Ultrafast Dynamics of the Photo-Excited Hemes *b* and c_n in the Cytochrome b_6f Complex.

Electronic Supplementary Information

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1. Double difference data

The transient signal from the heme *b* and c_n is isolated by subtracting the 2-dimentional time-wavelength transient data set corresponding to the complex while the heme *f* is fully reduced via ascorbate (Figure S1A) from that of the complex while all hemes are reduced via dithionite (Figure S1B). As mentioned in the manuscript, the resulting "double difference" data (Figure S1C) is used to better resolved the heme *b* and c_n signal that are otherwise buried underneath the signal from *β*-carotene (Car) and Chlorophyll *a* (Chl *a*). Spectra at selected time delays are shown in Figure S2.

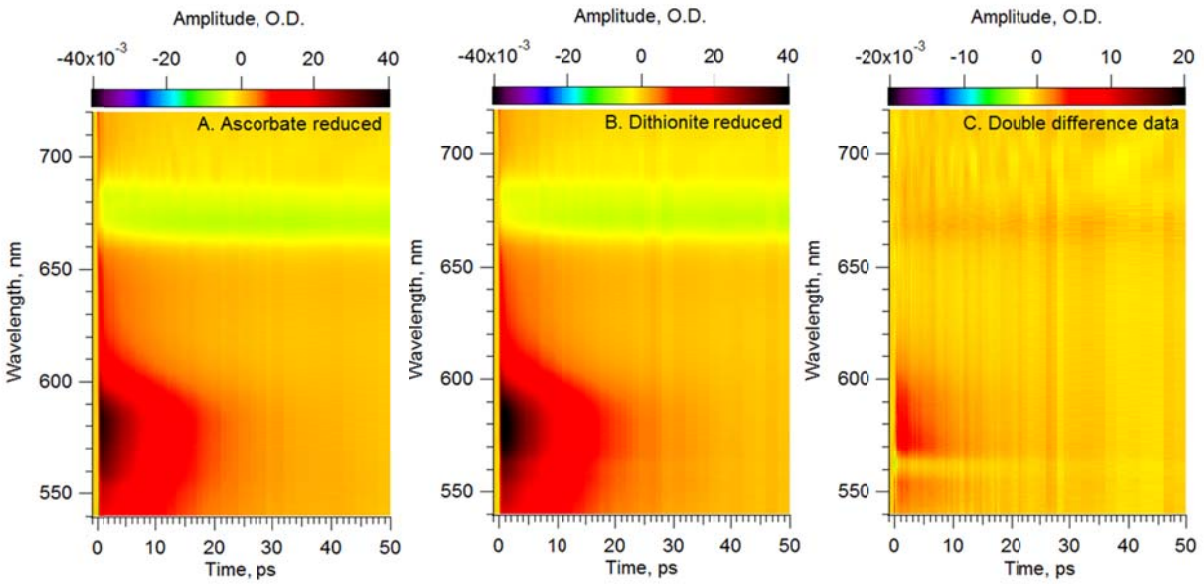


Figure S1: Two dimensional surface data of the cytochrome b_6f when the complex is partially reduced via ascorbate (A) and fully reduced via dithionite (B). Subtraction of A from B results in the so-called double difference data (C).

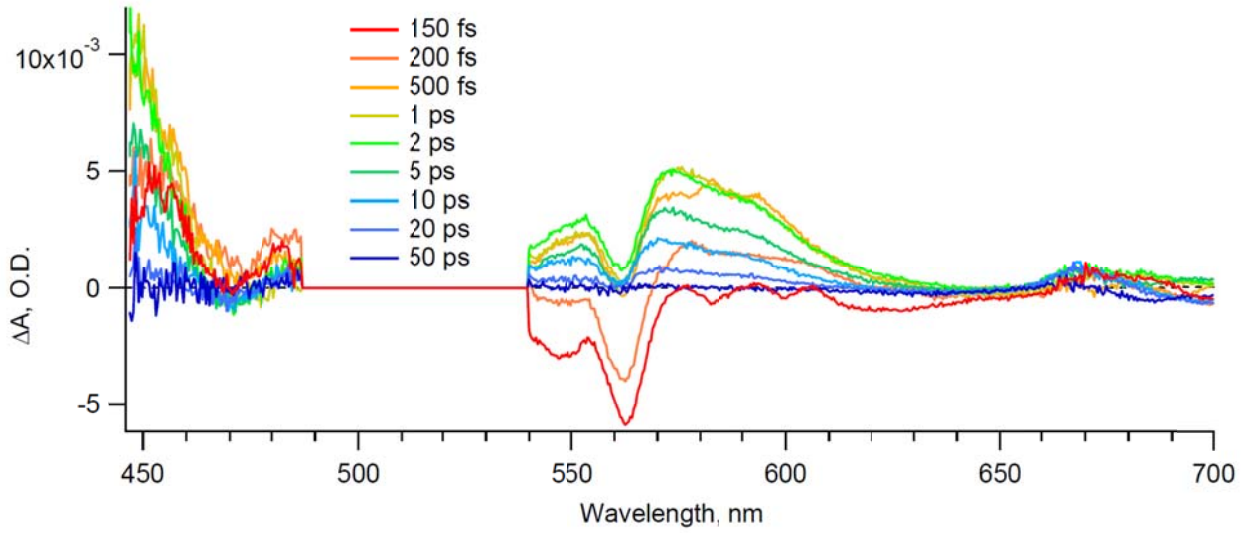


Figure S2: Full range transient absorption signal from the cyt b_6f complex after 515 nm excitation of the hemes *b* and c_n at selected time delays.

