Supplementary Information

for

Chemical diversity of bilin species defines the chiroptical landscape of phytochromes

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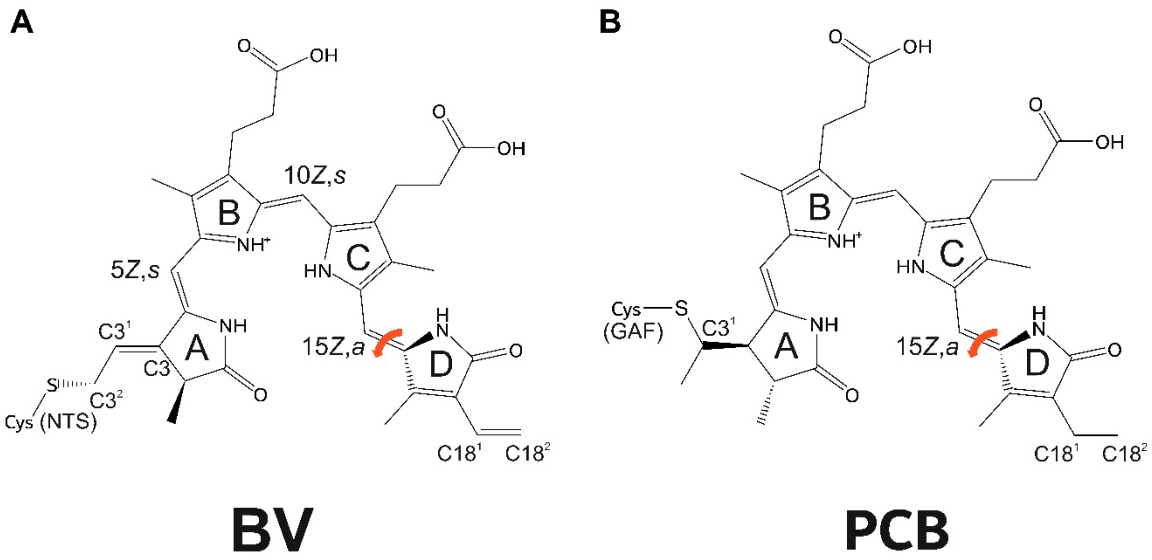
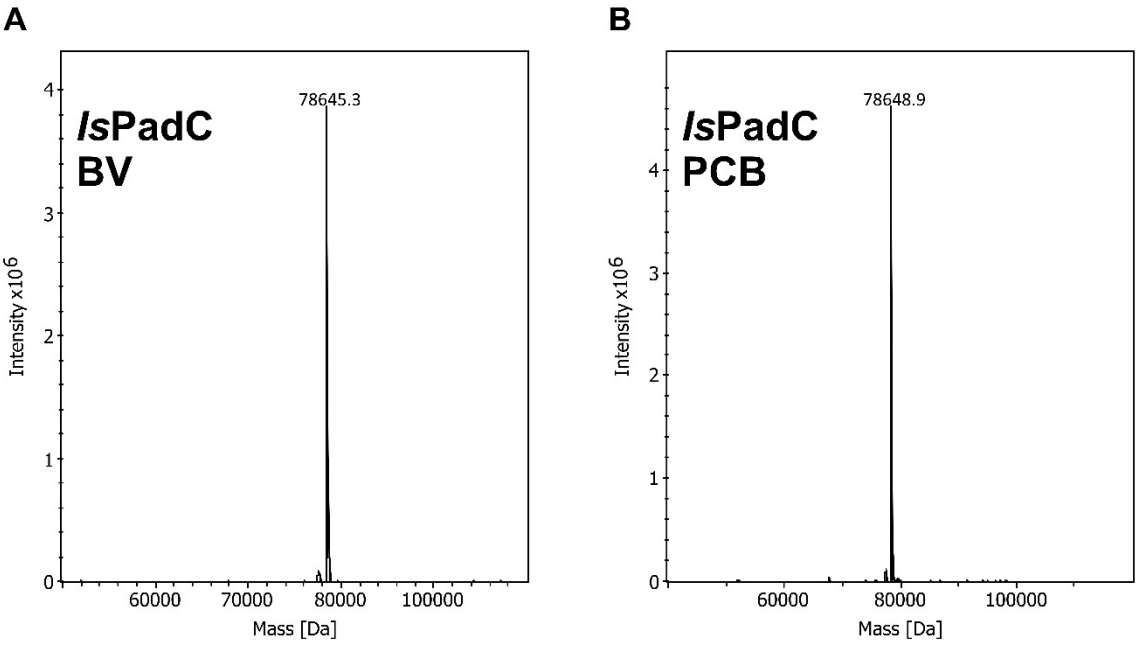
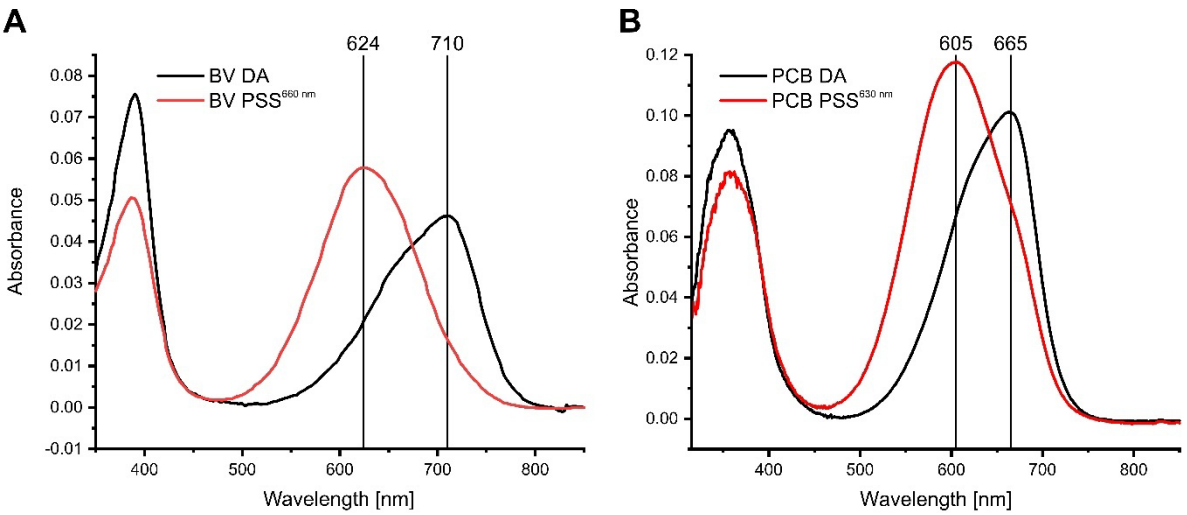


