Amino Acid Residues Associated with Enzymatic Activities of the Isomerizing Phycoviolobilin-lyase PecE/F[†]

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ABSTRACT: PecE and PecF jointly catalyze the covalent attachment of phycocyanobilin to Cys-α84 of PecA and its concomitant isomerization to phycoviolobilin. (a) An *Eschertchia coli* supernatant expressing *pecF* has a residual activity of 6%; compared to the holoenzyme, this activity is lost upon purification. (b) Functional domains of both subunits from the cyanobacterium *Mastigocladus laminosus* were evaluated by mutageneses and chemical modification of amino acids. When in PecE the two motifs Y29YAAWWL and D263DLL were deleted, the holoenzyme lost its activity; it is also inactivated upon deletion of a central part (R111 to A122). The three conserved cysteines C48, C91, and C161 have only minor effects on catalysis. When in PecF the 20 C-terminal and 56 N-terminal amino acids were truncated, the lyase-isomerase activity in combination with PecE decreased to 12% and 15%, respectively, compared to the native enzyme. The catalytic efficiency (k_{cat}/K_m) decreased 16-fold when the unique four histidine residues in PecF beginning at H53 were deleted. H121 and C122 of PecF are essential for the enzyme activity; they are part of a unique stretch extending from A104 to N125 which is absent in the β-subunit of related but nonisomerizing lyases. A single histidine and a single tryptophan are required for activity in both PecE and PecF, as judged from diethyl pyrocarbonate and *N*-bromosuccinimide modification and statistical analyses. Inactivation of PecE and PecF is also possible by arginine-specific reagents, while modifications of lysine, glutamate, and aspartate retained activity. (c) PecE and PecF, as well as most of the mutants, bind PCB covalently in substoichiometric amounts, as assayed by Zn^{2+}-induced fluorescence on denaturing gels.

Phycobilisomes (PBS),[1] the light-harvesting antennas in cyanobacteria and red algae, are supramolecular complexes of phycobiliproteins and linkers (for leading references, see refs *1*−*5*). The phycobilin chromophores of the different phycobiliproteins span an absorption range from 460 to 670 nm and transfer excitation energy with high quantum efficiency to the photosynthetic reaction centers. In cyanobacteria, four spectroscopic classes of biliproteins have been assigned on the basis of their visible absorption spectra: phycocyanin (PC) and allophycocyanin (APC) carrying phycocyanobilin (PCB) chromophores, phycoerythrin (PE) carrying phycoerythrobilin (PEB) and phycourobilin (PUB), and phycoerythrocyanin (PEC) carrying PCB and phycoviolobilin (PVB). They are all heterodimeric members of a closely related protein family, with the subunits carrying one to four covalent chromophores. Some of the linkers also carry bilin chromophores. Phycobilin chromophores are generally bound to the polypeptide chain via thioether bonds to conserved cysteines: there is always a bond to C-3[1] at ring A of the tetrapyrrole, while PEB and PUB often have an additional one to C-18[1] at ring D (*6*, *7*).

Of the four cyanobacterial and red algal phycobilins, PCB and PEB have been studied in considerable detail. They are synthesized from heme by ring opening at C-5 of the tetrapyrrole and subsequent reduction steps and then attached to the apoproteins by addition of a cysteine thiol to the common ethylidene group (*8*−*12*). In vitro, PCB and PEB can add thiols spontaneously and reversibly, including cysteines of apobiliproteins, forming a relatively stable thioether bond (*13*−*15*). Autocatalytic chromophore binding

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[1] Abbreviations: aa, amino acid(s); APC, allophycocyanin; BSA, bovine serum albumin; CD, circular dichroism; CHD, 1,2-cyclohexanedione; DEPC, diethyl pyrocarbonate; *E.*, *Escherichia*; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IPTG, isopropyl β-D-thiogalactopyranoside; KPP, potassium phosphate buffer; *M.*, *Mastigocladus*; NBS, *N*-bromosuccinimide; PBS, phycobilisome; PC, phycocyanin; PCB, phycocyanobilin; PEB, phycoerythrobilin; PEC, phycoerythrocyanin; PecA, apoprotein of α-PEC; PecE and PecF, subunits of PVB-α-PEC isomerase-lyase; PecE(x−y) or PecF(x−y), truncated PecE or PecF, respectively, extending from amino acid "x" to amino acid "y"; PecE(Δx−y) or PecF(Δx−y), deletion mutants of PecE and PecF, respectively, lacking aa x through y; PCR, polymerase chain reaction; PGO, phenylglyoxal; PLP, pyridoxal 5′-phosphate; PUB, phycourobilin; PVB, phycoviolobilin.

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has been demonstrated for phytochromes (for leading references, see refs *12* and *16*−*18*) and for the core-membrane linker of the phycobilisome, ApcE (*19*). In contrast, chromophore binding to the phycobiliproteins is catalyzed by site- and chromophore-specific lyases, of which only two types have hitherto been characterized in detail. The PCB:CpcA lyase reversibly adds PCB to cysteine-84 of the α-subunit of CPC. It is comprised of two subunits, CpcE/F, and similar lyases probably catalyze the attachment of phycoerythrobilin (PEB) (*8*, *20*). The phylogenetically related PVB:PecA lyase catalyzes not only the addition of PCB to cysteine-84 of the α-subunit of PEC but also an isomerization that generates the photoactive PVB chromophore which is characteristic for PEC. A similar reaction sequence would lead from PEB to the protein-bound phycourobilin (PUB), which is present in many marine cyanobacteria and red algae. A first example for such an enzyme may be the RpeE/F fusion protein (accession no. SYNW2025) from *Synechococcus* sp. WH 8102 (*21*). Both PVB and PUB are only known as protein-bound 3^1-thiol adducts; the presence of a Δ2,3 double bond precludes a second one at $\Delta 3,3^1$, which required an alternative mode of attachment. A new type of lyase has been reported recently (*22*); its binds PCB to the *β*-subunits of CPC, APC, and possibly other biliproteins.

The photochromism of PEC (*23*), which is unique among the light-harvesting phycobiliproteins, has been related to the PVB bound to cysteine-84 on the α-subunit (*24*, *25*). The isomerizing lyase PecE/F (EC 4.4.1.17) by which it is generated (*26*, *27*) is encoded by two genes (*pecE/F*) of the *pec*-operon downstream from the structural (*pec*B/A) and linker genes (*pec*C) (*28*−*30*). Neither the mechanism of the ligation catalyzed by the related but monofunctional CpcE/F nor that of the ligation/isomerization catalyzed by PecE/F is known. Sequence comparison between PecE/F and CpcE/F gave higher homologies for the α-subunits (PecE/CpcE) than for the *β*-subunits of the two types of α-84 lyases (PecF/CpcF; *31*). It has therefore been speculated that the isomerase activity is located on PecF, but both subunits were found to be involved in the lyase reaction.

The current work is aimed at validating this proposal and at mapping in more detail the functions to the protein sequence. Truncations, site-directed mutageneses, and chemical modifications were guided by a sequence comparison between several CpcE/F and PecE/F, focusing mainly at those regions which were homologous within each of the two classes of enzymes but differed between the two types. The reaction kinetics were analyzed, as well as the covalent chromophore binding of the two lyase subunits.

MATERIALS AND METHODS

Materials and Reagents. 1,2-Cyclohexanedione (CHD), phenylglyoxal (PGO), and *N*-bromosuccinimide (NBS) were from Sigma; diethyl pyrocarbonate (DEP), pyridoxal 5′-phosphate (PLP), and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDAC) were from Fluka. All other biochemicals and separation materials were of the highest purity available and were obtained from the sources described previously (*26*, *27*). Recombinant proteins were purified as before (*31*).

