Direct Evidence of an Excited State Triplet Species upon Photoactivation of the Chlorophyll Precursor Protochlorophyllide

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ABSTRACT: The chlorophyll precursor protochlorophyllide (Pchlide), which is the substrate for the light-driven enzyme protochlorophyllide oxidoreductase, has unique excited-state properties that facilitate photocatalysis. Previous time-resolved spectroscopy measurements have implied that a long-lived triplet state is formed during the excited-state relaxation of Pchlide, although direct evidence for its existence is still lacking. Here, we use time-resolved electronic paramagnetic resonance (EPR) in combination with time-resolved absorption measurements at a range of temperatures (10-290 K), solvents, and oxygen concentrations to provide a detailed characterization of the triplet state of Pchlide. The triplet decays in a biphasic, oxygen-dependent manner, whilst the first reported EPR signature of a Pchlide triplet displays both emissive and absorptive features and an antisymmetric spectrum similar to other porphyrin triplet states. This work demonstrates that the Pchlide triplet is accessible to various cryogenic spectroscopic probes over a range of timescales and paves the way for understanding its potential role in catalysis.

TOC GRAPHICS
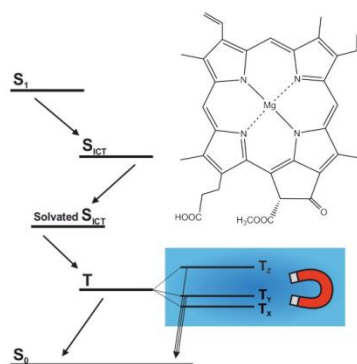
Protochlorophyllide (Pchlide) (Figure 1), a crucial intermediate in the biosynthesis of chlorophyll, is the predominant pigment found in seedlings and dark-grown plants.[1–3] As the substrate for the light-driven enzyme protochlorophyllide oxidoreductase (POR) Pchlide has unique excited state properties that allow it to capture sunlight and trigger the subsequent formation of chlorophyll and development of the plant.[3] The reaction catalyzed by POR involves a light-driven hydride transfer from NADPH to the C17 position of Pchlide,[4,5] followed by a thermally-activated proton transfer from a conserved Tyr residue to the C18 position.[6] Catalysis is dependent on excited-state processes in the Pchlide molecule, which occur on an ultrafast, picosecond, timescale. Previous time-resolved absorption and fluorescence spectroscopy measurements on the isolated Pchlide substrate have shown that it is an intrinsically reactive molecule with multi-phasic excited-state dynamics.[3,7–15]
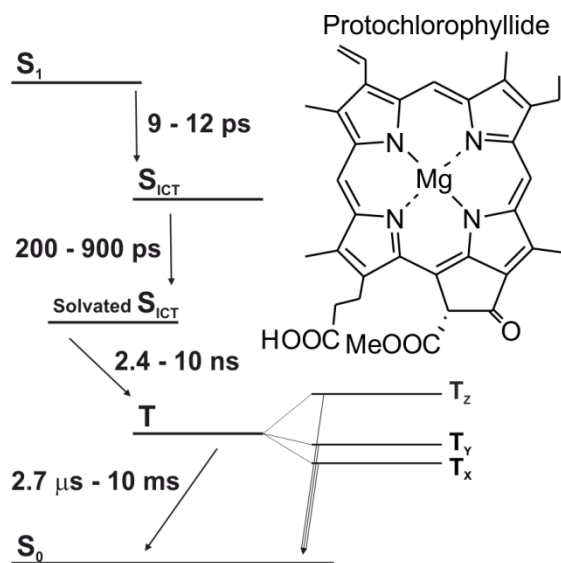

Figure 1. Decay lifetimes of protochlorophyllide's excited states.