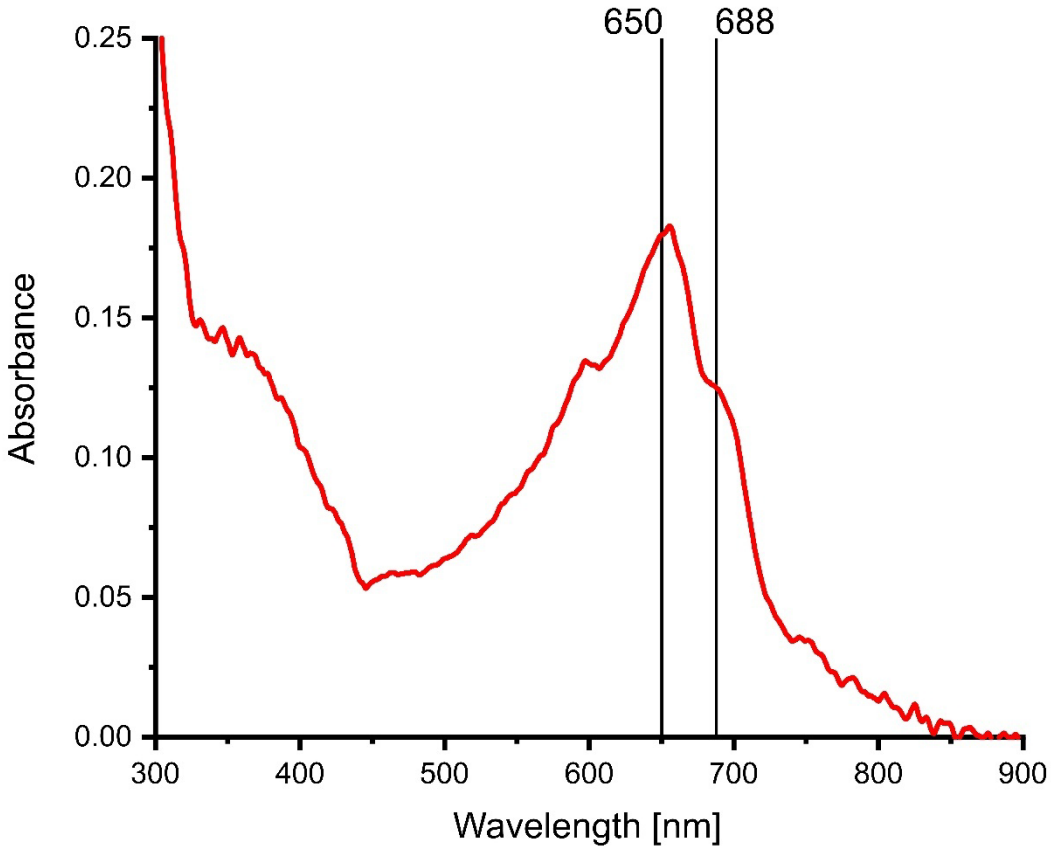
Supplementary Figure 1A+B: Chemical structures of biliverdin (BV) and phycocyanobilin (PCB), respectively. The location of the covalent attachment site to the protein is highlighted with either Cys (NTS) or Cys (GAF). In addition, the bilin atoms involved in the covalent attachment are shown which are either $C3^2$ (BV) or $C3^1$ (PCB). Note the nomenclature of the bilin A-D rings. Upon red light illumination, the C15=C16 double bond isomerizes from a 15Z to 15E configuration (red arrow).



