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Protochlorophyllide Reductase III: Synthesis of a Protochlorophyllide–Dihydroflavin Complex

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ABSTRACT

A mild and efficient method of linking a dihydroflavin to the C-17 carboxylic acid side chain of protochlorophyllide, without degradation of the sensitive E ring or loss of magnesium, is described. The appended dihydroflavin was shown to quench the fluorescence of protochlorophyllide. In contrast, a dihydronicotinamide moiety was unable to effect fluorescence quenching. The relevance of these findings to a possible mechanism of action of the enzyme protochlorophyllide reductase is discussed.

INTRODUCTION

Protochlorophyllide reductase is a membrane-associated enzyme required for the photoreduction of the magnesium porphyrin protochlorophyllide **1** to chlorophyllide **2** (Fig. 1). This reaction is the penultimate step on the biosynthetic pathway to chlorophyll (1). It is one of only two characterized light-dependent enzymes, the other being DNA photolyase (2). Dark-grown etiolated plants accumulate protochlorophyllide due to the fact that the enzyme is unable to carry out the reduction to chlorophyllide in the absence of light. The enzyme has a molecular weight of 37 000 and uses NADPH as a cosubstrate (3). The reduction results in the hydrogenation of the C-17, C-18 double bond of protochlorophyllide, the hydrogen atoms originating from NADPH and solvent (4).

The action spectrum of the enzyme closely follows the visible spectrum of protochlorophyllide (5), demonstrating that protochlorophyllide is the primary light-absorbing species. Recent reports have indicated that the enzyme may also utilize a flavin as a cofactor (6). Transient spectroscopy studies on this system have detected several short-lived, presently unidentified intermediates and have implicated the excited singlet state (S_1) of the metalloporphyrin as the first reactive species (7). A plausible mechanism to account for this reaction involves single electron transfer (SET)† from a cofactor to the photoexcited protochlorophyllide to generate the porphyrin π-radical anion **4**. Protonation of this species would give rise to a neutral radical species **5**. A second electron/proton transfer sequence would afford the fully reduced chlorophyllide species **2** (Fig. 2). The short lifetime of a metalloporphyrin S_1 state (1–10 ns) (8) necessitates that the reductant be capable of effecting rapid SET. In an earlier study, we demonstrated that a covalently linked dihydroflavin (FlH_2) is capable of quenching the fluorescence of a zinc methylpyrroporphyrin ester by electron transfer to the porphyrin (9). In marked contrast, a dihydronicotinamide moiety was unable to quench the fluorescence of the zinc porphyrin. Thus a possible mode of action of the enzyme would entail reduction of the flavin cofactor by NADPH followed by reduction of the photoexcited protochlorophyllide by the FlH_2.

Zinc and magnesium octaalkyl porphyrins possess similar ground-state redox potentials. Table 1 compares the redox potentials of a zinc porphyrin, and the cofactor models benzyl nicotinamidehydride (BNAH) and FlH_2. The one-electron reduction of photoexcited zinc porphyrin by FlH_2 is exergonic while that by BNAH is endergonic. Although these arguments suggest that one-electron reduction of a metalloporphyrin by an FlH_2 is thermodynamically facile, they say nothing about the kinetic competence of the reductant. Because the lifetime of the S_1 state of the metalloporphyrin is very short (1–10 ns) the reductant is required to effect electron transfer at a rate that is competitive with the rate of intrinsic decay of the S_1 state. Thus the feasibility of a mechanism involving an SET would depend largely on experimental verification of such an occurrence.

The porphyrin chosen for our earlier studies differs from protochlorophyllide, whose ground-state redox potential is not known, in that it contained zinc instead of magnesium and lacked the isocyclic E ring present in both protochlorophyllide and chlorophyllide. Furthermore, zinc methylpyrroporphyrin and protochlorophyllide differ in their visible spectra and hence in the redox potentials of their excited states. However the greater availability and stability of the zinc porphyrin made it a suitable starting point for our investigations.

