Heterologous Production of *Halorhodospira halophila* Holo-Photoactive Yellow Protein through Tandem Expression of the Postulated Biosynthetic Genes†

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ABSTRACT: The photoactive yellow protein (PYP) is a bacterial photoreceptor which is the structural prototype for the PAS domain superfamily of regulators and receptors. PYP is known to have a unique *p*-hydroxycinnamic acid chromophore, covalently attached to a cysteine. To date, it has not been shown how holo-PYP is formed in vivo. Two genes, nearby *pyp*, were postulated to encode the biosynthetic enzymes, but only one was previously isolated and shown to have the requisite activity. By using a dual plasmid system, one expressing the PYP from *Halorhodospira halophila* and the other expressing a two-gene operon, consisting of tyrosine ammonia lyase and *p*-hydroxycinnamic acid ligase, we are able to present evidence that a functionally active holo-PYP can be synthesized in *Escherichia coli*. Plasmids containing only one of the two enzymes failed to produce holoprotein. Thus, the two genes have been shown to be both necessary and sufficient for production of holoprotein, although the activating group remains unknown. This expression system not only holds great potential for mutagenesis studies but also opens new possibilities in the search for (a) signaling partner(s) of the PYP.

The photoactive yellow protein (PYP)[1] is a small cytoplasmic blue light receptor that is found in purple phototrophic bacteria. It was first isolated from *Halorhodospira halophila* (*1*) where it is presumed to be involved in negative phototaxis to avoid damage by UV radiation (*2*). Closely related proteins were also found in *Rhodothallasium salexigens* and *Halochromatium salexigens* (*3*). On the DNA level, somewhat more divergent *pyp* sequences were found in *Rhodobacter sphaeroides* (*4*) and *Rhodobacter capsulatus* (*5*; www.Integratedgenomics.com). Recently, the *R. sphaeroides* PYP has been chemically reconstituted and shown to have significantly different properties (*6*). Moreover, in *Rhodospirillum centenum*, a PYP−phytochrome chimera known as Ppr has been isolated which appears to be involved in the regulation of chalcone synthase (*7*). PYP is the structural prototype of the PAS domain superfamily, which consists of regulatory proteins in both prokaryotes and eukaryotes (*8*).

Studies on *H. halophila* PYP have provided a wealth of biophysical and structural data, which shows that this photoreceptor is a very good model to study the mechanism of light perception in biological systems (e.g., refs *9*−*12*). The chromophore, necessary for light activation of PYP, is *p*-hydroxycinnamic acid, which is bound to Cys 69 via a thioester bond (*13*, *14*). Upon illumination, PYP undergoes a photocycle that involves a trans to cis isomerization of the chromophore. Analogous photocycles have been seen in, e.g., the unrelated bacteriorhodopsin and halorhodopsin. Several intermediates of the photocycle have been characterized, both photochemically and structurally. The ground-state P (λ_{max} = 446 nm) is converted into I_0 (≤2 ps) and then into $I_0^‡$ (220 ps), both absorbing at ∼510 nm (*10*, *15*). Subsequently, $I_0^‡$ proceeds in about 3 ns to the I_1 intermediate (λ_{max} = 465 nm) and further to the blue-shifted intermediate I_2 (λ_{max} = 350 nm) in about 200 μs, which decays to the ground state with a lifetime of about 140 ms. The long-lived I_2 intermediate is the proposed signaling state of PYP. The interaction partner of PYP and the downstream signaling pathway are still unknown.

