Supplementary Information

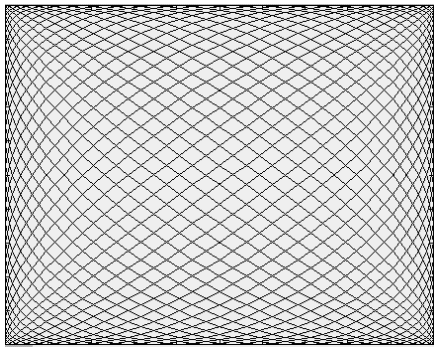

Figure S1. A pattern of the Lissajous sample scanner, the speed of which is such that it returns to the same spot after 1 minute. Sample cell was 2 cm diameter, and 200 mkm thick.

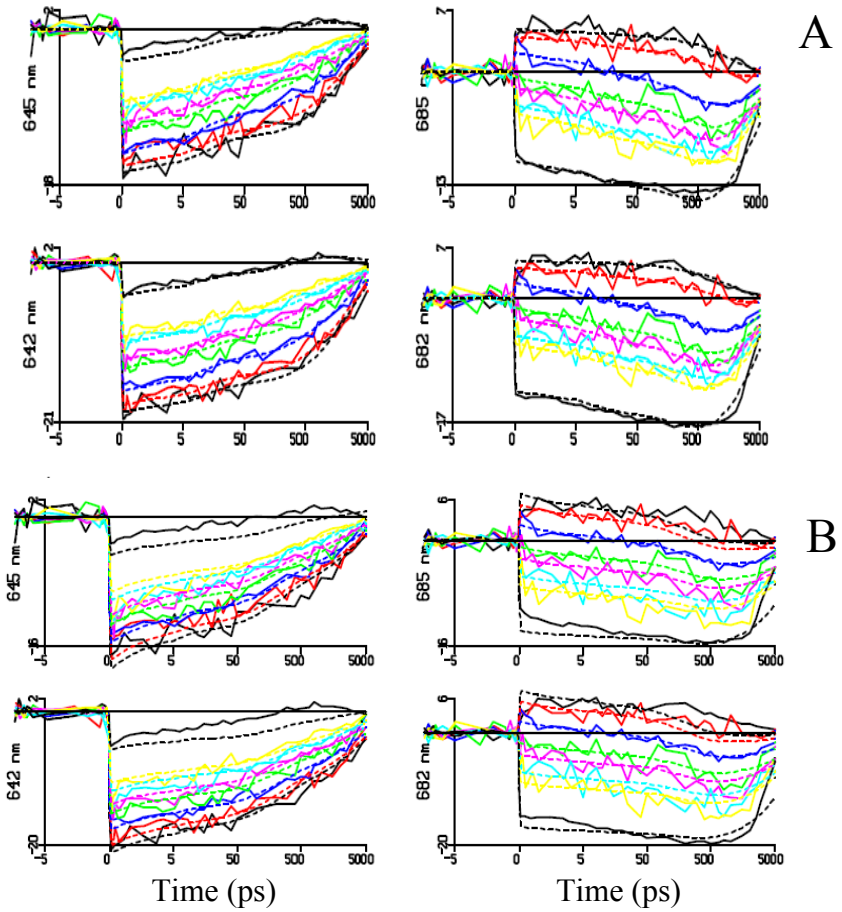

Figure S2. Time traces recorded at 642, 645, 682 and 685 nm. Scans 1-2 (black lines close to red), 3-5 (red), 6-18 (blue), 19-25 (green), 26-35 (magenta), 36-45 (cyan), 46-55 (yellow), 55+ CW illumination (black close to yellow). Scans 36-45 were measured in reversed-time direction to exclude that the illumination effect is caused by changing sample conditions during a scan. The time traces are the

average of traces recorded on three freshly prepared samples. The dotted lines are the fit resulting from the model described in figure 3.4 with inactive branch included in the model (**A**), and with inactive branch constrained to 0 (**B**).