In order to demonstrate that FlH_2 is capable of quenching the fluorescence of protochlorophyllide, we needed to synthesize protochlorophyllide with a covalently attached FlH_2 moiety. The C-17 propionate side chain of protochlorophyl-

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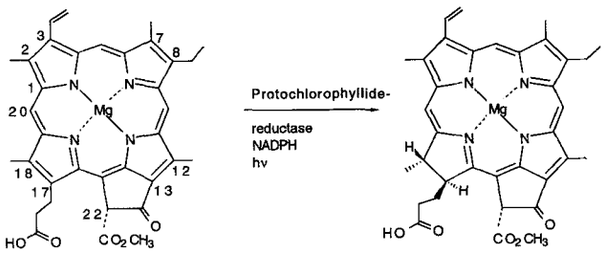
†*Abbreviations:* BNAH, benzyl nicotinamide hydride; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EI, electron impact; FAB-MS, fast atom bombardment-mass spectrometry; FlH_2, dihydroflavin; HPLC, high-pressure liquid chromatography; SET, single electron transfer; TLC, thin-layer chromatography.



Protochlorophyllide **1** Chlorophyllide **2**

Figure 1. Enzymatic photoreduction of protochlorophyllide **1** to chlorophyllide **2**.

lide offered a convenient point of attachment of the flavin, without perturbing the porphyrin skeleton. This paper describes the synthesis of the protochlorophyllide-FlH_2 model system **17**.

MATERIALS AND METHODS

Dimethylformamide (DMF) was freshly distilled from calcium hydride prior to use. 1,3-Dibromopropane, benzyl bromide, nitrosobenzene, 3-picolylchloride hydrochloride, nicotinamide and cesium fluoride were obtained from Aldrich Chemical Co. δ-Aminolevulinic acid hydrochloride was either obtained from Aldrich Chemical Co. or prepared by the method of MacDonald (10) and Benedikt and Köst (11). The NMR spectra were run on a Varian XL-200 or XL-400. Mass spectra were recorded on a Finnegan 3300 mass spectrometer using electron impact (EI) ionization. Fast atom bombardment mass spectra (FAB-MS) were obtained with a Kratos model MS890 instrument in the positive ion mode. Ultraviolet–visible spectra were recorded on a Hewlett Packard model 8451A diode array spectrophotometer. Preparative silica gel chromatography was performed on glass-backed silica gel plates from Merck of 1 and 2 mm thickness. Reverse-phase high-pressure liquid chromatography (HPLC) was carried out on a Waters system using a Supelco C18 semipreparative column. All compounds purified by column chromatography eluted as a single spot, when examined by thin-layer chromatography (TLC). Compounds purified by HPLC were shown to elute as a single peak, when reanalyzed by HPLC. The ^1H NMR analysis of the purified compounds showed no detectable impurities, besides trace amounts of solvent and water.

Steady-state fluorescence measurements were recorded on a Perkin-Elmer model MPF-44B in the energy mode. The solutions of the porphyrins were adjusted to equal absorbance values at the excitation values. Transient differential absorption spectra and lifetime measurements were recorded at the Center for Fast Kinetics Research, University of Texas at Austin. Singlet excited-state lifetimes were measured by the single-photon counting technique. A frequency-doubled, mode-locked Nd-YAG laser (Quantel YG 402) was used as the excitation source (full width at half maximum = 30 ps). A Tracor-Northern TN-6200 multichannel analyzer was used as the recorder. For photon counting a Tracor-Northern TN-7200 multichannel analyzer was used. Single first-order processes were linearized by plotting the natural log of the intensity *versus* time and a weighted least-squares analysis performed on the linearized data.

Protochlorophyllide was isolated from oat seedlings (Astro Oats obtained from Agway) grown on Fafard All Purpose Growing Mix. Oat seedlings were grown in the dark and harvested under a green safelight (Roscolux filters #74 and 75, Syracuse Scenery, 1423 North Salina Street, Syracuse, NY 13208).

Synthesis of 6-benzylaminouracil ***9***. 6-Chlorouracil (12) (**8**) (4 g, 27 mmol), was suspended in 20–30 mL of *n*-butanol and benzyl amine (8.7 g, 81 mmol) was added. The mixture was refluxed for 8–9 h, stored at −20°C for 10 h and filtered. The product, 6-benzylaminouracil (**9**) (4.9 g, 90%), which was obtained as an amorphous powder was washed with cold water and dried. ^1H NMR (DMSO-d^6, 200 MHz) δ: 4.25 (d, J = 5 Hz, 2H, benzylic), 4.35 (s, 1H, C-5), 6.65 (br, t, J = 5 Hz, 1H, N–H), 7.2–7.4 (m, ArH, 5H, aromatic).