Until now, the biosynthetic enzyme for the chromophore and the maturation enzyme for attachment to the apoprotein were unknown, and the only way to synthesize recombinant holo-PYP in *Escherichia coli* was to reconstitute the apoprotein with the chemically activated chromophore, e.g., as described by Imamoto et al. (*16*). On the basis of analogy to the phenylpropanoid pathway in plants (*17*), the chromophore is thought to be synthesized in vivo from tyrosine by a tyrosine ammonia lyase (TAL) and then to be activated by a *p*-hydroxycinnamic acid ligase (pCL) for binding to the apoprotein (Figure 1). We found a gene for a possible

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[1] Abbreviations: PYP, photoactive yellow protein; PAS, acronym formed of the names of the first three proteins recognized as sharing this sensor motif (*p*eriodic clock protein of *Drosophila*, *a*ryl hydrocarbon receptor nuclear translocator of vertebrates, *s*ingle-minded protein of *Drosophila*); TAL, tyrosine ammonia lyase; pCL, *p*-hydroxycinnamic acid ligase; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactoside; ACN, acetonitrile.

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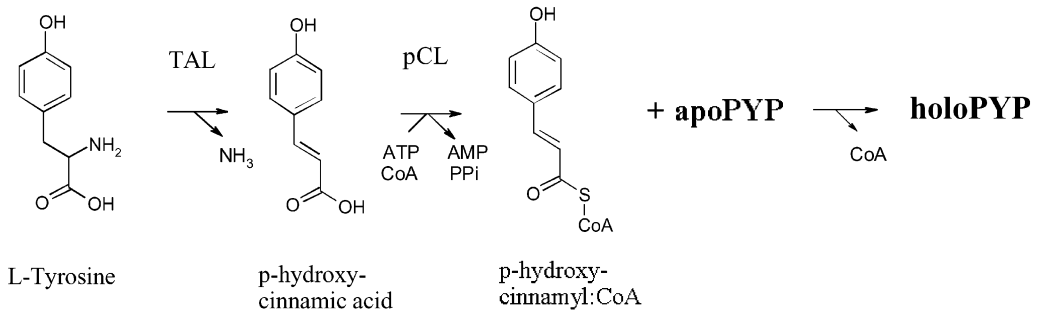

FIGURE 1: Biosynthesis of holo-photoactive yellow protein (PYP). Tyrosine ammonia lyase (TAL) catalyzes the conversion of L-tyrosine to *p*-hydroxycinnamic acid. Subsequently, the chromophore is most likely activated by binding to CoA at the expense of ATP, a reaction catalyzed by a presumed *p*-hydroxycinnamyl:CoA ligase (pCL) and the activated chromophore chemically attached to the apoprotein without the use of any additional enzymes.

TAL near the structural gene for PYP in *Rb. capsulatus* and expressed it in *E. coli* (*18*). The purified enzyme was shown to be specific for L-tyrosine and to convert it into *p*-hydroxycinnamic acid with good efficiency under physiological conditions. Genes encoding the proposed maturation enzyme (pCL) were found downstream of the *pyp* gene in *H. halophila*, *Rb. sphaeroides*, and *Rb. capsulatus* (*4*, *5*, *19*; www.Integratedgenomics.com), but the enzyme itself has not been characterized to date. Our own efforts to demonstrate activity were unsuccessful when coenzyme A was used as the activating group, perhaps because purified apoprotein is difficult to chemically reconstitute in vitro. *Rb. capsulatus* is the only organism in which both biosynthetic genes have been found to date.

The study described in this paper was performed to test the ability of the TAL and presumed pCL enzymes to produce holo-PYP in vivo. This was done by using a two-plasmid system. One of the plasmids contained a dual gene operon and produced the assumed biosynthetic proteins from *Rb. capsulatus*. The other plasmid expressed the *H. halophila* apo-PYP. By using the two plasmids, we established the feasibility of producing holo-PYP in the model bacterium *E. coli*. Plasmids containing only one of the two biosynthetic genes failed to produce holoprotein, thus proving that they are both necessary and sufficient. This expression system will facilitate mutagenesis studies and the search for downstream signaling partners of the PYP.

