Biosynthesis of Fluorescent Allophycocyanin α-Subunits by Autocatalytic Bilin Attachment†

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ABSTRACT: Allophycocyanin (APC) is one of the phycobiliproteins expressed in cyanobacteria. Phycobiliproteins contain a covalently bound chromophore, and thus, they are valuable as fluorescent probes. Biosynthesis of a functional phycobiliprotein is achieved by a bilin attachment process between the chromophore and apoprotein. Chromophore lyases are necessary to catalyze the chromophorylation of cyanobacterial phycobiliproteins, such as C-phycocyanin, and phycoerythrocyanin. To identify the lyase that catalyzes the chromophorylation of the APC α-subunit (ApcA), we searched the entire genomes of two cyanobacteria, *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC 7120; however, these genomes do not appear to encode an APC-specific chromophore lyase. In this study, chromophorylated ApcA (chromo-ApcA) was obtained via a spontaneous bilin attachment reaction. The absorption and fluorescence characteristics of chromo-ApcA were similar to those of the native APC α-subunit. The extent of chromophore attachment to apo-ApcA was comparable to that of the lyase-catalyzed reactions for other phycobiliproteins. These results indicate that ApcA has autocatalytic bilin:biliprotein lyase activity.

Phycobilisomes are unique photosynthetic light-harvesting complexes found in cyanobacteria and red algae (*1*−*5*). The main components of these complexes are phycobiliproteins (*1*, *4*), which are covalently attached with tetrapyrrol chromophores (bilin), giving them distinct absorption and emission spectra in the visible range (*6*, *7*). Allophycocyanin (APC),[1] C-phycocyanin (CPC), and phycoerythrocyanin (PEC) are the three most common phycobiliproteins in cyanobacteria, and each contains the covalently attached chromophore, phycocyanobilin (PCB) (*1*). Ferredoxin-dependent heme oxygenase (HO1) and phycocyanobilin:ferredoxin oxidoreductase (PcyA) are two key enzymes involved in the synthetic process from cellular heme to PCB (*8*). PCB reacts with a cysteine residue of the apophycobiliprotein to form a thioether bond, and this reaction is required for the biosynthesis of functional phycobiliprotein (*9*−*11*).

The attachment of chromophores to apobiliproteins has been studied extensively over the past 20 years (*9*−*19*). The attachment of PCB to cysteines of the CPC α-subunit is catalyzed by a specific lyase, CPC α-subunit PCB lyase (CpcE/CpcF) (*14*, *15*). In addition, the PEC α-subunit phycoviolobilin (PVB) lyase (PecE/PecF) catalyzes the attachment of PCB to the PEC α-subunit and isomerizes PCB to PVB (*16*−*19*). Genes encoding the chromophore lyases of CPC (*cpcE*/*cpcF*) and PEC (*pecE*/*pecF*) are located in the *cpc* and *pec* operons, respectively. The entire genomes of two cyanobacteria, *Synechocystis* sp. PCC6803 (*20*) and *Anabaena* sp. PCC 7120 (*21*), have been sequenced; however, on the basis of the Gene Category List of *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC7102 in the CyanoBase database, there is no lyase shown to catalyze the chromophorylation of APC. BLAST was used to perform a survey of different databases at NCBI, but no additional sequence which was similar to *cpcE*, *cpcF*, *pecE*, or *pecF* and might be the chromophore lyase of APC was found.

Cyanobacterial phytochromes (*22*−*25*) and the core−membrane linker (L_{CM}) (*2*, *3*) constitute other examples of proteins having covalently attached chromophores. Each phytochrome has a chromophore lyase domain in its N-terminal region, and thus, these proteins can autocatalyze the incorporation of chromophores (*26*−*28*). L_{CM} also is a PCB-bearing chromoprotein, and it is responsible for the attachment of phycobilisomes to the photosynthetic membrane. As reported recently (*29*), the reaction between L_{CM} and PCB results from the autocatalytic activity of L_{CM}. Thus, the other possible mechanism of chromophore attachment to apo-APC may be through autocatalysis.

Here we report the biosynthesis of the APC α-subunit (ApcA) and the attachment of PCB to the apo-ApcA. Native APC is a trimeric protein, consisting of three (α*β*) monomers (*30*). Each α- or *β*-subunit is covalently bound to a single PCB moiety (*31*, *32*). The sequences of APC and CPC are 50% similar, and they also contain the same chromophore, PCB (*1*). On this basis, we examined whether CpcE/CpcF participates in the biosynthesis of the APC α-subunit. We assembled fully active chromophorylated ApcA (chromo-ApcA), and it possessed the same absorption spectral and fluorescent characteristics as the purified holo-APC α-sub-

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[1] Abbreviations: APC, allophycocyanin; CPC, C-phycocyanin; PCB, phycocyanobilin; PVB, phycoviolobilin (phycobiliviolin); ApcA, APC α-subunit; chromo-ApcA, chromophorylated APC α-subunit.

