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Structure of the Krasnovskii Photoreduction Product of Chlorophyll *a*

(pulse Fourier transform proton magnetic resonance/dihydrochlorophyll)

HUGO SCHEER* AND JOSEPH J. KATZ

Chemistry Division, Argonne National Laboratory, Argonne, Illinois 60439

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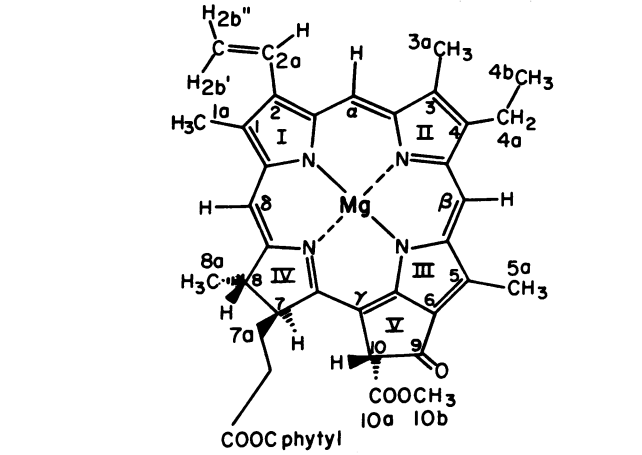
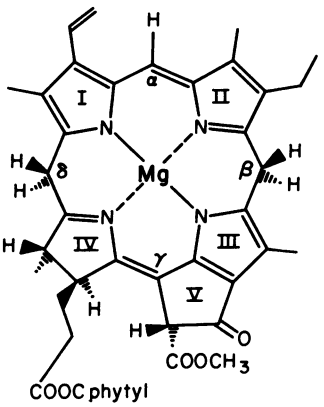
ABSTRACT **A proton magnetic resonance study at 220 MHz shows the Krasnovskii intermediate in the photoreduction of chlorophyll *a* by hydrogen sulfide to be β,δ-dihydrochlorophyll *a*.**

In 1948, Krasnovskii discovered that chlorophyll *a* (Chl *a*) (structure I) dissolved in pyridine can be reversibly reduced in light by ascorbic acid to a pink photoproduct (λ_{max} about 525 nm), which in the dark reverts to Chl *a* (1). In a long series of studies (2), Krasnovskii and his coworkers showed that photoreduction requires a base such as pyridine, imidazole, histidine, ammonia, or piperidine, and that compounds such as cysteine, hydrogen sulfide, dihydroxy maleic acid, or phenylhydrazine can be used as reductants. The reversible nature of the Krasnovskii light-induced chlorophyll oxidation–reduction has become the basis of an extensive literature (3–5) because of a possible role for photooxidation or reduction intermediates of chlorophyll in the light conversion step in photosynthesis. Despite the importance this famous reaction has acquired in the study of the photochemical conversions of chlorophyll, the chemistry of the reaction has remained obscure. On the basis of optical studies, it was early presumed (2, 3), that the photoreduction product was a dihydrochlorophyll *a*, but optical observations, even when aided by electrochemical and electron spin resonance studies, did not furnish structural information on the reaction intermediates. The application of nuclear magnetic resonance methods, which generally yield very detailed structural information, was for a long time restricted by the low sensitivity of conventional procedures to fairly stable chemical species in moderately concentrated solutions. Because the chlorophylls are strongly colored, the use of the concentrated solutions required to facilitate acquisition of proton magnetic resonance (^{1}HMR) data made it impossible to introduce enough light into the system to effect complete reaction. The great increase in sensitivity in recording ^{1}HMR spectra made possible by pulse Fourier transform (PFT) spectroscopy (6) permits the investigation of reaction intermediates with half-lives of several minutes in Chl *a* solutions dilute enough to be easily accessible to photochemistry (<1 mM). We have thus been able to study the Krasnovskii photoreduction of Chl *a* directly in the nuclear magnetic resonance sample tube by ^{1}HMR PFT spectroscopy at 220 MHz. In our experiments, the reaction was carried out in pyridine solution with ^1H_2S or ^2H_2S as reducing agents. Hydrogen sulfide has the considerable advantage that it possesses a simple ^{1}HMR spectra and, thus, does not obscure the chlorophyll ^{1}HMR spectrum. ^2H_2S is also readily obtained commercially (Merck, Canada). When ^2H_2S is used as reductant, the ^{1}HMR spectra are even further simplified, but proton exchange reactions between Chl *a* and ^2H_2S must then be taken into account. In the event, the exchange with ^2H_2S is found to aid the interpretation of