MATERIALS AND METHODS

Plasmid Construction. All molecular cloning experiments were performed according to Sambrook et al. (*20*). The cloning of the *pyp* gene from *H. halophila* has been described elsewhere (*21*). This gene was cloned into the pET20b vector (Novagen, Madison, WI; see Figure 2), where it was preceded by a pelB leader sequence. A periplasmic production of PYP was envisioned to facilitate the purification of the protein. To achieve cytoplasmic production of the PYP, we recloned the gene into pET15b (Novagen). This was done by digesting the pET20b(PYP) with *Nco*I and *Bam*HI restriction enzymes, which yielded the 375 bp *pyp* fragment (without the pelB leader sequence). The fragment was ligated into predigested pET15b, resulting in pET15b(PYP) (Figure 2). By cloning the *pyp* in this manner, the His-tag encoding sequence was removed from the pET15b.

To produce the dual biosynthetic gene operon, we first cloned the *Rb. capsulatus tal* gene into pACYC184 (NEBiolabs, Beverly, MA). Since the plasmid did not contain an inducible promotor or a multicloning site, we PCR-amplified a 200 bp region, which included the *tac* promoter, along with

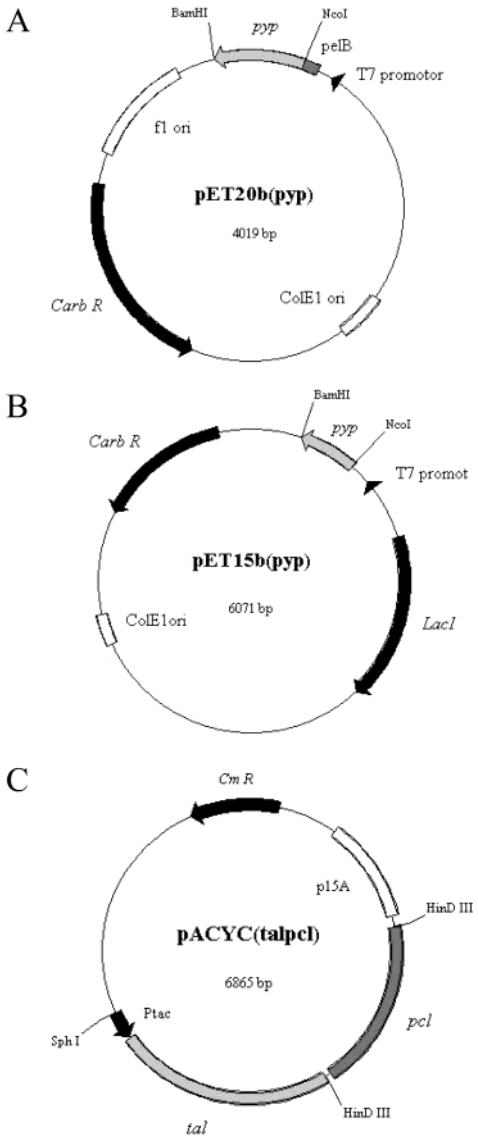

FIGURE 2: Expression vectors used in the dual plasmid system. (A) shows pET20b(pyp), constructed as described by Genick et al. (*21*) and containing a carbinicillin resistance gene (Carb R). There is a pelB leader in front of the *pyp* gene. (B) shows the pET15b-(pyp) construct, which also has a carbenicillin resistance gene. (C) shows the pACYC(talpcl) construct, on which a chloramphenicol resistance gene is encoded (Cm R). Both *tal* and *pcl* are under the control of an inducible *tac* promotor.

the *tal* gene. As template, the pKK223-3(TAL) plasmid was used (*18*). The primers used for this amplification were SphIpKK (ACATGCATGCGGAAGCTGTGGTATGGCTG) and TALHindIII (GCCCAAGCTTTCATGCCGGGGGATC) (restriction sites are underlined; additional nucleotides were added to the 5′ primer end to ensure efficient cleavage of the PCR product). We cloned the amplified 1826 bp fragment into the pACYC184 vector as a *Sph*I−*Hin*dIII fragment. This disrupted the tetracycline resistance gene of the vector and resulted in the pACYC(TAL) plasmid (not shown).