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Table 1: Cloning Materials

constructed vector	gene	primers	restriction enzymes	expression vector	antibiotic
pHPduet	*ho1*	5′-TCA TGA GTG TCA ACT TAG CTT CC-3′ and 5′-GAA TTC CTA GCC TTC GGA GG-3′	*Bsp*HI and *Eco*RI	pACYCDuet (Novagene)	34 μg/mL chlorophenicol
	pcyA	5′-CAT ATG GCC GTC ACT GAT TTA AGT TTG ACC-3′ and 5′-CTC GAG TTA TTG GAT AAC ATC AAA TAA GAC TTG GC-3′	*Nde*I and *Xho*I	pACYCDuet (Novagene)	34 μg/mL chlorophenicol
pEFduet	*cpcE*	5′-CAT ATG AGT GAA CCT AAC CTC AAC CCC G-3′ and 5′-TTA GAG TAA ACT ATC CAT TAA TTC C-3′	*Nde*I and *Bam*HI	pET23b (Novagene)	100 μg/mL ampicillin
	cpcF	5′-AGG AGG GCT AAC ATA TGG AGG GTA ATA GCG-3′ and 5′-CTA GAT TGG GCC GAT GTT TTC CAG G-3′	*Sal*I and *Xho*I	pET23b (Novagene)	100 μg/mL ampicillin
pSAa	*apcA*	5′-GGA TCC GAT GAG TAT CGT TAC CAA ATC C-3′ and 5′-GTC GAC TGA CAT TGC ACC AAT TAG G-3′	*Bam*HI and *Sal*I	pETDuet (Novagene)	100 μg/mL ampicillin

unit. Interestingly, the presence or absence of CpcE/CpcF during the synthesis of chromo-ApcA yielded the same chromoprotein adduct, suggesting that CpcE/CpcF is not a chromophore lyase for APC and that APC is chromophorylated via an autocatalytic mechanism. We also demonstrate an in vitro attachment reaction between pure apo-ApcA and PCB, confirming the autocatalytic mechanism for ApcA chromophorylation. The extent of attachment of PCB to apo-ApcA was comparable to that of the lyase-catalyzed reactions of other phycobiliproteins (*15*). These results reveal that our experimental conditions allowed the covalent assembly of PCB and apo-ApcA, suggesting that ApcA is an autocatalytic bilin:biliprotein lyase.

MATERIALS AND METHODS

Construction of Plasmids. The *Synechocystis* sp. PCC6803 *ho1*, *pcyA* (*8*, *33*, *34*), *cpcE*, and *cpcF* genes and the *Spirulina* sp. *apcA* gene were amplified by PCR using specific primers (Table 1) and Taq DNA polymerase. The chromosomal DNA of *Synechocystis* and *Spirulina* was isolated according to the method of Wu et al. (*35*). All the PCRs were run for 30 cycles (95 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s) and with an additional incubation at 72 °C for 10 min. The PCR products were then digested with the appropriate restriction enzymes (Table 1) and purified with a PCR Clean up kit (Qiagen).

To generate the pHPduet plasmid, a PCR-amplified DNA fragment containing the *ho1* gene was cloned between the *Bsp*HI and *Eco*RI sites of the first multicloning site (MCS) of pACYCDuet (Novagene). Another DNA fragment containing the *pcyA* gene was cloned between the *Nde*I and *Xho*I sites of the second MCS of pACYCDuet. The *cpcE* DNA fragment was cloned between the *Nde*I and *Bam*HI sites of vector pET23b (Novagene) to generate the pEmono plasmid. Sequentially, the DNA fragment of *cpcF* was cloned between the *Sal*I and *Xho*I sites of the pEmono plasmid to produce the bicistronic plasmid, pEFduet. To facilitate the expression and purification of His_6-tagged ApcA, the expression vector was constructed. The DNA fragment of *apcA* was cloned between the *Bam*HI and *Sal*I sites of vector pETDuet (Novagene) to generate the pSAa plasmid. Two ApcA mutations, A-C81A and A-C81S, were constructed with the QuikChange site-directed mutagenesis kit (Stratagene), respectively. All of the constructed plasmids were sequenced to check for the validity of gene sequences.

Overexpression and Purification of Proteins. The expression vectors described above were individually transformed into *Escherichia coli* (*E. coli*) strain BL21(DE3) (Novagene) for protein overexpression. A single colony of transformed BL21(DE3) was cultured in 20 mL of LB medium with antibiotics (Table 1) at 37 °C overnight. The bacterial culture was transferred into 400 mL of LB containing antibiotics and incubated with shaking at 30 °C. After the absorbance at 600 nm reached 0.6, the cultures were induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 30 °C for 4 h. The cultures were subsequently harvested by centrifugation (1000*g* for 30 min at 4 °C). Cell pellets were resuspended in 20 mL of buffer A [50 mM sodium phosphate and 150 mM NaCl (pH 7.4)]. After sonication, the cell debris was removed by centrifugation (17000*g* for 30 min at 4 °C).

Cell lysate containing His_6-tagged proteins were loaded into an open column containing 4 mL of TALON metal affinity resins (BD Biosciences). The resins were washed with 5 column volumes each of buffer A, buffer B (buffer A containing 10 mM imidazole), and buffer C (buffer A containing 25 mM imidazole). His_6-tagged proteins were eluted from the resin with 2 column volumes of buffer D (buffer A containing 100 mM imidazole) and then dialyzed against 50 mM sodium phosphate (pH 7.0). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories), after serial dilutions of the pure protein.

Preparation of Chromo-ApcA. The equal amounts of apo-ApcA and CpcE/CpcF in the cell lysate were estimated by SDS−PAGE. To determine the concentration of PCB in lysates, lysate was 10-fold diluted with methanol containing 5% HCl. The concentration of PCB was then quantified by absorption using an excitation coefficient of 37.9 mM^{-1} cm^{-1} at 690 nm (*36*).