Full-Length Proteins. Cloning and expression followed generally the standard procedures of Sambrook et al. (*32*). The integral genes *pecA*, *pecE*, and *pecF* were PCR-amplified from two strains of *Mastigocladus laminosus*, viz., PCC 7603 and Wuhan (*26*). Mutants of *pecE* and *pecF* from the strain PCC 7603, and of *pecE* from the strain Wuhan, were cloned first into pBluescript SK(+) (Stratagene) and then subcloned into pET30a (Novagen). PecF and mutants without the His tag were obtained by expressing pGEMEX containing the desired full-length or mutated *pecE* (*26*).

Deletion Mutants. Truncated mutants were prepared by the PCR method, using the following primers:

P1, 5′-TA**CCCGGG**ACTGCTGCTTGTTGTGCT-3′, upstream; P2, 5′-CG**CTCGAG**TTAAAGTTGAATTAATAAATCGTCAA-3′, downstream; P3, 5′-GAT**CCCGGG**GAAGATAATCAAATTCGTTAT-3′, upstream; P4, 5′-TCT**CCCGGG**AAACATCAAGTGCAAGCAGGT-3′, upstream; P5, 5′-AG**CCCGGG**CGCATTCCATCAGGAGGC-3′, upstream; P6, 5′-GCG**CTCGAG**TTAGTTATCTAATAAGGCTTCCAAAAT-3′, downstream; P7, 5′-CG**CTCGAG**TTACTGAAAAATTAACTGAGT-3′, downstream; P8, 5′-CG**CTCGAG**TTAAATTGCCTGAAAAATTAACTG-3′, downstream; P9, 5′-CG**CTCGAG**TTAATCGTCAATTGCCTGAAAAAT-3′, downstream; P10, 5′-CG**CTCGAG**TTATAAATCGTCAATTGCCTG-3′, downstream; P11, 5′-CG**CTCGAG**TTATAATAAATCGTCAATTGC-3′, downstream; P12, 5′-AG**CTCGAG**TTAAATTAATAAATCGTCAAT-3′, downstream; P13, 5′-AA**CCCGGG**CGCCATCACCATCATTCC-3′, upstream; P14, 5′-AA**CCCGGG**ATCGCAGCTCGCTTTACA-3′, upstream; P15, 5′-GG**CTCGAG**TTAGCAGGCGGGAATTTG-3′, downstream; P16, 5′-GG**CTCGAG**TTATTCGCCAGCTACAGCT-3′, downstream; P17, 5′-GG**CTCGAG**TTATTTCATTCGCGATCGCAC-3′, downstream; P18, 5′-AAA**CCCGGG**TCCTCCGTCGCTACTGCTG-3′, upstream. All upstream primers have a *Sma*I site (CCCGGG), and the downstream primers have a *Xho*I site (CTCGAG) (both marked in bold), which ensure correct ligation of the fragments to pBluescript.

Terminally truncated *pecE* mutants were generated using *pec*E of strain Wuhan (774 bp) as template and the following primers: P2 and P3 for *pec*E(23−269), P2 and P4 for *pec*E(37−269), P2 and P5 for *pec*E(57−269), P1 and P6 for *pec*E(1−234), P1 and P7 for *pec*E(1−260), P1 and P8 for *pec*E(1−262), P1 and P9 for *pec*E(1−264), P1 and P10 for *pec*E(1−265), P1 and P11 for *pec*E(1−266), and P1 and P12 for *pec*E(1−267).

A central deletion mutant of *Pec*E from strain PCC 7603 (807 bp) was generated using the two *Hin*dIII restriction sites. The plasmid pBlu-*pec*E(PCC 7603) was digested with *Hin*dIII and then ligated with T4 ligase to produce pBlu-*pec*E(Δ103−129), and subsequently pET-*pec*E(Δ103−129), coding for a PecE mutant lacking aa 103−129 near the center. The reading frame is not shifted by this deletion.

Terminally truncated *Pec*F mutants were generated using *pecF* from strain PCC 7603 (639 bp) as template with the following primers: P13 and P15 for *pec*F(52−212), P14 and P16 for *pec*F(1−191), P14 and P17 for *pec*F(1−201), and P18 and P15 for *pec*F(57−212). All mutations were verified by sequencing.

Site-Directed Mutants. Site-directed mutants in which the cysteine residues 48, 91, 161 (PecE), and 122 (PecF) were mutated individually were generated by the PCR-based megaprimer method (*33*, *34*), using the following primers:

P19, 5′-TACCCGGGA**CTG**CT**G**CTTGTTGTGCT-3′, upstream; P20, 5′-CGCTCGAGTTAAAGTTGAATTAATAAATCGTCAA-3′, downstream; P21, 5′-CGTCAAACAAGGCTTC**CTG**CA**G**AGCTG-3′; P22, 5′-CGCAATCCCAGATCTTCCTCT**AG**AGCCAAA- 3′; P23, 5′-GCAGC**CAGC**TG**G**ACTCGCTCGGAACTG-3′; P24, 5′-AACCCGGGATCGCAGCTCGCTTTACA-3′, upstream; P25, 5′-GGCTCGAGTTAGCAGGCGGGAATTTG-3′, downstream; P26, 5′-GCCAATCAC**CTG**CA**G**GGTAATGTC-3′. Restriction sites used for detection of the mutations are shown in bold.

P19 and P20 were used to clone the integral gene *pec*E from strain PCC 7603. P21, which carries the meaningless mutation CAG and a *Pst*I restriction site for detection, was used to produce PecE(C48Q). P22, which has a *Xba*I restriction site for detection, was used to produce the mutant protein PecE(C91L). P23, which carries the meaningless mutations GC**C** and TG**G** and a *Pvu*II restriction site for detection, was used to produce the mutant protein PecE-(C161L). P24 and P25 were used to clone the integral gene *pec*F from strain PCC 7603. P26, which carries the meaningless mutation CA**G** and a *Pst*I restriction site for detection, was used to produce the mutant protein PecF(C122L). All mutations were verified by sequencing.

Expressions. The pET-based plasmids were used to transform to *Escherichia coli* BL21(DE3). Cells were grown at 37 °C in Luria−Bertani medium containing kanamycin (30 $\mu g \cdot mL^{-1}$). When the cell density reached OD_{600} 0.5−0.7, IPTG (1 mM) was added. Five hours after induction, cells were collected by centrifugation, washed twice with doubly distilled water, and stored at −20 °C until use. PecA, PecE, and PecF of *M. laminosus* and all mutants were prepared using the methods described before (*27, 31*).

PCB and Protein Concentration Determinations. PCB was prepared as described before (*27*). PCB concentrations were determined spectroscopically using an extinction coefficient $\epsilon_{690} = 37900\ M^{-1} \cdot cm^{-1}$ in methanol/2% HCl (*27*). Protein concentrations were determined with the protein assay reagent (Bio-Rad) according to the instructions given by the supplier, using BSA as the standard (*35*).

SDS−Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed with the buffer system of Laemmli (*36*). The gels were stained with $ZnCl_2$ for bilin chromophores (*37*) and with Coomassie brilliant blue R for the protein.

Spectroscopy. Enzyme reactions and amino acid modifications were followed by UV−vis spectrophotometry (Perkin-Elmer model Lamda 25). Formation of the correct chromophore, PVB-PecA, in the lyase reaction, was monitored by the absorption at 570 nm and by double difference spectroscopy of the reversible photoreaction of the PVB chromophore as described previously (*25*). Briefly, the chromoprotein was first irradiated at 500 nm to convert it maximally to the 570 nm form and then irradiated at 570 nm to convert it to the 506 nm form. Photochemical activity was then defined as the amplitude $\Delta\Delta A_{570-500}$ of the s-shaped difference spectrum (sample irradiated at 570 nm *minus* sample irradiated at 500 nm), divided by A_{570} after the fist irradiation. Far-UV CD spectra were recorded at 25 °C with a Dichrograph VI (ISA, Jobin Yvon) using 1 mm cuvettes, five spectra were averaged, and the data were smoothed by 5 point averaging.

