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Electronic Supporting information

Cooperativity in Enzyme-Substrate Complex Formation in Nitrogenase-like Dark Operative Protochlorophyllide Oxidoreductase (DPOR)

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Raw Data

All raw data are available on Zenodo (DOI: 10.5281/zenodo.15051525).

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The Hill equation:

$$\Theta = \frac{[L]^{nH}}{(K_{\mathrm{d}} + [L]^{nH})}$$

Where Θ is the fraction of binding sites occupied, [L] is the ligand concentration, K_{d} is the dissociation constant, and nH is the Hill coefficient.[1–3]

BchNB and BchL protein purification

The protocol for the production and purification of BchNB and BchL has been previously described, although a brief protocol follows.[4,5] BchNB and BchL were expressed from the plasmids pHANB1 and pHAL1, respectively, encoding either BchNB with a Strep-tag or BchL with a Strep-tag. Both proteins were produced individually in *E. coli* BL21 Δ*iscR*.[6] Purification was performed in an anoxic glovebox (COY Laboratory Products, Michigan, USA; $[N_2] > 95\%$, $[H_2] < 5\%$, $[O_2] < 5$ PPM) using an Äkta Go system with StrepTrap XT columns (5 mL, Cytiva), and protein elution was realized with 50 mM biotin. Both proteins were eluted into Tris:HCl buffer at pH 8 (protein concentration ~10 mg/mL for BchNB and 6 mg/mL for BchL), and stored in liquid nitrogen until use. The specific activity of DPOR was determined to be 986 ± 13 $pmol_{Chlide}$ min^{-1} mg^{-1}.

Pchlide extraction from *Rhodobacter capsulatus*

As reported by Fujita *et al.*, protochlorophyllide (Pchlide) was extracted from the culture supernatant of *R. capsulatus* strain ZY5 (Δ*bchL*) and its concentration was determined in 80% v/v acetone, using the extinction coefficient $\varepsilon_{626\ nm}$ = 30,400 M^{-1} cm^{-1}.[7]

Real-time enzyme-substrate complex (ES-complex) formation

The assays were conducted in 2 mL of 100 mM HEPES buffer (pH 7.5) containing 150 mM NaCl at 22 °C. The experiments were conducted using 8 µM BchNB, and a total of 22 µM Pchlide was added in ~1 µM increments at each time point. The assays were performed in an Ar-filled anoxic glovebox (Jacomex, France, $[O_2] < 1$ ppm) using an Autolab UV/VIS/NIR spectrophotometer (Metrohm, Switzerland), with the spectrophotometer connected to the glovebox *via* 200 µm fiber optic cables. Absorbance was measured continuously, and spectra were recorded every 2 seconds.

Data processing

The data for kinetic constant assays and the investigation of cooperativity were processed using GNU Octave, which determines the contribution of each species to the absorbance spectrum based on the provided reference spectra (as reported previously[5]). Within the script, the reference spectra are smoothed using splines, and the following fitting function is created:

$$f(l) = p_1 \cdot S_S(l - p_6) + p_2 \cdot S_P(l - p_7) + p_3 \cdot S_{ES}(l - p_8) + p_4 + \frac{p_5}{l^3}$$

The spline-smoothed reference spectra for the substrate, product, and complex are denoted as S_S, S_P, and S_{ES}, respectively. The fitting function involves seven parameters. Parameters p_1, p_2, and p_3 represent the proportionality factors that define the contributions of each species to the analyzed spectrum. Parameters p_4 and p_5 account for any baseline shifts and possible precipitation in the sample. The remaining parameters are used to address potential wavelength shifts caused by spectrophotometer instability. Each parameter can either be optimized or fixed at a specific value. In our analysis, p_6, p_7, and p_8 are set to 0, and no significant wavelength shifts were observed. The Octave function "nonlin_curvefit" is employed to fit the function f(λ) to the analyzed spectrum, with the optimization results displayed at the end of the script.

Later, those curves were fitted with GraphPad by using exponential models involving a plateau.

- For one-phase kinetics the model used is “Plateau followed by one phase association”.
- For two phases kinetics, the model used is “two-phase association”.

Simulations

In the simulation, Gnu Octave was used to solve ordinary differential equations (ODE) to assess the change in concentration in function of time. Here, the ODEs for Models 1-3 are reported with the values of the applied rate constants.