Chlorophyll *a* I



β, δ-Dihydrochlorophyll *a* II

Abbreviations: Chl *a*, chlorophyll *a*; ^{1}HMR, proton magnetic resonance; PFT, pulse Fourier transform; ^2H_2S, deuterio-hydrogen sulfide.

* DFG-Stipendiate, permanent address: Gesellschaft fuer Molekularbiologische Forschung, D3301, Stockheim, West Germany.

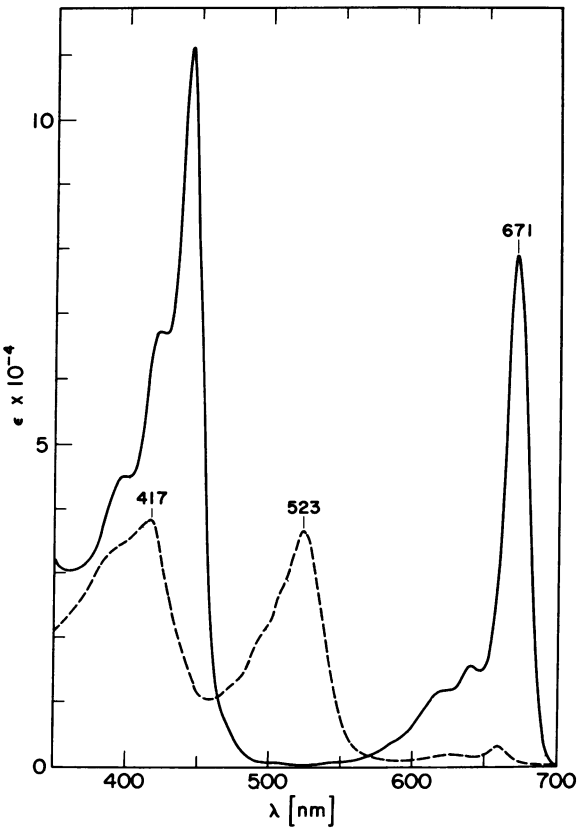


FIG. 1. Visible (electron excitation) spectra of chlorophyll *a* and β,δ-dihydrochlorophyll *a*. Concentration = 6×10^{-5} M in pyridine–H_2S.