Table 1. Relevant thermodynamic parameters for the photoreduction of zinc porphyrins

	Redox potential (E_0) V†	Singlet energy eV‡
Zn(OEP)	$Zn(OEP)/Zn(OEP)^{-\cdot}$ = −1.65§	2.15
BNAH	$BNAH^{+\cdot}$/BNAH = 0.76‖	—
FlH_2	$FlH^{\cdot}/FlH_2$ = −0.16¶	—

$Zn(OEP)^* + BNAH \rightarrow Zn(OEP)^{-\cdot} + BNAH^{+\cdot}$; E^0 = −0.26 V, ΔG^0 = 5.9 kcal/mol

$Zn(OEP)^* + FlH_2 \rightarrow Zn(OEP)^{-\cdot} + FlH^{\cdot} + H^+$; E^0 = 0.67 V, ΔG^0 = −15.4 kcal/mol

†Redox potentials are reported *vs* SCE.
‡Singlet energy value was determined from fluorescence spectroscopy(23).
§Zinc octaethylporphyrin (ZnOEP)(24).
‖Benzyl dihydronicotinamide (BNAH)(25).
¶Dihydroflavin (FlH_2)(26).

Synthesis of N10-benzylflavin ***10***. The compound was synthesized by the procedure of Yoneda et al. (13). 6-Benzylaminouracil (0.5 g, 2.3 mmol) and nitrosobenzene (0.73 g, 7 mmol) in 5 mL of acetic anhydride/acetic acid (4:1) were heated at reflux for 20 min. After cooling, excess methanol was added to destroy the acetic anhydride and the solvent was removed on a rotary evaporator. The crude reaction mixture was preadsorbed on silica and chromatographed using 1% methanol/chloroform to give the flavin **10** (0.11 g, 16%) as a bright-yellow powder. ^1H NMR (DMSO-d^6, 200 MHz) d: 5.9 (br, s, 2H, benzylic), 7.2–7.85 (m, 8H, aromatic), 8.15 (dd, J = 10, 2 Hz, 1H, aromatic). MS (EI): $C_{17}H_{12}N_4O_2$, M^+: 304 (found); 304 (calculated).

Synthesis of N3-(3-bromopropyl)-10-benzylflavin ***11***. Flavin **10** (35 mg, 0.115 mmol) was dissolved in 1 mL of DMF, and 1,3-dibromopropane (0.19 g, 0.98 mmol) and anhydrous potassium carbonate (0.13 g, 0.94 mmol) were added. The mixture was stirred under an atmosphere of argon for 4–6 h. The solution was evaporated to dryness under high vacuum and the residue purified by chromatography on silica (1% methanol/methylene chloride) to afford pure **11** (41 mg, 85%) as a yellow powder. ^1H NMR ($CDCl_3$, 200 MHz) δ: 2.35 (m, 2H, methylene), 3.5 (t, J = 8 Hz, 2H, bromomethylene), 4.25 (t, J = 9 Hz, 2H, N-methylene), 6.0 (br, s, 2H, benzylic), 7.2–7.85 (m, 8H, aromatic), 8.3 (dd, J = 10, 2 Hz, 1H, aromatic).

Isolation of protochlorophyllide. Two square feet of densely grown oat seedlings were germinated under conditions of ambient illumination and then transferred to the dark; 8–9 day-old, dark-grown etiolated seedlings were harvested and stacked upright in a crystallization dish containing 500 mL of 10 m*M* aminolevulinic acid in 2 m*M* phosphate buffer (pH 6.8). A gentle current of air was blown over the seedlings to facilitate uptake of aminolevulinic acid. All procedures were carried out under a green safelight to prevent the conversion of accumulated protochlorophyllide to chlorophyllide. After 10–12 h, protochlorophyllide was extracted from the seedlings using modifications of the procedure of Griffiths (14). The seedlings were cut into pieces 1–2 inches in length. These were macerated in acetone (500 mL) in a blender. After this stage it was no longer essential to work under a green safelight. However, to prevent low levels of photodegradation the pigment solutions were protected with aluminum foil. The acetone mixture was filtered through glass wool and the residue washed with ether (400 mL) to extract all the pigment. The mixture was transferred to a separatory funnel and extracted with 200–300 mL of brine. The lower aqueous acetone phase was discarded. The ether phase was washed with water to remove acetone and an equal volume of hexanes was added. This was then extracted twice with 150 mL of methanol/0.01 *M* NH_3

