Chlorophylls. VII. An ^{1}H NMR Spectroscopic Study of the Phase-Test Intermediate of Chlorophyll *a*

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The phase test intermediate of chlorophyll *a* was prepared by addition of methanolic potassium hydroxide to a degassed solution of chlorophyll in pyridine. ^{1}H NMR spectroscopic analysis showed that the intermediate is the enolate anion of chlorophyll *a*. Its high propensity for one-electron oxidation, manifested by its high reactivity with triplet oxygen in the allomerization of chlorophylls, is interpreted in terms of a relatively low oxidation potential presumably caused by the delocalization of the negative charge over the perturbed antiaromatic electron system of the enolate ion. The potential functioning of the enol derivatives of chlorophyll *a* or pheophytin *a* in the reaction centre(s) of photosynthesis is briefly discussed.

Solutions of chlorophylls and chlorophyll derivatives containing an intact isocyclic ring with a methoxycarbonyl group and a hydrogen atom at C-10 (Fig. 1) exhibit a transient reddish brown colour when treated with a strong base. This appearance of a characteristic colour is the basis of the well-known Molisch phase test[1] which has been traditionally used to determine whether or not a chlorophyll sample has become 'allomerized' (oxidized by triplet oxygen). The phase test intermediate (PTI) can be generated in several organic solvents by different bases.[2-6] In a solution containing oxygen, PTI disappears within a few seconds due to the formation of green oxidation products. However, in deoxygenated pyridine or *N,N*-dimethylformamide PTI can be detected for several hours.[4] It is suggested[4] on the basis of visible absorption spectra that the intermediate compound is an ionized diradical formed by separation of the two electrons which are left after the direct acid ionization of the CH group in position 10. This triplet state is stabilized by the possibility of a great number of resonating structures. There is both chemical and electron absorption spectroscopic evidence also for the hypothesis that PTI is formed by the enolization and ionization of the C-9 keto group of ring V resulting in the formation of a double bond between carbon 9 and 10.[7,8] Although it is evident that the chemical properties of the isocyclic ring are responsible for the phase test reaction, there is no definitive proof for any of the structures proposed for PTI in the literature. The question of the chemical nature of PTI is also important because it might be associated with the primary events of the photosynthesis. It

has been suggested that chlorophyll *a* enol has a role in green plant photosynthesis.[9,10] Experimental support for this idea is derived from studies in which Chl *a* *tert*-butyldimethylsilyl enol ether and 9-deoxo-9,10-dehydro-Chl *a* have been used as model compounds for Chl *a* enol.[11] In these studies it was concluded that monomeric Chl *a* enol provides a better model of the primary electron donor of photosystem I (P700) than Chl *a* special pair.

In our opinion, the β-keto ester system is one of the most important functional groups in the naturally occurring chlorophylls. Surprisingly little is, however, known about its functional role in the photosynthesis.[11] The chemical properties of the isocyclic ring of the chlorophylls are thus of considerable interest. This paper presents, for the first time, the ^{1}H NMR spectrum of the phase test intermediate of Chl *a*. On the basis of the information obtained from a careful analysis of the spectrum, the structure of the intermediate could be unequivocally determined.
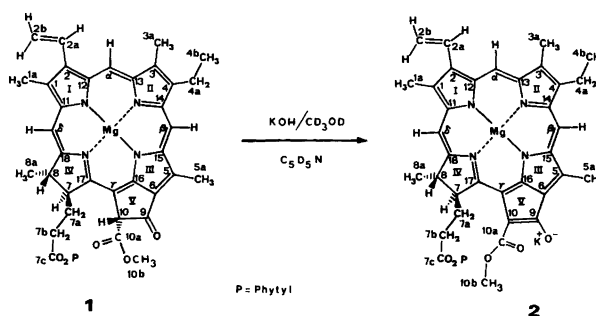

Fig. 1. The formation of PTI of chlorophyll *a*.

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Experimental

The phase test intermediate was prepared by the addition of 5 μl of 10 % (w/v) potassium hydroxide in methanol-d_4 to a pyridine-d_5 solution of chlorophyll a (Chl a, 1, prepared by method of Hynninen[12]). The chlorophyll solution was made in a 5 mm NMR tube. Both the potassium hydroxide and chlorophyll solutions (conc. 10 mg ml^{-1}) were degassed by bubbling argon through the solution for ca. 20 min. After the addition of methanolic potassium hydroxide to the Chl a solution, the red colour of the intermediate appeared immediately and remained for more than 1 h. The ^{1}H NMR spectra were recorded at ambient temperature on a Bruker instrument operating at 250 MHz. Measurement conditions were as follows: pulse width, 5.2 μs; repetition time, 2.0 s; spectral width 2700 Hz; number of data points 16 K.

