Tracing the Allomerization Pathways of Chlorophylls by ^{18}O-Labeling and Mass Spectrometry

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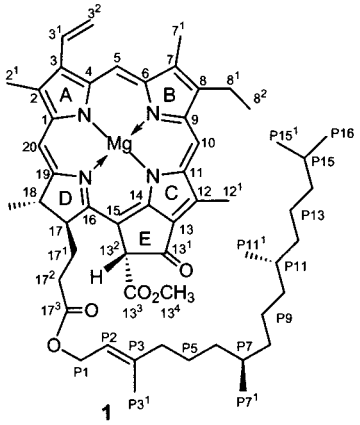
The Willstätter allomerization reaction of chlorophylls (Chl) has posed a difficult problem in Chl and photosynthesis research over the past 90 years. Here, we present strong additional evidence, based on ^{18}O-labeling and mass spectrometry, for the previously published free-radical allomerization (FRA) mechanism (Hynninen, *Z. Naturforsch.* **1981**, *36b*, 1010−1016). This mechanism is also complemented now by describing two alternative pathways for the formation of 13^2(*S*/*R*)-hydroxy-Chl *a*. The results from the $^{18,18}O_2$-experiments suggest that the predominant route for the formation of the 13^2(*S*/*R*)-hydroxy-Chl *a* under essentially anhydrous conditions (anhydrous Chl and thoroughly dried methanol) is the homolytic cleavage of the C-13^2-hydroperoxide intermediate. However, if Chl dihydrate and undried methanol are used in the reaction mixture, the direct route from the Chl C-13^2 radical to 13^2(*S*/*R*)-hydroxy-Chl *a* can be predicted to become significant. The results from the $^{18,18}O_2$-allomerization experiments described in this paper also verified that the 13^2(*S*/*R*)-methoxy-lactone derivatives and the 15-glyoxylic acid derivative of Chl *a* incorporated each a single ^{18}O-atom, whereas 13^2(*R*/*S*)-methoxy-Chl *a* remained unlabeled. Consequently, these allomers are formed via the pathways previously suggested in the original FRA mechanism. The possible factors contributing to the control of the allomerization reactions are considered. Finally, the relationship between the allomerization reactions of Chl *a* and those of Chl *b* and BChl *a* is briefly discussed.

Introduction

Chlorophylls (Chl) *a* (**1**) and *b* (**2**) and bacteriochlorophylls (BChl) *a* (**3**) and *b* (**4**) are Mg(II) complexes of cyclic tetrapyrroles, called chlorins (17,18-dihydroporphyrins) and bacteriochlorins (7,8,17,18-tetrahydroporphyrins; numbering of the atoms conforms to the semisystematic nomenclature of IUPAC−IUB[1]). These tetrapyrrolic compounds are ubiquitous in photosynthetic organisms,[2,3] not only because they can absorb light quanta effectively but also because they can release and take up electrons reversibly by means of their aromatic 18 π-electron [18]-diazaannulene macrocycle.[4,5] According to the widely spread theory (for reviews, see refs 6−9), light quanta impinging on a photosynthetic organism are absorbed by the antenna chlorophylls and accessory pigments and the



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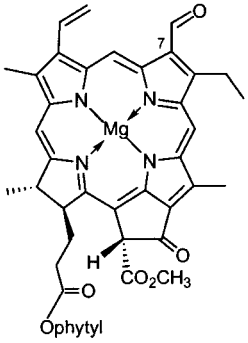

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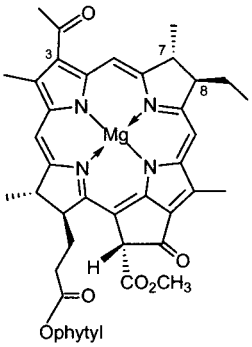

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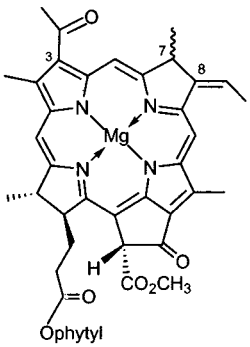

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excitation energy is funneled to a special environment, the reaction center (RC), where a (bacterio)chlorophyll "special pair" (P700 in the RC I and P680 in the RC II of green plants and P870 or P960 in the RC of photosynthetic bacteria) undergoes one-electron oxidation to yield a π-cation radical [$P700^{\bullet+}$, $P680^{\bullet+}$, $P870^{\bullet+}$, or $P960^{\bullet+}$]. In green plants, the first electron acceptor (A_1) is widely believed to be a Chl *a* (**1**) monomer in RC I and a pheophytin *a* (Pheo, Mg^{2+} replaced with 2 H^+ in **1**) monomer in RC II, which give the corresponding π-anion radicals. In photosynthetic bacteria, A_1 is presumably a bacteriopheophytin.