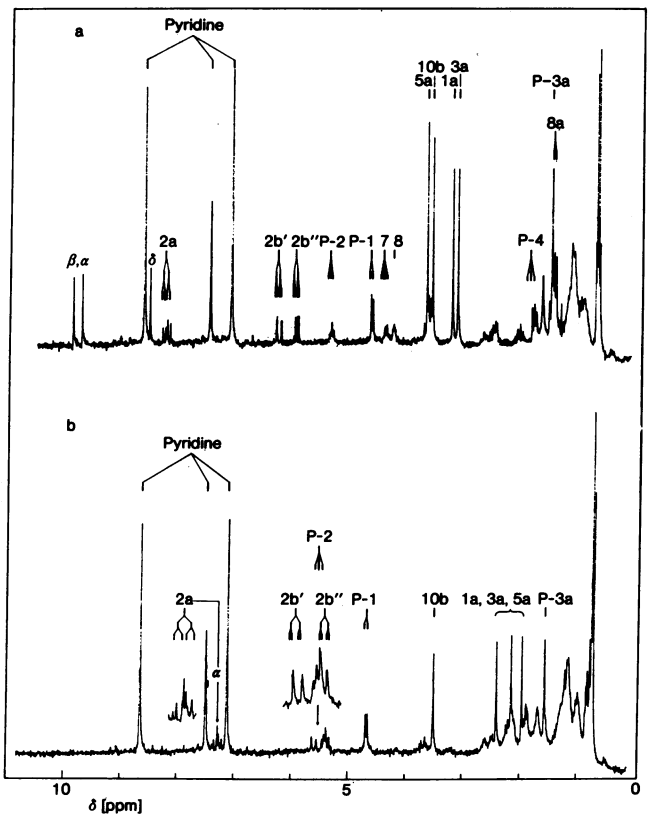


FIG. 2. PFT ^{1}HMR spectra (220 MHz) of: (*a*) chlorophyll *a*, 2×10^{-3} M in pyridine-$^2H_5$/^2H_2S; (*b*) β,δ-dihydrochlorophyll *a*, obtained from solution (*a*) after 10 min of light irradiation. 500 pulses, spectral width 2500 Hz, collected in 8192 channels.

the ^{1}HMR spectrum of the photoreduction product. The only product of the Krasnovskii reduction that is observed by ^{1}HMR in our experiments was β,δ-dihydrochlorophyll *a* (structure II).

EXPERIMENTAL METHODS

Ten milliters of H_2S gas (Matheson) were flushed from a syringe through 0.5 ml of pyridine-$^2H_5$ (Stohler). The H_2S–pyridine solution was then thoroughly deaerated by repeated freeze–thaw cycles on the vacuum line; the mixture was then distilled into an nuclear magnetic resonance sample tube containing about 1 mg of Chl *a*, and sealed off at a final vapor pressure of 10^{-5} torr at 77°K. All reactions described here were carried out in systems thoroughly free of air, although the general reaction course does not seem to be markedly affected by small amounts of either oxygen or water. In agreement with Zieger and Witt (7), we find the reduction rate is slowed by the presence of oxygen, and that irreversible side-reactions become more prominent when oxygen is present. The sealed sample was irradiated at 0°C with white light from a 500 W xenon lamp (Varian/Eimac 500×10S), filtered through 10 cm of water to remove infrared radiation. After 5–10 min of irradiation the reaction mixture lost its characteristic fluorescence and turned pink (Fig. 1). The first ^{1}HMR spectrum was taken immediately after the reaction was finished, and the rate of the reverse dark reaction, the regeneration of the Chl *a*, was followed by subsequent ^{1}HMR spectra recorded from time to time over a period of hours. Chemical shifts are given in δ, ppm, downfield from hexamethyl disiloxane (HMS).

RESULTS AND DISCUSSION

Fig. 2 compares the ^{1}HMR spectra of Chl *a* (see structure I for proton numbering) and the photoreduction product obtained upon reduction with ^2H_2S. It is immediately apparent that photoreduction of Chl *a* results in the loss of the ring current that so markedly affects the ^{1}HMR spectrum of Chl *a* (8). In the ^{1}HMR spectrum of the photoreduction product, most of the lower field resonances are shifted to fields substantially higher than those observed in Chl *a* itself, but the signals originating in the protons of the phytyl moiety remain essentially the same in both compounds. The upfield shifts in the photoproduct are most pronounced in the resonances of protons closest to the tetrapyrrole macrocycle. Thus, in the vinyl group the proton 2a, closest to the macrocycle, experiences a diamagnetic shift of 0.97 ppm and moves upfield to 7.22 ppm, while the more remote 2b″ proton is shifted upfield by 0.69 ppm to 5.55 ppm, and the 2b′ proton resonance is only shifted upfield by 0.61 ppm to 5.32 ppm. Three of the low-field methyl groups in the photoreduction product are likewise shifted upfield by 1.0–1.7 ppm and are now found at 2.40, 2.15, and 1.96 ppm. The chemical shift of the protons in the fourth methyl group on the photoreduction product remains at nearly the same value of 3.48 ppm that it possesses in Chl *a*, and this peak was, therefore, assigned to the protons of the 10b methyl group, which is expected to be only slightly dependent on the macrocyclic ring current. The C-10 proton is shifted in the photoreduction product relative to the C-10 proton of Chl *a* by 1.83 ppm and moves upfield to 4.78 ppm