Lyase Activity Assay. Chromophore reconstitution with PecA was assayed as described before (*31*). Either full-length PecE was complemented with mutants of PecF or full-length PecF was complemented with mutants of PecE, using the following standard reaction conditions: potassium phosphate buffer (KPP, 15−20 mM, pH 7.5) containing NaCl (150−200 mM), mercaptoethanol (ME, 5 mM), $MnCl_2$ (3 mM), Tris-HCl (100 mM), Triton X-100 [TX-100, 1% (v/v)], PecE and PecF or their mutants (6 μM each), and His_6-PecA (10 μM). PCB (final concentration = 5 μM) was added as a concentrated dimethyl sulfoxide solution; the final concentration of dimethyl sulfoxide was 1% (v/v). The mixture was incubated at 37 °C for 3 h in the dark. Products were quantified by absorption and double difference spectroscopy (*25*) as detailed in the previous paragraph. All ligations were carried out with three different preparations of each His-tagged PecE/F and mutants: (1) nonpurified proteins, i.e., the supernatants of the sonicated cells after centrifugation; (2) proteins purified by Ni^{2+}-chelating affinity chromatography as before (*27*); (3) co-renatured proteins, which were obtained by the following procedure. Purified PecE (or its mutant) and purified PecF (or its mutant) were denatured separately with urea (8 M) at room temperature. They were mixed in equimolar amounts (6 μM) and renatured slowly by repeated dialysis against KPP (50 mM, pH 7.2) containing NaCl (0.5 M) at 4 °C for 4 h. For untagged proteins, only method 1 was used.

For kinetic tests, only purified proteins were used. Again, either full-length PecE was complemented with mutants of PecF or full-length PecF was complemented with mutants of PecE. The purified subunits (6 μM) and different concentrations of substrate PCB were mixed in the reconstitution system (see above) and incubated at 37 °C. At regular time intervals, the reaction was terminated by rapidly cooling the samples on ice to 0 °C, and then the product was analyzed spectroscopically without delay (*31*). K_m, v_{max}, and k_{cat} were calculated from Lineweaver−Burk plots, using Origin V6 (Origin Lab Corp.).

Binding of PCB to PecE/F and Mutants. A mixture of PecE and PecF (1:1), individual subunits, or their mutants were incubated with PCB (20 μM) in the reconstitution system, as described above, but omitting PecA. The product(s) were purified by Ni^{2+}-chelating chromatography and analyzed by absorption (300−800 nm) and double difference spectroscopy as described before (*25*) and by SDS−PAGE using Coomassie and Zn^{2+} staining (*37*).

Complex Formation of PecE and PecF. Purified His-tagged PecF or its mutants were co-renatured (see Lyase Activity Assay) with untagged PecE. The mixtures were then loaded on a Ni^{2+} column and washed three times with 5 column volumes of KPP (20 mM, pH 7.2) containing NaCl (1 M), once with the same buffer containing in addition imidazole (50 mM), and finally with the same buffer containing 1 M imidazole. The eluate from the last wash was analyzed (a) by SDS−PAGE and (b) for enzymatic activity as described above. In the case of chemically modified subunits, the complementary, unmodified one was added as the supernatant of the *E. coli* lysate.

Chemical Modifications of Amino Acids. (a) Arginine. Aliquots of a stock solution of 1,2-cyclohexanedione (CHD, 150 mM) in sodium borate buffer (50 mM, pH 8.0) were incubated at 25 °C with PecE/F. Reactions were terminated

at defined times by cooling on ice to 0 °C. The mixtures were then dialyzed three times for 4 h against borate buffer (50 mM, pH 7.0), and the enzymatic activities were determined as above, but omitting Tris-HCl. Reversibility of the CHD modification was tested by the addition of neutralized hydroxylamine (1 M) (*38*, *39*). Phenylglyoxal (PGO) was added from a stock solution [1:1 (v/v) mixture of ethanol and KPP (50 mM, pH 7.2)], and the mixture was incubated at 25 °C with PecE/F (*38*, *40*). The reaction was stopped by adding arginine (50 mM), and the mixture was dialyzed three times for 4 h against KPP (50 mM, pH 7.2) and then analyzed for enzymatic activity as above. Kinetics of both reactions were analyzed using the equation $-\log(t_{0.5}) = \log(k') + n \log([I])$ (*41*), where k' is the first-order rate constant, [I] is the inhibitor concentration, and n is the reaction order giving the number of residues that are critical for activity.

(b) Lysine. Purified enzyme (6 μM) was dialyzed against KPP (50 mM, pH 7.2) containing EDTA (1 mM) and then treated without delay with aliquots of a freshly prepared, ice-cold pyridoxal phosphate (PLP) stock solution (100 mM) in KPP (50 mM, pH 7.2). After incubation in the dark at 25 °C, the amount of bound PLP was determined by addition of excess $NaBH_4$ and the subsequent measurement of the increase in absorbance at 325 nm using a molar extinction coefficient of 9710 $M^{-1}\cdot cm^{-1}$ for ϵ-pyridoxyllysine (*42*). After dialysis against KPP (50 mM, pH 7.2, three times for 4 h each), the enzyme activity was assayed as above.

(c) Carboxyl Groups. Purified enzyme (6 μM) in KPP (50 mM, pH 6.8) was incubated at 25 °C with varying concentrations of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) (*43*, *44*). The reactions were quenched by the addition of an equal amount of sodium acetate buffer (0.5 M, pH 5.5), the products were dialyzed three times for 4 h against KPP (50 mM, pH 7.2), and the enzymatic activity was assayed as above.

(d) Tryptophan. PecE/F (6 μM) was incubated at 30 °C with NBS in KPP (50 mM, pH 8.0). The reaction was stopped by adding an excess of tryptophan from a stock solution (50 mM) and ME (0.1 M) in the same buffer, and the product mixture was dialyzed against KPP (50 mM, pH 7.2). The number of oxidized tryptophan residues (n) was determined spectroscopically according to Spande and Witkop (*45*), using the equation $n = \Delta A_{280} 1.31/4500[E]$, where ΔA_{280} is the absorption change of the modified enzyme at 280 nm, 4500 $M^{-1}\cdot cm^{-1}$ is the molar extinction coefficient of tryptophan at 280 nm, and [E] is the protein concentration.

(e) Histidine. Modification followed Miles (*46*). Before each experiment, the exact concentration of a stock solution of diethyl pyrocarbonate (DEPC, ≈6.9 M) was determined by dilution into ethanol and measuring the absorbance increase at 230 nm (ϵ = 3000 $M^{-1}\cdot cm^{-1}$) following the addition of imidazole (10 mM) in KPP (20 mM, pH 7.2) containing NaCl (0.5 M). The reagent was diluted with ice-cold absolute ethanol and added immediately to a solution of PecE/F (6 μM) in KPP (20 mM, pH 7.2) containing NaCl (0.5 M), the reaction mixture was dialyzed against KPP (20 mM, pH 7.2), and the mixture was incubated at 25 °C. At defined time intervals, the reaction was quenched by addition of histidine (0.5 M) in KPP (50 mM, pH7.2), and the number of modified histidines was analyzed spectroscopically using an extinction coefficient ϵ_{242} = 3200 $M^{-1}\cdot cm^{-1}$ for *N*-carbethoxyhistidine. Reversibility of DEPC inactivation was tested by addition of neutralized hydroxylamine (1.0 M). After dialysis against KPP (50 mM, pH 7.2, three times for 4 h each), the enzyme activity was assayed as above.

