# Mechanism of the Allomerization of Chlorophyll: Inhibition of the Allomerization by Carotenoid Pigments

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Z. Naturforsch. 36b, 1010-1016 (1981); received May 19, 1981

Autooxidation of Chlorophyll, Free Radicals, Carotenoids, Photosynthesis

It is shown that the allomerization of chlorophylls a and b is effectively inhibited by carotenoid pigments. In the light of this finding, two possible mechanisms are considered for the allomerization. One assumes the addition of singlet oxygen to the 9,10-double bond of the chlorophyll (Chl) enolate anion to yield a C-10 hydroperoxide or a dioxetane derivative. The other assumes a free-radical chain reaction involving Chl enolate anion, triplet oxygen, Chl C-10 radicals and peroxide radicals, and Chl C-10 hydroperoxide. The observation that the allomerization and its inhibition by carotenoids occur under carefully controlled dark conditions precluding singlet oxygen formation provides substantial support to the latter mechanism. Further evidence for the free-radical mechanism is obtained by observing the increase of the allomerization rate when air is replaced with pure oxygen from a container. The rate increases to about two-fold in the methanolic Chl solution containing no carotenoid but remains close to zero in the Chl solution containing an equimolar amount of  $\beta$ -carotene. The relevance of the results to photosynthesis and cancer research is briefly discussed.

# Introduction

The oxidation properties of chlorophyll (1a, b)are of special interest, since photosynthesis involves as a primary reaction the ejection of an electron from photoexcited chlorophyll (Chl) molecule(s) to produce a Chl cation radical (Chl<sup>+</sup>) or a Chl special pair cation radical (Chl $t_{sp}$ ) [1-4]. It has been known for a long time that Chl is spontaneously oxidized by atmospheric oxygen in alcoholic solutions [5-8]. Willstätter [5] gave the name "allomerization" to this special kind of Chl oxidation, as some of its products are very similar to the parent compounds by their electronic absorption spectra. The investigations performed in later years have revealed that the allomerization involves a complicated series of reactions which may yield several oxidation products depending on the nature of the solvent [9-16]. All allomers, however, contain an oxygen atom at C-10 and they all react negatively to the Molisch phase test [7, 15]. Among the autooxidation products have been identified the 10-hydroxy- and

Abbreviations: Chl, chlorophyll; Chl<sup>+</sup>, chlorophyll cation radical; Chl<sup>+</sup><sub>sp</sub>, chlorophyll special pair cation radical; <sup>1</sup>H NMR, proton magnetic resonance; PS I, photosystem I; PS II, photosystem II, THF, tetrahydrofuran; TLC, thin-layer-chromatography; TMS, tetramethyl silane; UV/VIS, ultraviolet and visible region.

10-methoxy-chlorophylls, the purpurin 7-dimethyl phytyl ester (6) and the 10-hydroxy- or 10-methoxylactone derivatives (7, 8). The allomerization obviously differs essentially from the photo-oxidation ("photobleaching") of magnesium porphyrins [17 to 19] and chlorophylls [20-26], which seems to involve oxidation of the C-2 vinyl group, ring I or a methine bridge [17-19, 23, 25, 26]. The investigations by Sherman et al. [23] on the photooxidation of Chl a (1a) at low temperature and in solvents containing organic Lewis bases, suggest an addition of singlet molecular oxygen to C-1 (or C-2) and C-2b, thus yielding a labile cyclic peroxide which subsequently undergoes further transformation photochemically or thermally, Contrary to the allomerization, this oxidation does not require an intact cyclopentenone ring but does require a complexed metal atom (Mg or Zn) [23].

The mechanism of the allomerization has remained largely obscure, in spite of the fact that the chemical structures of most allomers are now known in detail [8, 10–14]. There is a good piece of evidence [7, 10, 12–14] supporting the view that the allomerization involves as a primary step the oxidation of small amounts of the Chl enolate anion (2). Fischer and Pfeiffer [8] have suggested the formation of a C-10 hydroperoxide intermediate (4) in the allomerization leading to the 10-alkoxy-lactone derivative (8) without giving details of the mechanism for its formation. The hydroperoxide intermediate could

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be generated through a free-radical chain reaction which is now known to be a common mechanism in many autooxidations [27-32]. However, the formation of the hydroperoxide (4) could also be possible via the direct addition of an oxygen molecule to the 9,10-double bond of the enolate anion (2) [13, 14]. The latter mechanism actually requires that the oxygen molecules are in the reactive singlet state ( $^{1}O_{2}$ ,  $^{1}\Delta_{g}$ ) [33-37]. No clear evidence supporting either of these mechanisms for the allomerization has been available in the literature thus far.