Culture lysates [in 50 mM sodium phosphate (pH 7.0)] containing an equal molar quantity of apo-ApcA and PCB were mixed and incubated in the dark for 2 h at 20 °C. To examine whether CpcE/CpcF is necessary for the PCB attachment reaction, cell lysates from *E. coli* bearing the pEFDuet plasmid were added to the reaction mixture, and then the experiment was performed under the same conditions. For spectral measurements, the PCB adduct was then purified using TALON metal affinity resins as described above and dialyzed against 50 mM sodium phosphate (pH 7.0).

Purification of PCB. To obtain pure PCB from *E. coli*, plasmid pHPduet was employed for production of *Synechocystis* HO1 and PcyA, after IPTG induction as described previously. PCB was then derived from cellular heme by

HO1 and PcyA (*8*) under this culture condition. The cell pellet was harvested and resuspended with 100% acetone, and PCB was released from the cells. Cell debris was removed by centrifugation (17000*g* for 30 min at 4 °C), and organic solvent was removed by a spin vacuum. The dry PCB product was dissolved in 100% methanol and stored at −80 °C. The concentration of PCB (in methanol containing 5% HCl) was quantified by absorption using an excitation coefficient of 37.9 mM^{-1} cm^{-1} at 690 nm (*36*).

Electrophoresis and Zn^{2+}−UV Fluorography. PCB covalently modified proteins were detected by Zn^{2+}−UV fluorography (*37*). Two micrograms of proteins was loaded and separated by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to Berkelman et al. (*37*) with modifications. The final concentration of 1 mM zinc chloride was added to both gels and running buffers. After electrophoresis, the chromophorylated protein bands on gels were visualized by UV fluorography before they were stained with Coomassie blue.

Spectral Analysis. The circular dichroism (CD) spectra of chromophorylated ApcA were obtained using an AVIV 202 spectropolarimeter. A 10 μM protein sample [in 20 mM sodium phosphate buffer (pH 7.0)] was scanned from 260 to 195 nm at 25 °C. Thermal unfolding was monitored at 222 nm over a range of temperatures (16−96 °C).

UV−vis absorbance spectra were recorded with a Hitachi U-3010 spectrophotometer using a 1 cm path length cuvette with protein concentrations of 3 μM [in 50 mM sodium phosphate buffer (pH 7.0)]. All spectra were obtained at 25 °C, using a bandwidth of 2 nm, and a scan speed of 600 nm/min.

Emission spectra were monitored from 600 to 700 nm with excitation at 600 nm using a Perkin-Elmer LS 55 luminescence spectrometer at 25 °C. Each protein sample concentration was 3 μM [in 50 mM sodium phosphate buffer (pH 7.0)]. Both excitation and emission slit widths were set at 5 nm, and a scan speed was 100 nm/min.

The masses of purified proteins and PCB were determined using a Micromass Quattro Ultima electrospray ionization mass spectrometer (ESI-MS). The samples were desalted with ZipTips according to the instructions of the manufacturer (Millipore) and finally prepared in 75% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA). Samples were flow injected (∼10 pmol/μL, 5 μL) into the source of the mass spectrometer at a rate of 20 μL/min utilizing a carrier solvent of H_2O and ACN (1:1, v/v). Data were acquired over a wide scan mass range of m/z ∼500−1500 with a cone voltage of 60 V and a capillary voltage of 2.5 kV. Output masses of different proteins were calculated and averaged using Micromass Maximum Entropy.

In Vitro Bilin Attachment Assay. The assembly assay was performed with 1 nmol each of purified PCB and purified apo-ApcA in 100 μL of 50 mM sodium phosphate (pH 7.0). After rapid mixing had been carried out, the absorption spectrum during the assembly was measured from 500 to 700 nm at 2 min intervals using a Hitachi U-3010 spectrophotometer. The fluorescence spectra during the assembly were also measured at 2 min intervals using a Perkin-Elmer LS 55 luminescence spectrometer. Emission spectra were monitored from 600 to 700 nm with excitation at 600 nm.

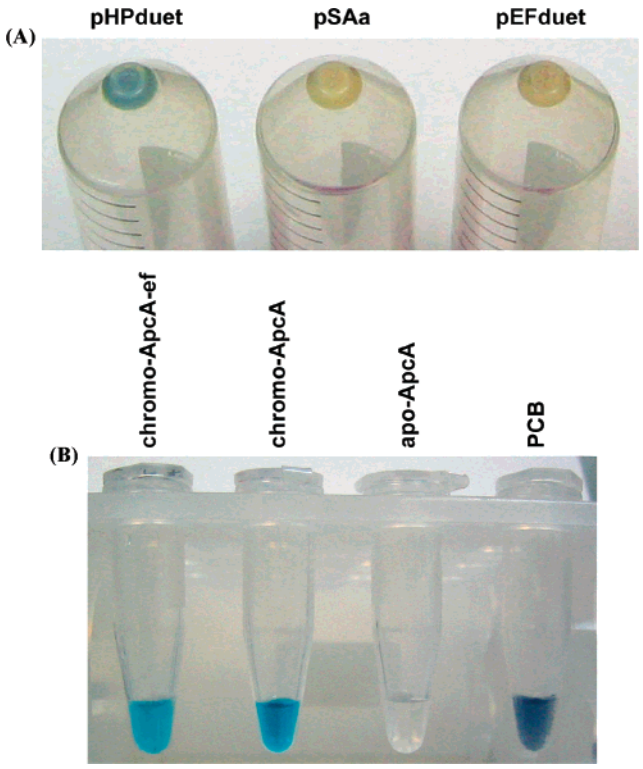

FIGURE 1: (A) IPTG-induced cell pellet and (B) purified PCB and protein samples.

