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Synthesis of phycocyanobilin in mammalian cells†

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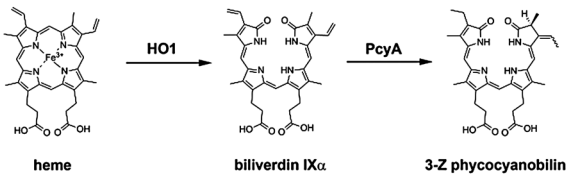
The chromophore 3-*Z* phycocyanobilin (PCB, (2*R*,3*Z*)-8,12-bis-(2-carboxyethyl)-18-ethyl-3-ethylidene-2,7,13,17-tetramethyl-2,3-dihydrobilin-1,19(21*H*,24*H*)-dione) mediates red and far-red light perception in natural and synthetic biological systems. Here we describe a PCB synthesis strategy in mammalian cells. We optimize the production by co-localizing the biocatalysts to the substrate source, by coordinating the availability of the biocatalysts and by reducing the degradation of the reaction product. We show that the resulting PCB levels of 2 μM are sufficient to sustain the functionality of red light-responsive optogenetic tools suitable for the light-inducible control of gene expression in mammalian cells.

Chromophores that translate the spectral properties of light into a biological readout have gained momentum with the advent of optogenetics aiming at the optically controlled, spatiotemporally resolved manipulation of individual cells and whole organisms.[1–11] Of special interest in this field are optogenetic tools responsive to red light, as this light spectrum can penetrate deeper into tissues and is less likely to induce unwanted side effects due to its lower energy content. Red-light-responsive optogenetic systems are based on bacterial or plant phytochrome proteins that undergo reversible structural rearrangements in response to illumination at 660 or 740 nm. Such phytochromes have successfully been applied to optically trigger gene expression and signalling in animal and human cells with unprecedented spatiotemporal resolution.[5,12] *In planta*, phytochromes perceive red light *via* the chromophore 3-*Z* phytochromobilin (PΦB, (3*Z*)-8,12-bis(2-carboxyethyl)-18-ethenyl-3-ethylidene-2,7,13,17-tetramethyl-2,3-dihydrobiline-1,19(21*H*,24*H*)-dione) by a red light-triggered *Z*–*E* isomerization about the C15–C16 double bond of the bilin. Far-red light returns the phytochromes to the ground state by re-isomerization of the chromophore. In place of PΦB, cyanobacteria use 3-*Z* phycocyanobilin (PCB, (2*R*,3*Z*)-8,12-bis(2-carboxyethyl)-18-ethyl-3-ethylidene-2,7,13,17-tetramethyl-2,3-dihydrobilin-1,19(21*H*,24*H*)-dione) as the chromophore. PCB can also replace PΦB in plant phytochromes and is autoligated to the phytochrome in optogenetic tools for mammalian systems, when exogenously added to the culture medium.

However, as PCB is tedious to extract from natural sources and is rather unstable in mammalian cell culture medium (1 h half-life time[12]) the further development of optogenetics would be significantly boosted by the direct synthesis of PCB within the target mammalian cell. In cyanobacteria, PCB is synthesized from heme in a two-step enzymatic reaction that comprises the oxidation of heme to biliverdin IXα by heme oxygenase 1 (HO1) and the reduction of this intermediate to 3*Z*-phycocyanobilin by PCB:ferredoxin oxidoreductase (PcyA) (Scheme 1). Although this biosynthetic process has been successfully reconstructed in *E. coli*,[13–16] its transfer to mammalian cells has so far not been achieved.

In order to develop a synthesis strategy for PCB in mammalian cells from the precursor heme, we introduced HO1 and PcyA from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1[16] on two separate plasmids into Chinese hamster



Scheme 1 Pathway of phycocyanobilin (PCB) biosynthesis in cyanobacteria. Heme oxygenase 1 (HO1) catalyzes stereospecific cleavage of heme to yield biliverdin IXα that is further reduced by PCB:ferredoxin oxidoreductase to phycocyanobilin (PCB).

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ovary (CHO-K1) cells. As an analytical tool to quantify the synthesis of bioavailable PCB, we applied our recently developed expression system in which a PCB-bound phytochrome mediates production of the reporter enzyme SEAP (secreted alkaline phosphatase) under illumination with 660 nm light while 740 nm light was shown to shut off reporter production.[12] One day after transfection with HO1 and PcyA genes, the cells were incubated at 30 °C for 48 h to allow for expression of HO1 and PcyA and the subsequent biosynthesis of PCB. After medium exchange, the cells were illuminated with 660 nm light or 740 nm light for 24 h at 37 °C prior to the quantification of the reporter enzyme SEAP. However, cells only showed minimally increased reporter expression suggesting that the PCB synthesis rate might not be sufficiently high (Fig. 1).

We reasoned that an insufficient supply of the precursor heme in the cytoplasm might represent a bottleneck as the final steps of heme biosynthesis are localized in mitochondria in eukaryotic cells.[17] For this reason we modified both biosynthetic enzymes for mitochondria localization by fusion to an N-terminal mitochondrial targeting sequence (MTS). In this configuration a significantly higher production of the reporter SEAP was observed under illumination with 660 nm light suggesting that the mitochondrial localization facilitated substrate supply (Fig. 1).

In order to increase co-expression of both enzymes, we placed the respective genes on a single plasmid as a bicistronic expression unit. This configuration further increased reporter expression and resulted in induction ratios of over 15-fold (Fig. 1).