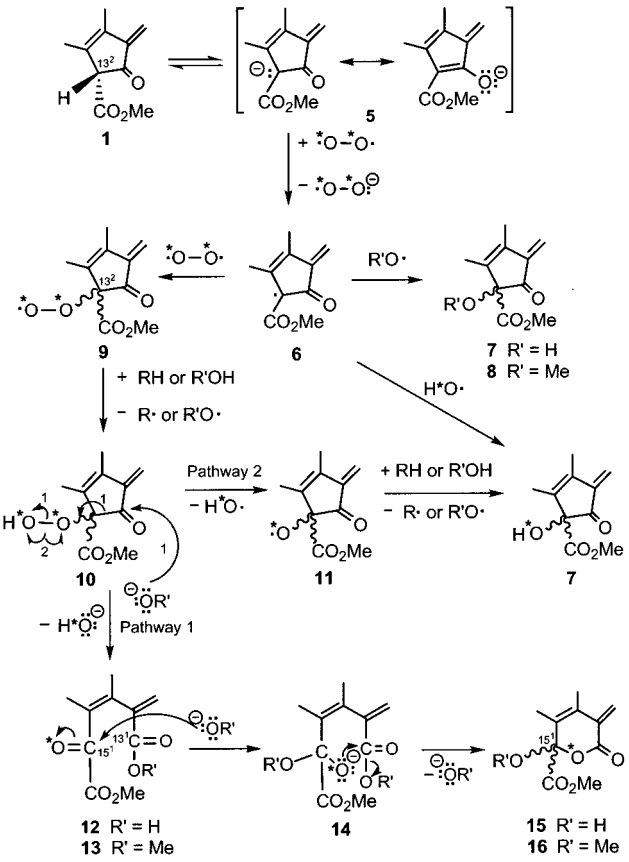
Release of an electron from the excited singlet state of Chl or BChl is just one special form of chlorophyll oxidations (these can be grouped into abstraction of electrons, addition of oxygen atoms = oxygenations, and abstraction of hydrogen atoms = dehydrogenations). Chlorophylls are also photooxidized in vitro and in vivo by a dioxygenation route, where the molecular singlet oxygen ($^1O_2$, $^1\Delta_g$) reacts with a methine bridge of the macrocycle by a cycloaddition mechanism, yielding finally an open-chain tetrapyrrole, a bilin, as a stable product. Apparently, this is the way through which chlorophylls are biodegraded in the natural senescence processes of plants (for a recent review, see ref 10).

The chlorophyll molecule is quite sensitive to a variety of other oxidants,[11] e.g., the ferric salts, I_2, and molecular ground-state oxygen. It has been known since 1911 that Chl is spontaneously oxidized by atmospheric oxygen, (i.e., by the ground-state or triplet-state oxygen, $^3O_2$, $^3\Sigma_g^-$) in alcoholic solutions in the dark.[12,13] Willstätter gave the name "allomerization" to this special kind of Chl oxidation, as some of its products were very similar to the parent compounds, the only clear difference being that the allomerized Chl reacted negatively to the Molisch phase test, whereas the original, intact Chl reacted positively in the test.[12,13]

The investigations performed in later years have revealed that the allomerization involves a complicated series of reactions, which may yield a variety of oxidation products depending on the nature of the Chl, solvent, etc. (see the reviews in refs 11, 14−17). Among the allomerization products derived from Chl *a* (**1**) have been identified the 15^1-diastereomers of the 15^1-methoxylactone derivative (**16**), the 13^2-diastereomers of the 13^2-hydroxy-Chl (**7**), and those of the 13^2-methoxy-Chl (**8**), as well as the 15-glyoxylic acid derivatives (**12**, **13**) (Scheme 1).

The allomerization reaction occurs with all Chls and their derivatives that have an intact β-keto ester structure in the isocyclic ring E (cf. compounds **1**−**4**). Several

Scheme 1



mechanisms for the Chl allomerization have been proposed during the past century (reviewed in refs 11 and 14−17). Nevertheless, a majority of the proposals are very incomplete and lack clear experimental evidence. The most detailed allomerization mechanism published thus far is the so-called free-radical allomerization (FRA), which was put forward by Hynninen in 1981 on the basis of the observation that β-carotene inhibits Chl allomerization in the dark.[18] A substantial amount of further evidence in support of the FRA mechanism is presented in the reviews of refs 15−17.

An essential feature in the FRA mechanism[15,18] is the Chl enolate anion (**5**), which is the oxygen-sensitive key intermediate. This argument is supported by the observation that the "pyro" derivatives, i.e., 13^2-demethoxycarbonylated derivatives of Chl, are not allomerized under the same conditions as Chl because their enolization needs more drastic conditions.[19] Also, acids inhibit the allomerization reaction, whereas bases (e.g., the sterically hindered organic base, 1,4-diazabicyclo[2.2.2]-octane, DABCO) increase its rate.[15] Further support for the important role played by the Chl enolate ion in the allomerization can be deduced from the oxidation potentials of the original Chl and its enol forms.[15,20,21] The oxidation potential of the Chl *a* enolate ion ($E_{1/2} \approx 400$ mV[15,21]) is expected to be lower than that of Chl *a* itself ($E_{1/2} = 860$ mV[20]) or its free enol form ($E_{1/2} = 500$ mV[20]).

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Such a reduction in the oxidation potential would make electron transfer from the Chl enolate anion to the ground-state oxygen a very feasible reaction. The Chl enolate anion is a resonance hybrid, where the negative charge is partially delocalized over the macrocyclic π-system, thus making the π-electronic structure of the Chl enolate anion ion substantially different from that of the Chl keto form. This structural difference between the two Chl forms appears in the noticeable differences between their electronic absorption spectra[11,15] and between their ^{1}H NMR spectra.[21] Furthermore, the following of the methanolic allomerization reaction of Chl *a* by sucrose TLC[22] has repeatedly shown that a significant amount of Chl *a*′ [= 13^2(*S*)-epimer of Chl *a*] is formed in the early stages of the reaction. It is well-known that epimerization involves the formation of the enol form intermediate.