RESULTS

Protein Expression. To investigate the biosynthetic process of APC, we used *E. coli* to produce cyanobacterial proteins, including the enzymes (HO1 and PcyA) that produce the chromophore PCB as well as the chromophore lyases for the CPC α-subunit (CpcE/CpcF) and the APC α-subunit apoprotein (apo-ApcA). To obtain the soluble proteins, we optimized the culture conditions, extraction, and purification processes. The solubility of the proteins and stability of PCB were increased by (a) reducing the concentration of IPTG from 2 to 0.5 mM, (b) lowering the incubation temperature from 37 to 30 °C during protein expression, and (c) harvesting the culture within 4 h of induction by IPTG. PCB is derived from heme by ferredoxin-dependent heme oxygenase (HO1) and phycocyanobilin:ferredoxin oxidoreductase (PcyA) (*8*). The *E. coli* cells containing plasmid pHPduet turned deep blue-green after a 4 h induction with IPTG at 30 °C (Figure 1A). The blue color of the cell pellets clearly revealed the production of PCB. The cells harboring either plasmids pEFduet or pSAa, in contrast to pHPduet, displayed no color change after induction with IPTG.

Reconstitution of Chromo-ApcA. Since no chromophore lyase responsible for chromophorylation of APC was known, we first examine whether CpcE/CpcF participates in the biosynthesis of ApcA and serves as the lyase. The *E. coli* BL21(DE3) cells bearing expression plasmids pSAa, pHPduet, and pEFduet were induced with IPTG. After cell lysis by sonication, the clear supernatants were obtained by centrifugation. Reconstituted mixtures from three different cell lysates were further incubated, and the His_6-tagged ApcA protein was purified with a metal affinity column. The eluted blue protein (Figure 1B) was named chromo-ApcA-ef and was then analyzed by SDS−PAGE in the presence of 1 mM