Table 1: Comparison of Enzymatic Activities of PecE/F and Their Deletion Mutants[a]

lyase/ substrate fraction	relative activity (%) non-purified	purified	co-renatured	without ME
PecE	0	0		0
PecF	6	0		0
PecE + PecF	100	100	105	0
PecE(57−269) + PecF	6	0	0	0
PecE(37−269) + PecF	6	0	0	0
PecE(23−269) + PecF	13	4	4	0
PecE(Δ103−129) + PecF	6	0	0	0
PecE(1−234) + PecF	6	0	0	0
PecE(1−260) + PecF	6	0	0	0
PecE(1−262) + PecF	6	0	0	0
PecE(1−264) + PecF	6	0	0	0
PecE(1−265) + PecF	24	17	6	0
PecE(1−266) + PecF	47	53	17	0
PecE(1−267) + PecF	100	100	105	0
PecE + PecF(52−212)	15	6	13	0
PecE + PecF(57−212)	49	35	24	0
PecE + PecF(1−191)	12	10	10	0
PecE + PecF(1−201)	23	10	10	0
PecE(C48Q)	66	78	78	0
PecE(C91L)	63	181	181	6
PecE(C161L)	70	64	70	0
PecF(C122L)	1	4	4	0

[a] See Materials and Methods for experimental details.

Determination of Essential Tryptophan and Histidine Residues. The statistical method of Tsou (*47*) was used to calculate the number of essential tryptophan and histidine residues for enzyme activity. Assuming that all modifiable residues (n), including essential residues (i), are equally reactive toward the modifier, the remaining enzyme activity A can be described by $(A/A_0)^{1/i} = (n - m)/n$, where A_0 is the activity of the unmodified enzyme and m is the number of modified residues. The number of essential tryptophan (i_W) or histidine residues (i_H) was determined from the slope of the line generated when $(A/A_0)^{1/i}$ was plotted against m (*47*).

RESULTS

Expression and Purification of Wild-Type and Mutant Enzymes. On account of the plasmids used in this study, most proteins carry N-terminal extensions containing a His and a S tag, plus two protease sites for thrombin and enterokinase. In the full-length proteins, these tags did not interfere either with the functions of the lyase (PecE/F) or with the reactivity of the apoprotein (PecA), but they facilitate their purification and improve their solubilities (*26*, *27*). A 1 L culture of *E. coli* yielded routinely ∼50 mg of PecE or its mutant proteins and about half that amount of PecF or its mutant proteins (Supporting Information, Figure S1). They were purified and concentrated to 6 μM by Ni^{2+} affinity chromatography. PecE (without the His tag) used for DEPC modification was purified by gradient ion exchange column chromatography and concentrated to 6 μM.

The enzymatic activities obtained with the mutated subunits are summarized in Table 1; they were quantified by the reversible photochemistry of the resulting α-PEC

($\Delta\Delta A$; see ref *25* and the section Spectroscopy in Materials and Methods) and expressed relative to those of α-PEC obtained by combining purified PecE and PecF. In these tests, a mutated subunit was always combined with the full-length complementary one.

Activities for reconstituting α-PEC were measured routinely with three preparations: with the nonpurified subunits (=supernatants of cleaved *E. coli*), with subunits purified via Ni affinity chromatography, and with purified subunits which were subsequently denatured with urea and then co-renatured by dialysis against urea-free buffer. As in previous studies (*31*), the full-length lyase showed highest activity when the purified subunits were renatured together from 8 M urea. However, the mutations may affect the interactions among subunits, and therefore all three procedures were tested. It is obvious from Table 1 that with the mutant proteins the nonpurified (=*E. coli* supernatants) samples generally showed higher activity than the purified proteins. Possibly, some factor(s) from *E. coli* assist(s) the lyase-isomerase activity of the mutated PecE/F (see below, the effect of serum albumin), or the mutated proteins are somewhat affected during the purification or denaturation/renaturation procedure.

Enzymatic Activity of PecF. Previous experiments had concluded that both subunits are required for the complete reaction sequence. It could now be shown that this statement has to be modified: under certain conditions, the presence of PecF alone is sufficient to catalyze both the lyase and the isomerase reactions, albeit with low activity. The crude extract of *E. coli* overexpressing *pecF* shows an activity of 6% compared to that of PecE/F. When PecF is purified by affinity chromatography and eluted with imidazole, this residual activity is lost but can partly (~50%) be restored by the addition of BSA. The residual activity is also retained when it is eluted from the Ni^{2+} column with EDTA. PecF has been distantly related to armadillo-type proteins such as karyopherins which are known to be involved in protein−protein interactions (*27*). It is possible that interactions with BSA or some protein retained by elution with EDTA somehow affect the activity of PecF. Alternatively, the chromophore could be modified by the accompanying protein. This could relate to the observation that the PCB absorption in the crude extract is blue shifted to 588 nm, as compared to the absorption in the presence of purified PecF in the same buffer (λ_{max} = 598 nm; not shown). The absorption of PCB is very sensitive to conformational changes (*5*, *48*, *49*), and conformational changes of PCB have been implied in the control of the nonenzymatic addition of chromophores to the bilin binding apoprotein, CpcB (*50*).

PecE Mutants. (a) Deletion Mutants. The N-terminally truncated mutants PecE(57−269) or PecE(37−269), in combination with full-length PecF, were inactive; they showed the same basal activity of 6% as PecF alone (*31*). The activity increased slightly (4−13%) if only 22 residues are removed N-terminally [PecE(23−269)], indicating that aa 23−32 are relevant for the activity. This region is homologous for the α-subunits of monofunctional (CpcE) and bifunctional lyases (PecE), with distinct differences of the first five aa (Figure 1).

Activity of PecE was also lost after deletion of a central stretch (aa 103−129). The cd of this mutant was not significantly different from that of the wild-type protein (not shown), indicating a similar folding. At the C-terminus, only the very last aa (266−269) can be deleted without complete loss of activity. The last three amino acids (IQL), which distinguish PecE from the respective subunit (CpcE) of lyases lacking the isomerase activity (Figure 1), were not necessary for the full activity of PecE. Further truncation led quickly to complete inactivation; C-terminal truncations beyond aa 265 proved inactive. A kinetic study (Table 2) showed that both v_{max} and the affinities for PCB decreased sharply from PecE(1−266), which had near-native activities, to PecE(1−265). These results point to an important function of L265 and L266 for the enzymatic activity.

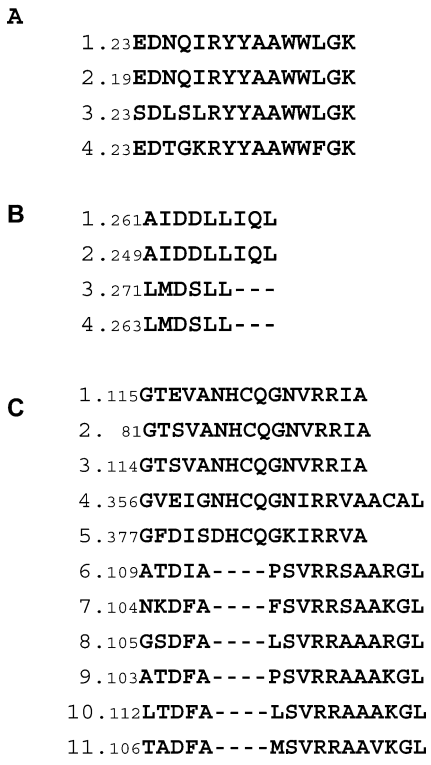

FIGURE 1: (A, B) Comparison of PecE distinctive domains with related domains in CpcE. Sequences shown are PecE from *M. laminosus* PCC 7603 (1) and *Anabaena* sp. PCC 7120 (2) and CpcE from *Anabaena* sp. PCC 7120 (3) and *Synechococcus* sp. PCC 7002 (4). (C) Comparison of the central domain which is distinctive for PecF, as compared to CpcF. Shown are the sequences of PecF from *M. laminosus* and *Anabaena* sp. PCC 7120, a sequence annotated as HEAT repeat from *Anabaena variabilis* ATCC29413 (accession no. ZP 00162268), the fused E/F-like protein from *Synechococcus* sp. WH 8102, a sequence annotated as HEAT repeat from *Crocosphaera watsonii* WH 8501 (accession no. ZP 00174981.2; starting G in sequence is aa 8226 in contig 166), RpcF from *Synechococcus* sp. WH 8020, CpcF from *Synechococcus* sp. PCC 7002, *Spirulina platensis*, *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and *Pseudoanabaena* sp. PCC 7409 (from top to bottom). The sequence positions of the first aa shown are given in front of the partial sequences.