A second highly reactive intermediate in the FRA mechanism (Scheme 1) is the Chl C-13^2 radical (**6**), which has the unpaired electron vicinal to the macrocyclic π-system. Evidence for the formation of the C-13^2 radical species is provided by the observation that, in addition to carotenoids, several other free-radical scavengers, such as tocopherols, BHT, and ascorbic acid, inhibit the allomerization, whereas the free-radical initiators, e.g., benzoyl peroxide, accelerate its rate.[15] Further support for the formation of the C-13^2 radical intermediate has been obtained from the horseradish peroxidase (EC 1.11.1.7, donor:hydrogen peroxide oxidoreductase) catalyzed H_2O_2 oxidation of Chl *a*, solubilized with Triton-X 100.[15,23] The major oxidation products were characterized as 13^2(*S*/*R*)-HO-Chl *a* (**7**), and minor amounts of other oxidation products, e.g., the15^1-hydroxylactone derivative (**15**), were found as well.[23] Because peroxidase is known to be capable of abstracting a labile hydrogen atom from the substrate, these results may be interpreted[15] as implying that the enzyme assisted in producing Chl C-13^2 radicals, thus accelerating the allomerization reaction. Our recent observation that several minor side products are formed in the allomerization of Chl *a* can be taken as a further piece of evidence for the free-radical character of the reaction.[24]

In 1998, Woolley et al.[25] identified 13^2-HO-Chl *a*, 15^1-HO-lact-Chl *a*, 15^1-CD_3O-lact-Chl *a*, and 13^1-CD_3O-15-Glyox-Chl *a* as allomerization products when Chl *a* reacted in CD_3OD. These results are important because they provide strong evidence for the later steps of the FRA mechanism (Scheme 1), postulating strict regioselective control for the nucleophilic attack by the methoxide or hydroxide ion.

The primary objective of this study was to find conclusive proof for the FRA mechanism, utilizing ^{18}O-labeling and mass spectrometry. To that end, the methanolic allomerization reaction of Chl *a* (**1**) was performed first in the presence of normal air (containing $^{16,16}O_2$) and then in the presence of $^{18,18}O_2$-enriched atmosphere. The ESI mass spectra of the chromatographically purified Chl *a* allomers[24] formed in these two reactions were compared to see which allomers contained the ^{18}O-label.

Results

On the basis of the performed HPLC and TLC analyses, the methanolic allomers, produced by Chl *a* (**1**) in the presence of $^{18,18}O_2$, were analogous to those produced by Chl *a* in the presence of normal air.[24,26] The main allomers were in both cases the 15^1-diastereomers (*S*/*R* ratio 46:54) of the Mg-15^1-methoxylactone derivative (52%) [15^1(*S*/*R*)-MeO-lact-Chl *a*, **16**], the 13^2-diastereomers (*S*/*R* ratio 79:21) of 13^2-hydroxychlorophyll *a* (21%) [13^2(*S*/*R*)-HO−Chl *a*, **7**], the 13^2-diastereomers (*S*/*R* ratio 38:62) of 13^2-methoxychlorophyll *a* (18%) [13^2(*S*/*R*)-MeO-Chl *a*, **8**], and the Mg-15-glyoxylic acid derivative (7%) [15-Glyox-Chl *a*, **13**] (Scheme 1; the IUPAC−IUB semisystematic names of these allomers can be found in refs 16, 17, 24, and 26).

The ESI mass spectrum of each unlabeled allomer displayed intensive $M^{\bullet+}$ (π-radical cation) and $[M + 1]^+$ peaks (Table 1, Figures 1 and 2). The $[M + 1]^+$ peak arises from a mixture of the heavy isotope $M^{\bullet+}$ ion (Chl allomer containing one ^{25}Mg or ^{13}C) and the $[M + H]^+$ ion. The ratio of the intensities of the $M^{\bullet+}$ and $[M + 1]^+$ peaks exhibited variation for different allomer samples. Inspection of the results in Table 1 and Figures 1 and 2 shows that the $[M + 1]^+$ ion is the most abundant one in the spectrum of each unlabeled allomer, except 13^2(*R*)-HO-Chl *a*, for which the $M^{\bullet+}$ is the most abundant ion. Because such variation in the abundances of the $M^{\bullet+}$ and $[M + 1]^+$ ions was observed even for two different samples of the same allomer (e.g., 13^2(*R*)-HO-Chl *a* and 15-Glyox-Chl *a*), the variation cannot be attributed to the differences in the oxidation potentials of the allomers. The variation is more likely associated with the mechanism of the delicate electrospray ionization process, which is sensitive to many factors, all of which are not yet completely understood.[27] In our case, one probable reason for the variation in the abundances can be attributed to differences in the allomer concentration in different MS samples.

