Recombinant holophytochrome in *Escherichia coli*

F.T. Landgraf[a], C. Forreiter[a], A. Hurtado Picó[b], T. Lamparter[b,*], J. Hughes[a]

[a] *Plant Physiology, Justus-Liebig-University Giessen, Das Zeughaus, Senckenbergstrasse 3, D-35390 Giessen, Germany*
[b] *Plant Physiology, Free University Berlin, Königin-Luise-Strasse 12–16, D-14195 Berlin, Germany*

Received 27 July 2001; accepted 21 September 2001

First published online 6 November 2001

Edited by Richard Cogdell

Abstract We have successfully co-expressed two genes from the bilin biosynthetic pathway of *Synechocystis* together with cyanobacterial phytochrome 1 (Cph1) from the same organism to produce holophytochrome in *Escherichia coli*. Heme oxygenase was used to convert host heme to biliverdin IXα which was then reduced to phycocyanobilin via phycocyanobilin:ferredoxin oxidoreductase, presumably with the aid of host ferredoxin. In this host environment Cph1 apophytochrome was able to autoassemble with the phycocyanobilin in vivo to form fully photoreversible holophytochrome. The system can be used as a tool for further genetic studies of phytochrome function and signal transduction as well as providing an excellent source of holophytochrome for physicochemical studies. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Holophytochrome; Heme oxygenase; Phycocyanobilin:ferredoxin oxidoreductase; Cyanobacterial phytochrome 1; *Synechocystis* sp. PCC6803

1. Introduction

Phytochromes were thought to be restricted to green plants until the discovery of cyanobacterial phytochrome 1 (Cph1) in *Synechocystis* [1]. This caused a paradigm shift in phytochrome research and revolutionized our view of the evolution and function of this class of biliprotein photoreceptors. Moreover, the ease with which Cph1 apoprotein can be overexpressed in *Escherichia coli*, autoassembled in vitro with appropriate bilins and purified to homogeneity opened the door to numerous genetic, biochemical and biophysical methods scarcely accessible with phytochrome extracted from plants.

In common with plant phytochromes, Cph1 has an N-terminal chromophore-binding region, the sensor module. An autocatalytic lyase function attaches appropriate bilin chromophores to a highly conserved cysteine residue (C259 in Cph1, see #380 in our alignment http://www.biologie.fu-berlin.de/phytochrome/align2x.htm or http://www.uni-giessen.de/~gf1251/Phytochrome/align2x.htm) [2]. The C-terminal domain comprises a histidine autokinase–phosphotransferase transmitter module typical of bacterial 'two-component' systems. Light regulates the autokinase activity of Cph1 in vitro; thereafter the phosphoryl moiety is transferred to the response regulator protein Rcp1, the 'second component' [3,4].

The natural chromophore of Cph1 is phycocyanobilin (PCB), a bilin abundant in *Synechocystis* [5]. Following admission to the acidic environment of the chromophore-binding pocket of apoCph1, PCB becomes protonated, and autocatalytically attached to the apoprotein via a covalent thioether bond, red-shifting its λ_{max} and increasing its extinction coefficient, finally producing a red/far-red photochromic holophytochrome [6].

PCB is derived from the tetrapyrrole heme. In the first biosynthetic step, the ring is cleaved between pyrroles A and D to form the linear tetrapyrrole biliverdin IXα (BV). In cyanobacteria and plants, this is accomplished by a ferredoxin-dependent heme oxygenase (HO) [7,8], whereas the mammalian homologue is cytochrome P450-dependent [9]. Although HO is also found in some other bacteria [9], there is no homologous sequence in the *E. coli* genome nor is a heme oxygenase activity detectable. The second conversion step, an unusual four-electron reduction of BV to PCB, is accomplished by a phycocyanobilin:ferredoxin oxidoreductase (PcyA) [10]. The corresponding gene from *Synechocystis*, *pcyA* (slr0116), was recently identified by its sequence homology to phytochromobilin synthase *HY2* of *Arabidopsis* [11].

2. Materials and methods

The *Synechocystis ho1* (sll1184) [7] and *pcyA* [10] genes were amplified from genomic DNA by PCR using an error-checking DNA polymerase (Pfu, Stratagene, La Jolla, CA, USA). The *ho1* 5′ and 3′ primers were GCGGTACCCATGAGTGTCAACTTAGCTTC and GGCGAAGCTTCTAGCCTTCGGAGGTG, incorporating *Hin*dIII and *Kpn*I sites, respectively. The PCR profile was: 25 cycles [94°C, 30 s; 65°C, 30 s; 72°C, 60 s] with a 100 ng genomic DNA template.

