Supporting Information

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Excited-State Charge Separation in the Photochemical Mechanism of the Light-Driven Enzyme Protochlorophyllide Oxidoreductase**

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Supporting Information

Experimental Section

Sample Preparation

Wild-type (1) and Y193F (2) POR from *Thermosynechococcus elongatus* were overexpressed in *Escherichia coli* and purified as described (1, 2). Pchlide was purified as described previously (1). Chlide was synthesized by the insertion of magnesium into pheophorbide *a* as described previously (3). For the time-resolved visible spectroscopy measurements samples contained 200 µM Pchlide in the presence and absence of 500 µM POR and 2 mM NADPH in activity buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 % Triton X-100, 0.1 % 2-mercaptoethanol). For the time-resolved IR spectroscopy measurements samples contained 350 µM Pchlide in the presence and absence of 500 µM POR and 2.5 mM NADPH in D$_2$O activity buffer (50 mM Tris pD 7.5, 100 mM NaCl, 1 % Triton X-100, 0.1 % 2-mercaptoethanol). Absorbance spectra were recorded using a Cary 50 UV/visible spectrophotometer (Agilent Technologies).

Visible Time-Resolved Spectroscopy

A Ti:sapphire amplifier (hybrid Coherent Legend Elite-F-HE) was pumped by a Q-switched Nd:YLF laser (Positive light evolution-30) and seeded by a Ti:sapphire laser (Spectra Physics Mai Tai). The amplifier output (1 kHz repetition rate, 800 nm centre wavelength, ~120 fs pulse duration) was split to generate the pump and probe beams. A non-collinear optical parametric amplifier (light conversion TOPAS white) was used to generate the pump beam centered at 450 nm, with FWHM of ca. 10 nm. A broad band ultrafast pump-probe transient absorbance spectrometer ‘Helios’ (Ultrafast systems LLC) was used to collect data (randomly) from ~5 ps to 3 ns with a time resolution of around 0.2 ps. The probe beam consisted of a white light continuum generated in a sapphire crystal, absorbance changes were monitored between 500 and 750 nm. Data from this set-up are referred to as the ‘fast’ data. A broad band sub-nanosecond pump-probe transient absorbance spectrometer ‘Eos’ (Ultrafast systems LLC) was used to collect data (randomly) up to 2 microseconds. A 2 kHz white-light continuum fibre laser was used to generate the probe pulses. The delay between pump and probe was managed electronically. For both sets of measurements samples were excited at 450 nm with 0.5 µJ power and a beam diameter of ~ 200 µm. Data from this set-up are referred to as the ‘fast’ data.

Samples were flowed at a rate of approximately 20 ml/min through a 0.2 mm pathlength quartz cell to ensure that a different area of the sample is excited with each pump laser pulse. After correcting the ‘fast’ data for spectral chirp the ‘fast’ and ‘slow’ datasets were combined by scaling the full
‘slow’ dataset by a fixed factor to match the intensity of ground state bleach feature in the ‘fast’
dataset at similar time points (datasets overlap between ~0.5 to 3 ns).

**Infra-Red Time-Resolved Spectroscopy**

These experiments were carried out at the Ultra facility (CLF, STFC Rutherford Appleton
Laboratory, UK), using the recently developed time-resolved multiple probe spectroscopy (TR<sup>MPS</sup>)
technique (4). Samples in D<sub>2</sub>O buffer were contained between two CaF<sub>2</sub> windows, separated by a
teflon spacer to give a pathlength of approximately 100 µm. The sample was flowed through the
cell and the sample holder rastered to avoid sample damage. For all samples an excitation
wavelength of 450 nm was used with 0.5 µJ pulse power and a beam diameter of ~ 150 µm set at
the magic angle with respect to the IR probe beam. Difference spectra were generated relative to the
ground state in the spectral window 1500-1800 cm<sup>-1</sup> at time delays ranging between 500 fs and 2
µs. Data were collected for approximately 10 mins per dataset, the spectral resolution was ~3 cm<sup>-1</sup>
and pixel to wavenumber calibration was performed as described previously (5).