(4:1, vol/vol). The lower green ammoniacal phase contained the protochlorophyllide. Any chlorophyll present in the mixture remains in the ether/hexanes phase, as do the carotenes and quinones. The combined ammoniacal methanol fractions were washed with ether/hexanes (1:1, vol/vol) to remove any nonpolar pigments and diluted with an equal volume of 100 m*M* phosphate buffer (pH 7.0). This was then extracted with ether until all the protochlorophyllide had been transferred to the ether phase. The ether solution was dried over anhydrous sodium sulfate and evaporated to dryness, initially on a rotary evaporator and subsequently on a high vacuum line. (Use of magnesium sulfate as a drying agent was accompanied by substantial demetallation of the protochlorophyllide. The vapor duct and condenser of the rotary evaporator were washed with hot water to remove any acidic impurity.) The yield of crude protochlorophyllide was estimated using the extinction coefficient reported by Koski and Smith (15) for protochlorophyll (ϵ = 22 000 M^{-1} cm^{-1}, 622 nm). Typically, 340 g of seedlings gave 3–4 mg of crude protochlorophyllide. The crude protochlorophyllide was dissolved in methanol (5 mL) and loaded onto a Waters C_{18} Sep-Pak in 0.5 mL aliquots. The adsorbed protochlorophyllide was washed with 20 mL of 50% methanol/water (vol/vol) and eluted with 100% methanol. The solvent was removed and the protochlorophyllide dissolved in methanol/water mixture (80:20, vol/vol) and purified on a Supelco C_{18} semipreparative HPLC column. Gradient elution was performed from an initial composition of 80% methanol/20% water to 100% methanol over 50 minutes (flow rate = 3 mL/minute). Protochlorophyllide eluted as a broad peak with a retention time of 30 min. Protochlorophyllide was converted to its benzyl ester **18**, which was used for spectroscopic analysis.

*Preparation of the benzyl ester of protochlorophyllide **18**.* Protochlorophyllide (1–2 mg, 1.6–3.2 μmol), shielded from ambient light, was dried on a high vacuum line for 10–12 h. Freshly distilled DMF (0.5 mL) was then added, followed by benzyl bromide (1.9 μL, 16 μmol) and cesium fluoride (2 mg, 16 μmol). The mixture was stirred for 2 h at room temperature under argon. Ethyl acetate (3–4 mL) was added and the solution extracted with water to remove excess cesium fluoride. The solvent was removed on a high-vacuum line. The crude product was then purified on a Supelco C_{18} semipreparative HPLC column. Gradient elution was performed from a starting composition of 80% methanol/20% water to a final composition of 100% methanol, over 50 min (flow rate = 3 mL/min). The solvent was removed by lyophilization and the deep-green amorphous powder analyzed by ^{1}H NMR and MS (400 MHz, C_5D_5N) δ 10.45 (s, 1H, *meso* proton), 10.4 (s, 1H, *meso* proton), 10.2 (s, 1H, *meso* proton), 8.59 (dd, J = 18 Hz, 12 Hz, 1H, C-3 vinylic), 7.63 (s, 1H, C-22), 7.46 (d, J = 8 Hz, 2H, aromatic), 7.31 (m, 3H, aromatic), 6.5 (d, J = 18 Hz, 1H, C-3 vinylic), 6.2 (d, J = 12 Hz, 1H, C-3 vinylic), 5.3 (m, 2H, benzylic), 4.5 (m, 1H, C-17 propionate), 4.4 (m, 1H, C-17 propionate), 4.0 (q, J = 8 Hz, 2H, C-8 ethyl), 3.88 (s, 3H, methyl), 3.82 (s, 3H, methyl), 3.66 (s, 3H, methyl), 3.54 (s, 3H, methyl), 3.49 (s, 3H, methyl), 3.42 (m, 1H, C-17 propionate), 3.3 (m, 1H, C-17 propionate), 1.8 (t, J = 8 Hz, 3H, C-8 ethyl). FAB-MS (POS): $C_{42}H_{38}N_4O_5Mg$: $(M+H)^+$: 703 (found); 703 (calculated). λ_{max} (methanol) = 430 nm, 635 nm.