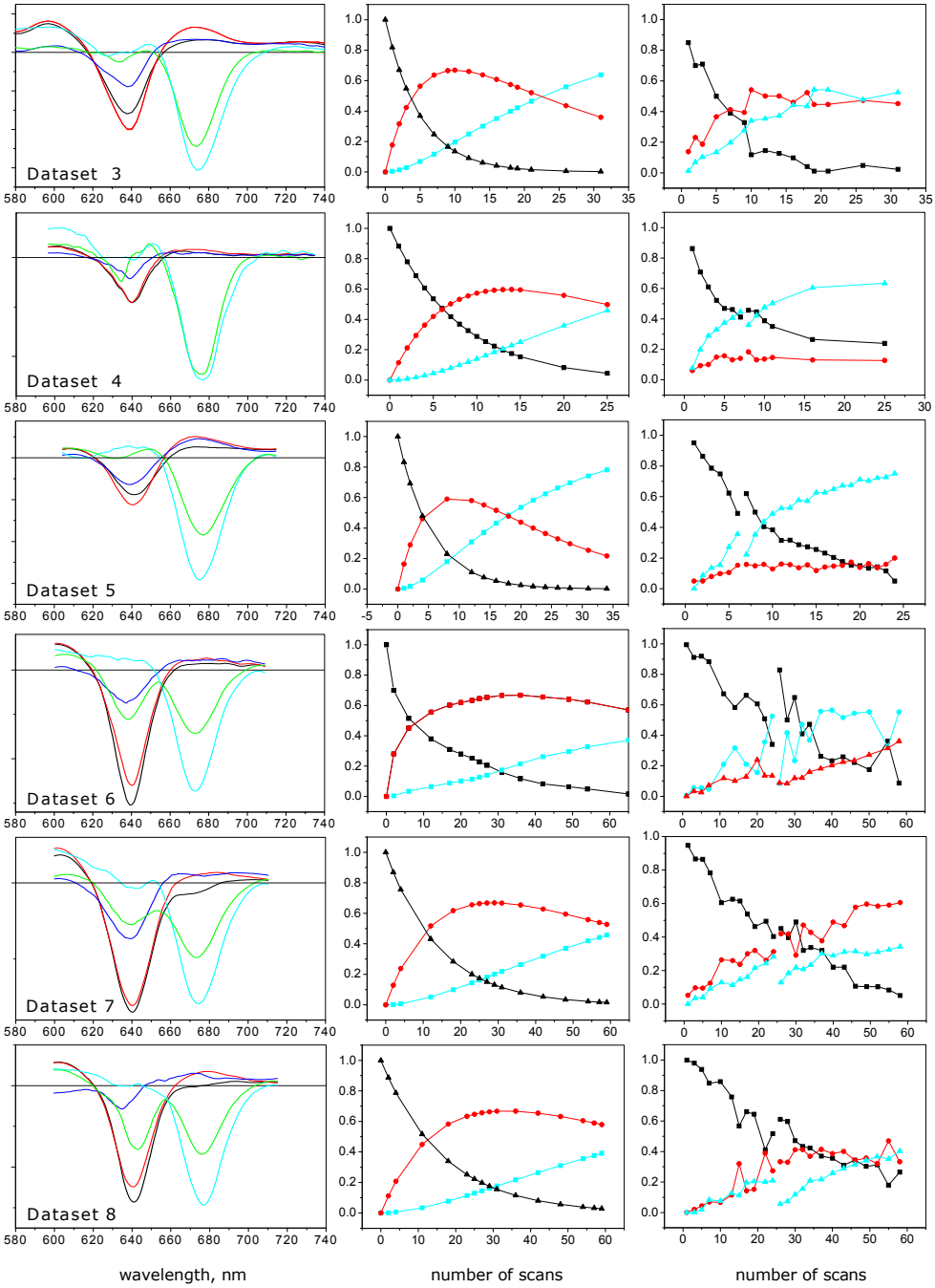

Figure S3. Left column: SADS estimated in the target analysis with analytical model. **Middle column:** relative concentrations as a function of scan number (analytical model). **Right column:** relative concentrations obtained in free model. In fraction fits black lines are unproductive enzymes, red lines are productive enzymes, cyan lines are accumulated Chlide.