The *pcyA* (slr0116) gene was amplified with GCGGATCCATGGCCGTCACTG and GCGCGGCCGCTTATTGGATAACATC, inserting a *Bam*HI site and a *Not*I site at the 5′ and 3′ termini, respectively. The PCR profile was: 50 cycles [94°C, 30 s; 55°C, 30 s; 90 s, 72°C] with a 100 ng genomic DNA template.

The PCR products were restricted appropriately (enzymes from New England Biolabs, Beverly, MA, USA) and sequentially cloned into the vector pPROLar.A122 (Clontech, Palo Alto, CA, USA) using the coli host DH5αPRO (Clontech), to yield the clones p40.1 and p45.2 respectively.

As the pQE12 (Qiagen, Hamburg, Germany) and pPROLar vectors carry compatible replication origins, pF10-His [12] containing the Cph1 gene (slr0473) could be co-transformed with p45.2 into DH5αPRO with kanamycin and ampicillin selection. 0.5 ml of [p45.2/pF10-His] overnight culture was transferred into 100 ml LB

*Corresponding author. Fax: (49)-30-838 54357.
E-mail address: lamparte@zedat.fu-berlin.de (T. Lamparter).

Abbreviations: BV, biliverdin; Cph1, cyanobacterial phytochrome 1; HO, heme oxygenase; PCB, phycocyanobilin; PcyA, phycocyanobilin:ferredoxin oxidoreductase; PEB, phycoerythrobilin; Pfr, far-red-absorbing form of phytochrome; Pr, red-absorbing form of phytochrome; PφB, phytochromobilin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

PII: S0014-5793(01)02988-X

containing antibiotics and grown with vigorous shaking at 37°C to $OD_{550\,nm}$ 0.4–0.5. Then 5 ml was transferred into a separate vial as a control. The rest was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG; Biomol, Hamburg, Germany) and 0.2% arabinose (Roth, Karlsruhe, Germany) and grown for 18 h at the appropriate temperature with vigorous shaking.

The overnight culture was collected by centrifugation (3000×*g*, 10 min, 4°C; Sorvall RC2-B, Du Pont, Bad Homburg, Germany) and washed twice with TES (50 mM tris-(hydroxymethyl)-aminomethane, 1 mM ethylenediaminetetraacetic acid, 300 mM NaCl, pH 7.6) before resuspension in 2 ml TES. Proteins were extracted with two passages through a French pressure cell at 16 000 psi. The cell debris were pelleted at 4°C, 50 000×*g* for 20 min (Stratos Biofuge, Heraeus, Germany).

Supernatants UV/Vis absorbance spectra were measured in an Agilent 8453 diode-array spectrophotometer for 100 ms at 1 nm resolution, following saturating irradiations at 658 ± 10 nm and 730 ± 12 nm from LED light sources.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the supernatant was performed according to Laemmli [13]. The gels were incubated for 15 min in 1 mM zinc acetate and visualized by UV fluorography before staining in Coomassie [14].

3. Results

In preliminary experiments we overexpressed rat *HO* in *E. coli* from pPROLar, analogously to the pBAHO30 alkaline phosphatase promoter expression system [15]. In both cases the resulting clones showed a pale green color after induction, indicative of BV accumulation. We then inserted *Synechocystis pcyA* [10] downstream of *HO* to allow bicistronic expression and checked for bilin synthase activity and bilin production by measuring red/far-red photochromicity after adding apoCph1 to the crude extract (see Fig. 1). The resulting spectrum, typical of the PCB-Cph1 adduct, indicated that *E. coli* is able to provide the reaction conditions and components for the action of HO and PcyA without co-expressing other genes such as ferredoxin (PcyA is ferredoxin-dependent [10]). It was, on the other hand, not possible to produce holoCph1 in vivo with this system: rat *HO* was not expressed effectively below 25°C, whereas apoCph1 is expressed as insoluble inclusion bodies at and above that temperature (Fig. 2A).