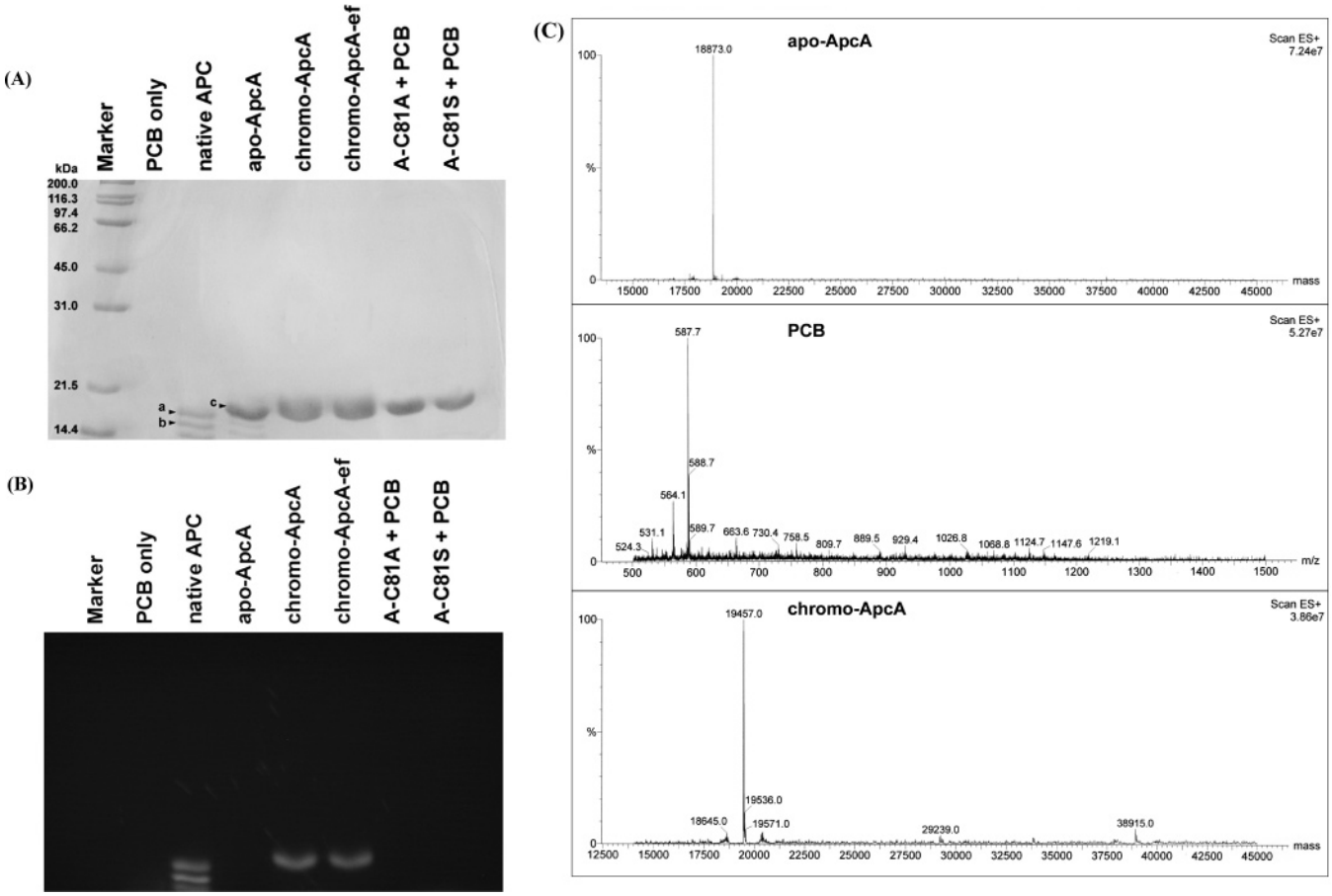

FIGURE 2: SDS−PAGE (12%) and Zn^{2+}−UV fluorography of chromo-ApcA. (A) Coomassie blue-stained gel. Arrows and labeling denote that a is the APC α-subunit, b the β-subunit, and c His_6-tagged ApcA. (B) Zn^{2+}−UV fluorography. (C) Mass spectra of purified apo-ApcA, PCB, and chromo-ApcA.

Zn^{2+}. The major Coomassie blue-stained band of 19 kDa corresponded to the calculated molecular mass of the His_6-tagged APC α-subunit (Figure 2A). A single bright band was visualized by UV fluorography before Coomassie blue staining (Figure 2B), and this band comigrated with the 19 kDa Coomassie blue-stained band, indicating that the chromophore was linked to the polypeptide. Interestingly, the chromo-ApcA protein purified from the lysate mixture in the absence of CpcE/CpcF had all the same properties as chromo-ApcA-ef, such as the elution conditions, the blue appearance (Figure 1B), and the results of PAGE and Zn^{2+}−UV fluorography (Figure 2A,B). These results indicate that CpcE/CpcF is not necessary for the biosynthesis of the PCB-attached APC α-subunit.

In Vitro Bilin Attachment Assay. The aforementioned data demonstrate that the attachment of apo-ApcA with PCB occurs in the absence of a cyanobacterial lyase enzyme. We further performed an in vitro assembly reaction using purified apo-ApcA and purified PCB to exclude the possibility that some contaminating chromophore lyase-like activity from *E. coli* catalyzed the bilin attachment. The purities of purified apo-ApcA and PCB were confirmed by electrophoresis and mass spectrometry. Apo-ApcA exhibits only a single band on the Coomassie blue-stained gel without detectable contaminations (Figure 2A), and one major component in the mass spectrum (Figure 2C). PCB shows no protein contaminations on the gel (Figure 2A) and one major component of *m*/*z* 587.7 in the mass spectrum (Figure 2C). Both results eliminate the existence of contaminating protein from *E. coli*.

The absorption spectra during the bilin attachment reaction were measured at 2 min intervals (Figure 3A). Purified PCB in the assembly buffer [50 mM sodium phosphate (pH 7.0)] had an absorption maximum at 610 nm, and purified apo-ApcA exhibited no absorption between 500 and 700 nm. During the assembly reaction, the absorption maximum of PCB shifted significantly from 610 to 620 nm (Figure 3A), indicating that PCB was transferred from the free state in buffer to the bound state (*9*). Figure 3B shows the increase in absorption over time at the λ_{max} of the final product, indicating that the attachment reaction was nearly complete in 30 min. Incubation of apo-ApcA with PCB also resulted in the induction of a red fluorescence of the emission maximum at 642 nm (Figure 3C). Chromophorylation of apo-ApcA by PCB during the assembly was also confirmed by PAGE and Zn^{2+}−UV fluorography (Figure 3D). In addition, the intensity of the chromo-ApcA band increased during the time course, indicating continuous production of the adduct over time.

Spectral Properties. We further examined chromo-ApcA with regard to its spectral characteristics using CD, mass spectrometry, and absorption and emission. The CD spectra of the native APC α-subunit and chromo-ApcA both exhibited double minima at 206 and 222 nm (Figure 4). Chromo-ApcA had a predominantly helical structure with a melting temperature (T_m) of 63 °C. The mass spectrum exhibited a component of 19 457 Da (Figure 2C), which matches that predicted for chromophorylated ApcA (apo-ApcA minus the N-terminal methionine and plus PCB) and