*Preparation of the protochlorophyllide–flavin complex **16**.* To a solution of protochlorophyllide (1–2 mg, 1.6–3.2 μmol) in DMF (1 mL) was added the flavin **11** (3 mg, 7 μmol) and cesium fluoride (2 mg, 13 μmol). The reaction mixture was shielded from light and stirred at room temperature for 2 h. Ethyl acetate (3–4 mL) was then added and the reaction mixture extracted with water (four times) and the solvent removed under vacuum. The product was purified on a Supelco C_{18} semipreparative HPLC column. Gradient elution was performed from an initial composition of 80% methanol/20% water to a final composition of 100% methanol (retention time = 27 min; flow rate = 3 mL/min). The product **16** was collected and repurified in a water/acetonitrile system on the same column (80% acetonitrile/20% water to 100% acetonitrile over 50 min; retention time 12 min; flow rate = 3 mL/min). The deep-green powder was analyzed by ^{1}H NMR and MS. ^{1}H NMR (400 MHz, C_5D_5N) δ 10.41 (s, 1H, *meso* proton), 10.38 (s, 1H, *meso* proton), 10.19 (s, 1H, *meso* proton), 8.55 (dd, J = 18 Hz, 12 Hz, 1H, C-3 vinylic), 8.22 (d, J = 10 Hz, 1H, aromatic), 7.68 (m, 1H, aromatic), 7.66 (s, 1H, C-22), 7.59 (m, 1H, aromatic), 7.4 (m, 3H, aromatic), 7.22 (m, 3H, aromatic), 6.5 (d, J = 18 Hz, 1H, C-3 vinyl), 6.18 (d, J = 12 Hz, 1H, C-3 vinyl), 6.06 (br s, 2H, *N*-benzylic), 4.55 (m, 1H, C-17 propionate), 4.35–4.48 (m, 5H, C-17 propionate [1H], flavin *N*-alkyl [4H]), 4.0 (q, J = 8 Hz, 2H, C-8 ethyl), 3.91 (s, 3H, methyl), 3.87 (s, 3H, methyl), 3.65 (s, 3H, methyl), 3.53 (s, 3H, methyl), 3.51 (s, 3H, methyl), 3.4 (m, 1H, C-17 propionate), 3.3 (m, 1H, C-17 propionate), 2.25 (m, 2H, flavin *N*-alkyl), 1.8 (t J = 8 Hz, 3H, C-8 ethyl). FAB-MS (POS): $C_{55}H_{48}N_8O_7Mg$ $(M+H)^+$: 957 (found); 957 (calculated). λ_{max} (methanol) = 430 nm, 635 nm.

Reduction of the protochlorophyllide–flavin complex. A 10–15 μ*M* solution of the protochlorophyllide–flavin complex **16** was reduced to the FlH_2 complex **17** using a 10-fold excess of sodium dithionite in 50% methanol/10 m*M* phosphate buffer (pH 7), as described below (Steady-state fluorescence quenching studies).

