that Chl *a*/Ag^+ = 120, (B) *Hydrangea macrophyla* leaf tissue ground without the desiccant, (C) sample (B) after standing for 100 min at 25°C, and (D) *Synechococcus* cells after standing wet for three weeks at 4°C in darkness. The Chl *a* peaks are normalized to a common intensity.

kneaded with $AgNO_3$ either in the absence or presence of leaf tissue.

The importance of the wet medium of crushed leaf tissue in promoting δ-chlorination of Chl *a* has been verified as follows. When *Hydrangea macrophylla* leaf tissue, which normally yields a very low level of Cl-Chl *a* in the standard extraction runs (fig.4), was ground without the desiccant and then extracted immediately with chloroform, a fairly large amount of Cl-Chl *a* was detected as trace (B) in fig.5 shows. When the pigments were extracted after leaving the ground tissue for about 100 min at 25°C, we obtained trace (C). In going from trace (B) to (C), we note remarkable progress of pheophytinization and δ-chlorination, and moderate promotion of epimerization. In addition to these, we observed the formation of fairly large amounts of allomers (not shown) which are eluted between the Chl *a* and Chl *b* peaks (cf. fig.1). In trace (C), Cl-Chl *a* amounts to as high as 1% of the total Chl *a*. Trace (D) in fig.5 shows the pigment composition of *Synechococcus* cells, after separating them from the culture medium and then allowing them to stand for three weeks at 4°C in darkness. The formation of Cl-Chl *a*, which was not at all detected in fresh cells, is clearly noted. These findings indicate that Cl-Chl *a* is a product of a chemical or enzymatic reaction, namely δ-chlorination of Chl *a* in decaying tissues.

δ-Chlorination of Chl *a* is an electrophilic process [11], and most of in vitro organic chlorinations are known to proceed via the action of Cl_2, Cl^+ or Cl radical. Further, it is well known that halogenations in cells of mammals [15] and fungi [16] are generally catalyzed by peroxidase which activates cytoplasm-derived halide ions into neutral molecules, cations, or radicales [16–18]. In view of these, the δ-chlorination of Chl *a* most probably takes place enzymatically. Senger et al. [5] and Katoh and Yasuda [19] indeed demonstrated an enzymatic synhesis of Cl-Chl *a* by use of chloroperoxidase [16,17]. The exact route of the δ-chlorination during plant tissue handling, however, is not yet clear at the present stage.

The 10-OH,Cl-Chl *a*, or Chl-RCI [1–5] is an oxi-

dation product of Cl-Chl *a*. We obtained no evidence for the presence of 10-OH,Cl-Chl *a*, which is eluted at around 45 min under the present HPLC conditions, in leaf tissue extracts. However, when an extract containing a sizeable amount of Cl-Chl *a* was applied to silica thin-layer chromatography (TLC) under air followed by scraping off the pigments and their HPLC analysis, we clearly noted the presence of hydroxylated Cl-Chl *a* (not shown). Consequently 10-OH,Cl-Chl *a* must also be an experimental artifact. No 10-OH,Cl-Chl *a* formation was observed as long as all the operations were conducted under a strictly oxygen-free atmosphere. Quite recently a similar observation has been made also by Senger and co-workers (private communication).

During TLC development under aerobic conditions, heavy molecular alterations including allomerization and pheophytinization of Chls *a* and *b* were observed in addition to the 10-OH,Cl-Chl *a* formation. Further, we have ample evidence that the choice of the extraction solvent is also crucial in ensuring the molecular integrity of Chl-type pigments: for instance, pigment extraction with hot methanol usually yields a pigment mixture, in which the relative amounts of Chl *a'* and Pheo *a* are variable and higher than in chloroform extracts, and fairly large amounts of Cl-Chl *a* and a series of allomers, which are not at all detected in carefully prepared chloroform extracts, are detected (unpublished). These indicate that due care has to be taken when one wants to delineate pigment composition as it is in vivo.

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