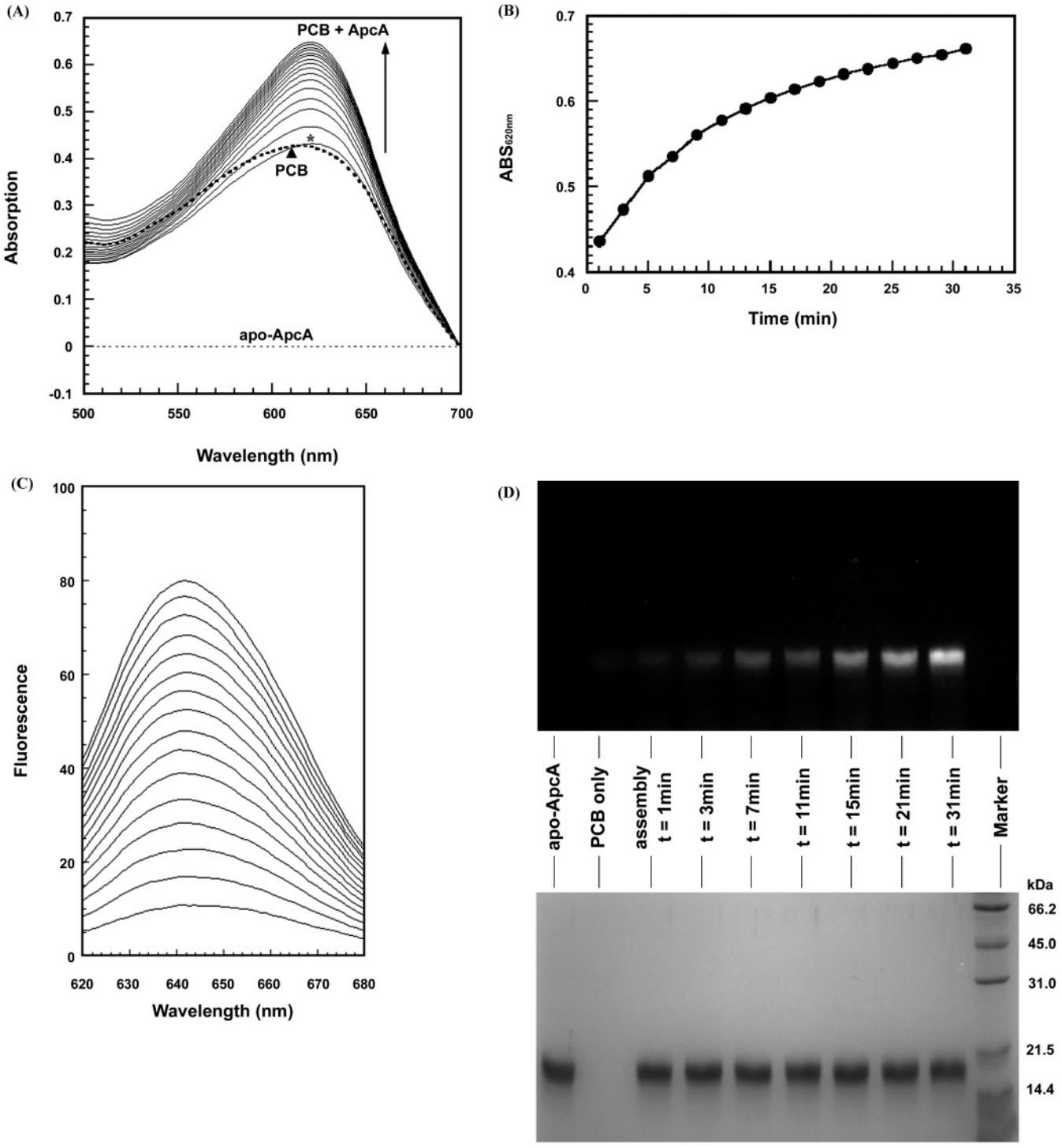

FIGURE 3: In vitro bilin attachment assay with PCB and apo-ApcA. (A) Spectra for the free PCB chromophore (thick dashed line) in assembly buffer [50 mM sodium phosphate (pH 7.0)] and the chromophore during the attachment reaction at 2 min intervals, from 1 min (bottom curve) to 31 min (top curve). During the reaction, the molar ratio of PCB to apo-ApcA was 1:1. The absorption maxima at 610 (▲) and 620 nm (*) are marked. Arrows indicate the direction of change with time. The thin, horizontal dashed line represents the spectrum of apo-ApcA. (B) Large increase in absorption over time at the λ_{max} of the final product. (C) Fluorescence spectra of the in vitro bilin attachment assay. The fluorescence spectra during the attachment reaction at 2 min intervals excited at 600 nm, from 1 min (bottom curve) to 29 min (top curve). During the reaction, the molar ratio of PCB to apo-ApcA was 1:1. (D) PAGE and Zn^{2+}−UV fluorography.

confirms that the modification of apo-ApcA by PCB was via a covalent link. Chromo-ApcA had an absorption maximum of 620 nm and an emission maximum of 640 nm (Figure 5A). The absorption and emission spectra were similar to that for native APC α-subunits (Figure 5B), indicating that chromo-ApcA had physical characteristics consistent with those of the native protein.

Identification of the PCB-Attached Residue. In native APC, PCB reacts with a cysteine residue to form a thioether bond. The APC α-subunit has only one cysteine residue, Cys81. To confirm that chromo-ApcA covalently linked with PCB via this cysteine, we constructed mutants in which this residue was replaced with alanine or serine (A-C81A or A-C81S, respectively). Wild-type and mutated apo-ApcA

