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TRANSVERSE MOTION OF CHLOROPHYLL DERIVATIVES IN PHOSPHOLIPID BILAYERS

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Summary

Chlorophyll derivatives were synthesized with spin labels attached to the porphyrin ring. These labels were incorporated into egg phosphatidylcholine vesicles in order to estimate the transbilayer motion (flip-flop) of this class of photosynthetic pigments. Using the ascorbate reduction method, the upper limit to the spin label half-life is $\tau_{1/2} \sim 4$ min at 0°C. The flip-flop rate is rapid compared to that of a phospholipid spin label under the same conditions. The presence or absence of magnesium in the center of the porphyrin ring had no measurable effect on the flip-flop rate.

Introduction

The primary events in photosynthesis involve the specialized pigments found in photosynthetic membranes (the chlorophylls *a* and *b*, the various bacteriochlorophylls, carotenoids, and related pigments). There is considerable evidence that the chlorophylls are associated with protein in both photosynthetic bacteria and in higher plants [1,2]. A number of different chlorophyll-binding proteins have been reported, but it is not yet well understood how the photosynthetic units are assembled in the membranes. The biosynthesis of chlorophyll is a stepwise process that occurs in the cytoplasm of photosynthetic bacteria (stroma of chloroplasts) up to the formation of protoporphyrin IX

Abbreviation: Me_4Si, tetramethylsilone.

or its precursor, followed by several steps that are thought to involve membrane-bound enzymes [3,4]. Knowledge of the rate at which chlorophyll and its precursors can diffuse from one side of the bilayer to the other (flip-flop) is of interest in understanding the assembly process and the dynamics of photosynthetic membranes. Although there have been numerous studies of phospholipid transverse motion [5–7], no data have been published on the flip-flop rates of chlorophyll or chlorophyll derivatives. In this study, we report measurements of the flip-flop rate of two spin-labeled chlorophyll derivatives in phospholipid vesicles. For this study, chlorophyll *b* was chosen because the aldehyde group on ring II provided a convenient functional group for the attachment of a nitroxide moiety.

Materials and Methods

Dipalmitoyl phosphatidyltempocholine was synthesized by using the method of Kornberg and McConnell [5]. Tempo palmitate (4-hexadecanoyloxy-2,2,6,6-tetramethylpiperidine-1-oxyl) was purchased from Molecular Probes, Inc. (Plano, TX). NMR spectra were recorded on a Varian XL-100 high-resolution spectrometer in C^2HCl_3. Chemical shifts are reported in parts per million (δ) downfield from internal $(CH_3)_4Si$. 9.5 GHz ESR spectra were recorded on a Varian E-line ESR spectrometer interfaced with a Varian 620L/100 computer. The number of spins per molecule was determined for the chlorophyll labels by comparing doubly integrated ESR spectra of the spin labels ($2 \cdot 10^{-4}$ M in $CHCl_3$) with doubly integrated spectra of analytically pure 1-oxy-4-carboxy-2,2,6,6-tetramethylpiperidine, also $2 \cdot 10^{-4}$ M in $CHCl_3$. All preparative work was carried out in subdued light.

Pheophytin b spin label synthesis

Chlorophyll *b* was isolated from spinach leaves by using the method of Strain and Svec [8]. The magnesium was removed by briefly shaking an ether solution of chlorophyll *b* with 6 M HCl [9]. The resulting pheophytin *b* was purified by sucrose column chromatography eluting with 0.5% *n*-propyl alcohol in petroleum ether. The aldehyde group attached to ring II of pheophytin *b* was reduced to the corresponding alcohol in the following manner: to a solution of 13.8 mg (16 μmol) of pheophytin *b* in 150 μl dry CH_3OH and 150 μl CH_2Cl_2 (passed over activity I alumina) were added 27 mg (430 μmol) sodium cyanoborohydride. After a 48 h stirring period at 25°C under N_2, the crude product was subjected to chromatography on a 1 mm preparative TLC plate (Analtech, silica gel GF_{254}, 3 × 10 cm) (ethyl acetate/cyclohexane (1 : 1) eluant) to yield 8.8 mg (62%) of the pure alcohol : EM Reagents silica gel 60 F-254 TLC (ethyl acetate/cyclohexane (1 : 1), R_f = 0.38; for pheophytin *b* in the same solvent system, R_f = 0.57). The NMR spectrum showed the loss of the δ 11.2 peak (aldehyde proton [10]) and the appearance of a new peak at δ 5.75, due to the methylene protons of the $-CH_2OH$ group.