(b) Site-Directed Mutations of Cysteines. The reaction mechanism of the PVB:PecA lyase-isomerase is currently unknown. Since the final chromophore acceptor in PecA is a cysteine (C84) (*51*, *52*), cysteines from the lyase may participate in the reaction. This was supported by the weak covalent bilin binding to PecE in the absence of PecA (see below). PecE contains three conserved cysteines. Individual mutations led only to moderate losses of activity (Tables 1 and 2); in the case of C91L it was even considerably enhanced (181% as compared to the native protein). The

Table 2: Kinetic Analyses for the Wild-Type and Mutated Lyase[a]

enzyme	K_M (μM)	v_{max} (nM·s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (μM·s^{-1})
wild type	10 ± 3.0	0.15 ± 0.03	2.1×10^{-5}	2.1×10^{-6}
PecE(C48Q)	16 ± 2.7	0.11 ± 0.04	1.8×10^{-5}	1.1×10^{-6}
PecE(C91L)	60 ± 2.9	0.96 ± 0.03	15×10^{-5}	2.5×10^{-6}
PecE(C161L)	5.9 ± 1.9	0.05 ± 0.01	0.7×10^{-5}	1.2×10^{-6}
PecE(1−265)	26 ± 2.5	0.01 ± 0.003	0.05×10^{-5}	0.02×10^{-6}
PecE(1−266)	11 ± 1.3	0.08 ± 0.02	1.3×10^{-5}	1.2×10^{-6}
PecE(1−267)	10 ± 2.5	0.08 ± 0.03	1.3×10^{-5}	1.3×10^{-6}
PecF(52−212)	22 ± 2.6	$10^{-4} \pm 0.5 \times 10^{-4}$	1.6×10^{-8}	0.7×10^{-9}
PecF(57−212)	30 ± 2.6	0.07 ± 0.01	0.4×10^{-5}	0.13×10^{-6}

[a] See Materials and Methods for details. The kinetics of mutant PecE(1−267) was practically identical to that of the full-length protein.

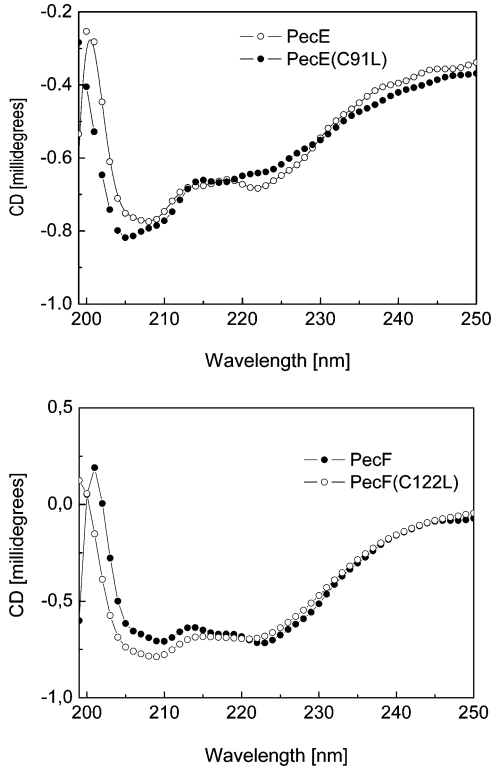

FIGURE 2: CD spectra of one cysteine mutant each of PecE (top) and PecF (bottom). Spectra of other cysteine mutants were the same within the limits of error.

affinity of this mutant is decreased to 16%, but this is more than compensated by the increase of v_{max}. Unlike the deletion mutants, and like the wild-type subunit, the site-directed mutants became more active if the protein was purified before the assay. The GOR4 algorithm (*53*) predicted no changes in secondary structures of the protein upon the cysteine mutations. This was supported by an analysis of the far-UV CD spectra, although the data are not very precise: in particular, PecE tends to aggregate, and even the purified protein shows scattering in the far-UV. Results from two mutants are shown as examples in Figure 2; they indicate the precision at which we were able to measure, and we do not consider the differences significant. All mutants for which the kinetics have been determined gave the same spectra as the respective wild-type protein within these limits of error.

The enzymatic reconstitution system of α-PEC requires Mn^{2+} or Mg^{2+} to achieve full catalytic activity (*26*). The activator ion Mn^{2+} may play the role of binding of the substrates; we therefore checked its effect on the mutants. Interestingly, the activity of the C91L mutant decreased to 38% if Mn^{2+} was omitted, while the activity of native PecE decreased much more (to 21%) under these conditions; PecE(C91L) is therefore almost as active (69%) in the absence of Mn^{2+} as the native protein in its presence (not shown). Possibly, the SH group of the conserved C91 interacts with the Mn^{2+}.

PecF Mutants. (a) Deletion Mutants. In the next series of experiments, mutated PecF was combined with full-length PecE. The mutants were designed on the basis of a sequence comparison of the two known PecF (*Anabaena* sp. PCC 7120, *M. laminosus* PCC 7603). Their homology is low from aa 1 to aa 52; this stretch is followed by an unusual motif of four conserved histidines 53−56. PecF(52−212) and PecF(57−212) were designed to investigate the significance of this His_4 motif. PecF(52−212) showed only little enzymatic activity (6−15%; see Table 1). Surprisingly, the activity increased to 24−49% when in addition the four histidines were deleted [PecF(57−212)]; compared to full-length PecF, however, this mutant has a decreased affinity to PCB and a reduced v_{max}, and the catalytic efficiency (k_{cat}/K_m) decreased about 16-fold (Table 2). Possibly, the presence of the His_4 motif leads in the absence of the more N-terminal aa to a misfolding of the protein, which is partly recovered when they are removed, too. To ascertain that the His tag did not affect the activity of the PecF(57−212), we also expressed PecF(57−212) without the His tag; this protein showed the same activity as the His-tagged one.

The two known PecF also have little homology over the 20 C-terminal amino acids. Accordingly, PecF(1−191) and PecF(1−201) were generated. The latter still had moderate activity (30% as compared to wild-type PecF), and even the removal of 10 more aa in PecF(1−191) did not abolish the activity (Table 1).

(b) Site-Directed Mutants. Work focused again on the mutation of conserved cysteines, of which there is only one in PecF(C122). The C122L mutant was nearly inactive in generating photochemically active α-PEC; this residue is critical for activity. However, it still promoted an absorbance increase at 610 nm (data not shown), indicating that at least part of the lyase activity is retained, while the isomerase activity is inhibited. C122 therefore seems to be required for the isomerization of the chromophore. It was therefore checked if there is any difference in the interaction of PCB with PecF and the C122L mutant. There is indeed a distinct red shift of the PCB adduct with PecF(C122L), as compared to the wild-type protein, while the increased absorption indicative of binding is nearly retained (Supporting Information, Figure 2). This indicates that more than one cysteine in PecF can bind PCB. There is also no significant change in the secondary structure of PecF upon the C122L mutation (Figure 2), as predicted by GOR4.