In the present paper, we provide the first experimental results supporting the view that the





allomerization of Chl proceeds via a free-radical mechanism. This evidence is based on the observation that the formation of the 10-hydroxy- and 10-methoxy-lactone derivatives (7, 8) is effectively inhibited by carotenoid pigments (e.g.  $\beta$ -carotene) in the dark.

### Experimental

#### Allomerization experiments

Nine mg of crystalline Chl a (8 mg of crystalline Chl b) were dissolved in 30 ml of acetone, and the solution was divided into two equal parts, A and B. These procedures were performed in dimmed light. The two flasks, containing solutions A and B, were provided with loose corks and wrapped with aluminium foil to avoid any effect of light. Eight mg of  $\beta$ -carotene (lutein) were dissolved in solution B, whereafter 35 ml of methanol were added to each solution. The obtained concentrations of chlorophyll and carotenoid were 0.2 and 0.3 mM, respectively. The solutions were allowed to stand in the dark at room temperature and under atmospheric pressure. Reactions were followed by UV/VIS spectroscopy and TLC on cellulose [38] employing pyridine-light petroleum, 1:15 (v/v), as the eluent. The solvents used in the allomerization experiment were of reagent grade purity.

The whole experiment was repeated with the difference that pure oxygen (triplet state,  ${}^{3}O_{2}$ ) was bubbled through solutions A and B from a steel container at a flow rate of *ca*. 50 ml/min.

# Spectroscopic measurements

The electronic absorption spectra (UV/VIS) were recorded on a Varian 634 spectrophotometer at 25 °C. The <sup>1</sup>H NMR spectra of chlorophylls were measured at ambient temperature with a Jeol FX-60 PFT instrument employing 5 mm sample tubes. The solvent was acetone-d<sub>6</sub> containing TMS as an internal reference. The sample concentration was ca.  $1.5 \times 10^{-2}$  M.

# Chlorophyll a (1a)

Chl a was isolated from clover leaves by the method described previously [39]. Repeated precipitation of the chlorophyll as its water adduct, (Chl a  $\cdot 2 \operatorname{H}_2\operatorname{O}_n$ , yielded a preparation free from colourless galacto- and other lipids as well as from chlorophyll a' and b'. TLC analysis on sucrose [40], yielded only one spot from the preparation. UV/VIS spectrum in THF,  $\lambda_{\max}$  [nm] ( $\varepsilon \cdot 10^{-3}$ ): 664 (91.2), 626 (15.9), 615 (14.1), 593 (8.50), 540 (3.15), 505 (1.74), 435 (122.0), 413 (70.6), 386 (45.3), 334 (28.1), 300 (21.4), 249 (24.5);  $\varepsilon$  [1·mol<sup>-1</sup>·cm<sup>-1</sup>] was calculated for molecular species Chl a  $\cdot \operatorname{H}_2\operatorname{O}$ . <sup>1</sup>H NMR (60 MHz, acetone-d<sub>6</sub>/TMS<sub>int</sub>),  $\delta$  [ppm]: 9.70 (s, 1H,  $\beta$ -H); 8.53 (s, 1H,  $\delta$ -H); 8.12 (dd, 1H, J = 11 Hz, 18 Hz, 2a-H<sub>X</sub>); 6.20 (dd, 1H,