Table 1 also shows the MS data obtained from the allomers produced when the allomerization reaction was performed under an $^{18,18}O_2$-enriched atmosphere. In addition, Figures 1 and 2 visualize the comparison between the MS spectra of allomers originating from the $^{16,16}O_2$-experiment (upper spectra) and those originating from the $^{18,18}O_2$-experiment (lower spectra). The comparison of the two sets of MS results demonstrates unequivocally that 13^2(*R*/*S*)-MeO-Chl *a* (**8**) carries no ^{18}O label, the MS data from the two experiments being almost identical. In contrast, the differences between the two sets of MS data for 13^2(*R*/*S*)-HO-Chl *a* (**7**), 15-Glyox-Chl *a* (**13**), and 15^1(*R*/*S*)-MeO-lact-Chl *a* (**16**) show with certainty that these allomers have incorporated a single ^{18}O-label. To recognize the presence of the ^{18}O-label in these allomers, it is important to see the differences between the whole molecular ion regions of the two sets of MS data, not just the difference between the two most intensive ion peaks in the two spectra, which is two atomic mass units for 13^2(*R*)-HO-Chl *a* and 15-Glyox-Chl *a*, but only one amu

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Table 1. Molecular Ion Regions of the ESI Mass Spectra of Chl *a* Allomers[a]

allomer	M	M + 1	M + 2 *M	M + 3 *M + 1	M + 4 *M + 2	M + 5 *M + 3	M + 6 *M + 4
13^2(*R*)-HO-Chl *a* (**7**)	908.5	909.5	910.5	911.5	912.5	913.5	914.5
	(100)	(92)	(57)	(25)	(9)	(3)	(0)
	[57]	[83]	*[100]	*[81]	*[55]	*[20]	*[15]
13^2(*S*)-HO-Chl *a* (**7**)	908.5	909.5	910.5	911.5	912.5	913.5	914.5
	(86)	(100)	(63)	(30)	(12)	(3)	(1)
	[56]	[81]	*[100]	*[87]	*[56]	*[26]	*[12]
13^2(*R*)-MeO-Chl *a* (**8**)	922.5	923.5	924.5	925.5	926.5	927.5	928.5
	(74)	(100)	(65)	(32)	(12)	(5)	(3)
	[94]	[100]	[65]	[33]	[13]	[8]	[0]
13^2(*S*)-MeO-Chl *a* (**8**)	922.5	923.5	924.5	925.5	926.5	927.5	928.5
	(78)	(100)	(62)	(28)	(10)	(4)	(0)
	[58]	[100]	[68]	[35]	[13]	[5]	[0]
15-Glyox-Chl *a* (**13**)	938.5	939.5	940.5	941.5	942.5	943.5	944.5
	(80)	(100)	(65)	(28)	(14)	(5)	(0)
	[36]	[92]	*[97]	*[100]	*[68]	*[33]	*[12]
15^1(*R*)-MeO-lact-Chl *a* (**16**)	938.5	939.5	940.5	941.5	942.5	943.5	944.5
	(96)	(100)	(55)	(25)	(8)	(2)	(0)
	[65]	[69]	*[100]	*[78]	*[47]	*[20]	*[7]
15^1(*S*)-MeO-lact-Chl *a* (**16**)	938.5	939.5	940.5	941.5	942.5	943.5	944.5
	(77)	(100)	(63)	(32)	(11)	(4)	(1)
	[71]	[71]	*[100]	*[78]	*[45]	*[21]	*[8]

[a] The relative abundances (%) are given as follows: (number) and [number] refer to the ion species of allomers formed in the allomerization reaction under normal air and under $^{18,18}O_2$-enriched atmosphere, respectively; * indicates the presence of ^{18}O.