Having addressed the supply of precursors and the co-production of both biosynthetic enzymes, we investigated the possibility of the active degradation of the reaction product in mammalian cells. We identified an eukaryotic cytoplasmically localized enzyme, biliverdin reductase A (BVRA),[18] that degrades biliverdin IXα to bilirubin and PCB to phycocyanorubin (PCR).[19] Indeed, expression of BVRA in plants correlated with a phytochrome

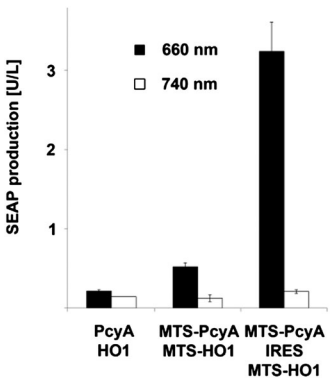

Fig. 1 Optimization of PCB biosynthesis. CHO-K1 cells were transfected for red light inducible gene expression. At the same time the cells were transfected with plasmids coding for HO1 and PcyA without (left) or with (middle) a mitochondrial targeting signal. Mitochondria-targeted HO1 and PcyA were also expressed as a bicistronic expression unit (right). After transfection the cells were incubated at 37 °C overnight and then switched to 30 °C incubation for 48 h to slow down the cell growth. Subsequently, the culture medium was exchanged and the cells were illuminated at 37 °C with 660 nm or 740 nm light for 24 h. Finally, SEAP was quantified in the cell culture supernatant. IRES, internal ribosome entry site; MTS, mitochondrial targeting signal. Data are means ± SD (n = 4).

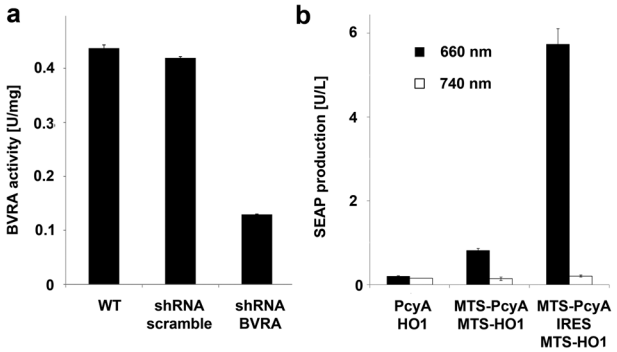

Fig. 2 Knock-down of BVRA and impact on PCB biosynthesis. (a) Knock-down of BVRA. CHO-K1 cells were transduced either with pKM048 ($shRNA_{BVRA}$) or with pLKO.1-puro scramble shRNA and selected with puromycin. BVRA activity 1 month after transduction is indicated as U per mg of total protein in cell lysates. (b) PCB biosynthesis in CHO-K1 with $shRNA_{BVRA}$. CHO-K1 cells with $shRNA_{BVRA}$ were transfected for red light-inducible gene expression and plasmids coding for HO1 and PcyA analogous to Fig. 1. After transfection the cells were incubated and illuminated as indicated in Fig. 1 and SEAP production was quantified. Data are means ± SD (n = 4).

knock-out phenotype.[20] In order to reduce this degradation, we performed a short hairpin RNA (shRNA)-mediated knockdown of BVRA in CHO-K1 cells. Cells were selected for integration of the shRNA expression cassette, which was shown to correlate with a BVRA activity reduced by 70% (Fig. 2a). This engineered CHO-K1 cell line (CHO_{shRNA}) was subsequently transfected with the expression vectors encoding the mitochondria-localized enzymes and assessed for PCB production using the enzyme-based readout system (Fig. 2b). It was shown that the knock-down resulted in a further two-fold increase in reporter enzyme production indicating that BVRA-mediated PCB degradation might significantly influence the intracellular concentration of this chromophore (Fig. 1 and 2b).

In order to obtain a quantitative picture of PCB synthesis, we determined its concentration in cells as a function of time using a previously developed quantitative model[12] and a calibration curve established by adding increasing concentrations of PCB to the enzymatic readout system[12] (Fig. 3 and ESI,† Fig. S1). We calculated the PCB concentration as a function of cultivation time and determined the steady-state concentration of PCB to be 2.0 μM. Furthermore, the rate of PCB synthesis was

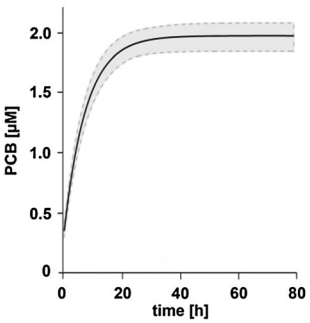

Fig. 3 Quantification of PCB synthesis. PCB concentration time course in CHO_{shRNA} cells transfected with the mitochondria-targeted biocatalysts encoded on a single bicistronic vector. The PCB concentration was calculated from a PCB calibration curve and a corresponding quantitative mathematical model.

calculated to be 0.27 μM h^{-1} with a 95% confidence interval of 0.24–0.30 μM h^{-1}.

In this study we applied multi-factor metabolic engineering to realize the synthesis of PCB in mammalian cells. Key factors were the targeting of the biocatalysts to the site of precursor production, their coordinated production from a single plasmid as well as the inhibition of product degradation. The resulting PCB levels of 2 μM were sufficient to sustain high red light-inducible gene activation.

We are convinced that this synthesis of PCB in mammalian cells will further boost the rapid expansion of optogenetics in mammalian cells[5,10–12,21] and whole animals[22] opening novel opportunities in many areas of fundamental and application-oriented research and development.

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