**Global Analysis**

The datasets were analyzed globally using the open-source software Glotaran (6). This procedure
reduces the matrix of change in absorbance as a function of time and wavelength, to a model of one
or more exponentially decaying time components, as described in the main manuscript, each with a
corresponding difference spectrum (species associated difference spectra (SADS)). Errors quoted
with the lifetime values are the standard errors calculated during the global analysis.

Previous studies on Pchlide systems have concluded that the fluorescence quantum yield is on the
order of 6-9% (7). The overall triplet yield (compared to the entire excited state population) has
been found to be around 30% (8, 9). Thus from the total initial excited state population it can be
assumed that around 65% returns to the ground state via non-radiative processes. Rather than try to
exactly fit these diverse processes, which would result in a model with so many parameters that
almost anything would have fitted, we have chosen to use a simplified model in which the that the
lifetimes quoted for the conversion between states also include contributions from the rates of
ground state recovery through both radiative and non-radiative processes. Differences in
experimental conditions (e.g. H<sub>2</sub>O vs. D<sub>2</sub>O) could be expected to affect the rates of non-radiative
relaxation, hence the apparent disparities in some of the rate constants described here. The purpose
of the global analysis is to distinguish the evolution of spectral features in order to determine the
mechanism of intermediate formation rather than precise kinetics. The model used to fit the ternary
POR-Pchlide-NADPH complex was settled upon after extensive testing. Starting with the simple
sequential scheme derived from the Pchlide only data, branches of varying length (1,2, or 3
components) to the ‘A696’ intermediate were tried from each of the S1, S<sub>ICT</sub>, Solvated S<sub>ICT</sub> and T
states. The final model was the only one which produced a set of physically sensible SADS and reasonable interconversion lifetimes.
Supplementary Figures

**Figure S1.** Ground state absorbance spectra of Pchlide only and POR-Pchlide-NADPH ternary complex samples.
Figure S2. Time-resolved visible spectroscopy data for Pchlide only after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 5 ps and 2 µs as described in the Experimental section.
Figure S3. Time-resolved IR spectroscopy data for Pchlide only after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 4 ps and 2 µs as described in the Experimental section.
Figure S4. Time-resolved visible spectroscopy data for a Y193F POR-Pchlide-NADPH ternary complex after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 5 ps and 2 µs as described in the Experimental section.
Figure S5. Time-resolved visible spectroscopy data for a wild-type POR-Pchlide-NADPH ternary complex after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 14 ps and 2 µs as described in the Experimental section.
**Figure S6.** Time-resolved IR spectroscopy data for a Y193F POR-Pchlide-NADPH ternary complex after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 1 ps and 2 µs as described in the Experimental section.
Figure S7. Time-resolved IR spectroscopy data for a wild-type POR-Pchlide-NADPH ternary complex after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 1 ps and 2 µs as described in the Experimental section.
Figure S8. Time-resolved IR spectroscopy data for Chlide only after photoexcitation with a laser pulse centred at ~450 nm. Time-resolved difference spectra were recorded between 1 ps and 2 µs as described in the Experimental section.
Figure S9. Gaussian fitting of SADS1-4 resulting from the global analysis of the transient IR absorption data for Pchlide (A-D), Y193F (E-H) and wild-type (I-L). The SADS (black dots) have been fitted with a sum (dark red line) of the following Gaussian functions of fixed position and FWHM (in brackets). Negative peaks: 1743 (30), 1687 (32), 1656 (32) (protein only) cm\(^{-1}\). Positive peaks: 1640 (27), 1612 (36), 1578 (28), 1550 (34), 1521 (10), 1505 (20) cm\(^{-1}\). Values of position and FWHM were derived from a fitting of free parameters to all datasets to find common values.
Figure S10. Kinetic traces at selected wavelengths with corresponding fits resulting from a global analysis of the time-resolved visible data for Pchlide only, the Y193F-Pchlide-NADPH ternary complex, and the wild-type POR-Pchlide-NADPH ternary complex after excitation at 450 nm. The data were fitted as described in the Supporting Information.
Figure S11. Kinetic traces at selected wavenumbers with corresponding fits resulting from a global analysis of the time-resolved IR data for Pchlide only, the Y193F-Pchlide-NADPH ternary complex, and the wild-type POR-Pchlide-NADPH ternary complex after excitation at 450 nm. The data were fitted as described in the Supporting Information.
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