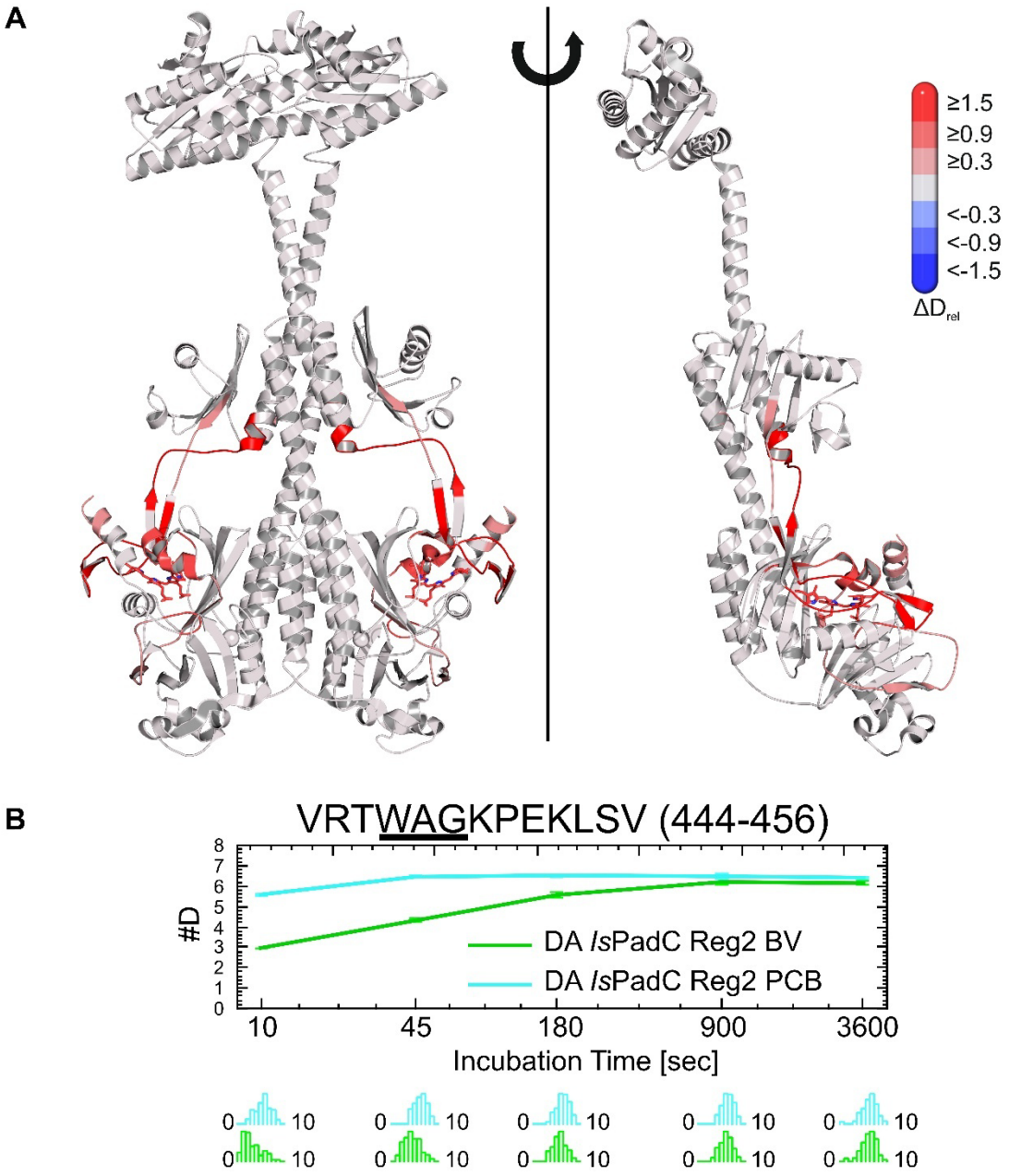
Supplementary Figure 2: Intact mass measurements prove complete loading of the proteins *Is*PadC BV (left) and *Is*PadC PCB (right) with their respective bilin cofactor. The theoretically calculated Holoprotein masses for *Is*PadC BV (78646,08 Da) and *Is*PadC PCB (78650,13 Da) agree well with the experimentally determined masses.