*Preparation of the nicotinamide salt **14**.* The hydrochloride salt of 3-picolyl chloride **12** (1 g, 6 mmol) was dissolved in the minimum volume of methanol and one equivalent of triethylamine was added to neutralize the hydrochloride salt. The precipitated triethylamine hydrochloride was filtered off, picolyl chloride **12** was extracted into ether and purified by flash chromatography on a silica column (10% methanol/chloroform). A solution of 3-picolyl chloride (0.7 g, 6 mmol) and nicotinamide **13** (2.2 g, 18 mmol) in 15–20 mL of DMF/acetone (1:2, vol/vol) was heated at reflux for 5 h. The solution was then concentrated and cooled to −5°C for 10–12 h. The resulting pale red precipitate was purified by column chromatography using C_{18} Whatman microparticle media in water (yield: 0.46 g, 31%). ^{1}H NMR (DMSO-d^6, 200 MHz) δ: 6.0 (s, 2H, N–CH_2–), 7.48 (dd, J = 8, 5 Hz, 1H), 8.08 (d, J = 8 Hz, 1H), 8.2 (br s, 1H, N–H), 8.3 (dd, J = 8, 5 Hz, 1H), 8.62 (d, J = 5 Hz, 1H), 8.8 (br s, 1H, N–H), 8.88 (s, 1H), 9.02 (d, J = 8 Hz, 1H), 9.38 (d, J = 5 Hz, 1H), 9.84 (s, 1H).

*Reduction of nicotinamide salt **14** to the dihydronicotinamide **15**.* The nicotinamide **14** (0.3 g, 1 mmol) and sodium carbonate (0.44 g, 4.1 mmol) were dissolved in 5 mL of water and the solution cooled to 5–10°C. Sodium dithionite (0.7 g, 4 mmol), dissolved in a minimum amount of water, was added dropwise *via* syringe over 15–20 min. The reduction was performed under an atmosphere of argon. The reaction mixture was stirred for 40 min, filtered and the solid residue washed with ethanol (10–20 mL). The combined filtrate and washings were lyophilized and the crude product was chromatographed (silica gel 10% methanol/chloroform, 1% triethylamine) to give **15** (yield: 0.086 g, 40%) as a pale yellow oil. ^{1}H NMR (DMSO-d^6, 200 MHz) δ: 2.93 (br s, 2H, C-4H), 4.35 (s, 2H, N–CH_2–), 4.62 (m, 1H, C-5H), 5.97 (d, J = 8 Hz, 1H, C-6H), 6.6 (br s, 2H, N–H), 7.0 (br s, 1H, C-2H), 7.3 (dd, J = 8, 5 Hz, 1H, aromatic), 7.7 (d, J = 8 Hz, 1H, aromatic), 8.48 (d, J = 5 Hz, 1H, aromatic), 8.52 (s, 1H, aromatic).

Steady-state fluorescence quenching studies. Solutions of the protochlorophyllide–flavin complex **16** were degassed in a custom-made apparatus comprised of a pyrex tube with a joint for attachment to a vacuum line. The pyrex tube was furnished with a side arm leading to a quartz fluorescence cuvette. The porphyrin–flavin complex was dissolved in 50% methanol/10 m*M* phosphate buffer (pH 7) and the concentration adjusted to an absorbance in the range of 0.1–0.15 at the excitation wavelength (580 nm). Sodium dithionite was dissolved in 10 m*M* phosphate buffer (pH 7) and aliquots were added to the degassed solutions to effect reduction of the flavin moiety. The operations were performed under an atmosphere of argon. Due to the tendency of FlH_2 to undergo rapid reoxidation in air, an excess of dithionite (20 equivalents) was added. Control experiments demonstrated that dithionite did not react with protochlorophyllide under the conditions of the experiment.

RESULTS AND DISCUSSION

Transient spectroscopy (7) and fluorescence lifetime measurements (16) on the enzyme-catalyzed reaction demonstrated that the excited state of protochlorophyllide (S_1), decays in 1–2 ns to an unidentified intermediate. A plausible reaction sequence for product formation is summarized in Fig. 2. To test the plausibility of this proposal, it was essential to demonstrate that the FlH_2 was capable of quenching the fluorescence of protochlorophyllide. We therefore syn-

