Mechanistic Studies on Protochlorophyllide Reductase: A Model System for the Enzymatic Reaction

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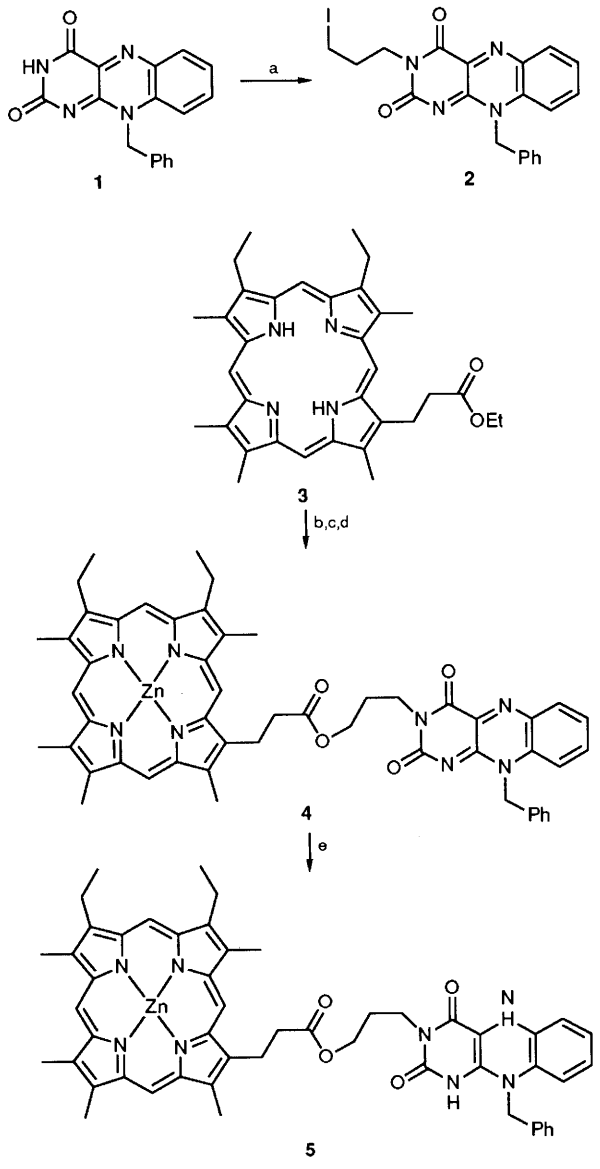
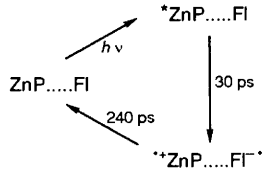
A model system for mimicry of the protochlorophyllide reductase enzymatic process, consisting of a zinc porphyrin covalently linked to a dihydroflavin, undergoes two successive one-electron transfer steps upon illumination, forming a zinc porphodimethene and the corresponding flavin.

Protochlorophyllide reductase catalyses the greening reaction which involves reduction of protochlorophyllide to chlorophyllide in a photochemical process.[1–4] This enzymatic reaction is one of only two characterised examples of light-dependent enzymes.[5,6] The chromophore is protochlorophyllide[7] and the enzyme utilises reduced nicotinamide[8] (NADPH) and flavin[9] cofactors. In attempting to clarify the mechanism of the *in vivo* enzymatic reaction, we have studied the photochemistry of a synthetic model system possessing covalently linked porphyrin and dihydroflavin subunits.



Scheme 1 Preparation of the various porphyrin derivatives. *Reagents and conditions*: a, 1,3-diiodopropane, K_2CO_3, dimethylformamide (DMF); b, 6 mol dm^{-3} HCl; c, **2**, K_2CO_3, DMF; d, $Zn(OAc)_2$, CH_2Cl_2; e, sodium dithionite, MeOH–H_2O.

The synthetic route used to obtain the various porphyrin derivatives **3–5** is depicted in Scheme 1; all new compounds gave satisfactory ^1H NMR, MS and elemental analyses. In deoxygenated aqueous methanol [25% v/v containing pH 7 buffer (0.01 mol dm^{-3})], the metalloporphyrin subunit in **3** fluoresced with a quantum yield (Φ_f) of 0.042 ± 0.004 and a lifetime (τ_s) of 1.90 ± 0.08 ns whereas the triplet state was formed in high quantum yield (Φ_t = 0.85 ± 0.07) and possessed a long lifetime (τ_2 = 200 ± 30 μs). Under the same conditions, fluorescence from **4** was extremely weak (Φ_f = 0.0008 ± 0.0002) and short-lived (τ_s = 30 ± 10 ps) while the triplet state could not be observed by ns laser flash photolysis techniques (Φ_t <0.005). Thus, the appended flavin is an extremely effective quencher for the excited singlet state of the zinc porphyrin, the unimolecular quenching rate constant being (3.3 ± 1.1) × 10^{10} s^{-1} and the degree of quenching being *ca.* 98%.