The excited-state dynamics depend strongly on solvent polarity[7–9] and have been interpreted by using both a branched model[8–13] and a sequential model[7] (Figure 1). However, both models involve the formation of an excited-state intramolecular charge transfer (ICT) state[7–15] and formation of a putative long-lived triplet state by intersystem crossing on a nanosecond timescale.[7,12] The triplet state has absorption bands at approximately 530 and 890 nm and is formed with a significant quantum yield (0.23-0.34).[7,12] However, the existence of this triplet species has only been based on circumstantial evidence, such as the long lifetime and absorption features in the near infra-red region.[12] Moreover, it is unclear whether the triplet state is directly involved in the reaction mechanism of POR[3,6,16] or if the enzyme has a photoprotective role in preventing non-productive side reactions of the highly reactive triplet intermediate.[12] As triplet states are paramagnetic we have used time-resolved electron paramagnetic resonance (EPR) spectroscopy to provide the first reported direct characterization of the excited-state Pchlide triplet species. These measurements have been combined with cryogenic time-resolved absorption measurements to investigate the thermodynamic properties and oxygen-dependence of triplet formation and decay.

Using EPR spectroscopy it is only possible to observe the triplet state at cryogenic temperatures. Hence, initially it was important to provide a direct comparison of the excited-state dynamics of Pchlide at both cryogenic and room temperatures by using time-resolved absorption measurements after laser excitation at 450 nm (ps to μs timescale) as described in the Supporting Information. The time-resolved difference spectra from these measurements (Figures S1-S8) were fitted to a sequential kinetic model by global analysis to yield evolution associated difference spectra (EADS) (Figure 2).[16] The time-resolved spectral changes at a range of temperatures between 10 K and room temperature, and in the presence and

absence of oxygen could all be fitted to four components with only minor differences in the lifetimes of the various excited state species (Table 1). The first three excited-state components have been described previously and correspond to the formation of an ICT state from the S_1 excited state (~5 ps), solvation of the ICT state (~200 ps) and decay of the S_1/S_{ICT} excited state into the long-lived triplet state on the nanosecond timescale (~4 ns).[7–13] Hence, it appears that temperature and presence of oxygen only have a minimal effect on these three excited-state processes, which are directly related to the formation of the triplet state. The remaining long-lived component (τ_4), which relates to the decay of the triplet state, occurs on longer timescales than the 80 μs time window of the pump-probe measurements.
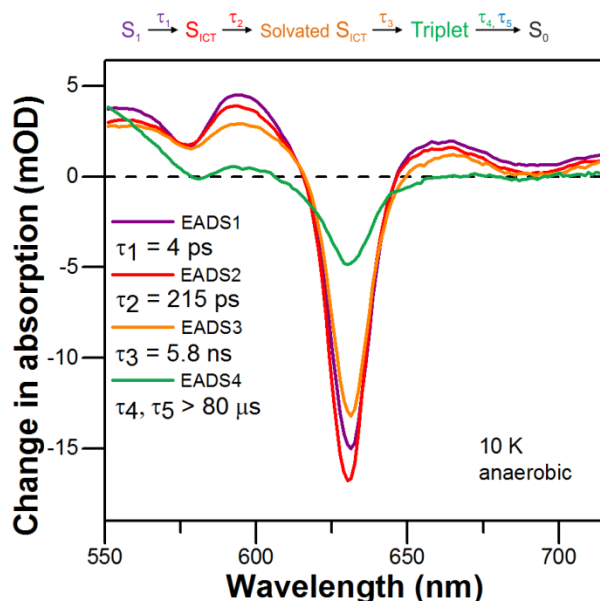

Figure 2. Evolution associated difference spectra (EADS) resulting from a global analysis of the time-resolved absorption data for an aqueous, anaerobic Pchlide sample after excitation at 450 nm at 10 K. The EADS were obtained from fitting the time-resolved absorption data to a sequential exponential model as described in the supplementary information. The triplet decay lifetime was set to infinity on the 80 μs timeframe of the measurement.

Table 1. Lifetimes of the S_1 (τ_1), S_{ICT} (τ_2), solvated S_{ICT} (τ_3) and triplet excited states of Pchlide (τ_4 and τ_5) measured in both aerobic and anaerobic aqueous buffer solutions at 10, 80, 150 and 290 K. For experimental conditions see Supporting Information.