The presumed *pcl* gene was cloned into the pKK223-3 vector (Amersham Biosciences, Uppsala, Sweden) as we described earlier for *tal* (*18*). With the resulting pKK(pCL) construct as template, PCR was used to amplify a 1311 bpfragment with the following primers: HindHYBpCL (TCCCAAGCTTGTGGAATTGTGAGCGGATAAC) and pCLHind (CGCAAGCTTCAGTCCCAATCCCG). The primers were designed in such a way that the amplified fragment contained the ribosome binding site of the pKK223-3 vector, followed by the gene for pCL. This fragment was cloned after the *tal* gene as a *Hin*dIII−*Hin*dIII fragment in the pACYC(TAL) plasmid. The resulting expression plasmid, pACYC(TALpCL), had both *tal* and *pcl* under the control of an inducible *tac* promotor (see Figure 2) and contained a p15A origin of replication and a chloramphenicol resistance encoding gene. The orientation of the *pcl* fragment was checked by restriction digest analysis and sequencing.

Protein Production and Purification. All expression experiments were performed with *E. coli* BL21(DE3). After transformation of both pET20b(PYP) or pET15b(PYP) and pACYC(TALpCL) to the bacterial cells, cultures were grown on carbenicillin (Cb, 100 μg/mL) and chloramphenicol (Cm, 25 μg/mL) antibiotics. An overnight culture was used to inoculate 1 L LB medium containing both antibiotics. This culture was grown at 28 °C and induced, at an OD_{600} of approximately 0.8, with a final concentration of 0.5 mM IPTG. The cells were pelleted by centrifugation after 16 h of induction. After resuspension in Tris-HCl buffer (50 mM, pH 9.0) the cells were freeze−thawed two times and further fractionated by sonication, followed by centrifugation to remove the cell debris. After dialyzing the supernatant, the sample was loaded onto a 10 mL Q-Sepharose Fast Flow column (Amersham Biosciences). A Tris-HCl buffer (50 mM, pH 9.0) was used to apply the sample, and proteins were eluted with the same buffer supplemented with an increasing amount of NaCl. The PYP eluted at approximately 250 mM NaCl. The yellow-colored fractions were pooled and concentrated on Ultrafree-4 centifugal filters (Millipore, Bedford, MA). The purification was continued by size exclusion chromatography on a Superdex 75 column (Hiload 16/60, Amersham Biosciences) with 100 mM Tris-HCl, pH 8.0, supplemented with 50 mM NaCl as running buffer. This step was performed using an ÄKTA Explorer HPLC system (Amersham Biosciences). After dialyzing the yellow sample, we proceeded with the purification on a MonoQ (HR 5/5, Amersham Biosciences) anion-exchange column. At this point, the PYP had a purity greater than 95%, and any nonreconstituted apo-PYP was absent.

Mass Spectrometry. Molecular mass determination was performed on a Q-TOF mass spectrometer (Micromass, Manchester, U.K.) equipped with a nano-electrospray source. Approximately 5−10 pmol of protein was dissolved in 5 μL of 50% ACN/0.1% HCOOH and loaded into a nanospray capillary. To perform MS/MS, the holo-PYP was digested with trypsin in 50 mM NH_4HCO_3 for 4 h. The ratio of trypsin to holo-PYP was 1/40 (w/w).

UV/Vis Spectroscopy. Absorption spectra were obtained by using a UVIKON spectrophotometer (Kontron, Herts, U.K.). Spectra were measured in Tris-HCl buffer of varying molarity and pH, according to the preceding purification scheme.

Time-Resolved Spectroscopy. The laser flash photolysis and spectroscopy apparatus, and the methods used for data analysis, were as previously described (*22*). The PYP sample was dialyzed against a universal buffer (20 mM MES, 20 mM HEPES, and 20 mM glycine, pH 8.0) prior to the experiment. We used 1 mL samples with an absorbance at 445 nm of approximately 0.2 for the kinetic measurements.