2. Extraction of the sole Chl *a* signal

Because the remaining Chl *a* signal is small and comparable to the noise level in the ~670 nm region, the associated components might not be faithfully represented in the global analysis. That is why we precede to an additional and more specific analysis of the Chl *a* signal, starting back from the raw data (Figure S1A and B). The aim is then to disentangle the Chl *a* Q_x signal in the 670 nm region from the underlying Car S_1 signal that extends from 600 up to 700 nm. In this region, the Car signal is broad and featureless,[1] and can be modeled as a first degree polynomial (black line in Figure S2). The fit is done on the region adjacent to the main Chl *a* Q_x signal, as shown in Figure S1. Subtraction of the polynomial function from the initial transient data results in the extracted Chl *a* signal. The same procedure is done independently at each time delay, giving rise to the time-wavelength data shown in Figure 9 in the main manuscript.

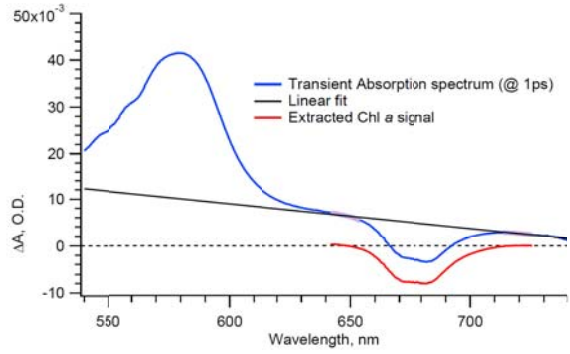


Figure S3: Transient data (blue) of the fully reduced cyt b_6f complex at 1ps after excitation superimposed with the fit of the featureless Car signal adjacent to the Chl *a* signal (purple regions) by a second order polynomial (black). The difference data represents the extracted Chl *a* signal (red).

3. Modelling of the static absorbance

Due to the small amount of sample used during the experiment, the absorbance spectra are taken directly from the probe beam. While the beam passes through the flow cell capillary the light scatters slightly, which adds a featureless background to the spectra. We acknowledge that this background is typically more intense in the UV region than in the near IR. However, for the purpose of the modeling, the assumption of a background with equal intensity over the considered spectral range is sufficient as a first approximation. This background is thus accounted for by setting the absorbance spectra to zero at 720 nm since the absorption of the protein complex is expected to be insignificant above this wavelength, as shown in Figure S4. In each case when the cyt b_6f complex (blue) is reduced by (A) ascorbate and (B) dithionite, the static spectrum is modeled by means of:

- the β-carotene absorbance spectrum in hexane taken from "PhotochemCAD".[2] (orange)
- the Chl *a* absorbance spectrum in methanol. (green)
- the cyt bc_1 spectrum (purple) in similar buffer and reducing conditions to the cyt b_6f complex. It is supposed to mimic the absorbance of the hemes *b*, *f* and Rieske protein.
- an extra Gaussian (pink) to represent the heme c_n spectrum in this region.

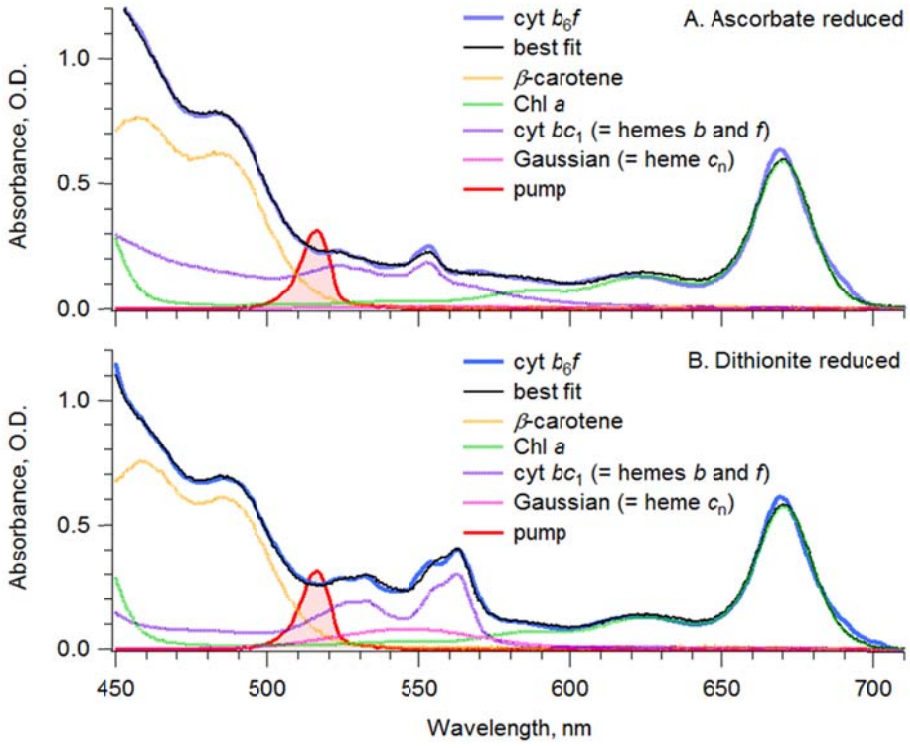


Figure S4: Modelling of the static absorbance of the cyt b_6f complex.

The amplitude and position of each spectral component is let free. The magnitude of each shift is shown in Table S1. Best fit is shown in black and in red is the spectrum of the excitation pump. Taking into account the roughness of the background subtraction, the fact that both the monomeric Car and Chl *a* absorbance spectrum are taken in environments that differs from their

embedded counterparts, and that the binding of the heme *f* differs from that of the heme c_1, the quality of the fit is nonetheless remarkable and sufficient for its purpose.

	Ascorbate reduced	Dithionite reduced
Chl *a*	- 1.4 nm	- 1.0 nm
β-carotene	+ 6.7 nm	+ 8.5 nm
Cyt. bc_1 complex	- 0.4 nm	- 1.2 nm

Table S1: Magnitude of the shifts with respect to the original spectra.