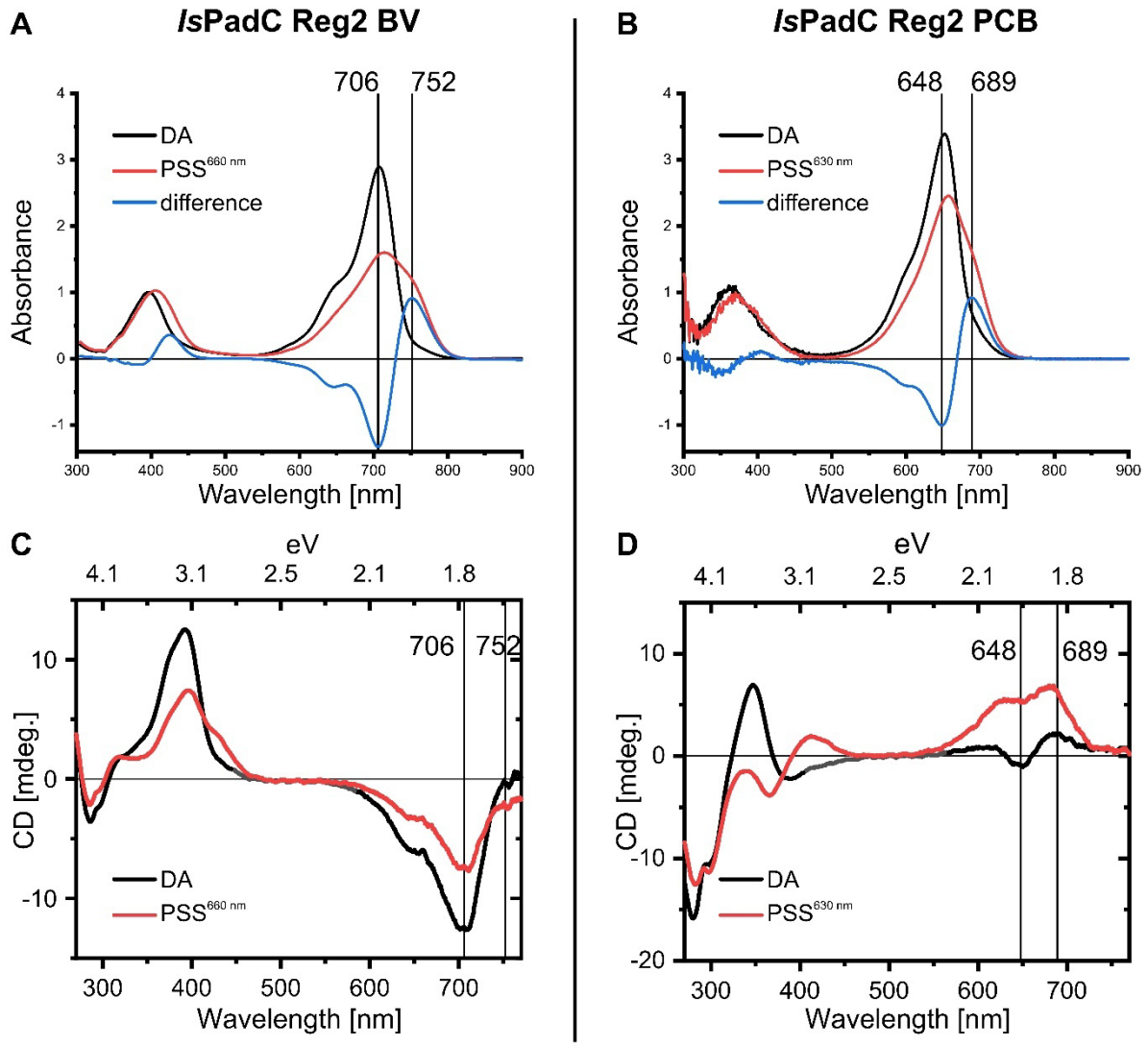
Supplementary Figure 3: A+B) Protein denaturation experiments of *Is*PadC BV and *Is*PadC PCB, respectively. Samples where either denatured using 0.1% TCA/Methanol (BV) or 6M Guanidinium HCl/1 % HCl (PCB). In both panels, the dark-adapted state (DA) is shown as a black line. For the illuminated samples (BV 660 nm, PCB: 630 nm), samples were treated with red light followed by immediate denaturation. Black vertical lines highlight the maxima of the respective UV/vis peak. BV data was taken from https://doi.org/10.7554/eLife.34815.