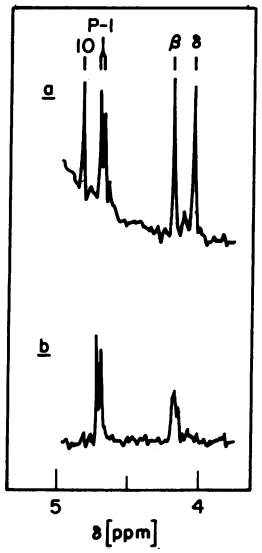


FIG. 3. PFT ^{1}HMR spectra (220 MHz) of β,δ-dihydrochlorophyll a (2×10^{-3} M in pyridine-$^2H_5/H_2S$). Region of the β and δ methylene and the 10 proton signals. (a) Reduced with H_2S; (b) reduced with ^2H_2S after 3.5 min of light irradiation. 500 pulses, spectral width 2500 Hz, collected in 8192 channels.

(Fig. 3a). The methine protons show even stronger high-field shifts, and the resonances due to these protons are now found at 7.42, 4.14, and 4.01 ppm (Fig. 3a). The integrated area of the first of these resonances corresponds to one proton, whereas, the latter two peaks correspond in area to two protons each. Evidently, two of the bridge positions are hydrogenated in the photoreduction product. The assignment of these three signals to the three methine bridge positions, and hence the sites of hydrogenation, was established by experiments in which the photoreduction was effected by ^2H_2S. The results of these experiments are summarized in Table 1 and Figs. 2b and 3. In the photoreduction product obtained with ^2H_2S, the singlet at 7.42 is unchanged relative to the product obtained with ^1H_2S, while the peak at 4.14 ppm is now split into a multiplet by spin–spin interactions with geminal ^2H that has been introduced from the ^2H_2S. Depending on the irradiation time and the detailed history of the sample, exchange of deuterium (^2H) for the methine proton originally present occurs to a variable extent. The intensity of the peak at 4.14 ppm varies between 1 and 0.20 of one proton, while the peak at 4.01 ppm practically disappears. It is known that the δ-proton in chlorophyll is rapidly exchangeable (9), and the resonance at 4.01 ppm was, therefore, tentatively assigned to the proton at the δ-position. All three signals, however, can be related directly to those of the three methine protons in the regenerated Chl a by their intensity pattern. On the basis of the experiments with ^2H_2S, the signal in the photoreduction product at 4.01 ppm is indeed assigned to the δ-methylene protons, the resonance at 4.14 ppm to the β-methylene protons, and the signal at 7.42 ppm to the α-methine proton. The chemical shift values for both the methine and methylene protons fall in the ranges characteristic for di- and tetrapyrrolic compounds in which an aromatic macrocycle is lacking. The chemical shift value of 7.42 ppm assigned to the α-methine proton corresponds satisfactorily to the methine resonances in zinc porphodimethenes obtained by reductive methylation of zinc porphyrins [7.05 for α,γ-dimethyl-α,γ-dihydro-octaethyl-porphyrin-zinc in $C_6{}^2H_6$ (10), 6.8 for the corresponding octamethyl-porphyrin in C^2HCl_3 (11)]. The resonances near 4 ppm are likewise similar in chemical shift to the methylene proton resonances in these compounds (4.32 and 4.22 ppm, respectively). On the basis of the chemical shift assignments of the protons in the photoreduction product, we therefore assign the structure II, β,δ-dihydrochlorophyll a, to the Krasnovskii photoreduction product of Chl a.

TABLE 1. *Chemical shifts (δ, ppm) of selected protons in chlorophyll α, β,δ-dihydrochlorophyll a, and regenerated chlorophyll a after dark reoxidation**

	α-H	β-H	δ-H	10-H
Chlorophyll a, starting solution				
H_2S	9.66	9.81	8.48	6.61
^2H_2S	9.66	9.82	8.48	—
β,δ-Dihydrochlorophyll a				
H_2S	7.42	4.14†	4.01†	4.78
^2H_2S (3.5 min of light)	7.42	4.13‡	—	—
^2H_2S (10 min of light)	7.42	4.12§	—	—
Chlorophyll a, dark regenerated				
H_2S	9.66	9.82	8.48	6.61
^2H_2S (3.5 min of light)	9.66	9.82¶	—	—
^2H_2S (10 min of light)	9.66	—	—	—

* Concentration = 2×10^{-3} M in pyridine-$^2H_5/H_2S$ or pyridine-$^2H_5/^2H_2S$.
† Singlet for two protons.
‡ Unsymmetrical triplet for 1 proton.
§ Multiplet, about 1/10 proton.
¶ Singlet, $^1/_2$ proton.

