Vibrational Fingerprinting of Protochlorophyllide Analogues and Implications for the Photochemical Synthesis of Chlorophyll

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Introduction

A key regulatory step in the chlorophyll biosynthetic pathway is the reduction by the light-driven enzyme protochlorophyllide oxidoreductase (POR) of the C17-C18 double bond of protochlorophyllide (Pchlide) to form chlorophyllide (Chlide; Figure 1).[1–3] The reaction catalyzed by POR involves a light-driven hydride transfer from NADPH to the C17 position of the Pchlide molecule,[4-5] followed by a thermally-activated proton transfer from a conserved Tyr residue to the C18 position, both reactions involving quantum mechanical tunneling.[6]

Although the chemical steps in the POR reaction cycle proceed on the microsecond timescale,[5] catalysis is dependent on excited-state processes in the Pchlide molecule. Time-resolved studies on the isolated Pchlide pigment have demonstrated that Pchlide is an intrinsically reactive molecule with multi-exponential dynamics[7-12] that depend strongly on solvent polarity.[7-8, 12] The identification of a number of Pchlide excited state species suggest a multi-phasic quenching of the Pchlide excited state emission via solvation of an intramolecular charge transfer (ICT) state[7-12] and the subsequent formation of a triplet state on the nanosecond timescale.[11-12] Moreover, time-dependent density functional theory (DFT) calculations have confirmed the ICT character of the Pchlide excited state in methanol and, in turn, this is thought to induce site-specific solvation of the photoexcited Pchlide molecule via strengthening of H-bonding interactions.[13] The highly reactive nature of the excited state is thought to be caused by the presence of a number of substituent groups on the Pchlide molecule, such as the electron-withdrawing carbonyl group on ring E,[12, 14-15] central Mg ion and the carboxylic acid sidechain at the C17 position, all of which have been shown to be important for enzymatic photoreduction (Figure 1).[16] We have now synthesized a number of Pchlide analogues that contain alterations to all of these positions (compounds A-F in Figure 1) and used time resolved infra-red (IR) spectroscopy to understand how the structural changes affect the photochemical and excited-state properties of the Pchlide molecule that are so crucial for POR catalysis.[17] Using this information has allowed us to identify the excited-state intermediates in the catalytic cycle of POR and to propose a mechanism for harnessing the light energy to drive the subsequent hydride and proton transfer steps on the microsecond timescale.[18]

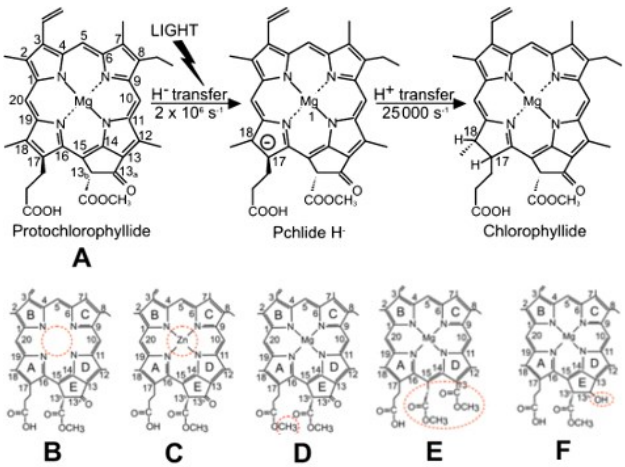

Figure 1. Light-driven reduction of the C17−C18 double bond of Pchlide to form Chlide is catalyzed by POR. The structures of the Pchlide derivatives described in the present study are shown, with modifications indicated by red circles.