Results and discussion

The ^{1}H NMR spectrum of PTI together with that of Chl a is shown in Fig. 2. The assigment of the spectrum of Chl a was based on the previous reports.[13–16] The spectrum of PTI resembled that of Chl a except that the C-10 proton signal was absent and that the spread of the spectrum has markedly diminished. This indicates a reduced ring current as compared with Chl a. Assuming that this reduction of ring current is approximately uniform throughout the molecule and taking into account the multiplicity of the signals, the spectrum of PTI could be assigned.

It can be seen from Table 1 that the incremental proton chemical shifts of PTI as compared with Chl a depend, with a few exceptions, on the distance of the proton from the aromatic system. The incremental shifts are largest for the methine bridge protons and smallest for the phytyl side-chain protons for which they are practically not observable at all (except for the P-1 and P-2 protons). The incremental shifts for protons separated by two and three bonds from the macrocycle are between the two extremes. The only substantial opposing incremental chemical shifts arise from the 7-CH, 8-CH$_3$, 10b-CH$_3$ and P-2-CH protons. The fact that none of the resonances from protons α, β and γ to the conjugated system show electron transfer broadening eliminates the diradical structures proposed by Weller[4] for PTI. On the other hand, our NMR results are in accordance with the enolate structure 2. This interpretation is supported by the very similar incremental proton chemical shifts reported for the peripheral magnesium complex of methyl pheophorbide a by Scheer and Katz.[17,18] The peripheral complex, in which the magnesium is chelated to the β-keto ester system of ring V, is structurally analogous to the enolate 2. Also the ^{1}H NMR spectroscopic data obtained by Hynninen *et al.* for the trimethylsilyl ether of the enol of methyl pheophorbide a[19] and by Eschenmoser's group for the cyclopheophorbide enols[20,21] are analogous to the NMR spectroscopic results here described for the phase test intermediate, thus providing further evidence for the enolate structure 2.

The observed incremental chemical shifts (Table 1) can be rationalized as follows. The movement of most signals to higher field (upfield shifts) may be accounted for by two parallel effects: (1) electron density is transferred from ring V to the macrocycle, implying increased screening; (2) aromaticity is decreased, i.e., ring current is reduced. It is difficult to decide which of these effects contributes more strongly to the upfield shifts. The opposite incremental shifts, observed for the 7-CH, 8-CH$_3$, 10b-CH$_3$ and P-2-CH protons, may be interpreted as arising from conformational

Table 1. ^{1}H chemical shifts (δ, in pyridine, ppm, relative to SiMe$_4$) for Chl a and for its phase test intermediate.

Proton (IUPAC-IUB notation[23])	Proton (Fischer notation[22])	Chl a	Intermediate	Δδ[a]	Proton (IUPAC)	Proton (Fischer)	Chl a	Intermediate	Δδ
10	β	9.85	8.79	−1.06	12^1	5a	3.62	3.20	−0.42
5	α	9.70	8.78	−0.92	2^1	1a	3.28	3.02	−0.26
20	δ	8.52	7.68	−0.84	7^1	3a	3.18	2.87	−0.31
3^1	2a H$_x$	8.23	7.94	−0.29	17^1	7a−	{2.78−	{2.42−	{−0.36−
13^2	10	6.64	−	−	17^2	7b	{2.07	{2.17	{ 0.10
3^2	2b{H$_b$	6.27	6.06	−0.21	P-4	P-4	1.86	1.94	0.08
3^2	{H$_a$	5.96	5.75	−0.21	8^2	4b	1.56	1.41	−0.15
P-2	P-2	5.36	5.68	0.32	P-3^1	P-3a	1.56	1.58	0.02
P-1	P-1	4.67	4.39	−0.28	18^1	8a	1.50	1.71	0.21
18	8	4.43	3.75	−0.68	P-CH$_2$'s		{1.34−	{1.45−	{0.11−
17	7	4.29	4.87	0.58			{0.92	{0.93	{0.01
13^4	10b	3.72	3.82	0.10	P-CH$_3$'s		{0.80−	{0.81−	{0.01−
8^1	4a	3.67	3.34	−0.33			{0.73	{0.76	{0.03