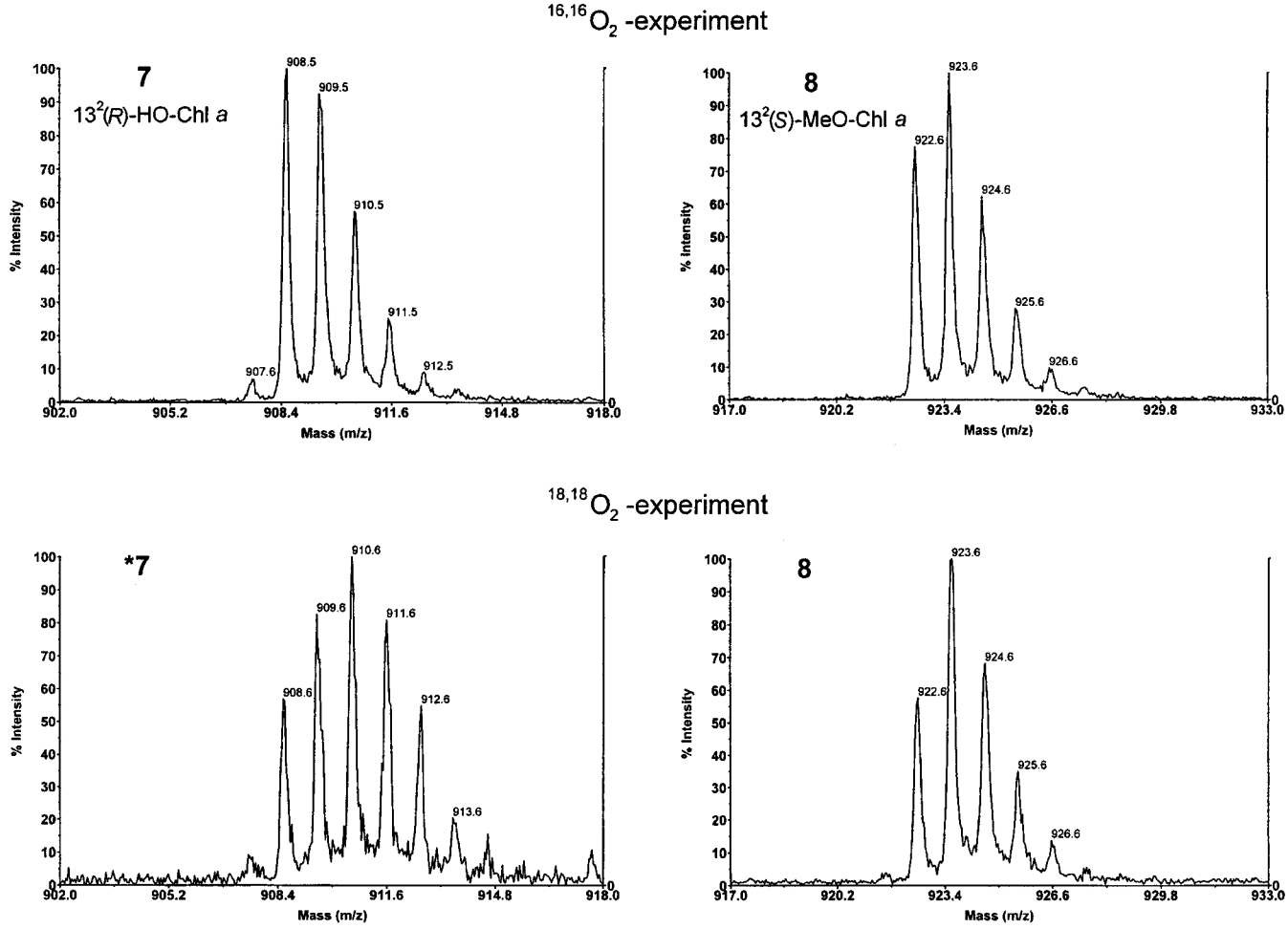


Figure 1. Molecular ion regions of the ESI mass spectra of the Chl *a* allomers **7** and **8**, formed in the allomerization reaction under normal air (top) and under $^{18,18}O_2$-enriched atmosphere (bottom).

for 13^2(*S*)-HO-Chl *a* and 15^1(*R*/*S*)-MeO-lact-Chl *a* (Table 1, Figures 1 and 2). Such variation in the peak intensities is understandable considering that the spectrum representing each ^{18}O-labeled allomer actually consists of two overlapping spectra, one arising from the ^{16}O-containing allomer and another arising from the ^{18}O-containing one. Despite the careful planning of the labeling experiment, obviously some $^{16,16}O_2$ remained in the reaction vessel, as a consequence of the incomplete deaeration of the methanolic Chl solution and/or the incomplete replace-