None of the ^{1}HMR spectra of β,δ-dihydrochlorophyll a showed additional or satellite peaks such as would originate in byproducts or possible isomers such as α,γ-dihydrochorophyll a. Thus, the photoreduction is not only selective for the two opposite methine bridge positions, but is regio-selective to at least 90% at the β and δ positions.

Products in which opposite methine positions are reduced are formed in the chemical (10) and photochemical (11–13) reduction of metalloporphyrins and phlorins (14). Reductive methylation of various octaethylporphyrin metal complexes yields α,γ-dihydro-α,γ-dimethyl porphyrins (10). Recently, one of the isomers obtained by Krasnovskii reduction of zinc-α,γ-dimethyloctamethylporphyrin was isolated and characterized by ^{1}HMR as the α,γ-dihydroproduct (11). Reduction at opposite sites is also probable for the photoreduction of zinc pheoporphyrins with ascorbic acid (12), and there is spectroscopic evidence that similar products are formed during the reaction of tin(IV) octaethylporphyrin with tin(II) (13). Opposite methine positions, therefore, appear to be principal points of attack in metalloporphyrins, although other reduction pathways are known (15–17) and subsequent isomerizations are not infrequently observed. In the two cases studied so far in which nonequivalent methine bridges are present, both isomers are formed (11, 12). However, in the photoreduction of pheoporphyrins (which contain the isocyclic Ring V characteristic of Chl a), formation of the β,δ-dihydroisomer is preferred (12), and the tendency for reduction by this pathway is even stronger in the photoreduction of Chl a.

Prolonged irradiation results in irreversible bleaching, and after long standing of the reaction mixture in the dark, irreversible side-reaction products are observed. The most promi-

nent byproduct in this case has a blue-shifted visible absorption spectrum (λ_{max} = 660 and 437 compared with 671 and 444 nm for Chl *a* in pyridine). The signals characteristic of the vinyl protons are missing in this substance, and the methine resonances are shifted to higher fields. A similar behavior is observed upon hydration of the vinyl group at position 2 to an hydroxy-ethyl group in pheoporphyrins (18); this irreversible reduction product is probably 2-desvinyl-2-mercaptoethyl chlorophyll *a*.

The Krasnovskii reduction product β,δ-dihydrochlorophyll *a* can be isolated in solid form by evaporation of the photoreduced reaction mixture to dryness on the vacuum line. This substance is exceedingly sensitive to oxidants such as O_2, I_2, or quinones, and even blowing the solvent off with high purity N_2 gas leads to complete reoxidation by the very small amounts of residual oxygen in the nitrogen gas. The yield of Chl *a* regenerated in this way, is quantitative, and the ^{1}HMR and visible absorption spectra of the product are indistinguishable (in terms of chemical shift) from the starting Chl *a*. As followed by ^{1}HMR, Chl *a* is regenerated in the dark at 8°C in a slow reaction with $\tau_{1/2}$ about 5 hr; at 48°C, the regeneration is faster, with $\tau_{1/2} \approx 1$ hr. It should be pointed out that the ^{1}HMR spectra of dark-regenerated Chl *a* generally have lower intensity than those of the original Chl *a* when the reaction is carried out with ^2H_2S. As the intensity of the residual ^{1}H in the solvent pyridine-$^2H_5$ is also reduced, the loss of intensity following reaction is probably to be attributed to exchange of ^{1}H with the reductant ^2H_2S. However, the possibility of side-reactions in which some of the starting Chl *a* is fragmented to such small entities that their ^{1}HMR spectra are lost in the noise, while unlikely, cannot be entirely dismissed. Experiments with [^{13}C]Chl *a* (19) should settle the role of exchange.

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