Model 1:

$$\frac{d\mathrm{S}}{dt} = -k_1\mathrm{S} \cdot \mathrm{E} + k_{1r}\mathrm{C}_1 - k_2\mathrm{S} \cdot \mathrm{C}_1 + k_{2r}\mathrm{C}_2$$

$$\frac{d\mathrm{E}}{dt} = -k_1\mathrm{S} \cdot + k_{1r}\mathrm{C}_1$$

$$\frac{d\mathrm{C}_1}{dt} = k_1\mathrm{S} \cdot \mathrm{E} - k_{1r}\mathrm{C}_1 - k_2\mathrm{S} \cdot \mathrm{C}_1 + k_{2r}\mathrm{C}_2$$

$$\frac{d\mathrm{C}_2}{dt} = k_2\mathrm{S} \cdot \mathrm{C}_1 - k_{2r}\mathrm{C}_2$$

The rate constant values used are:

- Positive cooperativity

$k_1 = 0.05$

$k_{1r} = k_1/10000;$

$k_2 = 1;$

$k_{2r} = k_2/10000;$

- Negative cooperativity

k1 = 1

k1r = k1/10000;

k2 = 0.05

k2r = k2/10000;

Model 2:

$$\frac{d\mathrm{S}}{dt} = -k_1\mathrm{S}\cdot\mathrm{E} + k_{1r}\mathrm{C}_1 - k_2\mathrm{S}\cdot\mathrm{C}_1 + k_{2r}\mathrm{C}_2$$

$$\frac{d\mathrm{E}}{dt} = -k_1\mathrm{S}\cdot\mathrm{E} + k_{1r}\mathrm{C}_1 + k_3\mathrm{C}_1\cdot\mathrm{C}_1 - k_{3r}\mathrm{C}_2\cdot\mathrm{E}$$

$$\frac{d\mathrm{C}_1}{dt} = k_1\mathrm{S}\cdot\mathrm{E} - k_{1r}\mathrm{C}_1 - k_2\mathrm{S}\cdot\mathrm{C}_1 + k_{2r}\mathrm{C}_2 - k_3\mathrm{C}_1\cdot\mathrm{C}_1 + k_{3r}\mathrm{C}_2\cdot\mathrm{E}$$

$$\frac{dC_2}{dt} = k_2\mathrm{S}\cdot\mathrm{C}_1 - k_{2r}\mathrm{C}_2 + k_3\mathrm{S}\cdot\mathrm{C}_1\cdot\mathrm{C}_{tot} - k_{3r}\mathrm{C}_2\cdot\mathrm{C}_{tot} + k_3\mathrm{C}_1\cdot\mathrm{C}_1 - k_{3r}\mathrm{C}_2\cdot\mathrm{E}$$

The rate constant values used are:

$k_1 = k_3/100$;

$k_{1r} = k_1/10000$;

$k_2 = 50*k_1$;

$k_{2r} = k_2/10000$;

$k_3 = 1$;

$k_{3r} = 100*k_3$;

Model 3:

$$\frac{d\mathrm{S}}{dt} = -k_1\mathrm{S}\cdot\mathrm{E} + k_{1r}\mathrm{C}_1 - k_2\mathrm{S}\cdot\mathrm{C}_1 + k_{2r}\mathrm{C}_2 - k_{3'}\mathrm{S}\cdot\mathrm{C}_1\cdot\mathrm{C}_2 + k_{3r'}\mathrm{C}_2\cdot\mathrm{C}_2$$

$$\frac{d\mathrm{E}}{dt} = -k_1\mathrm{S}\cdot\mathrm{E} + k_{1r}\mathrm{C}_1$$

$$\frac{d\mathrm{C}_1}{dt} = k_1\mathrm{S}\cdot\mathrm{E} - k_{1r}\mathrm{C}_1 - k_2\mathrm{S}\cdot\mathrm{C}_1 + k_{2r}\mathrm{C}_2 - k_{3'}\mathrm{S}\cdot\mathrm{C}_1\cdot\mathrm{C}_2 + k_{3r'}\mathrm{C}_2\cdot\mathrm{C}_2$$

$$\frac{d\mathrm{C}_2}{dt} = k_2\mathrm{S}\cdot\mathrm{C}_1 - k_{2r}\mathrm{C}_2 + k_{3'}\mathrm{S}\cdot\mathrm{C}_1\cdot\mathrm{C}_2 - k_{3r'}\mathrm{C}_2\cdot\mathrm{C}_2$$