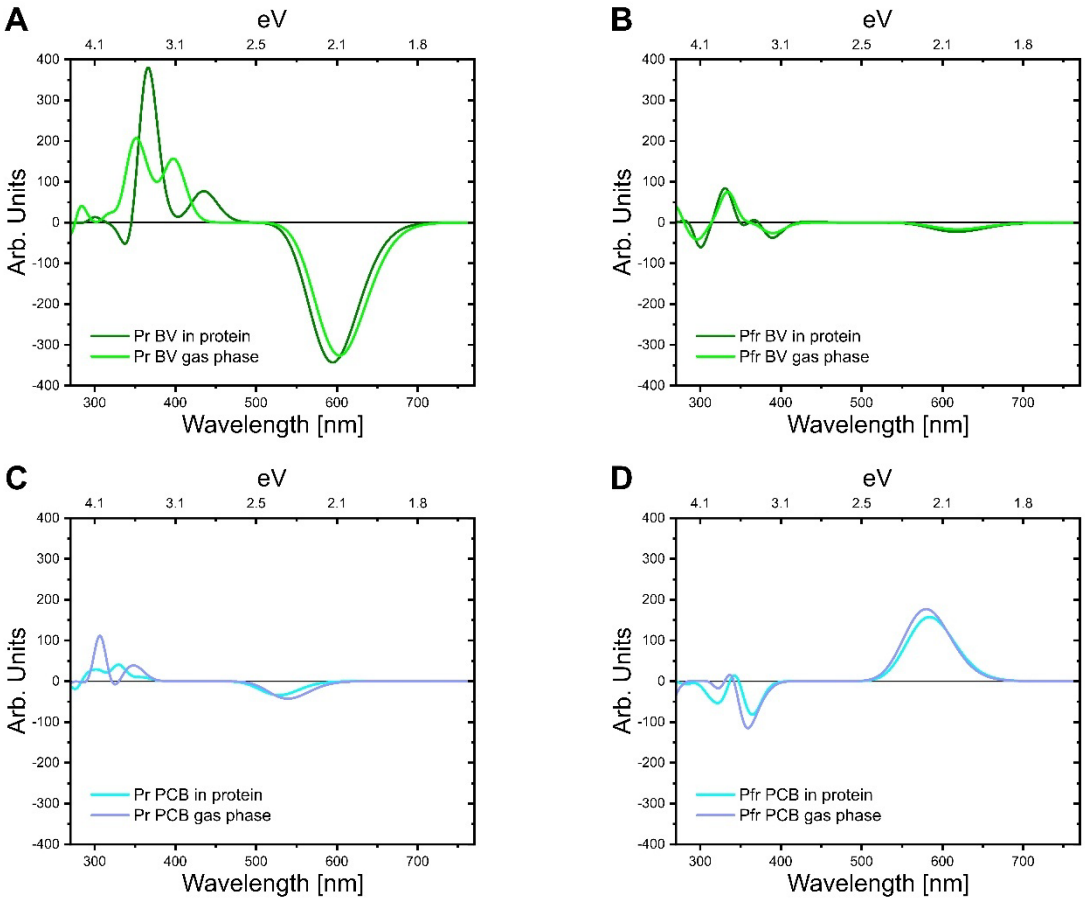
Supplementary Figure 4: *In crystallo* Optical Spectroscopy (icOS) data of *Is*PadC Reg2 PCB (C17L L251C S505V A526V) at 100 K. Before plotting, the spectrum was denoised. Note that the crystal was not illuminated but was kept in complete darkness. Nevertheless, the shape of the spectrum suggests the stabilization of a mixed Pr/Pfr state in the crystal.