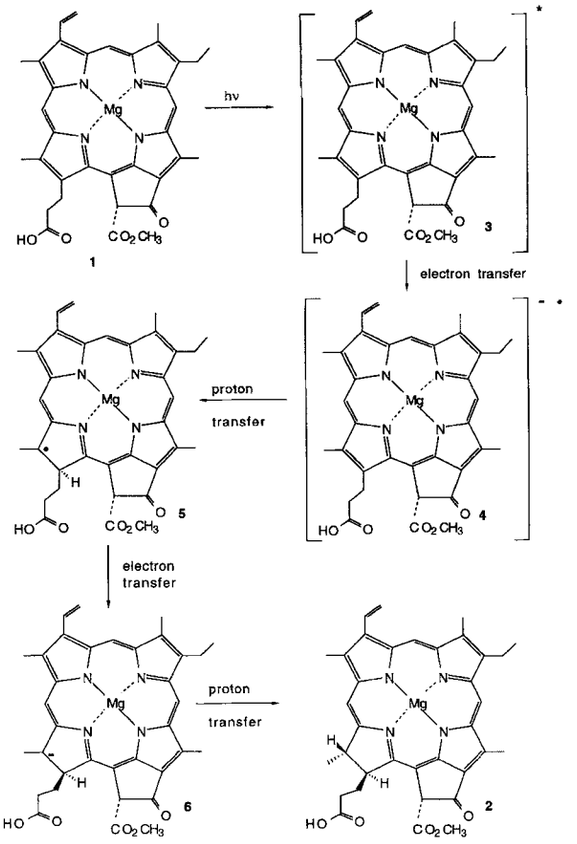


Figure 2. Proposed mechanism for the photoreduction of protochlorophyllide **1** to chlorophyllide **2**.

thesized the protochlorophyllide–FlH_2 complex **17** as a simple model for the enzyme–protochlorophyllide complex (Figs. 3 and 4). The covalent linkage was constructed to optimize the possibility of observing electron transfer by ensuring that a high effective concentration of the FlH_2 would be present, proximal to the photoexcited porphyrin moiety.

Although protopheophorbide (demetallated protochlorophyllide) has been methylated using diazomethane (17), a more general method of esterification suitable for the preparation of the FlH_2 complex **17** was needed. This proved to be a difficult synthetic challenge due to the high reactivity of protochlorophyllide. Attempts to carry out esterification using coupling agents such as carbonyl diimidazole and dicyclohexylcarbodiimide met with little success and led to either recovered starting material or decomposition products. Potassium carbonate-mediated reaction of the acid moiety with alkyl bromides was sluggish and on prolonged exposure led to scission of the exocyclic E-ring. The cesium fluoride-catalyzed (18) esterification described here was found to be a satisfactory method for the selective esterification of protochlorophyllide. The protochlorophyllide–flavin **16** complex was purified by reverse-phase HPLC and characterized by ^1H NMR and FAB-MS. Silica gel chromatography was avoided during purification as it has been implicated in the hydroxylation of the C–H bond at C-22 in chlorophyll (19).

The protochlorophyllide–FlH_2 complex **17** was generated *in situ* by sodium dithionite reduction of the precursor com-

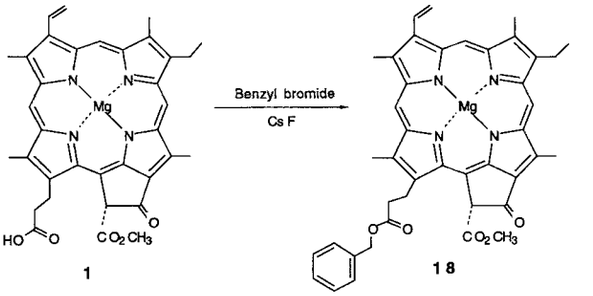


Figure 3. Synthesis of the protochlorophyllide-benzyl ester **18**.

plex **16**, in a methanol/water mixture. The fluorescence behavior of the porphyrin moiety was studied using an excitation wavelength of 580 nm where neither flavins nor FlH_2 possess an absorbance. Steady-state fluorescence measurements, carried out in the presence of excess sodium dithionite, demonstrated that the protochlorophyllide–FlH_2 complex displayed attenuated fluorescence intensity (≈10%) compared to protochlorophyllide. The fluorescence lifetime of protochlorophyllide itself was unchanged in the presence of sodium dithionite, indicating that sodium dithionite is not