In order to evaluate the relative absorption cross section of the different pigments, the pump spectrum is directly multiplied by the spectrum of each component as given by the fits. The absolute and relative integration of the resulting curve is given in Table S2:

	Total	Chl *a*	*β*-carotene	Cyt. bc_1 (= hemes *b* and *f*)	Gaussian (= heme c_n)
Ascorbate reduced	1.07	0.07	0.38	0.61	0.01
	100 %	6 %	36 %	57 %	1 %
Dithionite reduced	1.15	0.07	0.44	0.5	0.14
	100 %	6 %	38 %	44 %	12 %

Table S2: Relative absorption cross section of the light excitation by the different pigments.

4. Singular Value Decomposition analysis

a) Analysis of the double difference data

The double difference data (Figure S1C) is first analyzed by globally fitting (GF) a selection of kinetics representative of the major transient spectral features. The resulting exponential components are then used as initial parameters to fit the Eigen-kinetics that results from a Singular Value Decomposition (SVD) of the whole data-set (while omitting the portion containing cross-phase modulation).[3] The resulting Eigen-values associated with each Eigen-kinetic and Eigen-spectrum is depicted in Figure S5. In the present case, only the first three components are taken into consideration. Even so the subsequent Eigen-values are comparable to the third one considered, the associated Eigen-kinetic did not contribute to the refinement of the fitting parameters due to their poor signal-to-noise ratio.

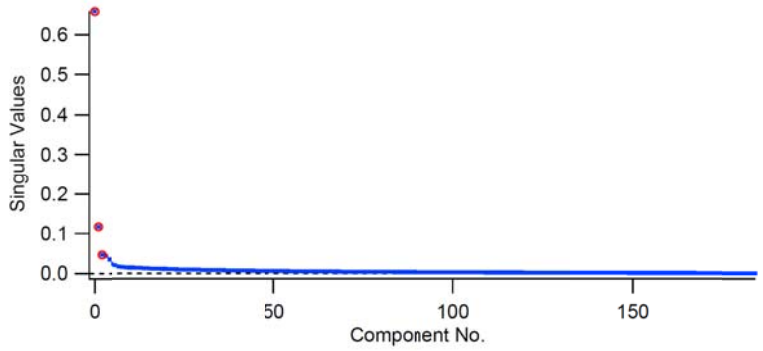


Figure S5: Eigen-values resulting from the SVD analysis of the whole double difference data set. Only the first three values are considered.

The number of exponential components as well as their values are then refined until they satisfactorily fit both GF and the set of Eigen-kinetic. The first three Eigen-kinetics and their fits are shown in Figure S6.

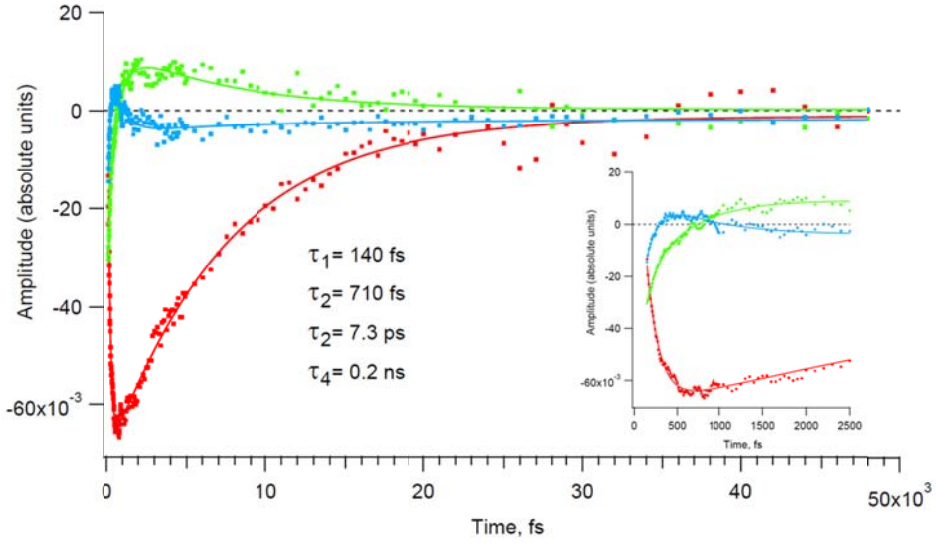


Figure S6: Eigen-kinetics resulting from the SVD analysis of the whole double difference data set and their fits (smooth curves). A close up of the first 2.5 ps is shown in the inset.

b) Analysis of the extracted Chl *a* signal

As mentioned in the manuscript, the analysis is done in the case in which the heme *b* and c_n are oxidized and in the case when the complex is fully reduced via dithionite. The extracted signals from Chl *a* are first analyzed by globally fitting the corresponding of kinetics. The resulting exponential components are then used as initial parameters to fit the Eigen-kinetics that results from the SVD (while omitting the portion containing cross-phase modulation).[3] The resulting Eigen-values associated with each data set in shown in Figure S7.

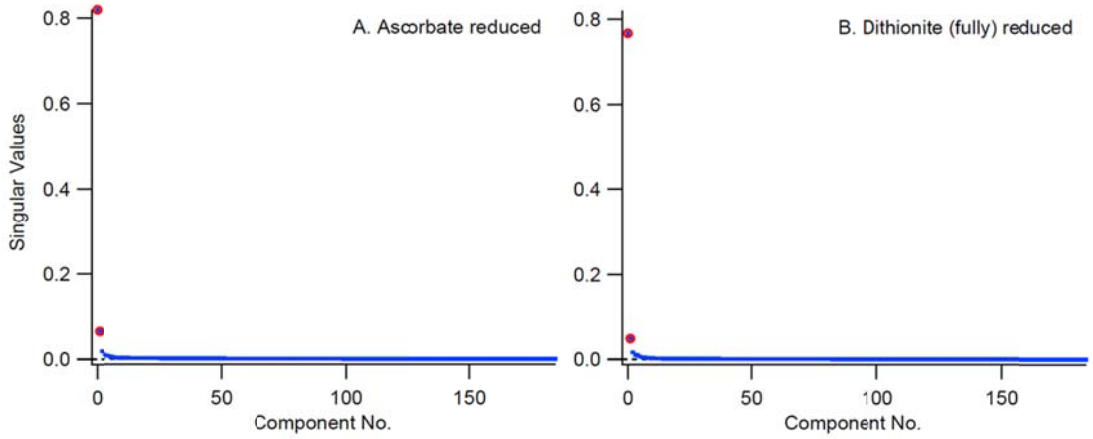


Figure S7: Eigen-values resulting from the SVD analysis of the extracted Chl *a* data set; only the first two values are considered.