The rate constant values used are:

$k_1 = 0.01$;

$k_{1r} = k_1 / 1000000;$

$k_2 = 0.02 * k_1;$

$k_{2r} = k_2 / 1000000;$

$k_{3'} = k_1 * 3;$

$k_{3r'} = k_{3'} / 1000000;$

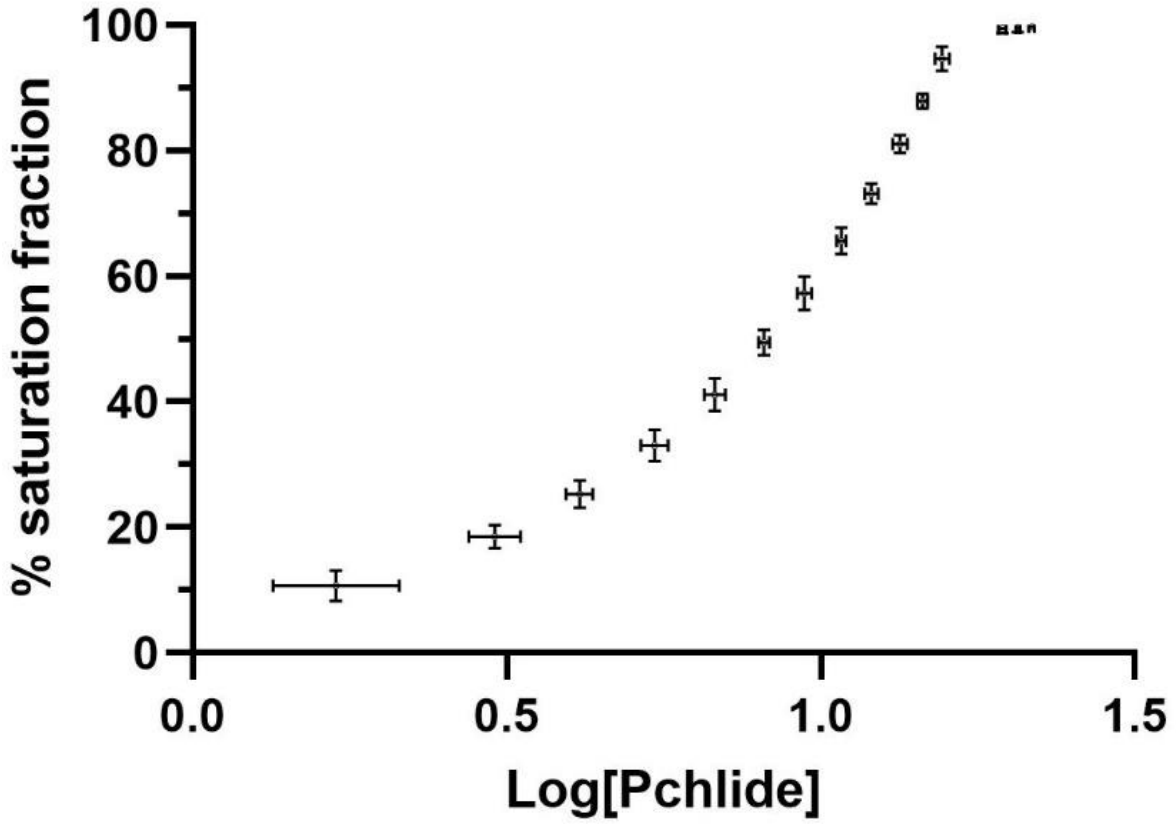

Figure S1. Percentage fractional saturation of BchNB, performed as detail in Figure 3 within the main article. A stirred solution of BchNB (8 μM) in 100 mM HEPES buffer (pH 7.5, containing 150 mM NaCl) was titrated with ~1 μM additions of Pchlide. Data presented as mean ± SD (*n* = 3).