Table T1. The lifetimes in picoseconds, $\theta 1^{-1}$, $\theta 2^{-1}$, $\theta 3^{-1}$, $\theta 4^{-1}$, for spectral evolutions of compartments and $\theta 5^{-1}$, $\theta 6^{-1}$ for I675* formation estimated in the target analysis of different experimental sessions. The parameter $\theta 7^{-1}$ was estimated in the models as 0 and not shown in table. Unless indicated otherwise, the numbers refer to thermophilic POR. Parameter λ denotes excitation wavelength in nanometers, used in each experimental session; **CW** indicates an illumination with the tungsten lamp. Parameter **P** indicates experimental protocol used in each experimental session, the protocol schemes are shown in figure 3.9. Parameter ***k1*** is excitation density. The expression for quantum yield ***QY*** of I675* formation is given in Discussion section. Parameter ***QY*** of Chlide is calculated as k2/k1, where k2 is derived from analytical model 2 for the Active → Product mechanism.

n	Sample	λ	**Number of fresh samples**	**Number of Sub-datasets**	**Number of scans in each sample**	$\theta 1^{-1}$	$\theta 2^{-1}$	$\theta 3^{-1}$	$\theta 4^{-1}$	$\theta 5^{-1}$	$\theta 6^{-1}$	***k1***	***QY* of I675***	***QY* of Chlide, *k2 / k1***
1	NADPH, H_2O	475	4	3	10	7	170	2700	4500	10	500	0.1	0.56	0.3
2	NADPH, H_2O	475	6	6	55+CW	7.5	650	5100	4500	8	289	0.04	0.84	0.35
3	NADPH, H_2O	475	1	15	31	7.8	600	8400	3796	12	378	0.2	0.76	0.20
4	NADPH, H_2O	475	4	17	25	4.3	270	2700	1200	11	350	0.12	0.59	0.30
5	NADPH, H_2O	475	2	15	34	4.4	240	1800	1800	8	578	0.18	0.54	0.30
6	NADPH, H_2O	640	1	15	61	6	460	2660	2600	12	462	0.05	0.66	0.20
7	NADPH, H_2O	640	1	15	59	6.2	340	1500	2300	15	370	0.13	0.63	0.15
8	NADPH, H_2O	640	1	15	59	5.9	710	4000	2000	9	357	0.06	0.79	0.20
9	NADPH, H_2O	475	1	17	CW+25	6	390	2300	1600	9	206	--	0.79	--
10	NADPH, H_2O	475	1	17	CW+70	5.7	120	2700	1400	9	256	--	0.57	--
11	NADPH, H_2O	475	1	17	CW+20	4.8	190	3000	1700	8	269	--	0.63	--
12	NADPD, H_2O	475	2	15	67	4.8	180	1900	2400	5	486	0.07	0.51	0.30
13	NADPD, H_2O	475	1	15	47	3.9	120	1800	2200	8	373	0.035	0.47	0.34
	Mean					**6**	**350**	**3100**	**2500**	**10**	**360**	**0.10**	**0.64**	**0.26**
	St.dev.					**1**	**200**	**1700**	**1050**	**2**	**100**	**0.06**	**0.11**	**0.06**
14	NADPH, H_2O	475	3	3	CW+1	7.5	230	2900	2120	8.4	149	-	0.79	-
15	NADPH, D_2O	475	3	3	CW+1	7.5	230	2900	2120	17	212	-	0.66	-

Table T2. The structure of composite dataset is illustrated in the following diagram, using the composite dataset n=1 from Table T1:

Sub-datasets used to generate composite dataset	**Fresh sample 1**	**Fresh sample 2**	**Fresh sample 3**	**Fresh sample 4**	**Fresh sample 5**
The first averaging among scans and samples, i.e. 5x3scans = 15 scans in total	Scan 1 Scan 2 Scan 3	Scan 1 Scan 2 Scan 3	Scan 1 Scan 2 Scan 3	Scan 1 Scan 2 Scan 3	Scan 1 Scan 2 Scan 3
The second averaging among scans and samples: 5x3scans=15 scans	Scan 4 Scan 5 Scan 6	Scan 4 Scan 5 Scan 6	Scan 4 Scan 5 Scan 6	Scan 4 Scan 5 Scan 6	Scan 4 Scan 5 Scan 6
The third averaging among scans and samples: 5x4=20 scans	Scan 7 Scan 8 Scan 9 Scan 10	Scan 7 Scan 8 Scan 9 Scan 10	Scan 7 Scan 8 Scan 9 Scan 10	Scan 7 Scan 8 Scan 9 Scan 10	Scan 7 Scan 8 Scan 9 Scan 10