In each case, only the first two components are taken into consideration. The number of exponential components as well as their values are then refined until they satisfactorily fit both GF (done of the kinetics shown in Figure 10 of the main manuscript) and the set of Eigen-kinetic. Each set of Eigen-kinetic and their best fit is shown in Figure S8 and Figure S9.

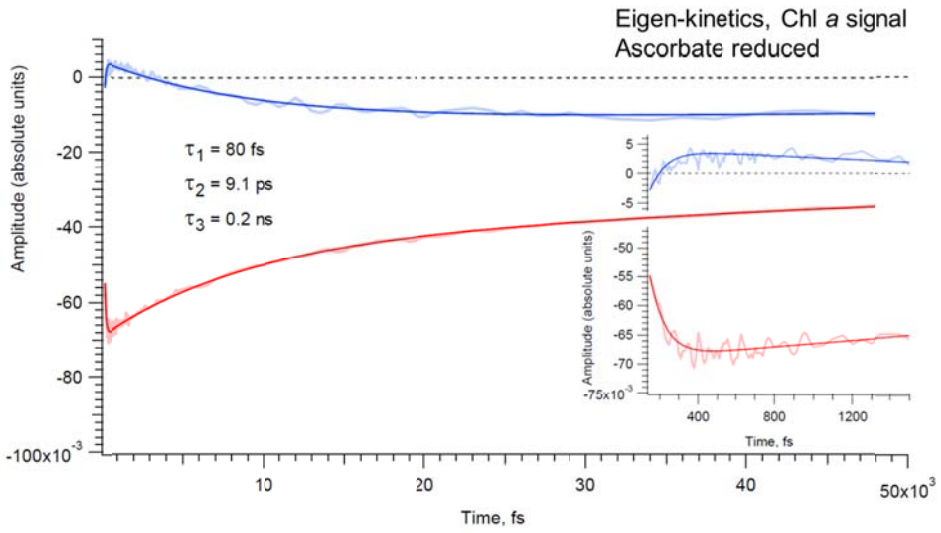


Figure S8: Eigen-kinetics resulting from the SVD analysis of the extracted Chl a data set while the complex is partially reduced via ascorbate, superimposed with the best fits (smooth curves). A close up of the first 1.5 ps is shown in inset.

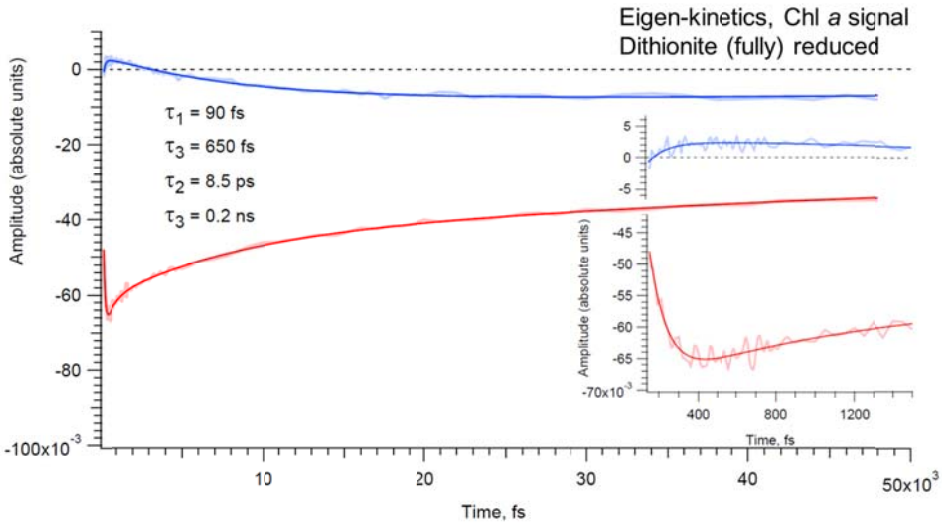


Figure S9: Eigen-kinetics resulting from the SVD analysis of the extracted Chl a data set while the complex is fully reduced via dithionite, superimposed with the best fits (smooth curves). A close up of the first 1.5 ps is shown in inset.

From each of these SVD analysis is generated the corresponding Decay Associated Spectra (DAS) shown in Figure 11 of the main manuscript.

5. Frame-by-frame analysis of the heme *b*

a) Fitting parameters

The heme *b* signal is assumed to be a superposition of a bleach (blue solid curve), characterized by the oxidized-minus-reduced spectrum, and a shift (green solid curve), characterized by the difference between the static heme *b*'s reduced-oxidized spectrum (pink dashed curve) and its shifted duplicate (purple dashed curve), as represented in Figure S 10. The fitting procedure was done using Matlab's minimizing function called "patternsearch" in order to avoid falling into local minimum.