Experimental Methods

All chemicals and solvents were purchased from Sigma Aldrich, except where specified, and were of analytical grade or better. All pigments were synthesized from commercially available pheophorbide a to yield the target compounds A-F and were verified by NMR and mass spectrometry. A full

description of the chemical syntheses, together with NMR and mass spectrometry analyses, can be found in the supporting information of reference 17. Wild-type and Y193F POR from *Thermosynechococcus elongatus* were overexpressed in *Escherichia coli* and purified as described previously.[1,2]

Infra-red transient absorption spectroscopy was carried out at the Ultra facility (Central Laser Facility, STFC, Rutherford Appleton Laboratory), using the time-resolved multiple probe spectroscopy ($TR^{M}PS$) technique.[19] Samples were contained between two CaF_2 windows with a pathlength of approximately 100 µm. The sample was flowed through the cell and the sample holder rastered to avoid sample damage. Difference spectra were generated relative to the ground state in the spectral window 1500-1800 cm^{-1} with a resolution of ~3 cm^{-1}. Samples containing each analogue (A-F) at OD ~0.07 at 430 nm in ^{2}H-methanol, were excited at 430 nm, data were collected for c.a. 30 minutes over a time range of 0.3 ps to 3 ns. Samples of 350 µM Chlide, and 350 µM. Pchlide in the presence and absence of 500 µM POR (wild type and Y193F variant) and 2.5 mM NADPH in D_20 activity buffer (50 mM Tris pD 7.5, 100 mM NaCl, 1 % Triton X-100, 0.1 % 2-mercaptoethanol) were excited at 450nm, data were collected for c.a. 10 minutes over a time range of 1 ps to 2 µs. All samples were excited with 0.5 µJ pulse power and a beam diameter of ~ 150 µm, set at the magic angle with respect to the probe beam.

Results and Discussion

Time-resolved spectral changes in the mid-IR region provide vibrational "fingerprints" of specific regions of the Pchlide chromophore (Figure 2).

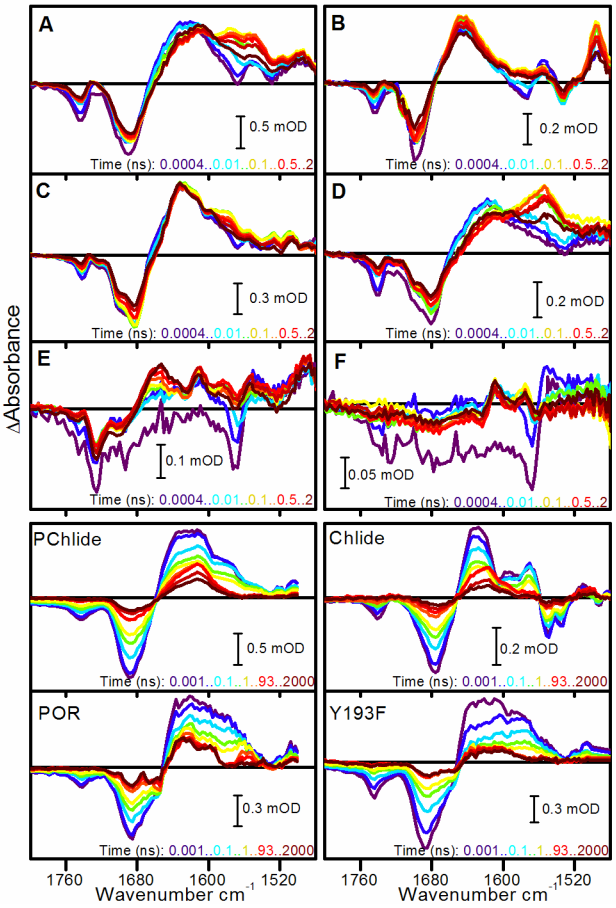

Figure 2. Transient IR absorption data for Pchlide derivatives A-F in methanol; and Pchlide, Chlide, and wild type and Y193F-Pchlide-NADPH ternary complexes in aqueous solution.

The transient absorption data for compounds A and D can be modelled with 4 component lifetimes whereas changes to the central metal ion (compounds B and C) yields data that are best fitted to 3 components.[17] In all cases the first lifetime, representing vibrational relaxation and reorganization of the initially excited S_n state, involves a decrease in the amplitude of the negative feature at ~1740 cm^{-1}. In compounds A and D the solvation of the ICT state is accompanied by an increase in signals in the 1500-1600 cm^{-1} region, which have been assigned to in-plain porphyrin ring vibrations (C=C and C=N modes).[20] The $S_{n/ICT}$ state is then converted to the long-lived triplet state on the nanosecond timescale.[12]