RESULTS

The genes encoding the two assumed biosynthetic enzymes of PYP were cloned into the low-copy pACYC184 vector. The construct was made in such a way that a synthetic operon, containing *tal* and *pcl*, was created (see Materials and Methods and Figure 2 for details). This allowed us to express both genes by inducing the strong *tac* promotor with IPTG. Since the pACYC(TALpCL) construct has a different origin of replication and antibiotic resistance encoding genes from pET20b(PYP) (see Figure 2 and ref *21*), it is possible to maintain both plasmids in the same cells by continuous selection with carbenicillin and chloramphenicol.

E. coli BL21(DE3) cells harboring both pET20b(PYP) and pACYC(TALpCL) were used to produce holo-PYP. After 4 h of induction, the cultures were centrifuged and the pellet already displayed a bright yellow color. The amount of yellow color increased with longer induction times, with an approximate maximum after 16 h of induction. Cells containing neither of the plasmids and cells harboring only pET20b(PYP) or only pACYC(TALpCL) served as controls. Centrifugation of these cultures after induction did not result in a yellow-colored pellet. Since the pCL enzyme has not been enzymatically characterized, it was important to determine whether pCL was necessary for holo-PYP synthesis. To this end, constructs were grown with TAL as the only biosynthetic enzyme. This was done by transforming pET20b-(PYP)-bearing cells with the pACYC(TAL) plasmid. After 18 h of induction, the cotransformants did not show any color change, which unambiguously shows that pCL is required for the in vivo formation of holo-PYP.

Crude cell extracts of the cotransformants containing all three genes were analyzed spectrophotometrically in the 350−550 nm region. Extracts of cells with only the pET20b-(PYP) construct served as a blank. The spectrum is shown in Figure 3. It can clearly be seen that there is an absorbance peak with a maximum around 446 nm, which is consistent with that for the wild-type *H. halophila* PYP. This is a first indication that the PYP has been reconstituted in vivo and has the expected conformation of the ground state. The maximum amount of holo-PYP present in these crude cell extracts was calculated to be 80 mg of protein/L of culture. We found that noninduced overnight cultures, which contained all three genes, also produced holo-PYP, but with a yield of approximately 15 mg of holo-PYP in crude cell extracts per liter of culture.

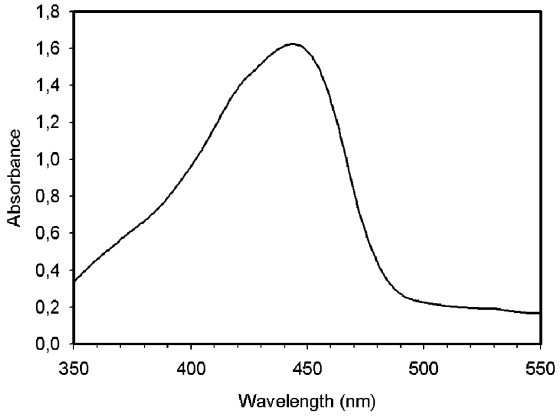

FIGURE 3: Crude holo-PYP spectrum. Cells were induced for 16 h and broken by sonication. Crude cell lysate from cells containing only the pET20b(pyp) served as a blank.

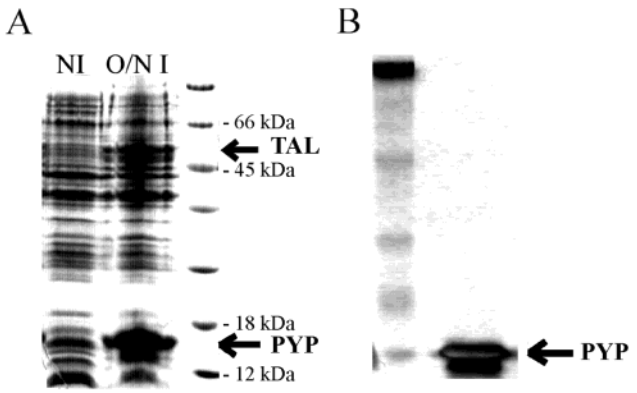

FIGURE 4: SDS−PAGE of (A) crude cell extracts before (NI) and after (O/N I) overnight induction. The proteins were visualized by Coomassie staining. The lower arrow indicates the 14 kDa PYP. Leakage of expressed PYP can be seen in the noninduced samples. The upper arrow indicates the production of TAL. (B) shows a silver-stained SDS−PAGE gel of the purified holo-PYP.