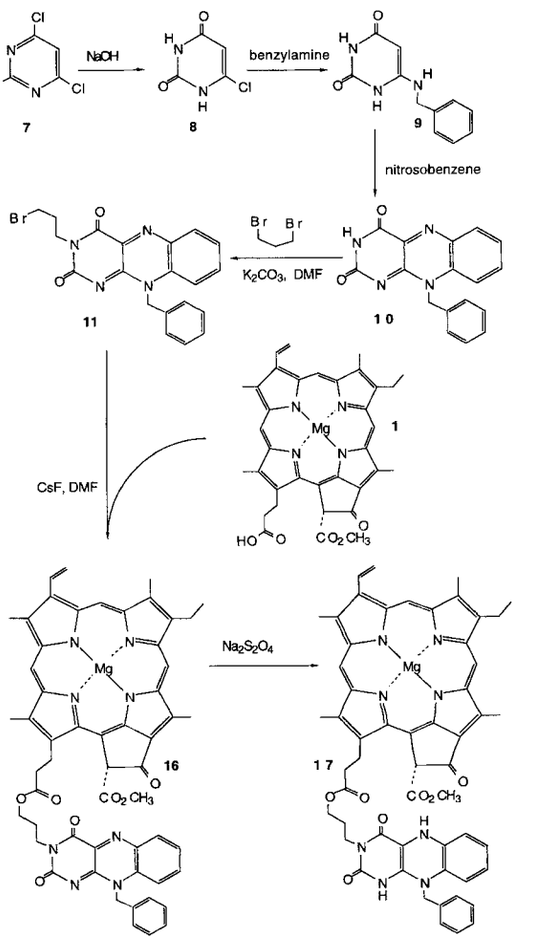


Figure 4. Synthetic route to flavin **11** and protochlorophyllide–dihydroflavin complex **17**.

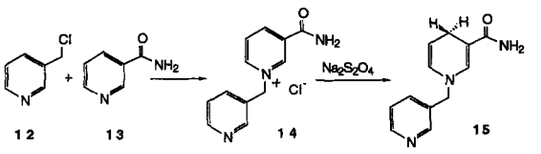


Figure 5. Synthetic route to dihydronicotinamide **15**.

reacting with the protochlorophyllide S_1 state at the concentrations employed. Picosecond fluorescence lifetime measurements demonstrated that the S_1 state of **17** decayed with a rate constant of 1.5×10^9 s^{-1} (τ_s = 0.66 ns). In contrast, the S_1 state of protochlorophyllide decayed with a rate constant of 2.3×10^8 s^{-1} (τ_s = 4.3 ns). It is interesting to note that fluorescence lifetime measurements carried out on the enzymatic reaction identified two forms of protochlorophyllide (16). One, a nonphotoactive species identified as free protochlorophyllide in solution had a fluorescence lifetime of approximately 5 ns, whereas a more short-lived species possessed a $\tau_s \approx 1$ ns.

Because the photochemical behavior of a protochlorophyllide–dihydronicotinamide complex was also of interest, the porphyrin fluorescence intensity was measured in the presence of the dihydronicotinamide **15** (Fig. 5). The use of a pyridyl group to position a reactant atop the porphyrin macrocycle *via* binding to the metal ion is documented (20). Therefore, we chose to examine the fluorescence intensity of protochlorophyllide in the presence of the dihydronicotinamide **15**, in methylene chloride. (Such a strategy could not be applied to the FlH_2 system, because the reduction conditions require an aqueous environment that would prevent coordination of the pyridyl ligand to the porphyrin.) The fluorescence intensity of protochlorophyllide was unchanged in the presence of an excess of dihydronicotinamide **15**.

These observations demonstrate that an FlH_2 is capable of quenching the short-lived S_1 state of protochlorophyllide, whereas a dihydronicotinamide is incapable of doing so. Earlier studies conducted in our laboratory (4) have shown that in the enzymatic reaction the pro-S proton of NADPH is transferred to C-17 of chlorophyllide with partial exchange (30%). This is consistent with the formation of an FlH_2 intermediate, which could undergo N–H exchange with the reaction buffer (21). This is well precedented in other flavoenzymes (22).

The porphyrin–FlH_2 complex described here represents a new type of porphyrin–donor complex distinct from the better known porphyrin–acceptor (porphyrin–quinone) systems. The complex **17** provides an opportunity to generate the putative protochlorophyllide radical anion intermediate in a controlled environment for comparison with spectroscopic data generated from studies on the enzyme.

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