	T (K)	τ_1 (ps)	τ_2 (ps)	τ_3 (ns)	τ_4 (ms)	τ_5 (ms)
anaerobic	**10**	4.0 ± 0.2	215 ± 5	5.8 ± 0.1	6.9 ± 0.1	24.9 ± 0.2
	80	6.3 ± 0.3	276 ± 5	4.9 ± 0.1	7.5 ± 0.1	22.3 ± 0.2
	150	5.0 ± 0.2	170 ± 3	4.1 ± 0.1	6.4 ± 0.1	19.4 ± 0.1
	290	3.4 ± 0.4	180 ± 6	3.5 ± 0.1	0.35 ± 0.01	1.82 ± 0.01
aerobic	**10**	4.6 ± 0.2	205 ± 8	5.9 ± 0.1	3.1 ± 0.2	-
	80	4.7 ± 0.2	597 ± 20	6.0 ± 0.1	3.6 ± 0.1	-
	150	5.6 ± 0.2	278 ± 9	5.5 ± 0.1	3.7 ± 0.2	-
	290	3.8 ± 0.2	364 ± 26	4.9 ± 0.2	0.11 ± 0.01	-

In order to investigate the kinetics of triplet decay in more detail the time-resolved absorption measurements were extended to the ms timescale by using laser flash photolysis experiments (Figures S9-S16). Consequently, the triplet state of Pchlide was found to decay in a biphasic process with τ_4 values of approximately 7 ms and τ_5 values of approximately 22 ms under cryogenic and anaerobic conditions (Figure 3A, Table 1). However, in the presence of oxygen the triplet decay kinetics become faster and more monoexponential in nature, displaying τ_4 values of ~3.5 ms at cryogenic temperatures. The oxygen dependence was investigated further by following the recovery of the Pchlide ground state (absorbance at 630 nm) over a range of oxygen concentrations at room temperature (Figure 3B). The triplet decay kinetics show a strong dependence on oxygen concentration, confirming that the triplet excited-state of Pchlide can interact with molecular oxygen, presumably to yield singlet oxygen or other reactive oxygen species

via triplet-triplet energy transfer.[17,18] In addition, the observed lifetimes show no dependence on temperature at cryogenic temperatures (Figure S33) suggesting that the decay of the triplet state is an adiabatic process in frozen samples.

As previous studies have shown that the excited-state dynamics of Pchlide depend strongly on solvent polarity[7–9] the transient absorption measurements were repeated in alcohol solutions. The ultrafast steps leading to the formation of the triplet state are very similar to those in water and show no noticeable dependence on oxygen or temperature (Figure S34, Table S1). However, the decay of the Pchlide triplet state now appears to follow single, monoexponential kinetics under both aerobic and anaerobic conditions with a lifetime of ~3 ms at cryogenic temperatures. Moreover, at higher temperatures the triplet excited state decays much more rapidly in polar solvent (lifetime of ~2μs) than in water (lifetime of ~0.1 ms). It has previously been shown that dimers/aggregates of Pchlide, formed in water-based buffers, display different excited state dynamics to monosolvated Pchlide found in polar solvents.[19,20] Hence, it is possible that the differences in aggregation state of Pchlide may also explain the changes in triplet decay kinetics observed in the present work.
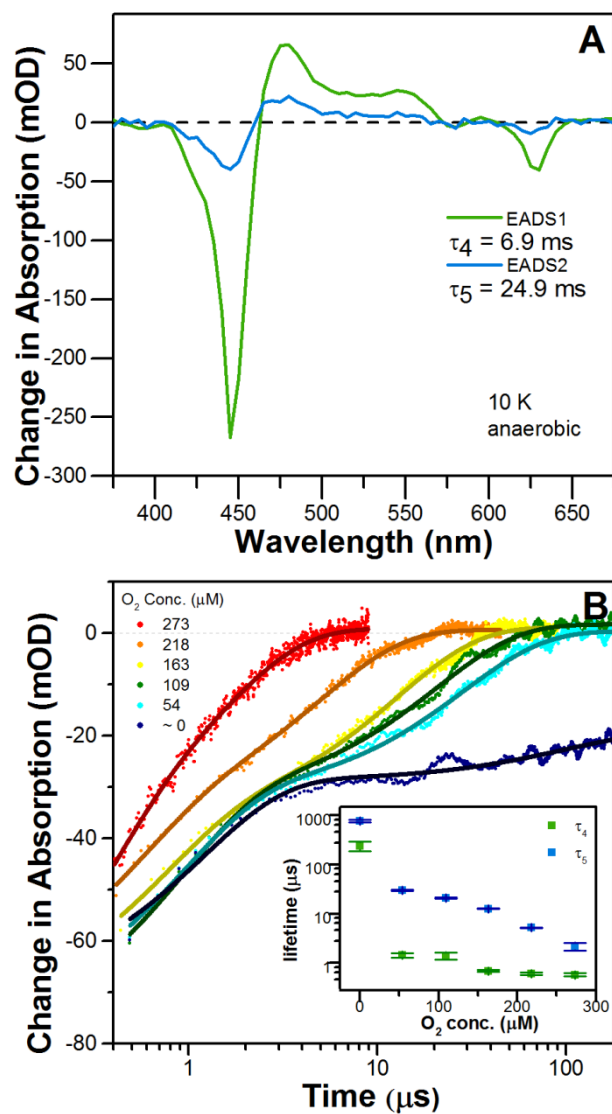
Figure 3. (A) EADS resulting from a global analysis of the laser flash photolysis data for an aqueous, anaerobic Pchlide sample after excitation at 450 nm at 10 K. The EADS were obtained from fitting the data to a sequential exponential model as described in the supplementary information. (B) Lifetime values (τ_4 and τ_5) obtained from fitting a double exponential decay to ground state recovery (630 nm) flash photolysis time traces. Samples consisted of Pchlide dissolved in mixtures of anaerobic and aerobic aqueous buffer at room temperature.