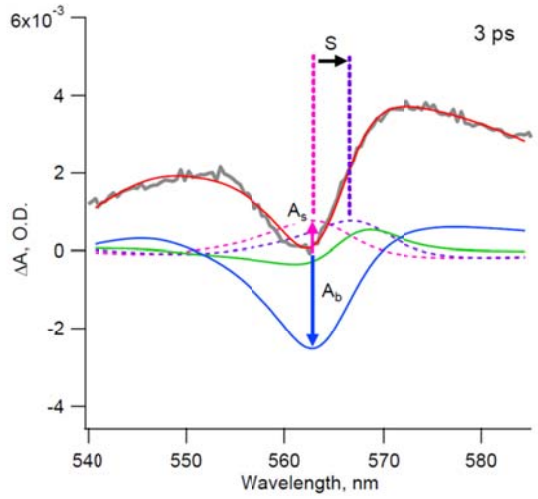


Figure S 10: Visual representation of the fitting parameters.

The time evolution of the different fitting parameters is plotted in Figure S 11.

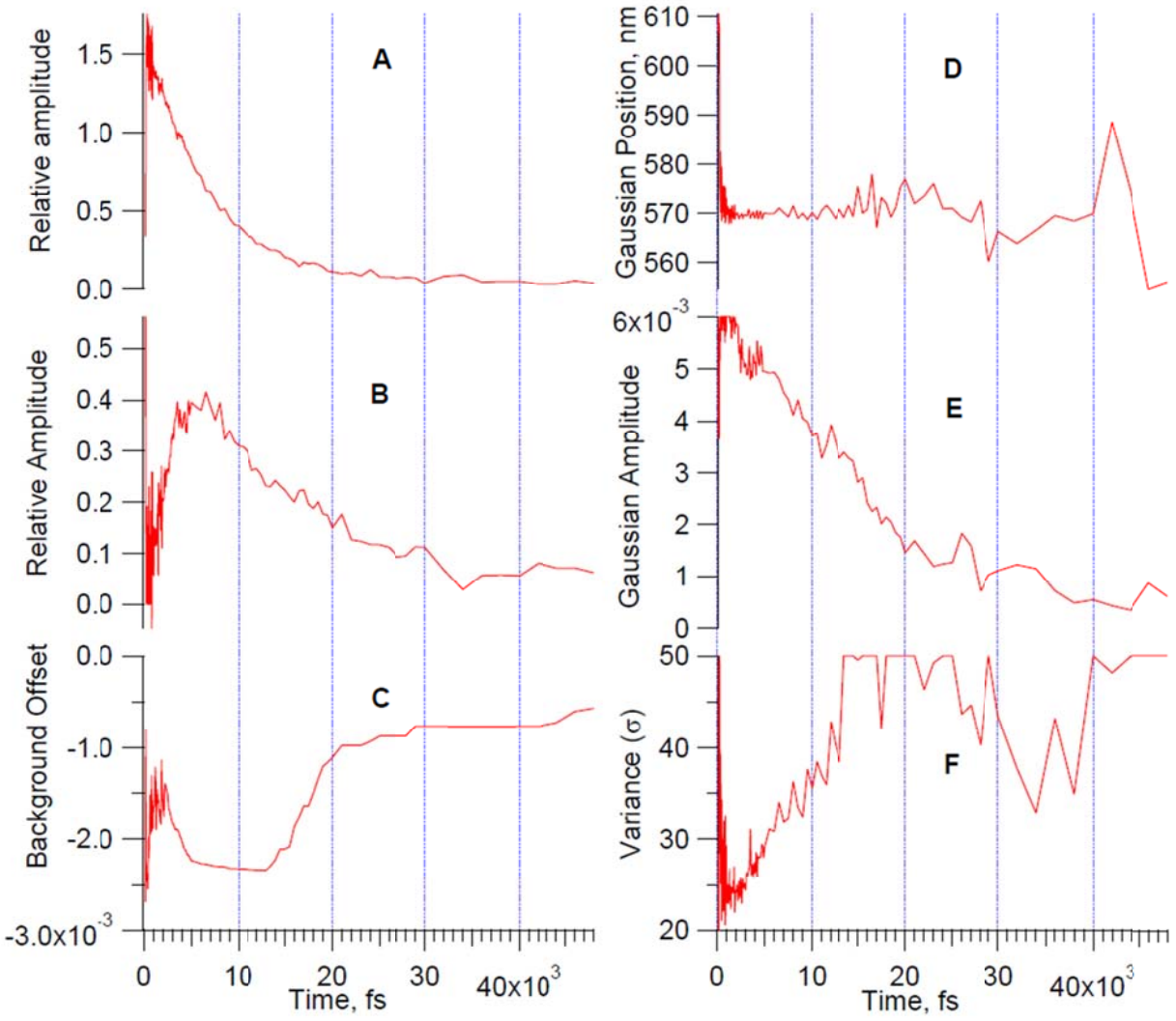


Figure S 11: Time evolution of the fitting parameters for a shift magnitude, i.e. parameter "S", set equal to +4.0 nm. Panel A shows the amplitude of the reduced-minus-oxidized heme *b* spectrum used for the reconstruction of the bleach, i.e. parameter "A_b", relative of the heme *f* static spectrum. Panel B show the amplitude of the reduced-minus-oxidized heme *b* spectrum used for the reconstruction of the shift, i.e. parameter "A_s", relative of the heme *b* static spectrum. Panel C shows the amplitude of the offset used in order to account for the background noise, in absolute units. Panel D shows the position of Gaussian function used to model the Car signal. Panel E shows the amplitude of that Gaussian function, in absolute units. Panel F shows the "σ" value of the Gaussian, in absolute units.

Note that the shift in the first hundreds of femtoseconds of the Gaussian's position (Figure S 11, Panel D) from 610 to 570 nm corresponds in fact to the interval conversion from the Car S2 to S1. The amplitude of the Car signal then decreases following the kinetic of Figure S 11, Panel E. A flat background is added to compensate for any possible fluctuation due to light scattering (Figure S 11, panel C), but is only relevant at long time delays (> 20 ps) when the signal amplitude approaches the noise level.

b) Fitting quality at multiple "S" values.