Scheme 2 Reaction sequence proposed for the photochemistry of the zinc porphyrin–flavin model compound **4**

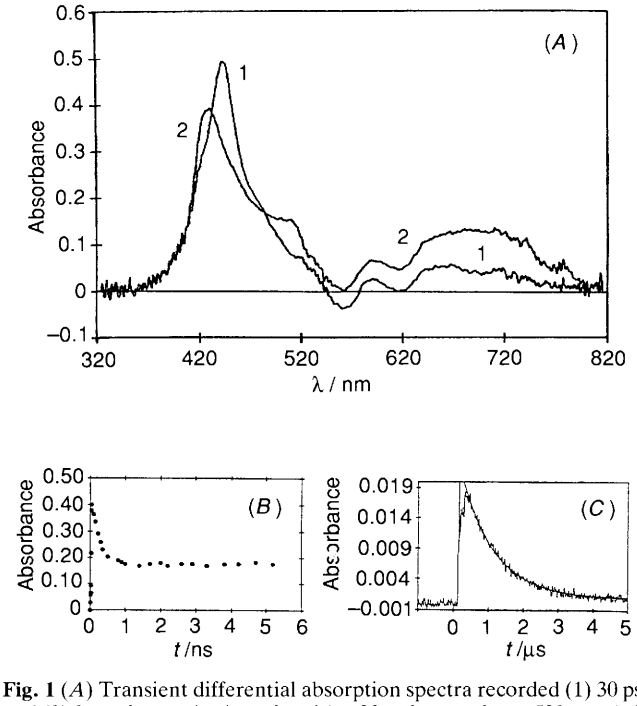


Fig. 1 (*A*) Transient differential absorption spectra recorded (1) 30 ps and (2) 2 ns after excitation of **5** with a 30 ps laser pulse at 532 nm. (*B*) Kinetic traces for the above experiment recorded at 450 nm. (*C*) Decay profile for the porphyrin π-radical anion at 680 nm as recorded after excitation of **5** with a 10 ns laser pulse at 532 nm.

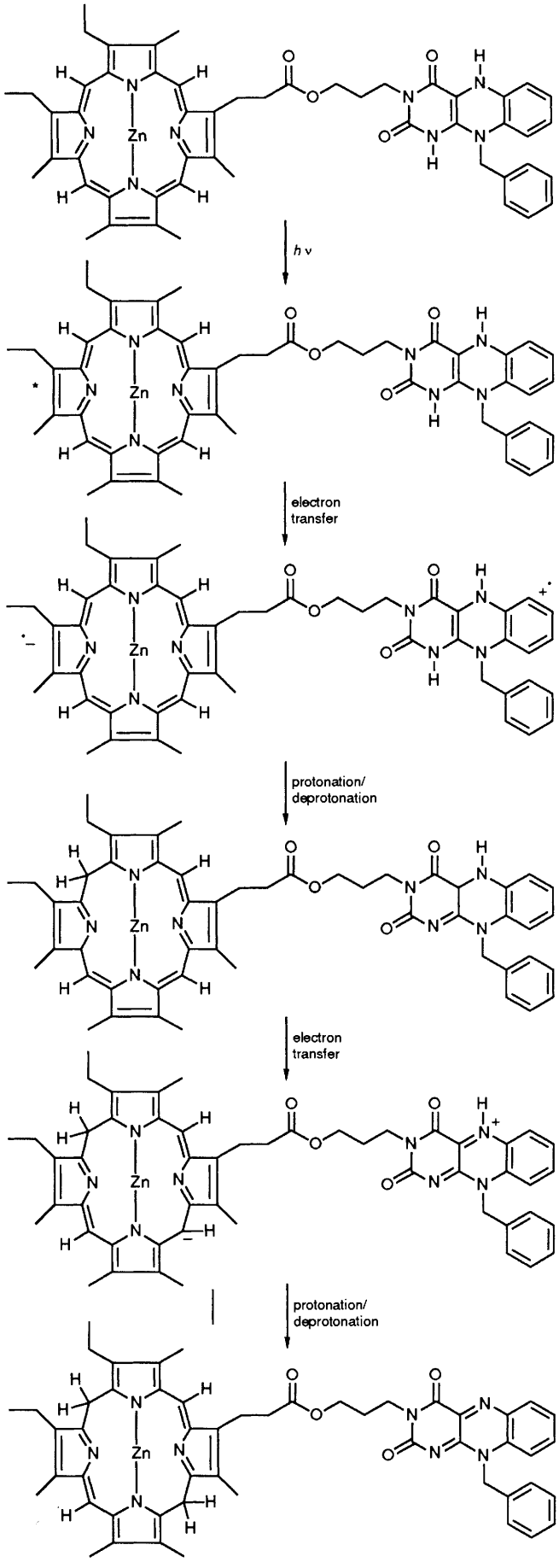
Immediately after excitation of **4** with a 30 ps laser pulse at 532 nm, where only the zinc porphyrin subunit absorbs, the porphyrin singlet excited state was detected[10] by transient differential absorption spectroscopy (TDAS). This species decayed rapidly ($\tau \approx 40$ ps) to form the π-radical cation of the zinc porphyrin, as identified[11] by TDAS. In turn, the π-radical cation decayed *via* first-order kinetics, with a rate constant of $(4.2 \pm 0.5) \times 10^9$ s^{-1}, to reform ground state **4** by way of charge recombination (the intermediate flavin π-radical anion[12] was not observed, presumably because it was masked by the more intense absorption spectral changes associated with the porphyrin subunit). Thus, the photochemistry of this system involves a highly reversible, one-electron transfer process and the kinetic records show no indication of conformational heterogeneity (Scheme 2).