We therefore substituted the rat *HO* gene with the sll1184 homologue *ho1* from *Synechocystis* [7]. The weak *Synechocystis ho1* expressor (p40.1) and *ho1/pcyA* co-expressor (p45.2) were thus constructed. Following induction at 22°C, crude extracts were acidified to protonate the bilin pigment and

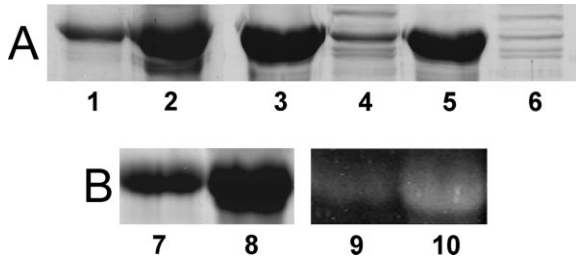

Fig. 2. A: SDS–PAGE/Coomassie of pF10-His cultures induced at 18°C (1: pellet; 2: supernatant); 25°C (3: pellet; 4: supernatant) and 30°C (5: pellet; 6: supernatant). B: SDS–PAGE of pF10-His/p45.2 culture at 22°C: left Coomassie (7: pellet; 8: supernatant), and right zinc stain (9: pellet; 10: supernatant).

extracted with chloroform as described [16]. UV-Vis spectra of these and similarly treated, HPLC-purified BV and PCB were measured (Fig. 3). λ_{max} values in the red region were 646 nm (sh 720 nm) and 650 nm (sh 710 nm) for BV and PCB respectively, with somewhat stronger UV-A peaks at 379 nm and 373 nm. The coli extracts showed weaker, broader red bands centered at 660 nm (sh 720 nm) and 655 nm (sh 710 nm) with two- and four-fold stronger UV-A peaks at 384 nm and 374 nm for p40.1 and p45.2 respectively.

Crude aqueous p45.2 extracts and HPLC-purified PCB were additionally assayed by adding excess recombinant apoCph1 [12] and measuring the subsequent red/far-red difference spectra (Fig. 4). The maxima, minima, and isosbestic points obtained matched exactly with each other and with previously published data [12] (Table 1).

Clearly therefore, p45.2 was able to synthesize PCB at temperatures favorable for soluble apoCph1 expression. An *ho1/pcyA/Cph1* co-expressor was thus constructed by co-transforming with pF10-His and p45.2. The resulting clone [pF10-His/p45.2] was induced with IPTG and arabinose at 22°C and affinity-purified over Ni-NTA [12]. The spectra following far-red and red irradiation and the associated difference spectrum are shown in Fig. 5. λ_{max} of the red-absorbing form of phytochrome (Pr) was at 663 nm and difference maximum, minimum and isosbestic point were at 660, 705 and 680 nm, respectively – values significantly different from those of conventional in vitro assembled PCB adducts of Cph1 (Table 1; Fig. 4).

The [pF10-His/p45.2] extracts were also analyzed by SDS–

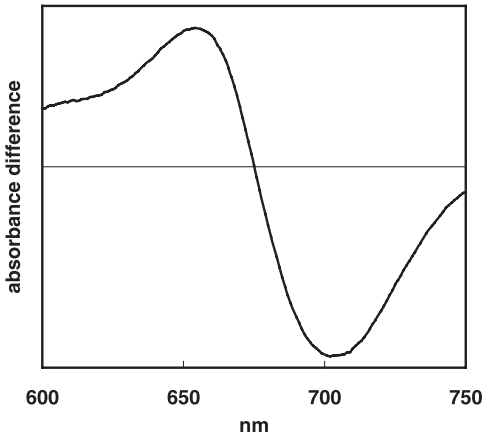

Fig. 1. Difference spectra obtained after assembly of apoCph1 and the induced crude extract of the rat *HO/pcyA* clone.

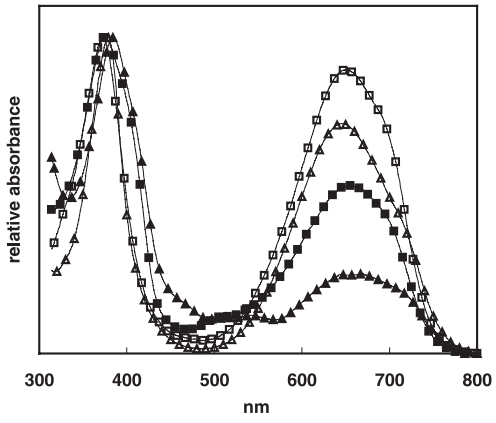

Fig. 3. Chloroform extracts of p40.1 (▲), BV (△), p45.2 (■) and PCB (□) (relative values).