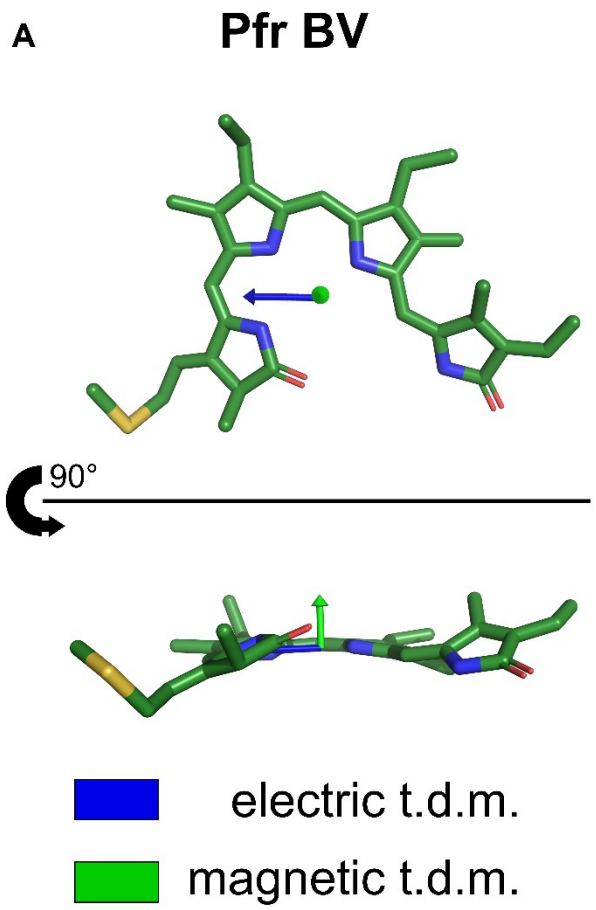
Supplementary Figure 5: Dark adapted (DA) HDX-MS data of a BV- and a PCB-bound *Is*PadC variant. A) Differences in deuterium exchange (ΔDrel) of *Is*PadC Reg2 PCB minus *Is*PadC Reg2 BV (t=10 s) mapped onto the crystal structure of *Is*PadC (PDB: 5llw). In the regions highlighted in red, the PCB variant is significantly more dynamic than the BV variant. B) Example PHY tongue peptide with diverging deuterium incorporation in the dark-adapted (DA) states. This peptide contains the WAG motif (underlined) and harbors strongly increased conformational dynamics for the PCB variant.