The same procedure is repeated for a range of fixed shift magnitudes ("S" parameter), from 0.4 to 7.9 nm. In each case, the fitting quality is assessed by calculating the mean-square deviation (χ^2) of the fit from the data, and plotted as a function of the shift magnitude in Figure S 12.

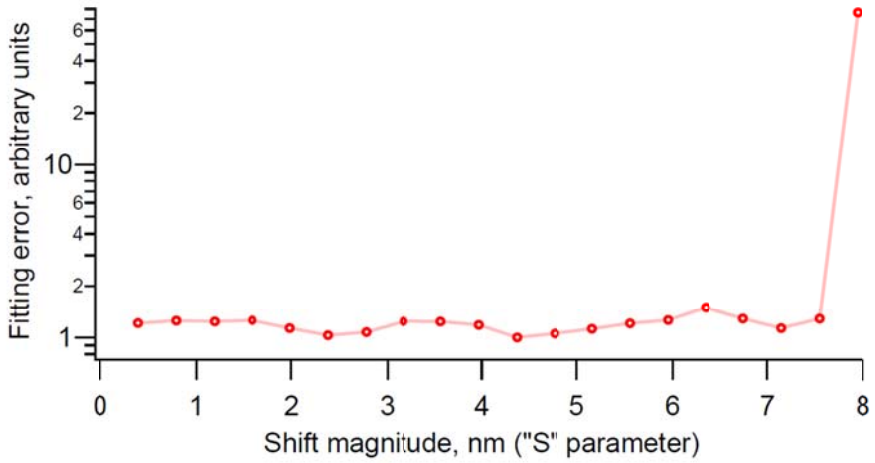


Figure S 12: Fitting quality is assessed by calculating the mean-square deviation of the fit from the data (χ^2).

It is interesting that regardless of the set "S" value, the fit quality is similar as long as "S" is smaller than 8 nm. This is due to the fact the "S" and "A_s" are complementary to one another, i.e. any variation in "S" is compensated by the opposite variation in "A_s" as illustrated subsequently. In order to select a particular "S" value, we refer to the kinetic model proposed in the main manuscript and check how well the model fits the kinetics of the "A_b" and "A_s" parameters. The degree of correspondence between the model and the "A_b" and "A_s" parameter's kinetics is plotted in Figure S 13.

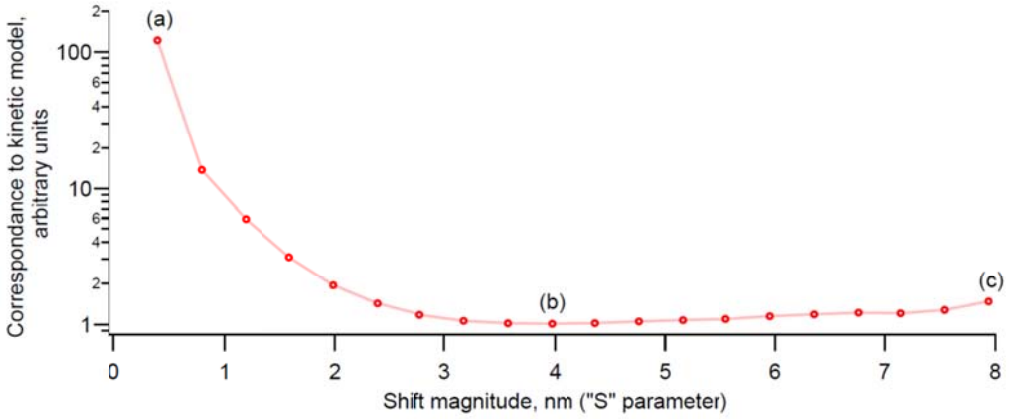


Figure S 13: Correspondence between the kinetics of the fitting parameters "A_b" and "A_s" with the kinetic model proposed, calculated via their mean-square deviation (χ^2), in function of the set "S" parameter.

The best correspondence between fit and model is found for "S" equal to 4.0 nm. The labels (a), (b) and (c), in Figure S 13, correspond to shift magnitude set to 0.4, 4.0 and 7.9 nm respectively. In each case, the corresponding "A_b" and "A_s" parameter's kinetics are plotted in Figure S 14.