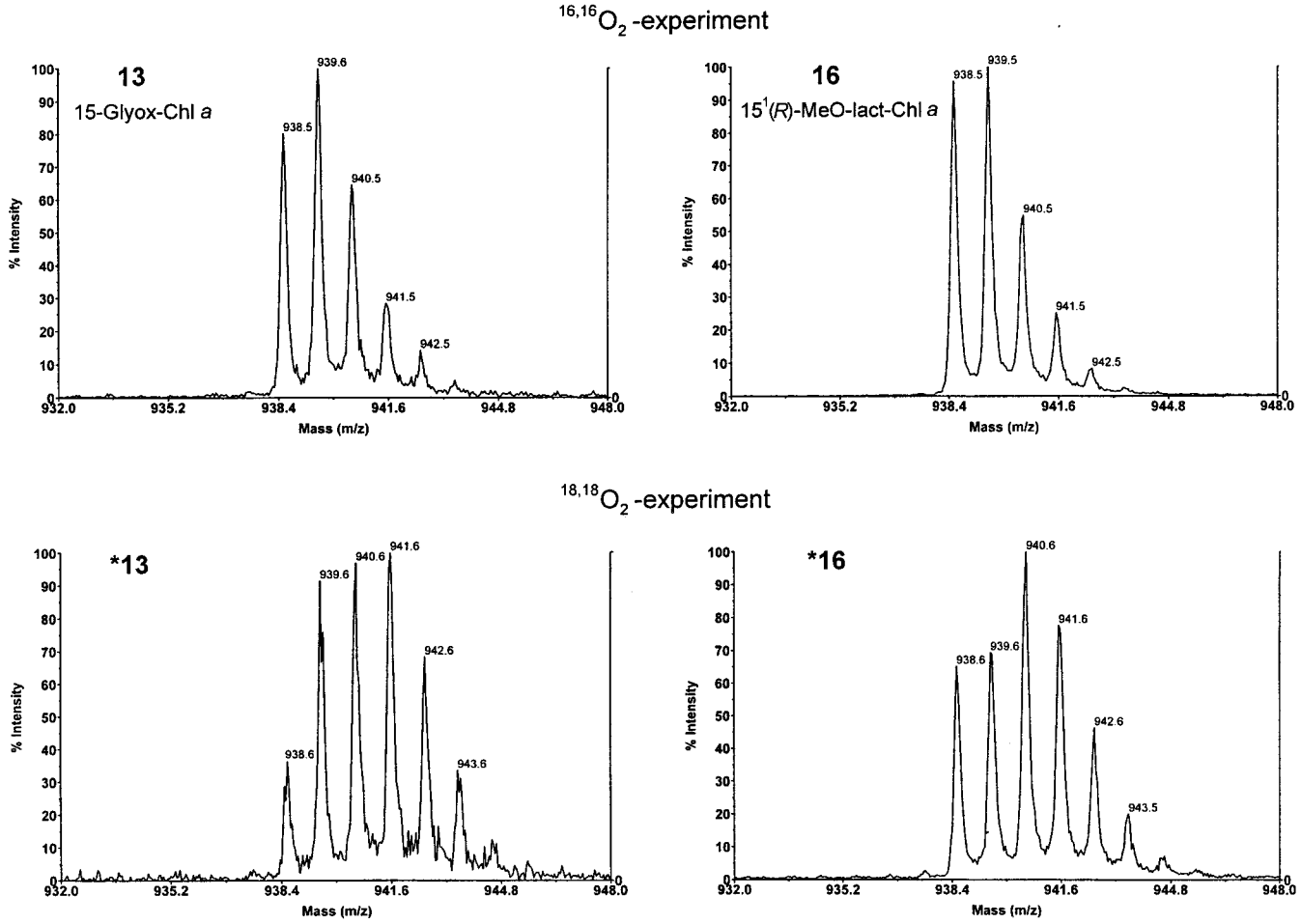


Figure 2. Molecular ion regions of the ESI mass spectra of the Chl *a* allomers **13** and **16**, formed in the allomerization reaction under normal air (top) and under $^{18,18}O_2$-enriched atmosphere (bottom).

ment of the air in the glovebox with argon gas. In addition, it is noteworthy that the relative abundances of the $M^{\bullet+}$ and $[M + 1]^+$ ions as well as those of the $*M^{\bullet+}$ and $[*M + 1]^+$ ions can vary in the individual spectra (see above), which also may influence the appearance of the overlapped spectrum.

The incorporation of the ^{18}O-label into the allomerization products was in general agreement with that predicted by the FRA mechanism.[15,18] The MS analyses verified that all the allomers, except $13^2(R)$- and $13^2(S)$-MeO-Chl *a* (**8**), were labeled when the allomerization reaction was performed in the presence of $^{18,18}O_2$. The pathways of the ^{18}O-label are shown with asterisks in Scheme 1 (only the changes occurring in the isocyclic ring are shown). In the FRA mechanism, the primary step is the autoxidation of the Chl enolate anion (**5**), formed in small amounts from Chl (**1**) in the methanol, containing trace amounts of base and metal impurities. The electron transfer from the Chl enolate anion to doubly labeled triplet oxygen ($^{18,18}O_2$) yields a Chl C-13^2 radical (**6**) in which the unpaired electron is vicinal to the macrocyclic π-system. The Chl C-13^2 radical can react along two alternative paths. It can give $13^2(S/R)$-HO-Chl (**7**) or $13^2(S/R)$-MeO-Chl (**8**) in a termination reaction with the $HO^{\bullet}$ or $MeO^{\bullet}$ radical, originating from water or methanol, respectively. Alternatively, it can react with $^{18,18}O_2$ to afford the Chl 13^2-peroxide radical (**9**), which after hydrogen-atom abstraction from another Chl molecule (RH) or solvent (R′OH) is converted into the Chl 13^2-hydroperoxyl derivative (**10**), bearing a doubly labeled hydroperoxyl group.

The 13^2-hydroperoxyl derivative (**10**) represents another important branching point in Scheme 1. According to the first alternative (pathway 1), the 13^1-carbonyl carbon of the hydroperoxyl derivative can undergo a nucleophilic attack by the methoxide or hydroxide ion, with concerted heterolytic cleavages of the C-13^1−C-13^2 bond and the peroxide bond, to result in the 15^2-monomethyl ester (**12**) or the 13^1,15^2-dimethyl ester (**13**) of the 15-glyoxylic acid derivative, bearing a labeled carbonyl oxygen at C-15^1. The 15^1-methoxylactone derivatives (**16**) are then formed through the nucleophilic attack by the methoxide ion to the 15^1-carbonyl carbon of the 15-glyoxylic acid derivative, followed by an intramolecular nucleophilic acyl substitution to afford the lactone ring. Hence, the 15^1-methoxylactone derivatives are expected to contain the label at the lactone-ring oxygen, which is in agreement with the MS finding. If the attacking nucleophile is a hydroxide ion, the reaction yields a C-15^1 *gem*-diol derivative (**14**, R′ = H), which then can be converted into the 15^1-HO-lactone derivatives (**15**). However, we were unable to detect these derivatives in this study, probably because the concentration of water, which is the main source of the hydroxide ion, was much lower than that of the methoxide ion in the reaction mixture (note that our Chl *a* was essentially anhydrous and that the methanol was thoroughly dried).

According to the original FRA mechanism,[15,18] 13^2-

(*S*/*R*)-HO-Chl (**7**) and 13^2(*S*/*R*)-MeO-Chl (**8**) are formed in the termination reaction between the Chl C-13^2 radical (**6**) and the HO$^\bullet$ or MeO$^\bullet$ radical, originating in H_2O or MeOH, respectively. Clearly, the methoxy-Chls are formed through this route, since no ^{18}O-label was observed in these derivatives. However, this route alone cannot explain the ^{18}O-label now observed by mass spectrometry (Table 1) in purified 13^2(*R*/*S*)-hydroxy-Chl *a* because, according to the former route, the hydroxyl radical is formed from water ($H_2{}^{16}O$). Therefore, the original FRA mechanism must be modified in the fashion shown in Scheme 1, which illustrates an alternative route (pathway 2) for the formation of 13^2(*R*/*S*)-hydroxy-Chl, namely the homolytic cleavage of the doubly labeled C-13^2-hydroperoxide (**10**), followed by a hydrogen atom addition to yield 13^2(*S*/*R*)-H*O-Chl (**7**).[24] The homolytic cleavage of the hydroperoxyl group also produces a labeled hydroxyl radical, which can react with the Chl 13^2-radical to yield the epimers of 13^2-H*O-Chl.