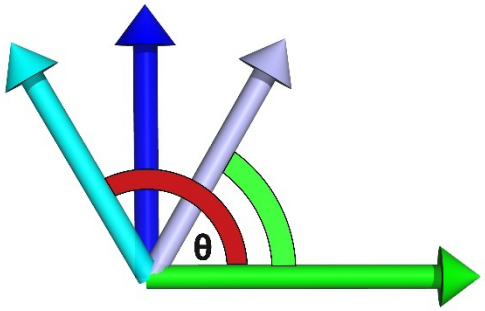


Supplementary Figure 6: Spectral properties of *Is*PadC Reg2 BV versus PCB. In all panels, spectra of dark adapted (DA) and red light illuminated (BV: $PSS^{660\ nm}$, PCB: $PSS^{630\ nm}$) samples are shown with black and red lines, respectively. A+B) UV/vis characterization of *Is*PadC Reg2 BV and *Is*PadC Reg2 PCB. Black vertical lines highlight the maxima and minima of the difference spectrum (PSS minus DA) in blue. Spectra are scaled to 1 according to their Soret band maximum (BV: 398 nm, PCB: 357 nm). C+D) Experimentally determined CD traces of *Is*PadC Reg2 BV and *Is*PadC Reg2 PCB. Black vertical lines indicate the maxima and minima of the UV/vis difference spectrum determined in A+B.



Supplementary Figure 7 A-D): Comparison of CD spectra calculated from bilin structures in the protein context versus isolated bilin structures in the gas phase. In green colors, the biliverdin (BV) data and in blue colors the phycocyanobilin (PCB) data is shown.





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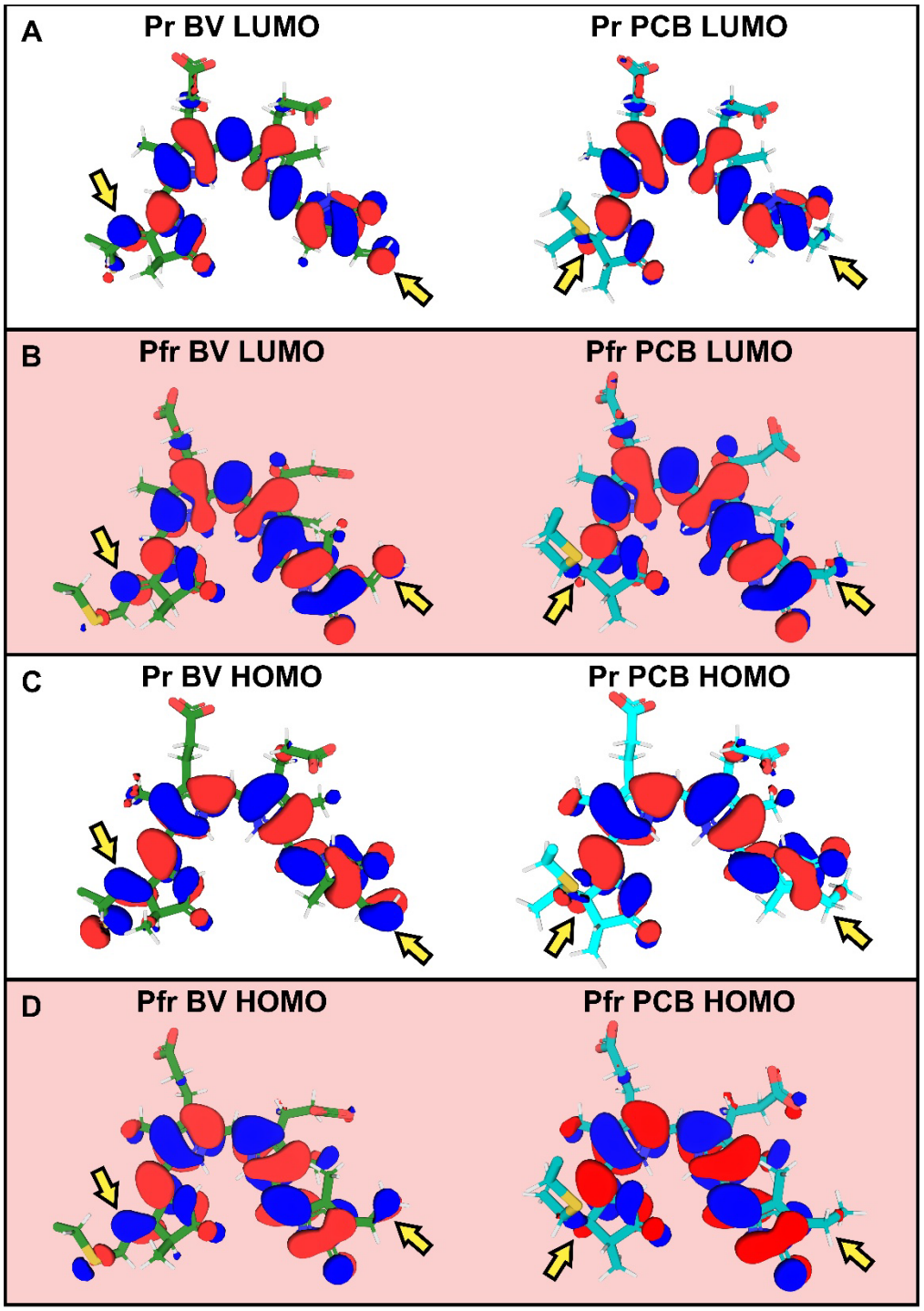
Structure	Angle θ	Cos(θ)
Pr BV	93.04°	− 0.053
Pfr BV	90.58°	− 0.010
Pr PCB	90.19°	− 0.003
Pfr PCB	87.90°	+ 0.036