In order to assign more of the IR modes to specific regions of the Pchlide molecule the initial S_n excited state species (the first difference spectra derived from global fitting) have been fitted with a combination of Gaussian components for all of the analogues (Figure 3).[17] As previously suggested[12,14,20] the major negative peak at ~1690 cm^{-1} and positive peak at ~1625 cm^{-1} represent the C13a=O carbonyl frequency as these features are absent in analogues that contain changes to the C13a keto group of the isocyclic ring (compounds E and F). As the majority of the IR signals in the region monitored originate from this C13a keto group, very few spectral changes are observed for compounds E and F. In most cases the spectral features associated with the C13a=O carbonyl group can be modelled with 2 separate sub-populations, with the negative peak at ~1690 cm^{-1} resolved into 2 distinct bands centred at 1689 cm^{-1} and 1706 cm^{-1} and the positive peak at ~1625 cm^{-1} resolved into 2 distinct bands centred at 1606 cm^{-1} and 1634 cm^{-1}. These are likely to represent differences in the solvent coordination or local environment around this group[12] and the frequencies of both species are influenced by changes to other regions of the Pchlide molecule (compounds B-E).

The negative feature at 1740 cm^{-1}, previously proposed to arise from C=O modes of the substituents at the C13b and C17 positions,[15] is almost identical to Pchlide when the C17 carboxylic acid group is changed to a methylester (compound D). However, the signal is downshifted by ~13 cm^{-1} upon opening of the ring E (compound E) and the peak, therefore, can be assigned entirely to the methylester at the C13b position. In addition, this band is only slightly sensitive to changes to the central metal ion (compounds B and C) or the C17 carboxylic acid (compound D) but removal of the C13a=O group (compound F) downshifts the C13b ester band by ~8 cm^{-1}, confirming that it is coupled to other vibrational modes in the ring E. Although these groups are the most intense IR markers in this region, due to their close proximity to the conjugated electronic macrocycle, the in-plane porphyrin ring vibrations in the 1500-1600 cm^{-1} region are influenced by alterations to the central metal ion. The C17 carboxylic acid group is located further away from the main macrocycle and therefore, changing this group to a methylester (compound D) only has a minor effect on the IR difference spectrum upon electronic excitation.

The time-resolved IR data for wild-type POR and the inactive variant Y193F ternary complexes reveal an extra ground state bleach, compared to free Pchlide, at 1656 cm^{-1}. This is likely to reflect additional hydrogen-bonding interactions between the protein and the bound Pchlide. The data for wild-type POR are much more complex than for free Pchlide and the Y193F variant with 2 additional components required to accurately model the data. The conversion between the S_n, S_{ICT}, solvated S_{ICT} and triplet states still occurs with very comparable lifetimes and spectral features to free Pchlide and the Y193F variant.[18] However, in contrast to free Pchlide and the Y193F variant there is an additional 'catalytic' pathway in wild-type POR from the S_{ICT} state to yield the hydride transfer intermediate.[18]

There are a number of different structural components in the 'catalytic' pathway components compared to the 'non-catalytic' pathway, which allow us to propose a model for the

photochemistry along the 'catalytic' branch. A down-shift in the peak at 1575 cm^{-1} to 1566 cm^{-1} suggests a potential loss of double bond character of the C17-C18 bond.[20] Similar features are observed in the analogues that contain changes to the C13a keto group of the isocyclic ring (compounds E and F), implying that this feature is not related to that area of the molecule. This conclusion is further supported by the time-resolved IR data collected for a Chlide only sample (i.e. C17-C18 bond is reduced), which show a significant feature at ~1570 cm^{-1} that is not present in the Pchlide data.[18] The 1575 cm^{-1} downshift is coupled to other excited state modes at ~1640, 1620 and 1580 cm^{-1} shifting by approximately 12-15 cm^{-1} to higher wavenumbers, suggesting that there is an increase in electron density in other regions of the Pchlide ring system. Moreover, there is a new negative feature at ~1708 cm^{-1}, which likely corresponds to loss of signal from the carbonyl stretch of the C17 COOH group,[20] which a study of the analogue spectral features suggested was at neither ~1740 nor ~1690 cm^{-1}.