Figure 4A shows an SDS−PAGE of whole cells containing both plasmids before and after overnight induction. The production of PYP can clearly be seen at 14 kDa. Although the above experiments showed that TAL and pCL production was necessary for holo-PYP production, we were unable to unambiguously show the expression of either *tal* or *pcl* on SDS−PAGE gels of different concentrations. This is likely to be a consequence of the low copy number of the pACYC vector, but even a limited amount of the biosynthetic enzymes seems to be sufficient to produce large amounts of activated chromophore and holoprotein. Yet, in some experiments a minor production of a protein around 56 kDa could be seen (see Figure 4A). N-Terminal sequencing identified the produced protein as the TAL enzyme.

To determine whether the PYP resulting from the two-plasmid construct has a normal photocycle and kinetics, we purified the yellow-colored protein as described in Materials and Methods. The purified protein was found to have a purity of about 98%, based on silver staining an SDS−PAGE gel (Figure 4B). As can be seen from Figure 5, the protein still has the characteristic 446 nm absorption. The yield of purified holo-PYP was approximately 48 mg/L of culture, which is more than twice as high as the yield reported by Genick et al. (*21*), who chemically attached the chromophore to the recombinant apo-PYP. The ratio of the 446 to 280 nm absorption is approximately 2.2 for the purified protein.

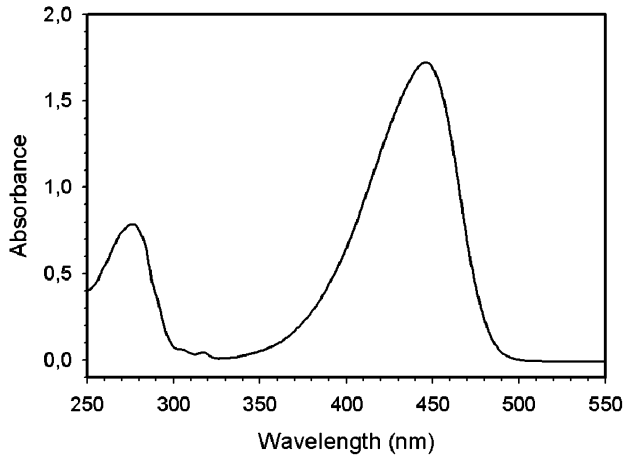

FIGURE 5: Absorption spectrum of the purified holo-PYP in Tris-HCl buffer at pH 9.0. No apoprotein could be detected by mass spectrometry. This spectrum is comparable to Figure 1 in ref *1*, which shows the absorption spectrum of purified wild-type PYP.

This is identical to the best ratios found for highly purified PYP from *H. halophila*.

To ascertain that the in vivo formed PYP has covalently attached *p*-hydroxycinnamic acid, we analyzed the purified protein by mass spectrometry. The mass of the denatured state was 14020 Da, which is 147 Da larger than the theoretically calculated mass of the apoprotein and corresponds to the mass of protein plus chromophore. This result is in agreement with the MS results for wild-type PYP reported by Van Beeumen et al. (*23*). Analysis of the PYP sample, after the size exclusion purification step, showed evidence of a small amount of nonreconstituted apo-PYP. After further fractionation on the MonoQ column, apo-PYP could no longer be detected by mass spectrometric analysis. This, together with the 446 to 280 nm absorption ratio of 2.2, indicates that the holo-PYP was fully separated from the apo form during the final purification step. After digesting the holo-PYP with trypsin, we performed MS/MS measurements, which confirmed that the *p*-hydroxycinnamic acid is attached to Cys 69, as was found with wild-type PYP (*23*).