The pheophytin *b* alcohol was then coupled to a six-membered ring nitroxide carboxylic acid using carbonyl diimidazole [11]. 16.1 mg (80 μmol) of dry 1-oxy-4-carboxy-2,2,6,6-tetramethylpiperidine (prepared by using the method of Raukman et al. [12]) and 15.7 mg (97 μmol) carbonyl diimidazole

were placed in a dry vial and dissolved in 400 μl of dry $CHCl_3$ under N_2. After a 1 h stirring period at 20°C, 20 μl of the red solution of the resulting acyl imidazolide were transferred to a vial containing 3.8 mg (4.3 μmol) of the alcohol derived from pheophytin *b*. Dry $CHCl_3$ (10 μl) was added, and the resulting solution was kept at 50°C for 2 h. Column chromatography of the crude product on powdered sucrose (2 × 50 cm column) eluting with petroleum ether containing 0.5% *n*-propyl alcohol yielded 1.7 mg (45%) of spin-labeled pheophytin *b*. On silica gel TLC (ethyl acetate/cyclohexane (1 : 1) eluant), one major component (R_f = 0.55) was evident; faint traces of impurities at lower R_f values were also visible. The broadened NMR spectrum showed no peak at δ 5.75 due to methylene protons of the -CH_2OH group of the alcohol starting material. This is evidence for the attachment of the nitroxide at the alcohol position, since acylation would normally be expected to shift these protons about 0.5—1.0 ppm to lower field. That the protons are not seen at all in the lower spectrum is consistent with the known broadening of nearby protons by a nitroxide group [13]. For the spin labeled pheophytin *b*, λ_{max} (ether) = 658 nm and ϵ = 28,200 $M^{-1} \cdot cm^{-1}$; unpaired spins per molecule, 1.0 ± 0.1.

Chlorophyll spin label synthesis

Because of the lower stability of magnesium-containing chlorophyll derivatives, reaction conditions were somewhat different for these compounds. 13.1 mg (14 μmol) chlorophyll *b* (dry) were dissolved in 300 μl dry $CHCl_3$ plus 200 μl absolute ethanol under N_2, and 32 μl (16 μmol) of 0.5 M $NaBH_4$ in ethanol were added. After a 2.5 h stirring period at 20°C, the solution was diluted with 3 ml of $CHCl_3$. The organic layer was washed three times with 1–2 ml of 0.1 M phosphate, pH 7.6, dried (Na_2SO_4) and rotary-evaporated to a green residue. The product was purified by sucrose column chromatography eluting with 0.5% *n*-propyl alcohol in petroleum ether to yield 9.5 mg (73%) of chlorophyll *b* alcohol. For TLC analysis (silica gel), about 0.1 mg chlorophyll *b* alcohol was converted to the magnesium-free analogue by treatment of a hexane/$CHCl_3$ solution with 6 M HCl. Elution with ethyl acetate/cyclohexane (1 : 1) gave a single spot, R_f = 0.4, which corresponded to the above pheophytin *b*-derived alcohol.

The nitroxide acid was coupled to the above chlorophyll *b*-derived alcohol as follows: 3.55 mg (17 μmol) 4-carboxy-2,2,5,5-tetramethylpiperidine-1-oxyl and 8.4 mg (41 μmol) of distilled dicyclohexylcarbodiimide were added to 200 μl of freshly distilled CH_2Cl_2 under N_2 and stirred for 10 min. After dilution with 100 μl of dry CH_2Cl_2, 175 μl of the resulting solution were transferred into a solution of 4.5 mg (5 μmol) of chlorophyll *b* alcohol in 100 μl of dry CH_2Cl_2. The mixture was stirred under N_2 at 25°C while 4-dimethylaminopyridine (3 μl of 0.4 M solution in CH_2Cl_2) was added. Three additional 3-μl portions of the 4-dimethylaminopyridine solution were added at 10-min intervals (total added, 5 μmol). The mixture was stirred for 1.5 h and then washed three times with 2 ml of 0.1 M phosphate, pH 7.6, and dried over Na_2SO_4. The solvent was removed by rotary evaporation and the residue was purified by sucrose column chromatography eluting with 0.5% *n*-propyl alcohol in petroleum ether. A total of 1.9 mg of spin-labeled chlorophyll *b*

was obtained: unpaired spins per molecule, 0.65. After removal of the magnesium from a small sample (less than 0.1 mg) of the chlorophyll spin label as described above, silica gel TLC (ethyl acetate/cyclohexane (1 : 1)) produced a single spot, $R_f = 0.53$, which corresponded to that of spin-labeled pheophytin *b*.