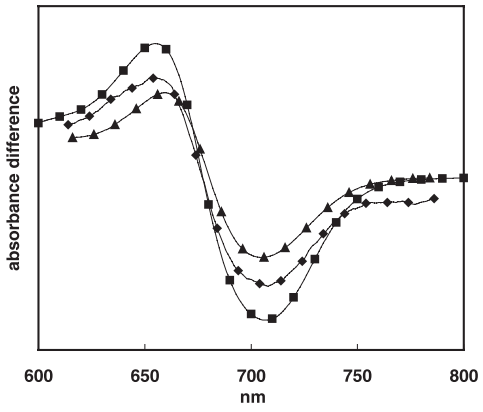

Fig. 4. Difference spectra of apoCph1 autocatalytically assembled in vitro with pure PCB (■) and crude extract of p45.2 (♦). Equivalent data for in vivo assembled holophytochrome from [pF10-His/p45.2] (▲), are included for comparison.

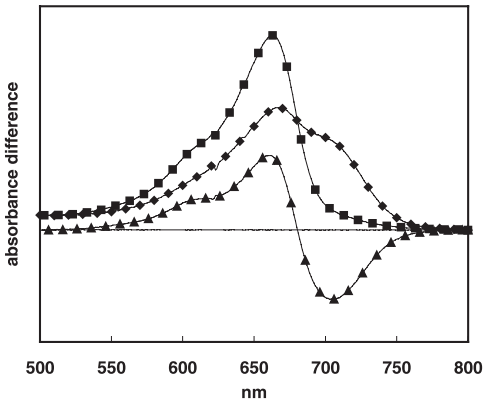

Fig. 5. Absorbance spectra of in vivo assembled holophytochrome from [pF10-His/p45.2] following affinity purification, after far-red (■) and red (♦) irradiation together with the corresponding difference spectrum (▲).

PAGE followed by Zn^{2+} and Coomassie staining (Fig. 2B). Approximately 80% of the Cph1 translational product was soluble and able to autoassemble with the PCB available in vivo as indicated by the zinc-induced fluorescence signal.

The resulting holophytochrome achieved through the overexpression of Cph1 with *ho1* and *pcyA* in *E. coli* was clearly apparent in the pelleted cells (Fig. 6).

4. Discussion

We were able to produce autocatalytically assembled holophytochrome in *E. coli* by co-expressing two genes for chromophore biosynthesis together with *cph1*. Although the absorbance spectra of the in vivo assembled holoCph1 resembled those of holoCph1 prepared conventionally by in vitro assembly with apoCph1 and PCB, interesting spectral differences were apparent (Table 1; Fig. 4). These might be due to a different final three-dimensional structure, perhaps resulting from assembly taking place during the translation process. In principle, such shifts could also arise were an alternative bilin to have been incorporated. Although we have not demonstrated directly that the chromophore of the in vivo adduct is PCB, we consider it most unlikely that the absorbance shifts result from the chromophore itself. Firstly, of the five naturally occurring bilins (see [2]) only PCB and phytochromobilin (PϕB) are known to form photochromic adducts with apophytochrome. Phycoerythrobilin (PEB) adducts are fluorescent rather than photochromic, while BV is not a substrate for the lyase function. Secondly, the spectra of the adduct formed from Cph1 and p45.2 extracts in vitro were identical to those with HPLC-purified PCB: conversely, PϕB adducts show pronounced bathochromic shifts especially in the far-red-absording form of phytochrome. Thirdly, although obviously impure, crude chloroform extracts of p40.1 and p45.2 following acid treatment showed spectra consistent with BV and PCB production. Nevertheless, a putative single-step conversion of BV to PCB by PcyA poses mechanistic problems as two double bonds need to be reduced. Models of this reaction generally assumed two enzymatic steps until the discovery of HY2 and PcyA. Clearly, further work is needed here.

The holoCph1 generated by [p45.2/pF10-His] overexpression has numerous potential uses in phytochrome research. Firstly, as the C-terminal oligohistidine tag permits easy purification via Ni-NTA affinity chromatography, it represents good starting material for biochemical and biophysical studies including crystallization. Secondly, the p45.2 plasmid allows mutant holoCph1 clones to be detected in mass colony screenings. For example, it is for the first time feasible to screen for amino acid substitutions which lead to fluorescence of PCB adducts (analogous to that seen when PEB is used as the chromophore [17]). Thirdly, holoCph1 in vivo might prove useful in elucidating the still unknown physiological function of Cph1 as in vivo signal transduction studies can now be performed in *E. coli*. Despite the clear functional divergence, Cph1 and plant phytochromes are likely to have a common ancestor, thus such information should be relevant to phytochromes in general.