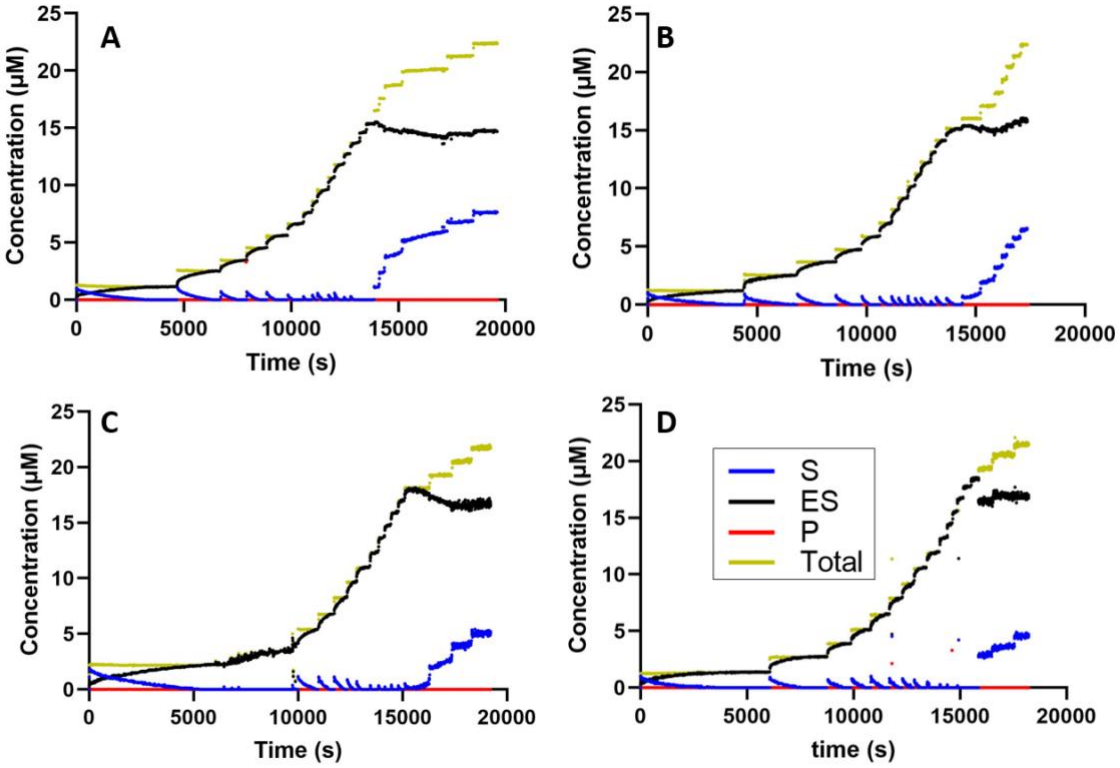

Figure S2. Repeats for titration of BchNB with Pchlide substrate (S). Experimental data showing the addition of Pchlide substrate (S, blue line) in ~1 µM additions to a stirred solution of substrate-free BchNB enzyme (E, 8 µM). Contributions from S, ES-complex (ES, black) and Chlide product (P, red) were deconvoluted, as described in SI; the total concentration of S + ES + P is given by the gold line. Here, the total concentration (gold line) validates the individual molar absorptivities and proportionality constants used herein. Each Pchlide addition was made immediately after having consumed all free Pchlide substrate (S) to form the ES-complex (until ~14,000 s). Performed in 100 mM HEPES buffer (pH 7.5) containing 150 mM NaCl.