To investigate whether the occurrence of apo-PYP is related to the periplasmic location of the PYP, we recloned the *pyp* gene into the pET15b vector, which did not contain the pelB leader sequence. By expressing the gene from this construct, we could achieve cytoplasmic production of the protein. Cotransformants containing both the pET15b(PYP) and the pACYC(TALpCL) were tested for their ability to produce holo-PYP, in a way analogous to what we described for the pET20b(PYP) construct. The yield of holo-PYP in crude cell extracts was approximately the same as with the periplasmic PYP production, and surprisingly, there was still a significant amount of apo-PYP present in the partially purified protein sample.

By flashing the PYP sample with laser light of 445 nm, we were able to determine the kinetics of the light-induced absorbance changes. Panels A and B of Figure 6 show the $I_1 \rightarrow I_2$ bleach reaction and the $I_2 \rightarrow P$ recovery of our in vivo formed holo-PYP. It can be seen that, after a flash with 445 nm light, the PYP went through a normal photocycle. After fitting the data, we found that the kinetics of both the bleach and recovery reactions were similar to those for wild-type PYP. The rate of recovery was 7.2 s^{-1}, compared to

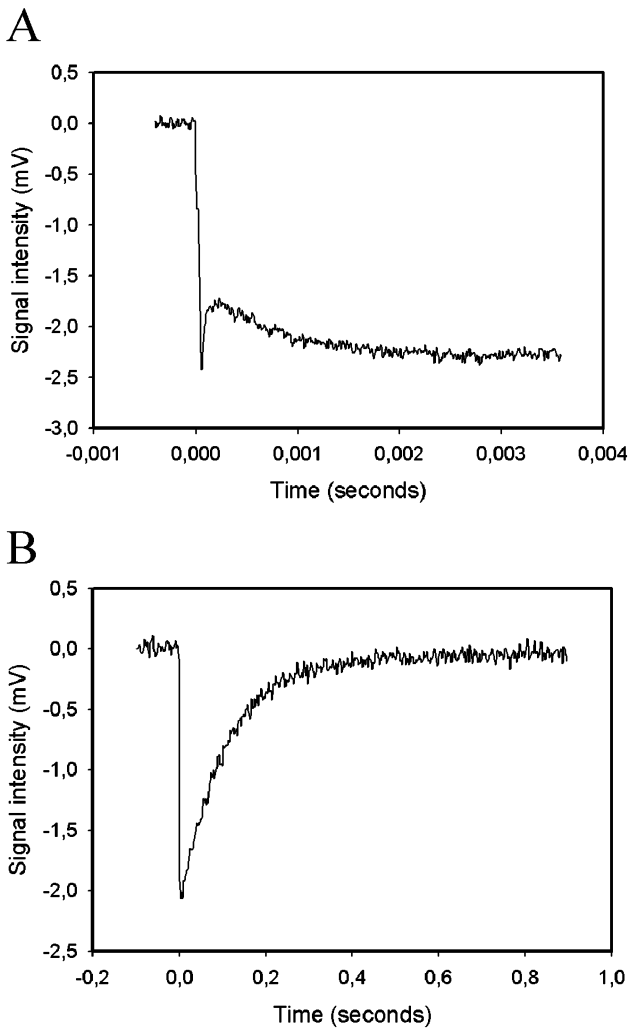

FIGURE 6: Kinetics of the light-induced absorbance changes: $I_1 \rightarrow I_2$ bleaching reaction (A) and $I_2 \rightarrow P$ recovery (B). The excitation wavelength was 445 nm. The sample contained PYP with an absorption at 446 nm of 0.2. Measurements were performed at pH 8.0 in universal buffer. These spectra are comparable to the measurements with wild-type PYP (see Figure 2 in ref *22*).