5. Note added during preparation

At the Missouri Symposium on Plant Photobiology from 30 May to 2 June 2001 (University of Missouri, Columbia, MO, USA), J.C. Lagarias reported that his group had been able to produce holophytochrome in *E. coli* using similar methods to those reported here.

Table 1

	λ_{max} Pr	$\Delta\Delta A_{max}$	$\Delta\Delta A_{min}$	$\Delta\Delta A_{isosb.\ p.}$
pF10-His/p45.2	663	660	705	680
p45.2+apoCph1		655	708	678
PCB+apoCph1		655	707	678
PCB+apoCph1[12]	658	655	708	677
PϕB+apoCph1[12]		670	719	680

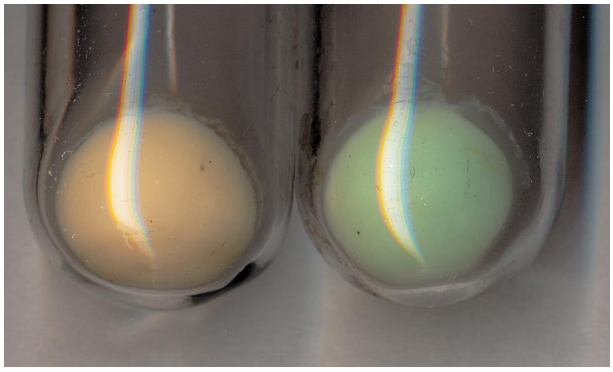

Fig. 6. Holophytochrome in *E. coli*. Clone [pF10-His/p45.2], uninduced control (left), and IPTG/arabinose-induced culture (right).

Acknowledgements: We wish to express our gratitude to Tanja Gans and Sigrid Ranostaj for excellent technical assistance. We also wish to thank Norbert Michael for the preparation of PCB. This work was funded by the Deutsche Forschungsgemeinschaft (DFG funding Hu703/2-1 and La799/6-1). КТО НЕ УСПЕЛ, ТОТ ОПОЗДАЛ.

References

[1] Hughes, J., Lamparter, T., Mittmann, F., Hartmann, E., Gärtner, W., Wilde, A. and Börner, T. (1997) Nature 386, 663.
[2] Hughes, J. and Lamparter, T. (1999) Plant Physiol. 121, 1059–1068.
[3] Yeh, K.C., Wu, S.H., Murphy, J.T. and Lagarias, J.C. (1997) Science 277, 1505–1508.
[4] Börner, T., Hübschmann, T., Wilde, A., Hughes, J. and Lamparter, T. (1998) in: Phototropic Prokaryotes (Peschek, G.A., Löffelhardt, W. and Schmetterer, G., Eds.), pp. 195–199. Kluwer Academic/Plenum, Dordrecht.
[5] Hübschmann, T., Börner, T., Hartmann, E. and Lamparter, T. (2001) Eur. J. Biochem. 268, 2055–2063.
[6] Lamparter, T., Esteban, B. and Hughes, J. (2001) Eur. J. Biochem. 268, 4720–4730.
[7] Cornejo, J., Willows, R.D. and Beale, S.I. (1998) Plant J. 15, 99–107.
[8] Muramoto, T., Kohchi, T., Yokota, A., Hwang, I. and Goodman, H.M. (1999) Plant Cell 11, 335–348.
[9] Ortiz de Montellano, P.R. (2000) Curr. Opin. Chem. Biol. 4, 221–227.
[10] Frankenberg, N., Mukougawa, K., Kohchi, T. and Lagarias, J.C. (2001) Plant Cell 13, 965–978.
[11] Kohchi, T., Mukougawa, K., Frankenberg, N., Masuda, M., Yokota, A. and Lagarias, J.C. (2001) Plant Cell 13, 425–436.
[12] Lamparter, T., Mittmann, F., Gärtner, W., Börner, T., Hartmann, E. and Hughes, J. (1997) Proc. Natl. Acad. Sci. USA 94, 11792–11797.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Sambrook, J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual, 3rd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Wilks, A. and Ortiz de Montellano, P.R. (1993) J. Biol. Chem. 268, 22357–22362.
[16] Ishikawa, K., Sato, M. and Yoshida, T. (1991) Eur. J. Biochem. 202, 161–165.
[17] Li, L., Murphy, J.T. and Lagarias, J.C. (1995) Biochemistry 34, 7923–7930.