Flip-flop experiments

Single-walled phospholipid vesicles were prepared using a modification of the ethanol-injection technique developed by Batzri and Korn [14]. 5 mg (650 μmol) of egg yolk phosphatidylcholine (prepared by using the method of Singleton et al. [15]) dissolved in ethanol and 6.5 μmol of spin label dissolved in $CHCl_3$ were dried under N_2. The mixture was then dissolved in 160 μl of ethanol and then rapidly injected into 2.2 ml of 50 mM Tris-0.1 M NaCl, pH 8.0, using a Hamilton syringe. The resulting vesicles were dialyzed against 50 mM Tris-0.1 M NaCl, pH 8.0, to remove the ethanol and then centrifuged at 159 000 × *g* for 3 h [16]. The centrifugation step is necessary for the removal of the few percent of multilamellar vesicles that are present in vesicles formed when the ethanol-injection technique is used. The homogeneous clear region above the pellet was removed and concentrated to about 100 μl using a collodian bag apparatus (Schleicher and Schuell, Inc., 25 000 mW cut-off). 20-μl of the spin-labeled vesicles were then placed in a melting-point capillary which was positioned in a quartz ESR tube. The ESR spectra were recorded at 0°C and the peak height of each low-field line was measured as indicated in Fig. 1. 5-μl of ice-cold sodium ascorbate solution (250 mg sodium ascorbate dissolved in 1 ml of 50 mM Tris-0.1 M NaCl, pH 7.1) were added with a cold syringe, the solution mixed by agitating with the syringe needle, and the low-field line was scanned repeatedly as a function of time after ascorbate addition to follow the destruction of the spin label. The initial peak height was scaled to take into account the sample dilution before plotting.

Results and Discussion

The experimental approach used in this study was to synthesize chlorophyll derivatives modified as little as possible from the naturally occuring plant pigment. Derivatives were prepared with and without magnesium in the center of the porphyrin ring. Control experiments were performed on phospholipid and single-chain lipid spin labels to eliminate the possibility of vesicle leakage to ascorbate, aggregation and complications due to multilamellar vesicles. The ESR spectrum of the chlorophyll spin label in egg phosphatidylcholine unilamellar vesicles at 0°C is shown in Fig. 1. The spectral line shape is generally characteristic of *x*-axis anisotropic motion (motion about the N-O bond) [17]. The ESR spectrum of the pheophytin *b* spin label is essentially the same, indicating that the magnesium has little influence on the molecular motions of the chlorophyll. There was no evidence of electron-electron spin exchange or dipolar effects. Thus, there was no detectable aggregation or phase separation of the chlorophyll or pheophytin derivatives under the conditions employed.

A plot of the ESR peak height of the chlorophyll derivatives vs. time is shown in Fig. 2A. The ESR spectrum rapidly decreases in amplitude when

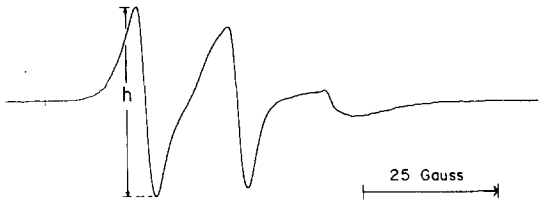

Fig. 1. ESR spectrum of single walled vesicles of egg phosphatidylcholine at 0°C containing 1% of the chlorophyll *b* spin-label derivative.