$$\begin{split} J &= 2 \; \text{Hz}, \; 18 \; \text{Hz}, \; 2b \cdot \text{H}_{\text{B}}); \; 5.99 \; (\text{dd}, \; 1 \; \text{H}, \; J = 2 \; \text{Hz}, \\ 11 \; \text{Hz}, \; 2b \cdot \text{H}_{\text{A}}); \; 6.16 \; (\text{s}, \; 1 \; \text{H}, \; 10 \cdot \text{H}); \; 5.01 \; (\text{t}, \; 1 \; \text{H}, \\ 2' \cdot \text{H}); \; 4.34 \; (\text{m}, \; 3 \; \text{H}, \; 1' \cdot \text{H}, \; 7 \cdot \text{H}, \; 8 \cdot \text{H}); \; 3.81 \; (\text{s}, \; 3 \; \text{H}, \\ 10 \; \text{b} \cdot \text{CH}_3); \; 3.80 \; (\text{q}, \; 2 \; \text{H}, \; J = 8 \; \text{Hz}, \; 4a \cdot \text{CH}_2); \; 3.58 \\ (\text{s}, \; 3 \; \text{H}, \; 5a \cdot \text{CH}_3); \; 3.33 \; (\text{s}, \; 3 \; \text{H}, \; 1a \cdot \text{CH}_3); \; 3.28 \; (\text{s}, \; 3 \; \text{H}, \\ 3a \cdot \text{CH}_3); \; 2.38 \; (\text{m}, \; 4 \; \text{H}, \; 7a, \; 7b \cdot \text{CH}_2); \; 1.76 \; (\text{d}, \; 3 \; \text{H}, \\ J = 7 \; \text{Hz}, \; 8a \cdot \text{CH}_3); \; 1.70 \; (\text{t}, \; 3 \; \text{H}, \; J = 7 \; \text{Hz}, \; 4b \cdot \text{CH}_3); \\ 1.51 \; (\text{s}, \; 3 \; \text{H}, \; 3a' \cdot \text{CH}_3); \; 1.28 \; (\text{s}, \; 3 \; \text{H}, \; 7' - 15' \cdot \text{CH}); \; 1.18 \\ (\text{s}, \; \text{broad}, \; 18 \; \text{H}, \; 4' - 14' \cdot \text{CH}_2); \; 0.889, \; 0.797 \; (\text{s}, \; \text{s}, \; 12 \; \text{H}, \\ 7a' - 16' \cdot \text{CH}_3). \end{split}$$

#### Chlorophyll b (1b)

Chl b was obtained simultaneously with Chl a using the method described previously [39]. TLC on sucrose [40] revealed only one component in the preparation. UV/VIS spectrum in THF,  $\lambda_{max}$  [nm]  $(\varepsilon \cdot 10^{-3})$ : 643 (56.0), 593 (10.5), 564 (7.52), 543 (6.85), 453 (155.6), 428 (56.0), 375 (21.1), 356 (25.1), 332 (30.1), 308 (28.4), 253 (30.1);  $\varepsilon$  [l · mol<sup>-1</sup> · cm<sup>-1</sup>] was calculated for molecular species Chl b  $\cdot$  H<sub>2</sub>O. <sup>1</sup>H NMR (60 MHz, acetone- $d_6/TMS_{int}, \delta$  [ppm]: 11.21 (s, 1H, 3a-CHO); 10.09 (s, 1H,  $\beta$ -H); 9.83 (s, 1H, a-H); 8.42 (s, 1H,  $\delta$ -H); 7.99 (dd, 1H, J = 11 Hz, 18 Hz, 2a-H<sub>x</sub>); 6.26 (dd, 1H, J = 2 Hz, 18 Hz, 2b-H<sub>B</sub>); 6.00 (dd, 1H, J = 2 Hz, 11 Hz, 2b-H<sub>A</sub>); 6.13 (s, 1H, 10-H); 5.05 (t, 1H, 2'-H); 4.24 (m, 3H, 1'-H, 7-H, 8-H); 3.83 (s, 3H, 10b-CH<sub>3</sub>); 3.80 (q, 2H, 2H); 3.85 (s, 2H); 3.8  $4a-CH_2$ ; 3.57 (s, 3H, 5a-CH<sub>3</sub>); 3.27 (s, 3H, 1a-CH<sub>3</sub>); 2.35 (m, 4H, 7a, 7b-CH<sub>2</sub>); 1.84 (d, 3H, J = 7 Hz, 8a-CH<sub>3</sub>); 1.81 (t, 3H, J=8 Hz, 4b-CH<sub>3</sub>); 1.54 (s, 3H, 3a'-CH<sub>3</sub>); 1.29 (s, 3H, 7'-15'-CH); 1.19 (s, broad, 18H, 4'-14'-CH<sub>2</sub>); 0.891, 0.799 (s, s, 12H, 7a' to 16'-CH<sub>3</sub>).

#### Carotenoids

 $\beta$ -Carotene and lutein were isolated by chromatography on a sucrose column from the chloroplast extract remaining after the precipitation of the chlorophylls [39].  $\beta$ -Carotene,  $\lambda_{\max}$  (acetone): 478, 452 nm; lutein,  $\lambda_{\max}$  (acetone): 474, 446.5, 422 (sh) nm.