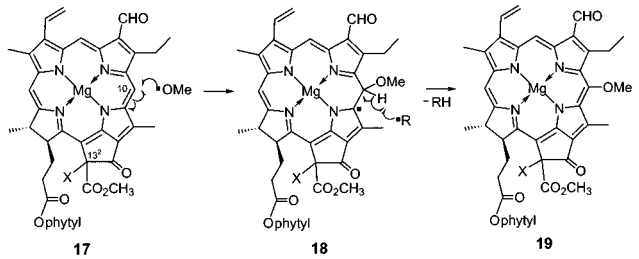
Discussion

The performed $^{18,18}O_2$ allomerization experiments have provided strong additional evidence in support of the FRA mechanism and have also complemented it by describing two alternative pathways for the formation of 13^2(*S*/*R*)-HO-Chl *a* (**7**). The MS results of the allomers from the $^{18,18}O_2$ experiment suggest that the predominant route for the formation of the hydroxychlorophylls under essentially anhydrous conditions (anhydrous Chl and thoroughly dried MeOH) is the homolytic fission of the C-13^2-hydroperoxide (**10**), i.e., pathway 2 in Scheme 1. However, if Chl dihydrate (cf. "microcrystalline Chl") and undried methanol are used in the allomerization reaction, the direct route from the C-13^2 radical (**6**) to 13^2(*S*/*R*)-HO-Chl *a* (**7**) can be predicted to become significant.

It would also be of interest to know which factors determine the proportions observed for the different allomers in the product mixture? Apparently, in addition to the steric factors, also the electronic effects of the macrocyclic π-system are strongly involved in directing the allomerization reaction. Such a situation is expected considering that, in the FRA mechanism, the important reactive sites are the C-13^2 and C-13^1 positions, which are conjugated with the macrocyclic π-system. Therefore, electron delocalization or charge transfer from these sites over the π-system is possible.[15]

The observation that the pheophytins (Mg-free Chl derivatives) are less susceptible to allomerization than the corresponding chlorophylls indicates that the electron structure of the macrocycle contributes to the control of the allomerization reaction. In addition, the results from our recent allomerization experiments with Chl *b* (**2**)[28,29] provide direct evidence for the view that the electronic effects of the macrocyclic π-system are strongly involved in the allomerization reaction. The seemingly small structural difference between Chl *a* (**1**) and Chl *b* (**2**) has a profound effect on the composition of the allomer mixture. The allomers produced by Chl *b* [15-Glyox-Chl *b* (36%), 15^1(*S*/*R*)-MeO-lact-Chl *b* (46%), 13^2(*S*/*R*)-HO-Chl *b* (≪0.5%), 13^2(*S*/*R*)-MeO-Chl *b* (7%), and 13^2(*S*)-HO-10-MeO-Chl *b* (8%)][29] show clear differences as compared

Scheme 2



with the allomers yielded by Chl *a* [15-Glyox-Chl *a* (7%), 15^1(*S*/*R*)-MeO-lact-Chl *a* (52%), 13^2(*S*/*R*)-HO-Chl *a* (18%), and 13^2(*S*/*R*)-MeO-Chl *a* (21%)].[24,26] The percentages in parentheses indicate that the allomerization of Chl *b* produces 15-Glyox-Chl *b* + 15^1(*S*/*R*)-MeO-lact-Chl *b* in a yield of 82%, whereas the allomerization of Chl *a* produces the corresponding *a*-series allomers only in a yield of 59%. This difference in the allomer proportions suggests that pathway 1 in Scheme 1 is more favored in the allomerization of Chl *b*. A likely reason for this is that the macrocyclic π-system mediates the electron withdrawing effect of the 7-formyl group to the C-13^1 carbonyl carbon of the 13^2-hydroperoxyl derivative corresponding to **10**, thus rendering this carbonyl carbon more susceptible to attacks by the methoxide-ion nucleophile.

Another special feature in the allomerization of Chl *b* is that an entirely new type of Chl allomer, the 13^2(*S*)-HO-10-MeO-Chl *b* (**19**, X = HO),[28,29] was formed instead of 13^2(*S*)-HO-Chl *b*. These results make it understandable why, in the earlier allomerization studies,[30] a substantial amount of 13^2-HO-Chl *a* was formed when Chl *a* reacted in methanol, but not even traces of 13^2-HO-Chl *b* could be observed when Chl *b* reacted under the same conditions.

A reasonable explanation for the formation of the 13^2-(*S*)-HO-10-methoxy-Chl *b* is provided by the bimolecular aromatic radical substitution (S_R 2 Ar) mechanism (Scheme 2).[16] In this mechanism, an important precursor is 13^2(*S*)-X-Chl *b* (**17**), where X is a hydroxyl or a hydroperoxyl group. The MeO$^\bullet$ radical attacks regioselectively the C-10 of **17**, producing the C-11 radical intermediate **18**. From **18**, the C-10 hydrogen atom is abstracted by some radical species in the reaction mixture (e.g. an organic radical or the triplet oxygen, a diradical) to restore the macrocyclic aromatic π-electron system and to form the product **19**. The MeO$^\bullet$ attacks regioselectively the C-10 of the intermediate **17** probably because the total electron-withdrawing effect of the 7-CHO, 13^1-C=O, 13^2-X, and 13^2-COOCH$_3$ groups is stronger at C-10 than at the other methine bridge positions.