Binding of PCB to PecE and PecF. Previous results on the interaction of PCB with the lyase or its subunits, PecE and PecF, were inconclusive. This has now been tested by two methods: The wild-type and mutant proteins were incubated with PCB under reconstitution conditions but omitting the acceptor, PecA. Excess PCB was then washed off the Ni^{2+} affinity column with KPP (pH 7.2) containing NaCl (1 M), and the spectra were recorded of the protein fractions eluted with the imidazole (1 M) wash buffer

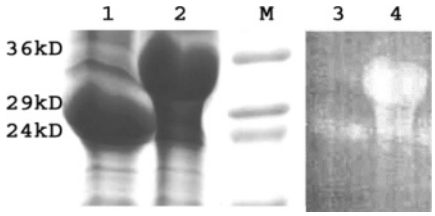

FIGURE 3: Covalent binding of PCB to PecE and PecF. SDS−PAGE of His-tagged PecF (lanes 1 and 3) and His-tagged PecE (lanes 2 and 4) after incubation with PCB (see text for details). M = protein marker; lanes 1 and 2 were stained with Coomassie and lanes 3 and 4 with Zn^{2+} and observed by fluorescence according to Berkelman and Lagarias (*36*).

(Supporting Information, Figure 2). The high absorption at <400 nm is due to the presence of imidazole (1 M) in the elution buffer. The chromophores are lost after dialysis, and the eluates are only weakly colored, corresponding to a substoichiometric binding. To further test if the binding is covalent, the proteins were subjected to SDS−PAGE and subsequent chromophore staining (Figure 3). The fluorescence of the two protein bands suggests even covalent binding but, again, only in substoichiometric amounts; a rough estimate comparing the staining intensities and absorptions with those of native biliproteins gives less than 10% binding in PecE and even less than 1% in PecF. This low amount of bound PCB and the loss during sample preparation can be rationalized if the binding is weak and reversible, but it has not been investigated in detail.

Chemical Modifications of Amino Acids. Arginine modification by CHD and PGO (*38*−*40*) resulted in inactivation of PecE and PecF (Figure 4) by various concentrations of CHD and PGO as shown. The semilogarithmic plots of the remaining enzyme activity against reaction time are linear, indicating that the inactivations followed pseudo-first-order kinetics. The following second-order rate constants were obtained from the linear plots of the pseudo-first-order rate constants of inactivation against modifier concentrations: 0.84 ± 0.05 and 1.66 ± 0.13 $M^{-1}\cdot min^{-1}$ for the reactions of PecE with CHD and PGO, respectively, and 0.27 ± 0.04 and 0.36 ± 0.06 $M^{-1}\cdot min^{-1}$ for the reactions of PecF with CHD and PGO, respectively. The numbers of modified residues were obtained from plots of $\log(1/t_{0.5})$ against log-[PGO] or log[CHD]. They resulted in straight lines (insets of Figure 4) with slopes 1.02 and 0.82 for the reactions of PecE with CHD and PGO, respectively, and 0.91 and 0.82 for the reactions of PecF with CHD and PGO, respectively. It is concluded that in each subunit one arginine residue is required for the catalytic activity.

The modifications may interfere with the interactions of PecE and PecF and thereby affect the lyase activity. This was tested by binding His-tagged PecF to a Ni^{2+} column and analyzing for a retention of unlabeled PecE by SDS−PAGE (see below, Figure 7) and activity assays. According to this criterion, arginine modification on either subunit did not interfere with complex formation between the two chromophores. It should be noted that, due to the "natural" His tag present in PecF (H53HHH), the reverse experiment is ambiguous: untagged PecF binds weakly to the Ni^{2+} column, from which it elutes with 100−200 mM imidazole, while the proteins with engineered His tags elute at >500 mM imidazole (not shown). For routine analyses of complex formation using a His-tagged protein as a bait for untagged PecF, this difference is not strong enough, however, to allow for a clear distinction. Therefore, other methods will be required to analyze the interactions of the two subunits more thoroughly and quantitatively.

Lysine residues were modified with PLP and EDAC (*42*, *43*). Treatment of PecE and PecF with an excess of these reagents for 30 min resulted only in minor activity changes (94−98% vs control; not shown), indicating that none of the accessible lysines in PecE and PecF are required for the catalytic activity.

Tryptophan residues were modified by NBS (*45*). The activity of PecF decreased sharply after the modification of two of the three tryptophans (Figure 5A). This indicates a sequential modification of these residues, with the third one being critical for the activity of PecF. With PecE, there was a gradual decrease of activity. Assuming comparable reactivity of the tryptophans, it was analyzed for the number of critical residues, *i*, by the statistical method of Tsou (*47*). The data can be fitted to a straight line ($i = 1$, Figure 5B), suggesting that a single tryptophan residue is critical, too, for the activity of PecE. The location of the tryptophan residues was tested by fluorescence quenching experiments. Addition of PecE with KI (50 mM) caused loss of fluorescence with PecE but not with PecF (data not shown), indicating that only PecE has accessible tryptophan residues on the surface. This would explain the different titration behavior of the two subunits (Figure 5).

Histidine residues were modified by DEPC. The data were again analyzed by the statistical method of Tsou (*47*) to determine the number of essential residues (Figure 6). In both PecE and PecF, there is accordingly one histidine residue critical for the enzymatic activity. Modification of PecF did not interfere with binding of PecE, as judged by the secondary affinity assay using His-tagged PecF as the bait (Figure 7). The inverse experiment using His-tagged PecE was inconclusive, because PecF binds weakly to the Ni^{2+} column due to its "natural His tag", H53 through H56 (see above). The fact that the deletion mutant PecF(57−212) still had enzymatic activity indicated that H121 in PecF should be the key residue. It is neighboring to the cysteine residue required for activity, and a sequence comparison showed that it is more conserved than the other two histidine residues. A final proof of the critical role of H121 was obtained by chemical modification of PecF(57−212): DEPC caused this mutant to lose activity, and this inhibition could be reversed by neutralized hydroxylamine, which proves that the only remaining histidine, H121, is a key residue.

DISCUSSION

The two subunits of the isomerizing PVB:PecA lyase, viz., PecE and PecF, are homologous to the respective nonisomerizing subunits of the PCB:CpcA lyase. There are currently only two of the former enzymes known, in contrast to >10 of the latter even though only a few have been studied as proteins (*8*, *16*, *20*, *26*). The present analysis is aimed at defining those parts of PecE/F from *M. laminosus* PCC 7603 that catalyze the complete reaction, viz., the covalent attachment and the migration of the Δ4,5 double bond of the PCB chromophore to the Δ2,3 position. As a guide, we have concentrated on regions which are homologous among