#### **Results and Discussion**

# Inhibition of the allomerization by carotenoids

The electronic absorption spectra obtained from the allomerization experiments with respect to Chl a are shown in Fig. 1. After standing for 24 h under atmospheric oxygen and in the dark, the methanolic chlorophyll solution A which did *not* contain  $\beta$ carotene (Fig. 1, A), exhibited  $\lambda_{\text{max}}$  (R) in acetone at: 654 (1.77), 611 (8.77), 570 (17.3), 523 (27.4) and 418 (1.00) nm;  $\mathbf{R} = A_{\text{soret}}/A\lambda_{\text{max}}$ . These spectroscopic properties are quite similar to those previously reported for the lactone derivatives 7 and 8 [14]. The TLC analysis on cellulose [38] revealed that solution A contained principally 10-methoxy-lactone



Fig. 1. Electronic absorption spectra of (A) Chl a and (B) Chl  $a + \beta$ -carotene after standing in the dark in acetone-methanol, 3:7, v/v, for 0 h (---) and 24 h (--). The spectra were measured in acetone.

derivative (8: Mg-10(R,S) methoxy-purpurin 7lactone-methyl phytyl ester) and a small amount of 10-hydroxy-Chl a. This result is consistent with that previously obtained for the allomerization of Chl a in methanol [13]. The methanolic chlorophyll solution B which contained  $\beta$ -carotene (Fig. 1, B), on the contrary, had undergone very little change during 24 h. Also the TLC analysis from solution B, revealed only one yellow and one green spot, the latter migrating at the same speed as that of Chl a.

The results obtained from a corresponding experiment where Chl b and lutein were used, are shown in Fig. 2. These results are analogous to those in Fig. 1, though lutein appeared to be somewhat less effective an inhibitor than  $\beta$ -carotene. Furthermore, the allomerization of Chl b was found

to be slower than that of Chl a. First after 62 h, most of the Chl b had been allomerized (Fig. 2, A). The TLC on cellulose [38] from solution A (no lutein) yielded three spots representing residual Chl b/b', 10-methoxy-lactone derivative of Chl b and presumably Mg-rhoding<sub>7</sub>-triester (a solvolysis product). No spot corresponding to 10-hydroxy-Chl b was visible [13].

When the experiment of Fig. 1 was repeated using pure oxygen from a container instead of air, the allomerization rate in solution A (no  $\beta$ -carotene) increased by a factor 2, whereas in solution B, the  $\beta$ -carotene still effectively inhibited the allomerization. The chemical character of the allomerization products was, however, similar to that obtained when air was used.



Fig. 2. Electronic absorption spectra of (A) Chl b and (B) Chl b + lutein after standing in the dark in acetone-methanol, 3:7, v/v, for 0 h (---) and 62 h (--). The spectra were measured in acetone.

### Mechanism of the allomerization

The results described above unequivocally show that the allomerization of chlorophylls is efficiently inhibited by carotenoid pigments, in particular, by  $\beta$ -carotene. As  $\beta$ -carotene is known to be the most efficient quencher of singlet oxygen [33, 41-43], the above results might be interpreted, at first sight, as indicating that the reaction sequence,  $1 \rightarrow 2 \rightarrow 3 \rightarrow 5 \rightarrow \cdots$ , is involved in the allomerization. However, as the experiments were performed under carefully controlled dark conditions, the formation of adequate amounts of singlet oxygen through the fotosensitizing action of chlorophyll [34, 41-43] seems very unlikely. On the contrary, the present experiments suggest that it is the triplet oxygen  $({}^{3}O_{2})$  that is involved in the allomerization. This conclusion is supported by the observation

that the allomerization rate increased by a factor 2 in solution A (but not in solution B), when pure oxygen from a steel container was bubbled through the solutions. To account for the observed inhibition of the allomerization by  $\beta$ -carotene at the experimental conditions precluding singlet oxygen formation, the following two basic assumptions are found necessary:

- β-carotene is also an efficient scavenger of free radicals, not only a quencher of singlet oxygen;
- 2. The allomerization proceeds via a free-radical chain reaction mechanism involving triplet oxygen and chlorophyll C-10 peroxide radicals.

Assumption (1) is in accordance with the results of a recent report [44] showing that  $\beta$ -carotene reacts rapidly with trichloromethyl peroxy-radicals generated by the pulse radiolysis technique. A reaction mechanism which takes into consideration the above facts, is shown in the scheme by the sequence: Chl  $(1) \rightarrow$  Chl enolate anion  $(2) \rightarrow \rightarrow \rightarrow$ Chl 10-hydroperoxide  $(4) \rightarrow \cdots$ . A nucleophilic attack at C-9 of 4 by hydroxyl or methoxyl ions subsequently leads to the formation of Mg-purpurin 7-monomethyl phytyl ester (5) or Mgpurpurin 7-dimethyl phytyl ester (6), respectively. The lactone derivatives (7, 8) can be formed from 5 through solvation and elimination as previously proposed [10, 13].