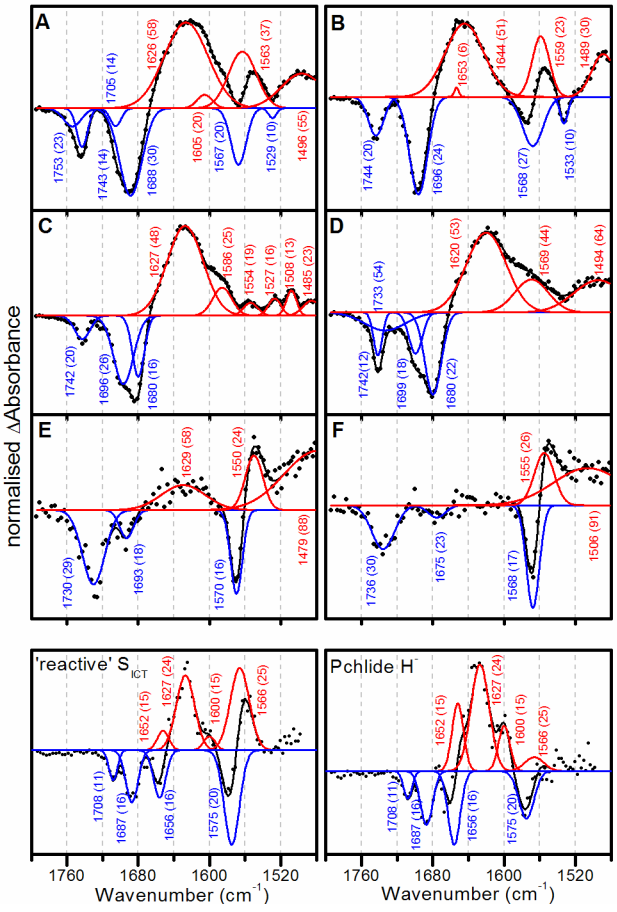

Figure 3. Fitting, by a sum of Gaussian functions, of the initial S_n excited state difference spectra from the global analysis of the transient IR absorption data for Pchlide derivatives A-F, and of the difference spectra of the 'reactive' S_{ICT} and initial photoproduct (Pchlide H^-) for the wild-type POR-Pchlide-NADPH ternary complex. The data (black dots) have been fitted with a sum (black line) of Gaussian functions (negative shown as blue lines, positive as red lines). Positions and FWHM (in brackets) are indicated.

As the 'catalytic' pathway does not occur in the Y193F variant it suggests that the Tyr residue is involved in formation of the 'reactive' excited state species. Taken together, it is likely that excited state H-bonding interactions between the OH group of the Tyr residue and the carbonyl at the C17 position have an electron-withdrawing effect on the neighbouring C17-C18 double bond to create an electron-deficient site. The dipolar nature of the ICT state in free Pchlide is caused by the presence of the electron-withdrawing carbonyl group on the isocyclic ring of Pchlide,[11] as indicated by the negative peak at ~1743 cm^{-1}. However, this peak is absent in the 'reactive' ICT state, providing further evidence that the electron-deficient site at the C17-C18 double bond is formed as a result of excited state interactions with the C17 carboxyl group.

Conclusions

Previous studies have shown that an ICT state is the major driving force behind the excited state reactivity of the molecule[6-8] and a combination of time-resolved spectroscopies and DFT calculations has now shown that the dipolar nature of this species is caused by the presence of the carbonyl group at the C13a position of ring E.[17] In addition to ring vibrations from the main porphyrin macrocycle, the frequencies of both groups are also influenced by changes to other regions of the Pchlide molecule. These assignments provide direct confirmation of previous models for the involvement of specific vibrational modes in the excited state dynamics of Pchlide.[12] These data now provide strong mechanistic evidence for how light energy is captured to drive catalysis in POR. Excited state interactions between the Pchlide molecule and active site residues are required to decrease the local electron density across the C17-C18 double bond, which facilitates the transfer of the negatively charged hydride ion from NADPH. More generally, these findings will now be crucial for mapping the coupling of any vibrational modes to the hydride and proton transfer chemistry in POR and for understanding the role of specific regions of the Pchlide molecule in POR catalysis.

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