Reduction of the flavin subunit, with dithionite, forms the dihydroflavin derivative **5**. Fluorescence from the zinc porphyrin subunit in **5** was quenched relative to **3**; $\Phi_f = 0.0050 \pm 0.0008$ and $\tau_s = 0.22 \pm 0.04$ ns. These values correspond to a unimolecular quenching rate constant of $(4.0 \pm 0.5) \times 10^9$ s^{-1} and a total fluorescence quenching of *ca.* 88%. Under identical conditions, triplet state formation was inefficient ($\Phi_t = 0.08 \pm 0.01$) but the triplet lifetime ($\tau_t = 175 \pm 25$ μs) remained similar to that of **3**. Quenching by the appended dihydroflavin, therefore, is less pronounced than found for the corresponding flavin but still it competes effectively with the inherent nonradiative deactivation of the porphyrin excited singlet state.

Excitation of **5** in deoxygenated aqueous methanol with a 30 ps laser pulse at 532 nm resulted in immediate population of the excited singlet state of the zinc porphyrin subunit, as evidenced[10] by TDAS (Fig. 1*A*). The species decayed rapidly *via* first-order kinetics with a rate constant of $(4.0 \pm 0.6) \times 10^9$ s^{-1} to generate the porphyrin π-radical anion[13] (Fig. 1*A*, *B*), which did not decay within 10 ns of the laser pulse. Using molar differential extinction coefficients derived for the excited singlet state ($\varepsilon_{450} = 48000$ dm^3 mol^{-1} cm^{-1}) and π-radical anion ($\varepsilon_{440} = 45500$ dm^3 mol^{-1} cm^{-1}) of **3**, the quantum yield for generation of the porphyrin π-radical anion upon excitation of **5** was calculated as 0.80 ± 0.10. Excitation of the solution with a 10 ns laser pulse at 532 nm showed that this π-radical anion decayed *via* first-order kinetics with a rate constant of only $1.1 \pm 0.2 \times 10^6$ s^{-1} (Fig. 1*C*). The final product was assigned a zinc porphodimethene structure on the basis of its absorption spectral profile,[14] which showed a strong band at 445 nm and weak, structureless absorption stretching across the near-IR region. Conventional analysis is difficult because the porphodimethene is extremely air-sensitive and, most probably, exists as a mixture of isomers.

The overall photochemistry of **5** is depicted in Scheme 3. An initial electron transfer from dihydroflavin to porphyrin excited singlet state produces the porphyrin π-radical anion and the corresponding dihydroflavin π-radical cation. This latter species is strongly acidic[15,16] and, in competition to reverse electron transfer, it loses a proton from one of the aza N-atoms. Porphyrin π-radical anions are known[17,18] to be protonated in neutral aqueous solution so that the overall process consists of hydrogen-atom transfer from dihydroflavin to porphyrin, generating two neutral radicals. The flavin radical possesses reducing character[19] and transfers a second electron to the appended porphyrin π-radical anion; this electron transfer process is preferred over bimolecular reactions such as dimerisation of the flavin radical or disproportionation of the porphyrin radical. Protonation of the resultant phlorin anion generates the porphodimethene. The rates of each electron transfer step are quite disparate, possibly because of differences in thermodynamic driving forces or reorganisation energies for the two steps.

On the basis of these studies, we propose a similar mechanism for the enzyme catalysed reduction of protochlorophyllide to chlorophyllide.[1-8] Laser flash photolysis



Scheme 3 Reaction sequence proposed for the photochemistry of the zinc porphyrin–dihydroflavin model compound **5**

studies have shown that the primary electron acceptor is the singlet excited state of the porphyrin and that a dihydroflavin is capable of functioning as a one-electron reductant, under such conditions. Addition of two protons and a further electron to the protochlorophyllide nucleus completes the process. That reduction occurs at the pyrrole ring in the enzymatic process rather than at the *meso*-position, as in our model system, serves to illustrate the additional regio- and stereo-chemical control elements inherent in natural systems. Current work is aimed at improving the directionality of the reduction process by stereochemical control of the porphyrin nucleus.

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