[a] δinterm − δchl a.
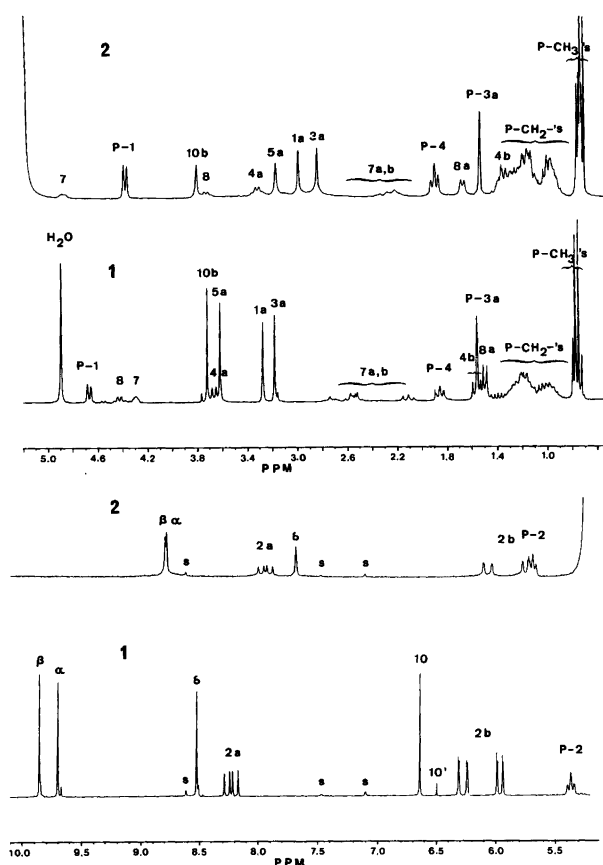
Fig. 2. The ¹H NMR spectra of chlorophyll a (1) and its PTI (2).

of the electron density is expected to lower further the oxidation potential ($E_{1/2}$ for the Chl enolate ion has been estimated to be ca. 400 mV[24]) compared with that of the trialkylsilyl enol ether ($E_{1/2}$ = 500 mV[11]). The high propensity of the Chl enolate ion for one-electron oxidation makes this derivative an attractive model compound for photosynthetic reaction centre(s). It should be noted that the functioning of the Chl special pair as a primary electron donor,[25,26] does not make the low-energy monomeric Chl derivatives such as the Chl a or pheophytin a enol derivatives, unnecessary in the reaction centre(s).

The delocalization (conjugation) of the Chl enolate ion negative charge over the perturbed π-system also explains the red colour of the phase test intermediate.[27] In this context, it is interesting that the Chl enol derivatives (trialkylsilyl enol ether or the peripheral Mg-chelate of pheophytin a) where such a charge delocalization is not possible, exhibit a green or yellow–green colour. This colour difference corresponds to some distinct differences (e.g. the right half of the split Soret band shifts to the red by ca. 100 nm) in the electronic absorption spectra of these enol derivatives.[24]

changes occurring in the periphery of the molecule on the enolization. The formation of new double bond between C-9 and C-10 imposes a planarity demand for ring V and the 10-methoxycarbonyl substituent. This trend to planarity is opposed by the increase of steric crowding (strain) between rings V and IV and their peripheral substituents.

On the basis of the ¹H NMR spectroscopic data it can be concluded that the phase test intermediate, prepared by the addition of methanolic potassium hydroxide to a degassed pyridine solution of Chl a, is the enolate anion of Chl a. As none of the signals in the spectrum of PTI exhibits excessive broadening, the diradical structures proposed by Weller[4] can be definitely excluded at least in the ground state of the molecule. Consequently, the high reativity of the intermediate with triplet oxygen (a diradical) cannot be accounted for, as did Weller,[4] by assuming a diradical electron structure for the substrate. A more likely explanation for the high oxygen reactivity is based on a lowered oxidation potential ($E_{1/2}$) of the Chl enolate ion as compared with that of original Chl a ($E_{1/2}$ = 860 mV[11]). The Chl enolate ion possesses a perturbed π-system, which is antiaromatic (Fig. 1, structure 2). The negative charge of the Chl enolate is delocalized over the perturbed π-system thus increasing its electron density. This is in the line with the observed upfield incremental chemical shifts. The increase

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