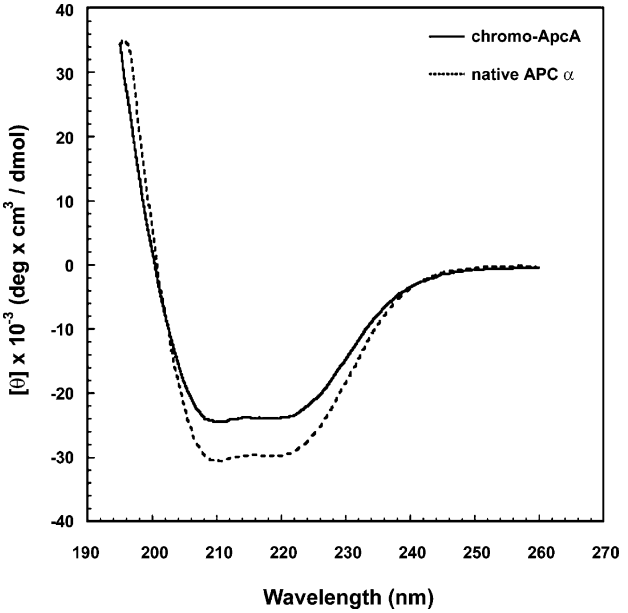

FIGURE 4: CD spectrum of chromo-ApcA (10 μM) in 20 mM phosphate buffer (pH 7.0) (dashed line) and the native APC α-subunit (10 μM) in 20 mM phosphate buffer (pH 7.0) (solid line).

proteins were overexpressed in *E. coli*, and the cell lysates were employed in the reconstituted experiment. After incubation with the cell lysate containing PCB, the His_6-tagged ApcA proteins were purified with a metal affinity column. Two purified mutants did not have the blue appearance caused by PCB attachment, absorption in the visible range, or fluorescence characteristics of chromophorylated proteins (data not shown). Among chromo-ApcA, A-C81A+PCB, and A-C81S+PCB, only the chromo-ApcA band exhibited fluorescence via PAGE and Zn^{2+}−UV fluorography (Figure 2B). The comparison between chromo-ApcA and mutants therefore confirms that recombinant ApcA covalently linked with PCB via Cys81.

DISCUSSION

In this work, we investigated the biosynthesis process of the APC α-subunit in vitro, so we used *E. coli* to produce several recombinant proteins of *Synechocystis* sp. However, since we failed to obtain the *Synechocystis* ApcA, we used the ApcA from *Spirulina* sp. in this experiment, because these two proteins are highly similar (they are 93% similar and 80% identical in amino acid sequence).

Our results constitute the first demonstration that apo-ApcA can become covalently modified by PCB to form a fluorescent chromoprotein, and no known chromophore lyase was necessary to mediate the modification reaction. We had supposed that CpcE/CpcF might be a chromophore lyase for APC; however, the fact that the biosynthesis of chromo-ApcA does not require CpcE/CpcF indicates that the modification of apo-ApcA by PCB occurs via an autocatalytic mechanism.

Results of the in vitro bilin attachment assay performed with the purified apo-ApcA and PCB excluded the possibility that some contamination from *E. coli* catalyzed the reaction, thereby confirming autocatalysis by ApcA. Previous reports have mentioned that nonenzymatic attachment of PCB to CPC or PEC in vitro results in products whose spectral properties differ from those of the native biliproteins (*9*, *38*).

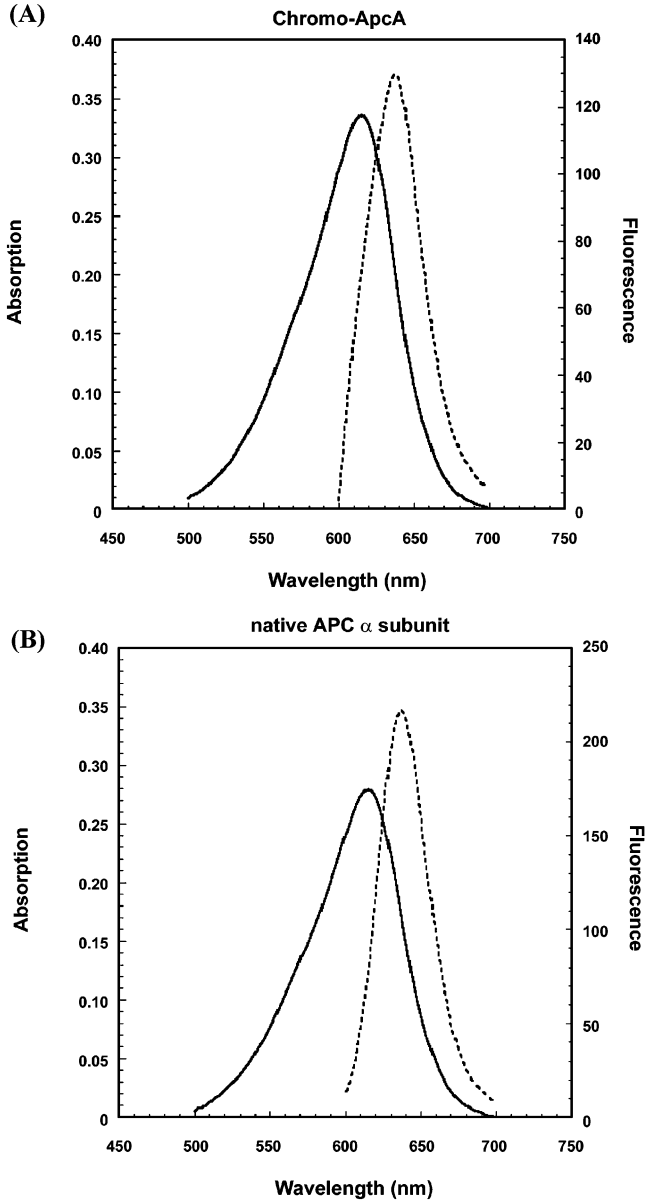

FIGURE 5: Absorption and emission spectra. (A) Spectra of chromo-ApcA (3 μM) in 50 mM phosphate buffer (pH 7.0). (B) Spectra of the native APC α-subunit (3 μM) in 50 mM phosphate buffer (pH 7.0). Each absorption spectrum is shown as a solid line, and each fluorescence spectrum is shown as a dashed line. The excitation wavelength for emission measurements was 600 nm.