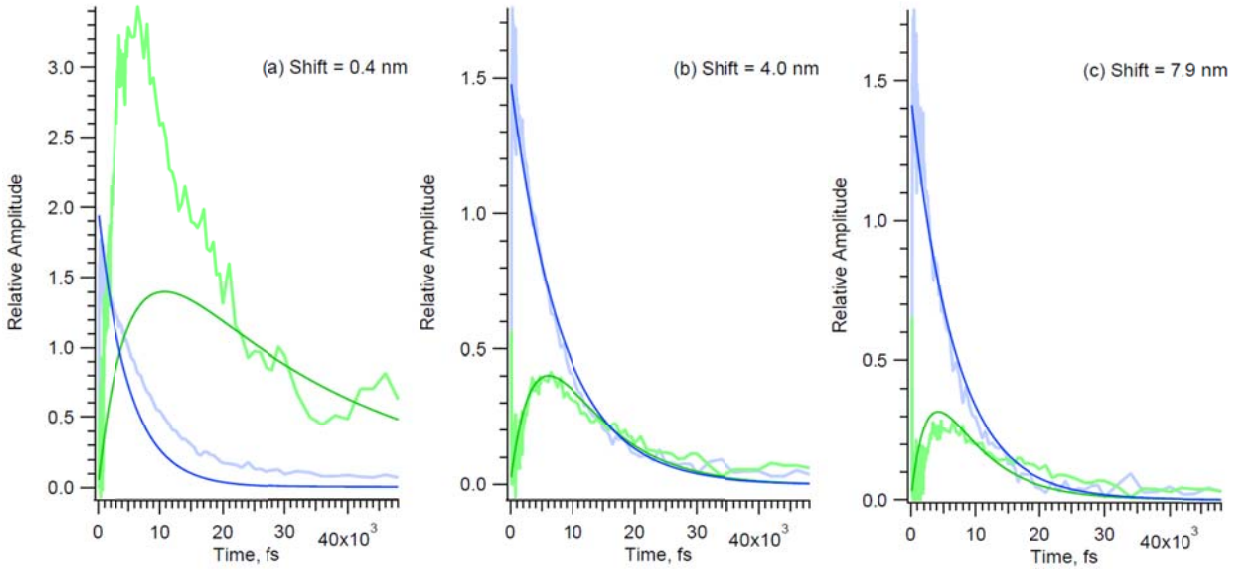
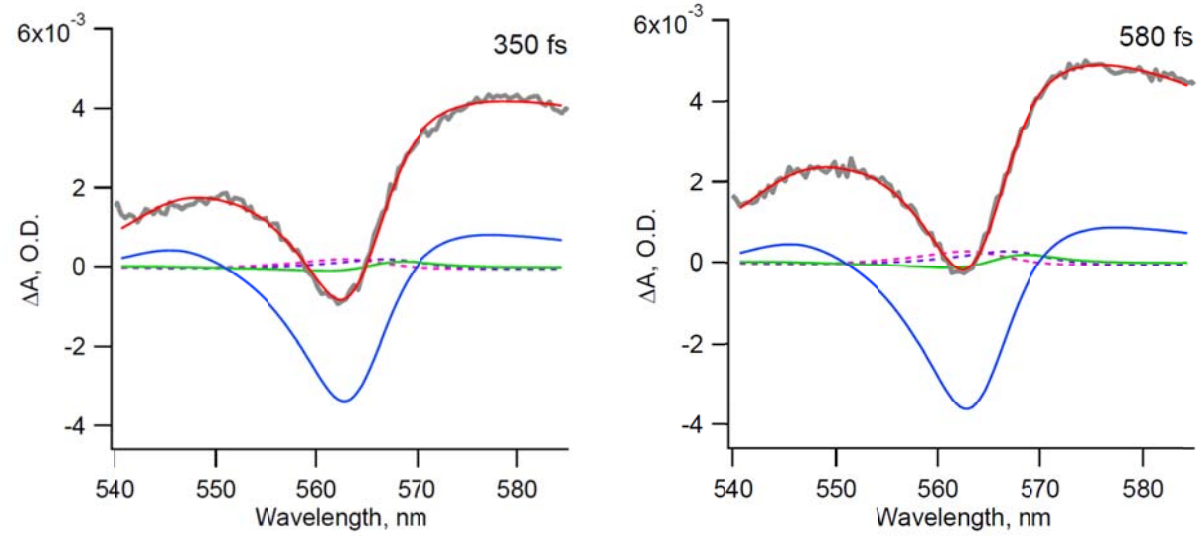


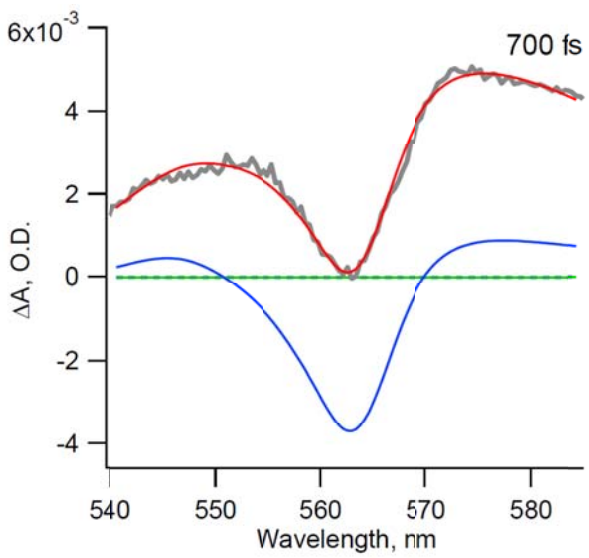
Figure S 14: Kinetics of the "A_b" (blue) and "A_s" (green) parameters superimposed with their best fit (smooth lines) while using the kinetic model described by equation (1) and (2) from the main manuscript.

We note that, while the evolution of the "A_b" parameter remain quasi identical in each case, the amplitude of the "A_s" differs greatly. The overall shape of the "A_s" parameter however remains quasi identical in each data set, as if the kinetic was simply weighted by the inverse of the "S" value: multiplying "S" by 2 is approximately equivalent to dividing "A_s" by 2.

c) Fitting results at selected time delays (while "S" = +4 nm).

For all following spectra, the grey curve is the data, red is the fit, blue and green are the bleach and the shift contribution to the fit, respectively. The dashed pink and purple curves represent the initial and final (shifted) α-band contribution to the shift (green curve), respectively. We show only the fits of 8 spectra out of a total of the 185 time delays.