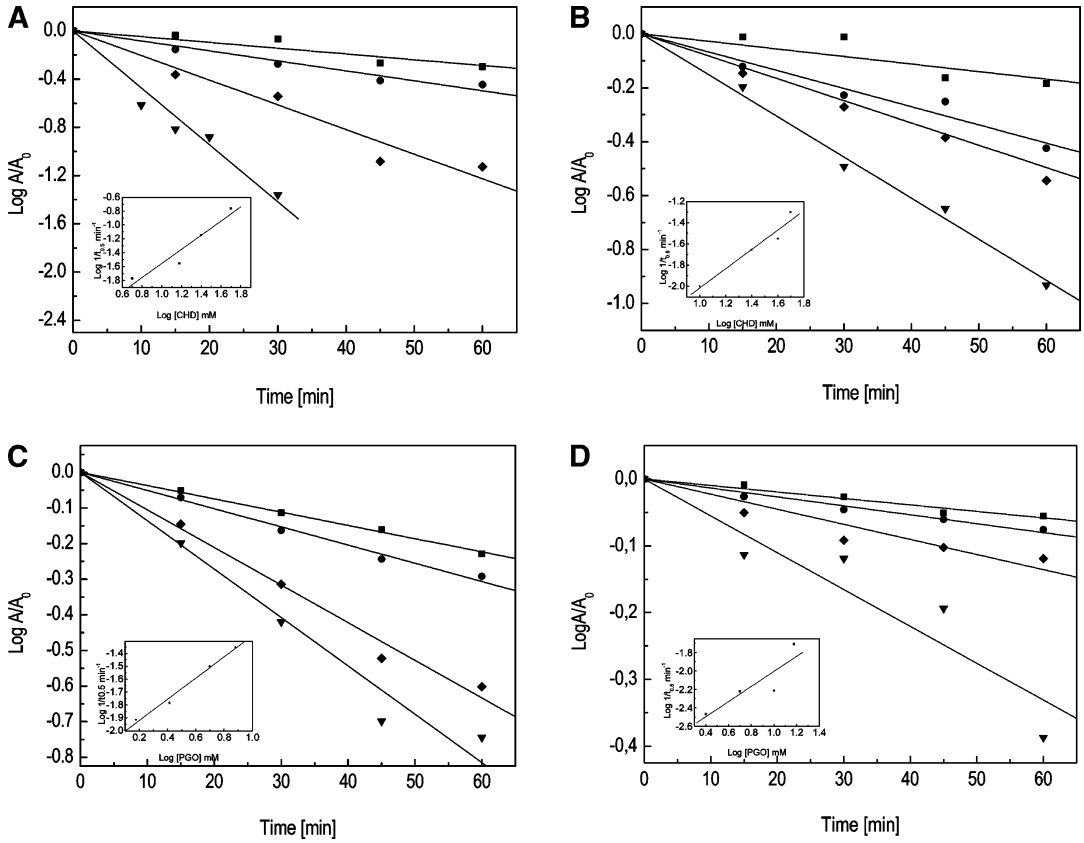

FIGURE 4: Kinetics of inactivation of PecE by CHD (A) and PGO (C) and of PecF by CHD (B) and PGO (D). The insets show plots of log($1/t_{0.5}$ of inactivation) against log(modifier concentration). Final CHD concentrations were (■) 5 mM, (●) 15 mM,(◆) 25 mM, and (▼) 50 mM in (A) and (■) 10 mM, (●) 25 mM, (◆) 40 mM, and (▼) 50 mM in (B); final PGO concentrations were (■) 1.5 mM, (●) 2.6 mM, (◆) 5 mM, and (▼) 7.6 mM in (C) and (■) 2.5 mM, (●) 5 mM, (◆) 10 mM, and (▼) 15 mM in (D).

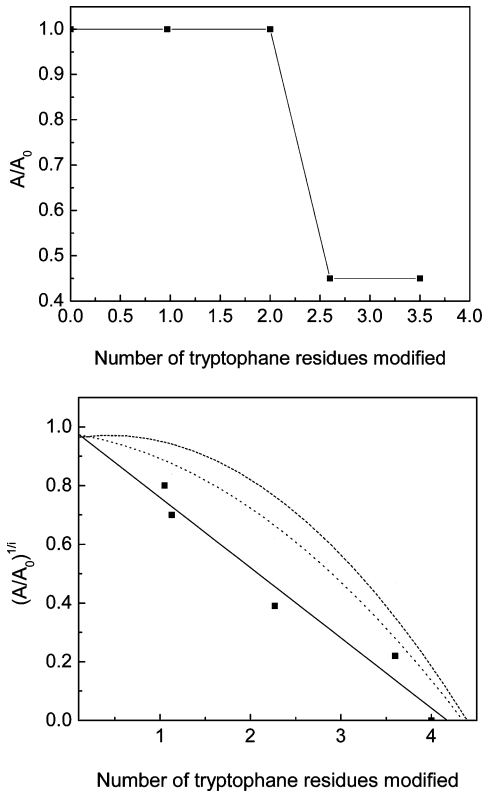

FIGURE 5: Relationship between residual enzyme activity and number of tryptophan residues modified by NBS. Top: PecF. Bottom: PecE. The squares (■) are the experimental values; the lines are obtained from the equation of Tsou (*47*) with $i = 1$ (—), $i = 2$ (• • •), and $i = 3$ (- - -).

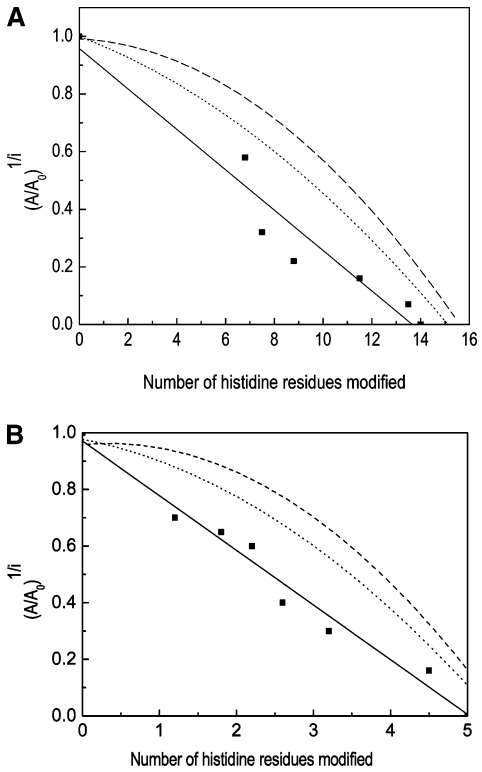

FIGURE 6: Correlation between the number of histidine residues modified and the remaining enzyme activity (Tsou plot) (*47*). (A) DEPC-modified PecF (with His tag): $i = 1$ (—); $i = 2$ (• • •); $i = 3$ (- - -). (B) DEPC-modified PecE (without His tag): $i = 1$ (—); $i = 2$ (• • •); $i = 3$ (- - -). The squares (■) are the experimental values; the lines are obtained from the equation of Tsou (*47*).

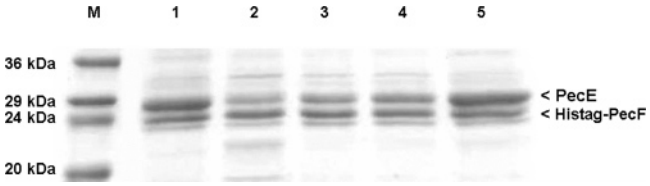

FIGURE 7: Complex formation of modified PecF with unmodified PecE. SDS−PAGE of fractions eluted with 1 M imidazole from the Ni^{2+}-chelating columns. Lane assignments: M, protein marker; 1, His-tagged PecF modified by CHD with PecE; 2, His-tagged PecF with PecE modified by DEPC; 3, His-tagged PecF modified by PGO with PecE; 4, His-tagged PecF modified by NBS with PecE; 5, His-tagged PecF with PecE (control).

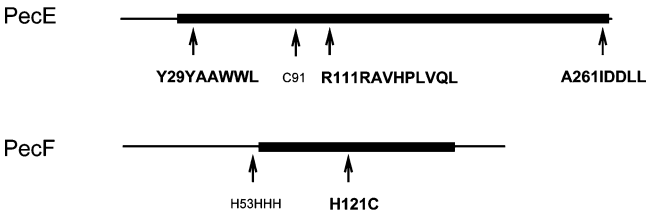

FIGURE 8: Schematic representation of elements (in bold) identified as critical for the catalytic function of the two subunits of PVB: PecA isomerizing lyase. Light face letters indicate aa that affect function but are not essential.

the two known PVB:PecA lyases but differ from regions which are homologous among the cyanobacterial PCB:CpcA lyases. These were then probed by deletions and site-directed mutation.

The results of the mutations are summarized in Figure 8. The α-subunits of CPC and PEC lyases, CpcE and PecE, respectively, are very homologous among each other and within the two classes. In PecE, there are two homologous motifs which distinctly differ from the respective subunits of nonisomerizing lyases, CpcE: one is N25QI near the N-terminus which is followed by a more homologous region; the other is A261IDDLLIQL, very close to the C-terminus (Figure 1). N-Terminal truncations that lacked the former motif [PecE(57−269) and PecE(37−269)] were inactive in the complementation assay, while PecE(23−269) that completely contains the former motif has enzymatic activity. Obviously, aa 23−36 are important for the reactivity of PecE. However, even this is still strongly reduced compared to the full-length subunit, indicating that also the more N-terminal parts are effective. At the C-terminus, the activity is rapidly lost if only a few aa are removed. Here, removal of the two leucines at the end of the C-terminal sequence motif A261IDDLL caused complete loss of activity; this motif is therefore also essential for the activity of PecE.