Supplementary Figure 8: Specific calculations of transition dipole moments determining the sign of the rotatory strength and thus the CD behavior. A) Example structure of biliverdin (BV) in the Pfr configuration. Although the calculations were conducted in the protein context, the amino acids are not shown to aid visibility. Displayed at the center of mass of the molecule, the rescaled electric (blue) and magnetic (green) transition dipole moments (t.d.m.) are visible. B) Scheme to visualize the dependency of the sign of the rotatory strength with the angle between the electronic (blue shades) and magnetic (green) transition dipole moment. Due to the properties of the cosine function, angles greater than 90° lead to negative cos(θ) values whereas angles smaller than 90° lead to positive cos(θ) values. C) Overview of angles θ and cos(θ) calculated for the biliverdin (BV) and phycocyanobilin (PCB) structures in the Pr or Pfr geometry, respectively.



Supplementary Figure 9: Calculated molecular orbitals of *Is*PadC BV and PCB in Pr and Pfr, respectively. A-D) HOMO and LUMO orbitals were calculated from the QM/MM optimized starting structures utilized for the *in silico* CD simulations. Positive phases are shown in red whereas negative phases are displayed in blue. Differences in orbitals between the BV and PCB cofactor are highlighted with yellow arrows. In BV, the C3=C3^1 interaction on the A-ring (left)and the C18^1=C18^2 interaction on the D-ring (right) has double bond character. The equivalent positions in PCB have single bond character.

Supplementary Table 1: Primers used to generate the *Is*PadC C17L L251C construct.

primer name	**primer sequence**
IsPSM_C17L_fwd	GCAGCATTGGATCAAGAACCGATTCATATTCCGAATGCAATTC
IsPSM_C17L_rev	TTGATCCAATGCTGCAATCAGTTTGCTGATATCATCACTACCC
IsPSM_L251C_fwd	GTTCTGCGTGCAGTTAGCCCTTGTCACATGCAGTATCTGCGTAATTTTGG
IsPSM_L251C_rev	AACTGCACGCAGAACACCGCTGCTCAGATTAACTGC

Supplementary Table 2: HDX-MS dataset statistics for the shown datasets according to the conventions of the HDX-MS community

	***Is*PadC Reg2 BV**	***Is*PadC Reg2 PCB**	**Peptides occurring in both datasets**
Light conditions	Dark-adapted (DA)	Dark-adapted (DA)	-
HDX reaction details	10mM HEPES, 150mM NaCl, 2mM $MgCl_2$, pD=7.0, 20°C		-
HDX time course (s)	10, 45, 180, 900, 3600		-
HDX control samples	Unlabeled control (Reg2 BV DA)	Unlabeled control (Reg2 PCB DA)	-
Back-exchange	Not measured		-
# of Peptides	180	161	120
Sequence coverage	92%	93%	85%
Avg peptide length / Avg. redundancy	13/3	13.6/3	13.5/2
Replicates	3	3	-
Repeatability (average SD for each time point)	0.09, 0.06, 0.08, 0.07, 0.06	0.05, 0.05, 0.04, 0.06, 0.07	-
Significant differences in HDX	ΔHDX > 0.3 D		-