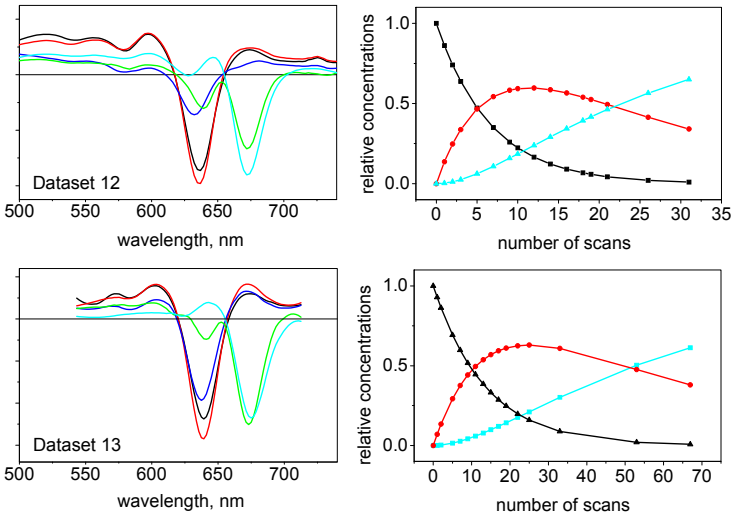

Figure S4. SADS and fractions obtained in analytical model 2 for the protonated enzyme in combination with NADPD, i.e. in H_2O Tris/Triton POR:PChlide:NADPD sample (datasets 14, 15) . The corresponding time constants are shown in table T1.

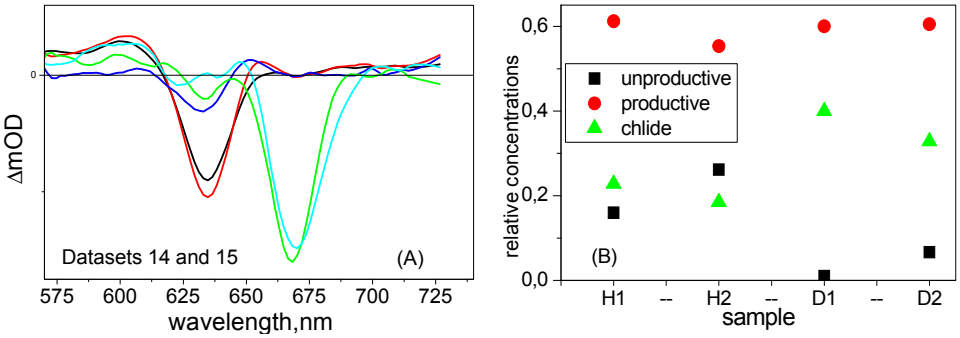

Figure S5 SADS obtained in the simultaneous target analysis with free fractional model 1 of protonated and deuterated enzyme in combination with NADPH. **(B)** Relative concentrations obtained in free fractional model 1 for two solvents. The samples H1, H2 are indicated as dataset 16 in table T1, samples D1, D2 are indicated as dataset 17 in table T1.

Excitation densities

Besides the target analysis, which enabled us to estimate the parameter of excitation density k_1 for each dataset individually, as detailed below, there are two experimental methods to determine the fraction of excited molecules in each laser shot. One can calculate the number of absorbed photons relative to the total number of molecules in the laser focus, using the following expressions:

$$N_{photons} = \frac{E - E \cdot 10^{-OD}}{E_{onephoton}}$$

where E is the laser pulse energy (100 nJ in most of experiments), OD is the optical density of the sample at the excitation wavelength (475 nm or 640 nm); The number of molecules is:

$$N_{total} = N_A \cdot C \cdot V$$

where N_A is Avogadro's number, and C is the sample concentration:

$$C = \frac{OD}{\varepsilon \cdot path}$$

The concentration can be determined from the absorption of the sample at a wavelength where an extinction coefficient is known, in our case this was 640 nm for PChlide bound to protein, and 630 nm when PChlide is unbound, with an extinction coefficient:

$$\varepsilon = 30.1\frac{L}{mM \cdot cm}$$

V is the illuminated volume, which depends on the focus size, in our case it was 160 µm. Applying these formulas to,for example a sample with the absorption spectrum shown in figure 2, one obtains an excitation density of 0.05 (or 5%). It is slightly underestimated as compared to the result from the target analysis (on average 0.1) because in target analysis the fraction of unbound PChlides is neglected and $N_{bound} < N_{total}$.

A similar result can be obtained when the ratio of the total number of photons which the sample absorbs after one sample scan, relative to the total number of molecules in the sample cell is calculated using the following expression:

$$N_{total} = N_{photons} \cdot F \cdot T_{scan} \cdot N_{scans}$$

where F is pump repetition rate, 500 Hz, T_{scan} is the duration of one sample scan, i.e. the total time that the sample cell is illuminated by the pump, usually one scan of the delay line takes 1 minute; N_{scans} is number of scans of a sample. The number of molecules in a sample cell can be estimated in the same manner as given above, where V is the sample volume corresponding to the illuminated area of the entire sample cell, which was 80 µL. Using this method one can obtain an excitation density of 0.045 (or 4.5%) of molecules which are excited in one sample scan.

Another possibility is to determine the excitation density from a calculation of the ratio of the integral of the bleached signal immediately after photo excitation to the integral of the Q_Y absorption in the steady-state regime, multiplied by 0.5 to account for the contribution of the stimulated emission to the absorption difference signal. This estimate results in ≤0.05 (or, 5% which slightly depends on the spectral window selected for integration) when 100 nJ power was used.

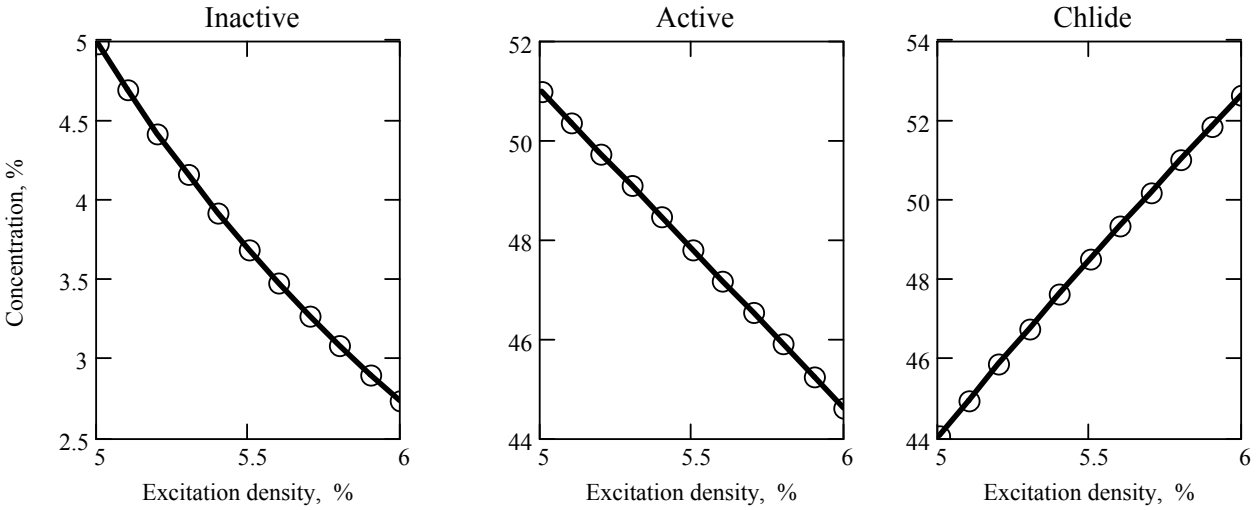

Figure S6. Concentrations of Inactive, Active fractions and Chlide as a function of excitation density obtained with analytical model for 60 sample scans.