Despite the appreciable advances achieved in the allomerization studies of Chls *a* and *b*, there are still questions to be answered. One difficult question concerns the stereochemical control of the allomer formation: Which factors determine the *S*/*R* ratios of the 15^1- or 13^2-epimers of the 15^1-MeO-lact-Chl *a* (**16**, *S*/*R* = 46:54), 13^2-HO-Chl *a* (**7**, *S*/*R* = 79:21), and 13^2-MeO-Chl *a* (**8**, *S*/*R* = 38:62)?[24,26] The *S*/*R* ratio for the 15^1-epimers of **16** is as would be expected according to Scheme 1. The approximately reversed 13^2-epimer ratios observed for

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allomers **7** and **8** can be rationalized on assuming that the relatively large MeO• radical attacks the C-13^2 of the radical intermediate **6** preferably from the more open backside of the intermediate, thus leading to the predominance of the 13^2(*R*)-configuration for **8**. In contrast, the smaller HO• radical and O_2 diradical presumably prefer to attack the radical intermediate **6** from the front side, which results in the predominance of the 13^2(*S*) configuration in the final product **7**. It is noteworthy that, if the attacking species is O_2, also the intermediates **9**, **10**, and **11** have predominantly the 13^2(*S*) configuration (pathway 2). It seems reasonable to assume that steric hindrance is involved in the stereochemical control, but not alone. Probably also the electronic effects of the macrocyclic π-system contribute to the stereochemical control of the allomerization reaction in some so far unidentified fashion.

Only a few investigations dealing with the allomerization of BChls can be found in the literature.[25,30−34] 13^2-(*S*/*R*)-HO−BChl *a* is reported in these investigations as the major methanolic allomer of BChl *a* (**3**). Woolley et al.[25] observed that BChl *a* is easily dehydrogenated by triplet oxygen into 3-devinyl-3-acetyl-Chl *a* (also called bacterioviridin, BVir), because the hydrogen atoms at the positions C-7 and C-8 are weakly bonded. They reported the 15^1-MeO-lactone derivative of BVir as the major allomer, while the 15^1-HO-lactone derivative of BVir and 13^2-HO−BVir were minor products, (the stereochemical configurations at C-15^1 or C-13^2 were not specified). The formation of these allomers conforms to the reactions of Scheme 1. Woolley et al.[25] also concluded that the nature of the C-7−C-8 bonding influences the conformation of the macrocycle and through it the reactivity of the isocyclic ring E in the allomerization. Nevertheless, studies on the allomerization of BChl *b* (**4**) seem highly desirable as well, to see clearly how the structural features inherent in sub-rings A and B influence the allomerization of the bacteriochlorophylls as compared with that of Chl *a*.

Experimental Section

Preparation and Purity of Chl *a* (1). Chl *a* was isolated from clover leaves by the method described previously,[35] but since then modified for large-scale preparation.[36] The purity of the of the Chl *a* was analyzed by UV−vis spectroscopy,[37] ^{1}H NMR,[37] TLC on sucrose,[22] and normal-phase HPLC.[24] The spectroscopic properties of the preparation were identical with those described previously.[37] The ^{1}H NMR spectrum showed that the only impurity was water (present in a ratio smaller than 1:1). The sucrose TLC with fluorescence detection under UV light (λ = 366 nm) and the NP-HPLC revealed the precence of trace amounts (<1%) of Chl *a*′ [13^2(*S*)-epimer of Chl *a*] and pheophytin *a* (Mg-free Chl *a*).

Preparation of Chl *a* Allomers. The ^{16}O-containing Chl *a* allomers were prepared and purified as described previously.[24,26] The $^{18,18}O_2$-allomerization experiment was carried out according to the following procedure. A 150 mL volume of methanol (dried over 3 Å molecular sieves) and a bar magnet were placed into a 250 mL round-bottomed, one-necked flask, and oxygen was thoroughly removed from the system by using an oil-pump and the freezing-thawing technique. In a glovebox, filled with argon gas, 300 mg of pure Chl *a* was added into the flask, which was then connected by means of a ca. 50 cm long PVC-tubing to the opened, outer extension tube of a glass ampule, containing $^{18,18}O_2$ gas (99 atom % O-18, 100 mL; the tip of the outer extension tube of the ampule was cautiously cut open, so that the melted tip of the inner glass capillary remained intact). The oxygen was released from the ampule by dropping a bar magnet, placed beforehand into the PVC-tubing, onto the melted tip of the glass capillary inside the outer extension tube. The solution in the flask was continuously stirred magnetically at room temperature, keeping a slight overpressure for argon inside the glovebox during the whole reaction. After 7 days, the reaction mixture was evaporated to dryness. The ^{16}O-containing and ^{18}O-labeled Chl *a* allomers in the product mixture were pre-fractionated by column chromatography (CC) on sucrose and more thoroughly purified by normal-phase, high-pressure liquid chromatography (NP-HPLC) as described previously for the ^{16}O-containing allomers.[24,26]

Mass Spectrometry. The mass spectra (MS) were measured on a Mariner time-of-flight (TOF) mass spectrometer using the positive mode electrospray ionization (ESI). The spray tip potential was set at 4006 V and the nozzle potential at 100 or 300 V. The nozzle temperature was 140 °C. The sample, dissolved in acetone (pro anal.) was introduced into the spectrometer by means of a syringe pump operating at 2−5 μL/min. The mass spectra were recorded (3 s/spectrum) between 400 and 1000 mass units, and many spectra were summed together.

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