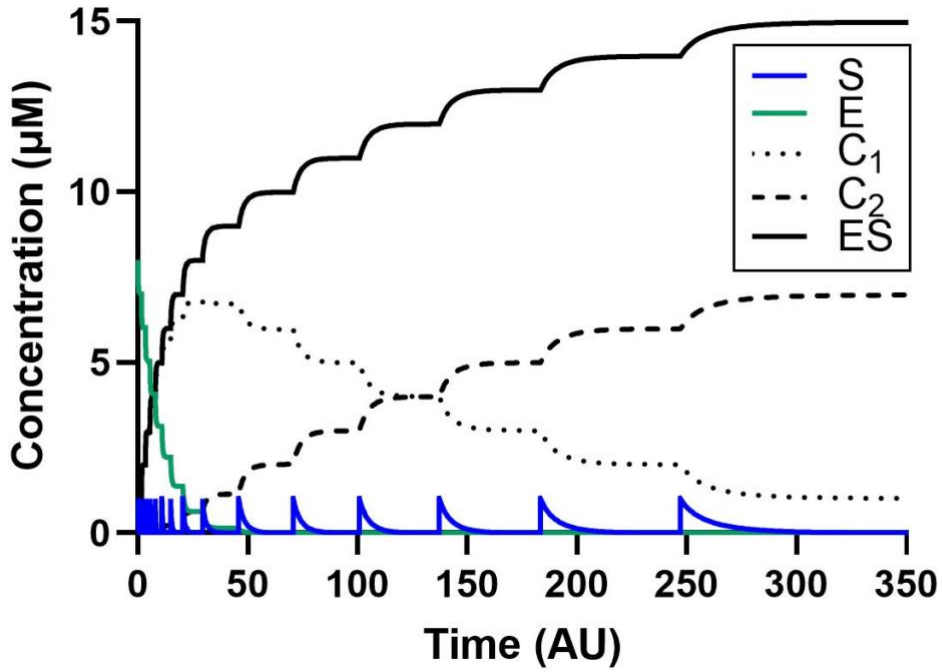

Figure S3. Results of simulations of Model 1 to understand DPOR's behavior during enzyme-substrate complex (ES-complex) formation. In this simulation of Model 1 (as presented in Figure of the main article) $k_2 = k_1/20$, where The concentration of free Pchlide substrate (S) is shown in blue, free BchNB enzyme (E) in shown green, BchNB singly occupied with Pchlide substrate (C_1) is shown in black dotted, and doubly occupied BchNB (C_2) is represented by the black dashed line. The ES concentration of $C_1 + C_2$ is represented by the black line. The time is given in arbitrary units.

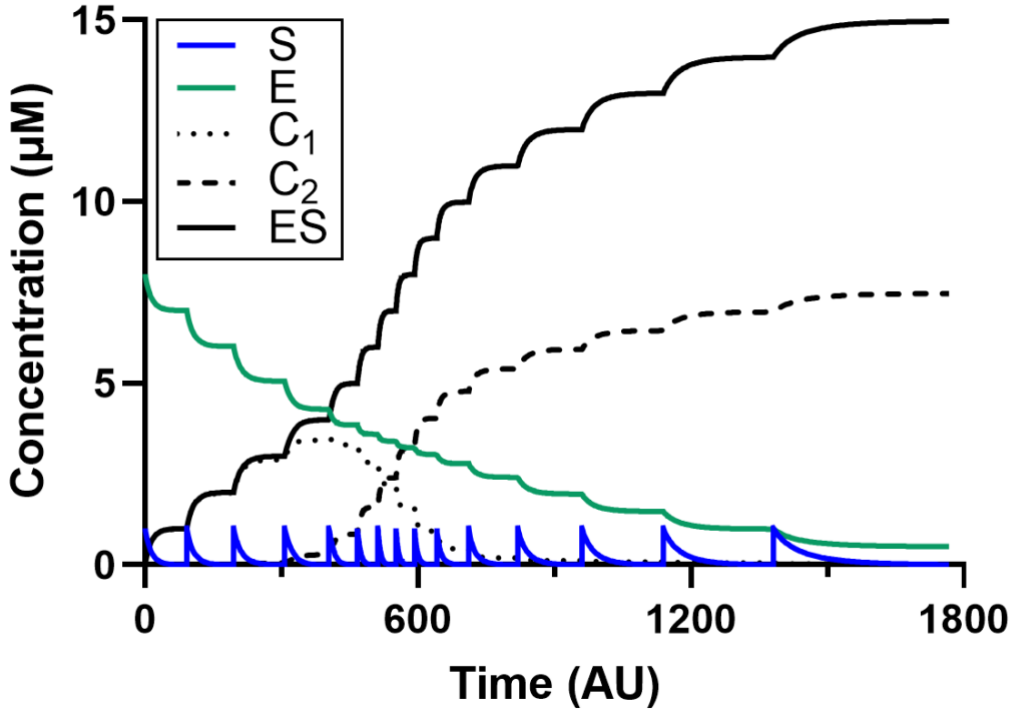

Figure S4. Simulations of Model 3 to describe ES-complex formation between BchNB and Pchlide. The concentration of free Pchlide substrate (S) is shown in blue, free BchNB enzyme (E) in shown green, BchNB singly occupied with Pchlide substrate (C_1) is shown in black dotted line, and doubly occupied BchNB (C_2) is represented by the black dashed line. The ES concentration of $C_1 + C_2$ is represented by the black line. The time is given in arbitrary units.

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