In order to provide direct experimental evidence of the existence of an excited-state triplet of Pchlide time-resolved EPR has been used after photoexcitation with laser pulses at 532 nm (Figure 4) and 450 nm (Figure S36). The EPR signature of the photoexcited triplet state Pchlide was obtained from integrating direct-detection EPR time traces over a range of magnetic field positions. The EPR spectrum displays both emissive and absorptive features and an antisymmetric lineshape (Figure 4A), which are all features that are characteristic of a triplet state.[21–23] The lifting of the degeneracy of spin states in the absence of an applied magnetic field is referred to as zero-field splitting (ZFS). The value of the ZFS parameter is usually defined via D and E parameters. D describes the axial component of the magnetic dipole-dipole interaction, and E the transversal component. The values of the ZFS splitting parameters obtained from a simulation of the spectrum (Figure S37) are of a rhombic symmetry and were found to be $|D| = 860 \pm 8$ and $|E| = 182 \pm 5$ MHz. These values are comparable to those of chlorophyll a ($|D| = 818 \pm 24$ and $|E| = 120 \pm 6$ MHz) and chlorophyll b ($|D| = 878 \pm 27$ and $|E| = 156 \pm 3$ MHz)[24], suggesting that the energy states of the triplet state of Pchlide are similar to those found in the chlorophylls. Changes in the E value reflect some differences in asymmetry. Density functional theory calculations incorrectly predicted ZFS parameters as $|D| = 648$ and $|E| = 166$ MHz, although this error might be due to the inability of the method to accurately take into account various molecular orbitals in highly delocalized systems, such as photoexcited Pchlide.[25]

Electron spin echo (ESE) field-swept spectra (Figure 4A) measured at different delay-after-flash (DAF) values reflect the anisotropic decay of the triplet sub-levels of the Pchlide triplet state, as previously observed in other organic triplets.[26–28] Triplet sub-level

decay rates were calculated from measuring the intensity of the two-pulse spin echo EPR signal at increasing DAF values. A representation of the ESE intensity vs the DAF is shown in Figure 4B. A double exponential decay with a positive and a negative component was fit to the time evolution traces. These were measured at the high-field Z^+ orientation of the triplet state EPR spectrum for Pchlide samples in aerobic and anaerobic aqueous and alcohol solutions, providing the decay constants from T_0 for that orientation as per the following formula:

$$I = A_1 exp(-t/\tau_1) + A_0 exp(-t/\tau_0)$$

with I being the ESE intensity, t the DAF, $|A_1|$ and $|A_0|$ being the initial relative populations of T_{+1}/T_{-1} and T_0, with τ_1 and τ_0 as their respective decay lifetimes. A_0 is positive for the "+" canonical orientations and negative for the "-" orientations, representing absorption and emission from T_0 respectively. The opposite is true for A_1 and T_{+1}/T_{-1}. The triplet EPR decay lifetimes in different solvents show the same trends as the optical experiments, with faster decays in the alcohol solutions and in the presence of oxygen. However, it should be noted that the EPR signal intensity for the aerobic aqueous sample was too weak to measure accurately.
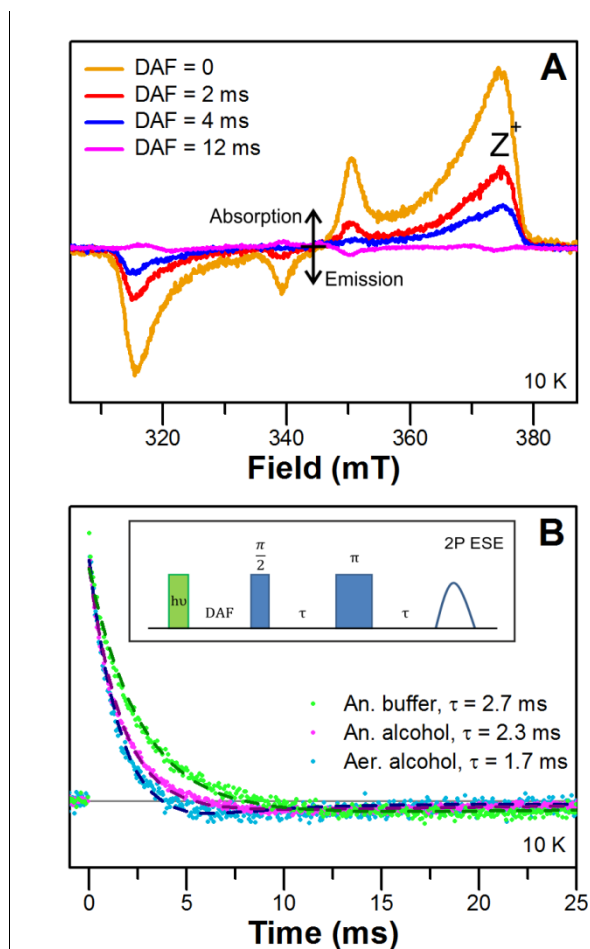
Figure 4. (A) ESE field-swept spectra of Pchlide recorded at 10 K in anaerobic aqueous buffer at different DAF values displaying an anisotropic decay of the triplet state spectrum features. (B) ESE-detected kinetics of the Pchlide triplet in anaerobic aqueous buffer and both anaerobic and aerobic alcohol solutions at 10 K at the high-field Z^+ canonical orientation. Lifetimes correspond to the decay from T_0. **Inset:** Pulse sequence for the EPR two-pulse electron spin echo (ESE) experiment consisting of a laser pulse (in green), microwave pulses (in blue at a delay after flash DAF and separated by a delay τ) and the generated spin echo signal.

This work provides the first direct evidence that a long-lived triplet state is formed during the excited-state relaxation of Pchlide. Time-resolved EPR measurements confirm that the Pchlide

triplet, which forms on the nanosecond timescale and decays on the millisecond timescale, is similar to other porphyrin triplet states. The Pchlide excited-state triplet species has been shown to react with oxygen, suggesting that highly reactive oxygen species could be formed, which may lead to photo-damage in the cell. Hence, it is likely that specific chromophore-protein interactions occur when Pchlide is bound to the enzyme, which would help to minimize any undesirable effects of Pchlide triplet formation. These findings open up an exciting new range of pulsed EPR spectroscopy studies such as ESE envelope modulation (ESEEM), hyperfine sublevel correlation (HYSCORE) or electron nuclear double resonance (ENDOR), which will be essential to gain further information on the electronic couplings of Pchlide in the excited triplet state. In addition, measurements on POR-bound Pchlide will provide important insights into how the enzyme active site affects such electronic properties and the potential role of the triplet state in POR catalysis.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge. Detailed experimental methods, raw transient absorption data, thermodynamic data, EPR data (PDF).

AUTHOR INFORMATION

Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENTS

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