In the case of the ApcA, however, the assembled and native proteins had very similar spectral characteristics such as CD spectra (Figure 4), absorption, and fluorescence spectra (Figure 5). As a control, we included an in vitro assembly reaction mixture containing CpcA (CPC α-subunit) and PCB in the absence of the chromophore lyase, CpcE/CpcF; no significant chromoprotein was synthesized even after an overnight incubation (based on the absorption spectrum of the purified product). Thus, these data indicate that the apo-ApcA indeed autocatalyzes the attachment of PCB.

The observed mass of chromo-ApcA reveals that PCB is covalently linked to the ApcA polypeptide. In native APC, bilin attachment is via a thioether bond between PCB and a cysteine residue. The comparison between chromo-ApcA and Cys81 mutants via PAGE and Zn^{2+}−UV fluorography (Figure 2B) and apparent molecular masses (Table 2) confirm

Table 2: Apparent Molecular Mass of Proteins

protein sample	modification	theoretical molecular mass[b] (Da)	observed molecular mass[c] (Da)
native APC α-subunit		17 847.63	17 847
apo-ApcA	−	18 873.29	18 873
	with PCB[a]	19 460.29	19 457
	with PCB and CpcE/CpcF[a]	19 460.29	19 457
ApcA-C81A	−	18 841.23	18 842
	with PCB[a]	19 428.23	18 842
ApcA-C81S	−	18 857.23	18 858
	with PCB[a]	19 444.23	18 858

[a] All the His_6-tagged protein samples interacted with PCB at 20 °C for 2 h and then purified by metal affinity resins. [b] The theoretical molecular mass (without the initial methionine residue) was computed with PeptideMass (http://tw.expasy.org/tools/peptide-mass.html). The native APC α-subunit is covalently bonded with PCB. Three different ApcA proteins are His_6-tagged recombinant protiens. [c] The observed molecular masses were determined by ESI-MS, and the initial methionine residue had been removed in all of the ApcA samples.

that chromophorylated recombinant ApcA covalently linked with PCB via Cys81, as in the native APC α-subunits.

Native APC was used as the positive control in the analysis via polyacrylamide gel electrophoresis (Figure 2A,B). Two bright bands derived from native APC in the PAGE and Zn^{2+}−UV fluorography (Figure 2B) confirmed that PCB covalently linked proteins were observed by UV excitation. Band a and band b (Figure 2A, lane 3) were identified as APC α- and *β*-subunits, respectively, by MALDI-TOF peptide mapping (data not shown). This native APC sample was further checked with an ESI mass spectrometer; only two major components with molecular masses of 17 847 and 17 970 Da were shown in the spectrum and corresponded to the APC α-subunit and APC *β*-subunit, respectively. In addition, the questionable third band in lane 3 was the dye front only.

Spectroscopic analysis of the reaction between pure apo-ApcA and PCB showed a significant change in absorption, with a shift in the absorption maximum from 610 to 620 nm, corresponding to the absorption maximum of free PCB versus that of PCB linked to the APC α-subunit. The attachment of PCB to the apo-ApcA in vitro was nearly complete in 30 min, a rate that is comparable to those of lyase-catalyzed reactions involving CPC (*15*) as well as the autocatalytic bilin attachment of phytochromes (*36*). The fact that the attachment of PCB to the apo-ApcA occurs in the absence of a lyase leads us to conclude that ApcA has chromophore lyase activity.

The absorbance change in Figure 3B reminded us of a question of whether the autocatalytic attachment of PCB to apo-ApcA was part of a biphasic reaction. We have shown that when the absorption signal approximated saturation, the fluorescence signal did not reach it. The time delay between the absorption change and fluorescence change during the chromophore assembly may be caused by the fine arrangement of the chromophore and the residues around it. We have tried to calculate the kinetic parameter of the PCB assembly by fixing the concentration of the apo-ApcA and changing the concentration of PCB. The V_{max} was ∼16.2 pmol/min and the K_m ∼9.29 *μ*M, and these values approximated the parameters of CPC α-subunit PCB lyase. Since ApcA is an autocatalytic enzyme and does not exactly obey the classical Michaelis−Menten model, further study of the detailed enzyme kinetics of ApcA is needed.

The amino acid sequence of ApcA is not significantly similar to those of known chromophore lyases or phytochromes, both of which catalyze chromophorylation. On the other hand, ApcA is similar in sequence to the core−membrane linker (L_{CM}). ApcA and L_{CM} have a low degree of sequence similarity in overall sequence but a high degree of similarity in two regions comprising residues 16−73 and 87−171, the latter of which contains the chromophore-bonding cysteine. As reported recently (*29*), the reaction between L_{CM} and PCB results from the autocatalytic activity of L_{CM}; this activity was considered a novel type of bilin: biliprotein lyase. Our results strongly suggest that ApcA can also serve as an autocatalytic bilin:biliprotein lyase. The consensus sequence of two aligned regions may serve as a signature of this novel type of autocatalytic chromophore lyase, which remains further confirmation by discovering more members of this family.

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