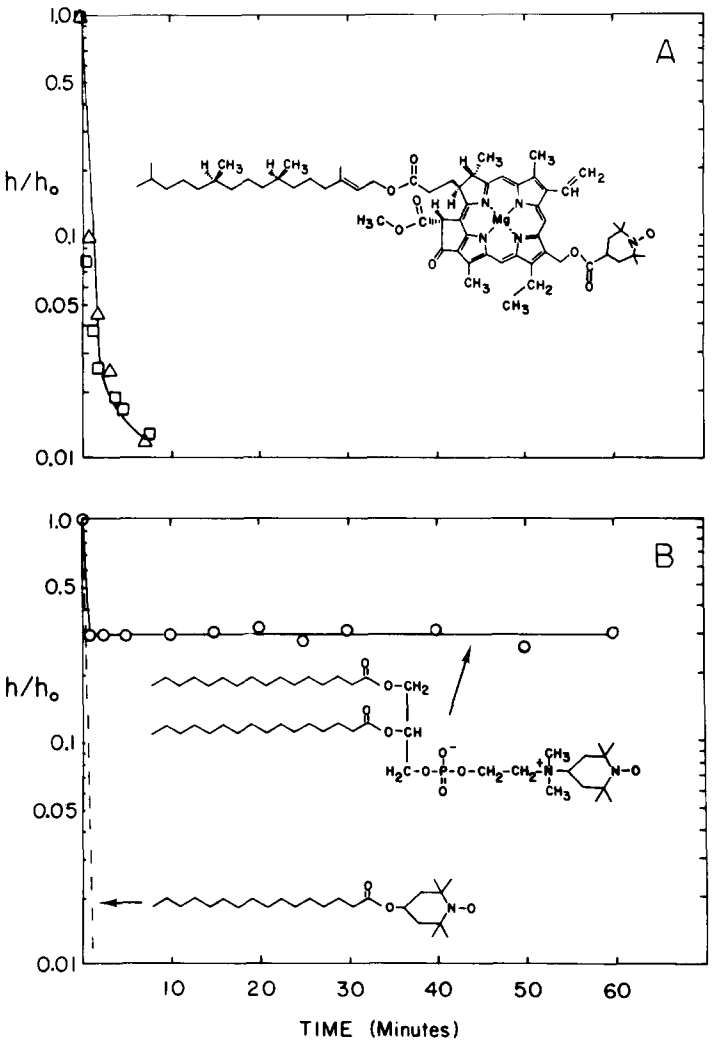

Fig. 2. ESR signal as a function of time in small unilamellar vesicles of egg phosphatidylcholine at 0°C after addition of sodium ascorbate. (A) Signal decay curves for spin labels derived from chlorophyll *b* (△) and pheophytin *b* (□). The structure of the chlorophyll *b* spin label is shown. The structure of the pheophytin *b* spin label is identical except that the magnesium has been replaced by two hydrogens. (B) Signal decay curves for dipalmitoyl phosphatidyltempocholine (○) and tempo palmitate (— — —).

sodium ascorbate is added to the solution at 0°C, indicating that the nitroxide moieties are readily accessible to the reducing agent. The most probable explanation is that the chlorophyll-derived spin labels rapidly flip-flop from one side of the bilayer to the other so that the entire population of labels is exposed to the reducing agent on the outside of the vesicles. There are two other possibilities that must be considered: the chlorophyll spin labels are located only on the outer half of the bilayer or the chlorophyll makes the vesicles leaky to ascorbate. The chlorophyll may well be asymmetrically distributed in the vesicles, but considering the method of preparation, it is highly unlikely that all of the chlorophyll labels are on one side of the membrane. The initial samples are a homogeneous mixture of the phospholipid and chlorophyll in an organic solvent. Destruction of the ESR signal is essentially complete within 10 min, and no significant population of spin label (less than 1%) remains. The second possibility is eliminated by the control experiment discussed below using a phospholipid spin label with added chlorophyll present.

We conclude that the transbilayer motion of the chlorophyll derivatives in model bilayer systems is rapid at 0°C *. From Fig. 2A, the upper limit to the half life is $\tau_{1/2} \sim 4$ min. The plots for the chlorophyll derivatives with and without magnesium are superimposable within experimental error (Fig. 2A). Thus, the presence of the magnesium has little influence on the flip-flop rate. This may not be the case in lipid model systems containing high concentrations of chlorophyll or lipid-protein complexes where the presence of magnesium may affect the extent of aggregation or binding.

For comparison, the corresponding ascorbate reduction curve for a phospholipid spin label in the same vesicle preparation is shown in Fig. 2B. A rapid destruction of the label in the outer half of the bilayers is followed by a flat plateau region, indicating that the remaining spin label population is shielded from the reducing agent. This behavior indicates that the flip-flop rate of the phospholipid spin label is very slow under these conditions ($\tau_{1/2} > 10$ h). This conclusion is consistent with several studies of phospholipid flip-flop rates in the literature [5,19]. The experiment was repeated with pheophytin *b* present in the bilayers (1—5%) and the reduction curve remained essentially the same as in Fig. 2A. This control demonstrates that the presence of a chlorophyll derivative does not make the vesicles leaky to ascorbate.

Also shown in Fig. 2B is the ascorbate reduction curve for an uncharged fatty acid ester label. The ESR signal of this single-tail lipid becomes undetectable in less than 1 min (the earliest time sampled), indicating that there is not a significant population of multilamellar vesicles. When multilamellar vesicles are present, the ESR signal of this spin label decreases rapidly at first but then levels off, reflecting a population of labels inaccessible to the ascorbate. These two controls, the phospholipid and the single-tail ester spin labels, performed in the same system, strengthen our conclusion that the flip-flop

* The flip-flop rates of these chlorophyll derivatives appear to be slower in bacterial photosynthetic membranes (chromatophores [18] isolated from *Rhodopseudomonas sphaeroides*). However, the ESR spectra were difficult to quantitate because of several technical factors (exchange interactions, intrinsic ESR signals of the chromatophores, and some signal destruction by components of the chromatophores).

rates of the chlorophyll derivatives were relatively rapid in fluid phospholipid bilayers.

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