In the central region, there is a stretch of aa from around A104 to P126 which has relatively little homology among and between the CpcE and PecE, followed by a gap in the PecE where 12 aa are missing which are contained in most CpcE. Deletion of 26 aa in this region [PecE(Δ103−129)] caused the complete loss of activity. It is noteworthy that this mutant, as well as most of the other PecE mutants, caused a loss of the residual activity of PecF when the two purified subunits were denatured with urea and then co-renatured. Apparently, these mutants can still interact with PecF but in an inhibitory fashion. The amino acids in the deleted region (103−129) may be part of the interaction domain between PecE and PecF, which in the deletion mutant imposes an improper conformation on the latter, and the same might be true for part of the C-terminal aa between G235 and the N-terminus.

The finding that photochemically active PVB-PecA is formed in the presence of only PecF renders this subunit particularly interesting in defining the regions involved in catalysis of the lyase-isomerase reaction. The β-subunits of CPC and PEC lyases, CpcF and PecF, respectively, show considerably less homology among each other and between them. The two known PecF are 67% homologous over a central stretch beginning with a characteristic H53HHH sequence that provides a natural (but relatively weak; see above) His tag to the protein and extending to L172. The four histidines are not required for catalysis, however, as shown by the N-terminal deletions: PecF(52−212) containing the four histidines is even less active than PecF(57−212) which lacks them.

Also the C-terminus of PecF shows little homology to various CpcF and even to PecF from *Anabaena* sp. PCC 7120. The C-terminal deletion mutants PecF(1−201) and PecF(1−191) support that it is not essential to function. The region between aa 57 and 191 is therefore sufficient to maintain a basic lyase-isomerase activity.

In this central part of the protein, the region starting around A104 and ending abruptly after the motif N120HCQGN is particularly distinctive for PecF (Figure 1C). Two amino acids of this motif were proven essential for the lyase activity of PecF: mutation of C122 resulted in complete loss of activity and so did chemical modification of the only histidine (H121) in the truncated mutant PecF(57−212). Besides the two PecF, the same motif is also contained in several other sequences in the databases. One is coded by a gene from the marine cyanobacterium *Synechococcus* sp. WH 8102. It has been annotated as coding for a E/F fused phycoerythrobilin lyase (*54*), but its location in the Rpc operon renders it possible that it is rather involved in the synthesis of a phycocyanin (*21*). The homology of this region and, to a lesser extent, also other regions of PecF may then point to a different function: marine cyanobacteria such as *Synechococcus* WH 8102 contain large amounts of phycourobilin (PUB) (*2*), which like PVB has never been found as a free chromophore in cyanobacteria. Since PUB is isomeric to phycoerythrobilin in a similar way as is PVB to PCB, it may also be the result of an isomerizing lyase that converts PEB to PUB. The two other sequences have been annotated as HEAT repeats: one from *Anabaena variabilis* ATCC 29413 (accession no. ZP 00162868) and the other from *Crocosphaera watsonii* (formerly *Synechocystis* sp.) WH 8501 (accession no. ZP 174981). This annotation may be misleading, however. A distant and patchy relationship of the PEC lyases with armadillo-type proteins containing HEAT repeats had already been noticed before (*27*). The sequences show, on the other hand, high homology with the known PecF over most of its length. That of *A. variabilis* is by this criterion clearly a conventional F-subunit, that of *C. watsonii* appears to code for a E/F fusion protein similar to that of *Synechococcus* sp. WH 8102. *A. variabilis* contains PEC (*55*, *56*), and *C. watsonii* contains an unusual phycocyanin bearing a PUB chromophore at the conserved α-84 binding site for which the EF-type lyases are specific (*57*). On the basis of this evidence, we suggest that both genes code for lyases and probably isomerizing ones.

The binding cysteine of the "classical" chromophore binding site of phytochromes near aa 300 (*12*, *17*) is frequently C-terminally flanked by a histidine. The latter is even conserved and functional in the nonclassical phytochromes that bind a biliverdin chromophore to a cysteine near the N-terminus (*17*). In a helical context, binding to HC and CH could be quite similar if the chromophores were inverted, too. The motif might therefore be derived from a binding site, which now functions as an isomerization site.

The chromophores of phycobiliproteins and phytochromes are bound to cysteines. The work presented here gives evidence that both subunits of the PVB:PecA lyase can bind PCB. It is unclear, however, if this binding is part of the catalytic reaction sequence. The binding is quite weak compared to the binding at the "proper" cysteine, if judged from the fluorescence intensity of the Zn^{2+}-treated SDS−PAGE gels. This would be compatible with a transient binding during catalysis, where the bound chromophore is eventually released to the final acceptor, PecA. Apparently, all three cysteines (C48, C91, C161) can bind PCB in substoichiometric quantities, and among them C48 most strongly. The PVB:PecA lyase of *M. laminosus* PCC 7603 contains seven cysteine residues in PecE and five in PecF. Only three of PecE and one of PecF are conserved between this lyase and the corresponding one of *Anabaena* sp. PCC 7120. Individual site-directed mutageneses of these cysteine residues showed that none of the three of PecE is essential; in the case of C91L there is even an increase in enzymatic activity. It could then be that the covalent binding is only a side reaction, which is readily visible only if the proper substrate is not present. 2-Mercaptoethanol (ME) has been identified as a cofactor of the enzyme (*26*, *27*, *31*). Since it can also bind to the 3-ethylidine group of PCB, its function may be to maintain binding reversible until the proper site has been reached and the chromophore isomerized.

The chemical modifications add more information to the involvement of particular aa residues: none of the 14 lysines (6 in PecE, 8 in PecF, plus 3 from the vector pET30a) or of the acidic aa seem to be involved. One residue each of arginine, tryptophan, and histidine is involved in PecE and one tryptophan plus the already discussed H122 in PecF.

PecE and PecF form a relatively stable complex (*31*). If judged from affinity chromatography tests using the His-tagged F-subunit as the target, all mutants were still capable of forming this binary complex (not shown). However, quantitative data are needed to possibly correlate the binding with the activities. As a further complication of the affinity method used, untagged PecF binds moderately strong to the Ni^{2+} column on account of the four contiguous histidines which are present already in the wild-type enzyme, and therefore experiments using His-tagged PecE as a bait were inconclusive.

The importance of the C-terminal leucines for activity is reminiscent of the identification of one hydrophobic residue, isoleucine, for the autocatalytic lyase of phytochromes (*16*, *58*). Since conformational control seems to be a critical factor for the chromophore binding (*50*), the large hydrophobic residues may be involved in such a control by sterically defining a binding template.

In the current analysis, a black box approach has been used to identify the core regions of the two subunits, PecE and PecF, and several critical amino acid residues within that are responsible for the complete reaction sequence by which PCB is attached to PecA and isomerized to PVB. Weak binding of the chromophore seems possible to both subunits. An important finding is furthermore that PecF alone is capable of the complete reaction sequence, although with low yield. In view of the diversity of biliprotein lyases known to date, including autocatalytic binding, as well as the variety of substrates and binding sites, it will be critical in the future to separate in detail the lyase and the isomerase activities and to identify their mechanisms. The (N/D)HCQG(N/K) motif suggested from this work as being specific for the isomerase activity is a first step in this direction.

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SUPPORTING INFORMATION AVAILABLE

Two figures showing (1) expression of wild-type and mutant proteins in *E. coli* and SDS−PAGE of samples after the first enrichment by Ni^{2+} affinity chromatography and (2) interaction of PCB with wild type and PecE/F mutants and absorption spectra after incubation of the His-tagged proteins with PCB. This material is available free of charge via the Internet at http://pubs.acs.org.

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