The free-radical chain reaction may involve the following steps:

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where RH is Chl (1);  $R|^{\ominus}$ , Chl enolate anion (C-10 carbanion) (2); R, Chl C-10 radical; ROO, Chl C-10 peroxide radical; ROOH, Chl C-10 hydroperoxide (4);  ${}^{3}O_{2}$ , triplet molecular oxygen (a diradical);  $[|\overline{Q}-\overline{Q}\cdot]^{\ominus}$ , superoxide anion radical;  $[|\overline{Q}-\overline{Q}H]^{\ominus}$ , hydroperoxide anion; R'OH, water (R'=H) or methanol ( $R'=CH_{3}$ ); and R'O, hydroxyl radical ( $R'=CH_{3}$ ).

An essential point in the above mechanism is the initiation of the chain (reactions (1) and (2)). The reactive species is thought to be the Chl enolate anion  $\leftrightarrow$  Chl C-10 carbanion (2) which reacts with triplet molecular oxygen to yield a Chl C-10 radical and a superoxide anion radical. The latter can abstract a hydrogen atom from a Chl molecule resulting in a Chl C-10 radical and a hydroperoxide anion (reaction (3)). The Chl C-10 radical can alternatively react with 3O2 thus yielding a Chl C-10 peroxide radical or with methanol (water) resulting in a methoxyl (hydroxyl) radical and a Chl molecule (reactions (4) and (5)). The Chl C-10 peroxide radical is able to abstract a hydrogen atom from Chl or a solvent molecule (methanol or water) (reactions (6) and (7)). Both of these reactions yield a Chl C-10 hydroperoxide (4) which presumably is the important intermediate in the allomerization. Reactions (3) to (7) represent various possibilities for the propagation of the chain reaction. Among the alternatives shown for the termination, reaction (9) is very important, as it yields C-10 methoxy- or hydroxy-Chl. Reaction (8) is unlikely to occur owing to steric hindrance.

The results from the carotenoid inhibition experiments provide substantial support for the above mechanism. However, more definitive evidence is still required for the final establishment of the now proposed free-radical mechanism. Experiments to this end utilizing  $^{18,18}O_2$  and mass spectrometry are underway in our laboratory.

#### Some biological implications of the results

The relevance of the present investigation to photosynthesis was already mentioned in the Introduction. In connection with photosynthesis, an important question is: How are the carotenoids organized relative to chlorophylls and how do they function in photosynthetic membranes ?  $\beta$ -Carotene and lutein are known [45] to be the major carotenoids in the thylakoid membranes of higher plant chloroplasts. Recent investigations suggest that  $\beta$ -carotene is associated with the antenna pigments of PS I and PS II, while the polar carotenoids (lutein, violaxanthin and neoxanthin) are located primarily in the light-harvesting auxiliary pigment system [45]. Two aspects of function are generally recognized for the carotenoids in photosynthetic membranes. First, carotenoids assist chlorophylls to harvest light energy, i.e. they absorb light quanta and transfer the excitation energy to the chlorophyll system [46, 47]. Second, carotenoids protect the cell against photodynamic destruction by deactivating triplet chlorophyll and singlet oxygen states [34, 41-43, 48]. In addition to these, it has also been suggested that  $\beta$ -carotene could play a role in photochemistry [49].

Considering the results of the present investigation, an interesting question is whether the carotenoids can react with chlorophyll  $\pi$ -cation radicals (Chl<sup>+</sup> or Chl<sup>+</sup><sub>sp</sub>) produced in the photosynthetic reaction centers by photoexcitation [1-4]. The present work is also of considerable interest in relation to the mechanism of oxygen evolution in PS II [50-52] and the photoreduction of molecular oxygen by the primary electron acceptor in PS I [53]. It should be noted that the superoxide anion radical  $(O_2^{\overline{i}})$  is the primary product of the photoreduction [53].

Finally, the present investigation is also relevant to the question whether  $\beta$ -carotene and related pigments can function as protective agents against

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cancer as suggested by a recent epidemiological report [54].

This work was supported by the Research Council for the Natural Sciences of the Academy of Finland.

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