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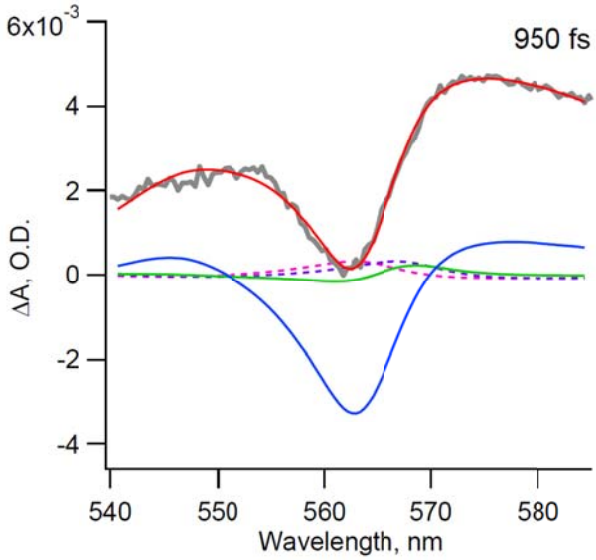
4
2
0
-2
-4
ΔA, O.D.
540
550
560
570
580
Wavelength, nm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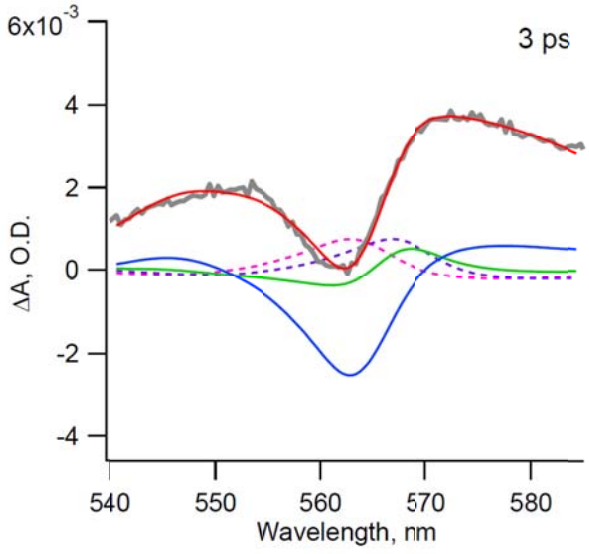
Wavelength, nm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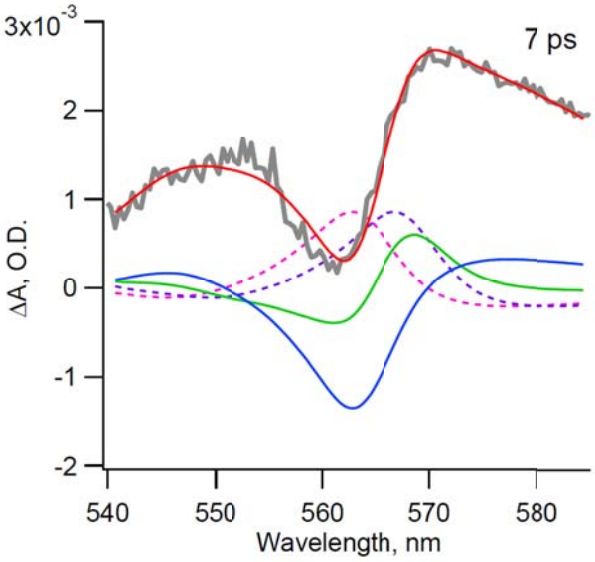
Wavelength, nm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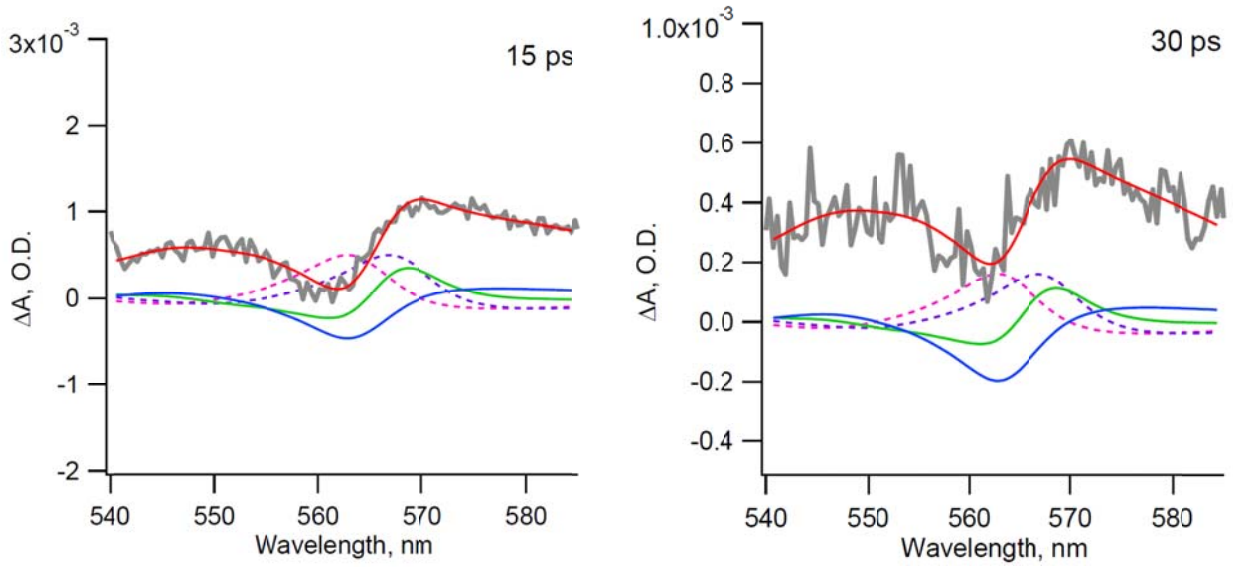

Figure S 15: Fitting results at multiple time delays. The fit (red curve) to the data (grey curve) consists of the oxidized-minus-reduced spectrum of heme f (blue curve) and the heme f band shift (green curve). The band shift itself is the difference between the fixed heme f reduced-minus-oxidized spectrum (pink dotted curves) and its shifted duplicate (purple dotted curves). The Gaussian used to model the background signal of the Car molecule is omitted for clarity.

References:

1. T. Lenzer, F. Ehlers, M. Scholz, R. Oswald and K. Oum, *Phys. Chem. Chem. Phys.*, 2010, **12**, 8832-8839.
2. J. M. Dixon, M. Taniguchi and J. S. Lindsey, *Photochem. Photobiol.*, 2005, **81**, 212-213.
3. J. Helbing, L. Bonacina, R. Pietri, J. Bredenbeck, P. Hamm, F. van Mourik, F. Chaussard, A. Gonzalez-Gonzalez, M. Chergui, C. Ramos-Alvarez, C. Ruiz and J. López-Garriga, *Biophys. J.*, 2004, **87**, 1881-1891.