6.3 s^{-1} for wild-type PYP and chemically reconstituted PYP (*21*, *22*). The rate constant of the bleach was found to be 1.6 ms^{-1}. Since this is significantly different from the 3.4 ms^{-1} reported by Genick et al. (*21*), we repeated the experiment with native PYP under the current conditions and found the rate to be 1.3 ms^{-1}. This is approximately the same as what we measured with recombinant PYP. The small differences in kinetics are not particularly significant since the kinetics have been shown to be sensitive to environmental effects (*22*). The fact that the recombinant PYP is photoactive, with kinetics that closely resemble the wild type, establishes that the recombinant PYP is correctly folded and functional.

DISCUSSION

This study documents the production of a photoactive yellow protein in its fully reconstituted form, in the model bacterium *E. coli*, through coexpression of the gene for apo-PYP and the genes for the two assumed biosynthetic enzymes, namely, a tyrosine ammonia lyase and a *p*-hydroxycinnamic acid ligase. Our work thus establishes that both biosynthetic enzymes are functional in *E. coli* cells and are required for attachment of the chromophore. This implies that the *p*-hydroxycinnamic acid, which is formed from L-tyrosine (*18*), can serve as a substrate for the pCL, which activates the chromophore for covalent attachment to the apo-PYP. Whether the pCL uses coenzyme A or some other thiol-containing substrate to activate the *p*-hydroxycinnamic acid and the underlying reaction mechanism for activation is currently under investigation. In any case, our experiments have shown for the first time that pCL is the maturation enzyme for attachment of *p*-hydroxycinnamic acid to the apo-PYP.

Approximately 80 mg of photoactive yellow protein could be recovered in crude cell extracts per liter of culture, which enabled the purification of as much as 48 mg of holo-PYP. It was found that noninduced overnight cultures also produced a substantial amount of holo-PYP (approximately 15 mg in crude cell extracts per liter of culture). This "leakage" in the expression system may be a consequence of a limiting amount of the $LacI^q$ repressor, used by both the *tac* promotor and the T7 RNA polymerase promotor. Providing the cells with a higher level of the repressor (either by a different host or by incorporating an extra copy of the *lacI*^{q} gene on one of the plasmids) might prevent this leakage from occurring.

Laser flash experiments together with mass spectrometric analysis showed that the in vivo formed PYP was reconstituted and photoactive. The photocycle kinetics were found to be similar to those for wild-type or chemically reconstituted PYP from *H. halophila* (*21*, *22*), with the rate constants for the bleach and recovery reactions being 1.6 ms^{-1} and 7.2 s^{-1}, respectively. These data, together with the absorption spectra, establish that the heterologously produced holo-PYP is correctly assembled in vivo.

The MS experiments showed evidence for the presence of some nonreconstituted apo-PYP. We assumed this could be due to the artificially periplasmic expression of the apo-PYP, while the biosynthetic enzymes are located in the cytoplasm. We therefore expressed the PYP in the cytoplasm as in the native *H. halophila* but found that the yield of holo-PYP was essentially the same as obtained by the periplasmic expression and that again apo-PYP was present in the partially purified protein sample. This can be a consequence of the misfolding of some of the apo-PYP, since chemical reconstitution from purified apo-PYP leads to low yields (unpublished results). Induction of the biosynthetic genes prior to that for the *pyp* gene may yield a higher ratio of holo- to apo-PYP, or expression of the biosynthetic genes on a high copy number plasmid may increase the amount of the biosynthetic enzymes and, therefore, of holo-PYP. Experiments to clarify these issues are in progress.

The ability to produce large amounts of holo-PYP in *E. coli* will facilitate mutational studies of PYP, which will provide further insight into its photocycle and related structural issues. A second beneficial aspect of the in vivo expression of holo-PYP is that it opens the way for two-hybrid-based studies to search for PYP signaling partners by screening a library of potential targets in *E. coli* since such experiments were unsuccessful using the apoprotein.

Further studies with the biosynthetic enzymes using apo-PYP proteins from other bacteria are under way and have been successful in cases where chemical reconstitution of apo-PYP failed, e.g., with the PYP from *R. capsulatus*

(unpublished results). In general, we believe that the in vivo production of holo-PYP will lead to a better understanding of the mechanism and physiological roles of this intriguing photoactive protein.

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