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Occurrence of P-Flavin Binding Protein in *Vibrio fischeri* and Properties of the Protein[1]

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In previous studies involving *Photobacterium* species we proposed that (i) P-flavin is the product of luciferase, (ii) the physiological function of the *lux* operon is not to produce light but to produce FP_{390} (*lux*F protein), including its prosthetic group, P-flavin, and (iii) FP_{390} reactivates oxidatively inactivated cobalamin-dependent methionine synthase similar to flavodoxin but at relatively high ionic strength. It seems difficult to extend this idea to all luminous bacteria because the *lux*F gene is not present in the *lux* operon in *Vibrio* or *Xenorhabdus*. But we predicted that a luciferase fragment which binds P-flavin should function like FP_{390} in these species. In this study, we isolated P-flavin binding protein from *Vibrio fischeri* ATCC 7744. The obtained protein was a modified luciferase as expected, in which the β-subunit was intact but about 25 amino acid residues at the C-terminus of the α-subunit were deleted and the prosthetic group was the fully reduced P-flavin. These results strongly support that the physiological function of the *lux* operon is as described above even in luminous bacteria other than *Photobacterium* species. We propose that chromophore B reported by Tu and Hastings [Tu, S.-C. and Hastings, J.W. (1975) *Biochemistry* 14, 1975-1980] is the reduced P-flavin.

Key words: flavodoxin, FP_{390}, *lux* operon, P-flavin, *Vibrio fischeri*.

Although bacterial bioluminescence was recognized by Aristotle in the form of the phosphorescence of flesh (*1*), its physiological function is not yet fully understood. After extensive research the reaction process catalyzed by bacterial luciferase was thought to be:

$$CH_3(CH_2)_{12}CHO + FMNH_2 + O_2 \longrightarrow CH_3(CH_2)_{12}COOH + FMN + H_2O + h\nu,$$

and the function of the *lux* operon to be the production of light. This reaction scheme is given in almost all current papers dealing with bacterial luciferase, and even some textbooks in the fields of bacteriology, biochemistry and others. The *in vitro* light-emitting reaction certainly proceeds as above but many questions, posed below, can not be answered using this reaction scheme. Why do bacteria emit light even though they have no photoreceptors (eyes)? Why does a salty environment (*2*) or acyl derivatives of homoserine lactone (*3, 4*) induce bacterial bioluminescence? Why does luciferase from *Photobacterium* species bind P-flavin, mainly 6-(3″-myristic acid)-FMN (*5, 6*)? Although the *lux* operon in all luminous bacteria comprises two genes coding for the two luciferase subunits (*lux*A and *lux*B) as well as three genes coding for three myristic acid reductase subunits generating tetradecanal from myristic acid (*lux*C-E) (*7-9*), why does the *lux* operon in *Photobacterium* species contain an additional gene, *lux*F (*lux*ABN or *lux*G) (*10-12*), of which the product, FP_{390} (non-fluorescent flavoprotein or green flavoprotein), does not directly participate in light emission? Why does FP_{390} or a corresponding protein also bind P-flavin (*6, 13, 34*)? We sought answers to these questions through studies involving luminous *Photobacterium* species (*P. phosphoreum*), and proposed that P-flavin is the product of luciferase and that the light is a by-product in the cells (*6*):

$$CH_3(CH_2)_{12}CHO + FMN + O_2 \longrightarrow \text{P-flavin} + H_2O + h\nu.$$

We also proposed that the physiological function of the *lux* operon is to produce FP_{390} including its prosthetic group, Q(P)-flavin (*6*). Finally we proposed that FP_{390} reactivates oxidatively inactivated cobalamin-dependent methionine synthase similar to flavodoxin in *Escherichia coli* (*14-19*), but at relatively high ionic strength (*20*). These proposals have not yet been completely confirmed and we should accumulate more evidence to substantiate them. One of the questions regarding these proposals may be how FP_{390} or an FP_{390} equivalent protein is synthesized in luminous bacteria other than *Photobacterium* species, *Vibrio* and *Xenorhabdus*, because these species have no *lux*F gene in the *lux* operon (*8, 21, 22*). To answer this question, we predicted that a luciferase fragment should function as FP_{390} in these species (*20*), because the homology between FP_{390} and β-subunit of luciferase is very high (*10, 11*). To confirm this, we attempted to isolate this predicted protein from *Vibrio fischeri* ATCC 7744. This protein was designated as P-flavin binding protein (PFBP) because it should bind to

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Abbreviations: PFBP, P-flavin binding protein; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ESI, electro spray ionization.

P-flavin. Here we report the presence of PFBP in *Vibrio* and evidence supporting that this protein functions as flavodoxin as expected.

MATERIALS AND METHODS

Bacteria—ATCC *Photobacterium* broth 101 (Difco 0417) was modified slightly: 1 liter of the liquid medium contained 5 g of polypepton (Nihon Pharmaceutical), 2.5 g of yeast extract (Oriental Yeast), 6 g of glycerol, 30 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 3 g of KH_2PO_4, and the pH was adjusted to 7.0 with NaOH. *V. fischeri* ATCC 7744 was cultured at 26°C with aeration. Cells at the early stationary phase were harvested by centrifugation at $17,780 \times g$ for 3 min and stored in a freezer at −20°C. The yield of packed wet cells ranged between 100 to 120 g from a 16-liter culture.

Preparation of PFBP—The frozen cell paste (220 g) was osmotically lysed at 3°C for 20 min in 800 ml of 50 mM phosphate buffer, pH 7.0, and then the cell debris was removed by centrifugation at $42,500 \times g$ for 10 min. The supernatant (900 ml) was applied to a column of DEAE-Sepharose Fast Flow (5.5×35 cm; Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.0. PFBP was eluted with a linear gradient consisting of 800 ml of 200 mM phosphate buffer, pH 7.0, and an equal volume of 500 mM phosphate buffer, pH 7.0, and subsequently the column was eluted isocratically with 500 mM phosphate buffer, pH 7.0. Fractions (18 ml each) containing PFBP, as monitored by TLC as described below, were pooled. PFBP was precipitated by adding ammonium sulfate (75% saturation) to the pooled fractions. The precipitate was collected by centrifugation at $42,500 \times g$ for 15 min and then dialyzed against 50 mM phosphate buffer, pH 7.0. After removing insoluble material by centrifugation, the clear solution of PFBP obtained was applied on a column of Sephadex G-75 (5.5× 80 cm; Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.0. PFBP was eluted with the same buffer and 18-ml fractions were collected. PFBP was precipitated as described above. The precipitate was collected by centrifugation and then washed quickly with a minimum volume of 50 mM phosphate buffer containing 1.2 M ammonium sulfate, pH 7.0. The washed precipitate was dissolved in 20 ml of the same buffer and then insoluble material was removed by centrifugation. The clear supernatant was applied on a column of Butyl Cellulofine gel (3.6×90 cm), prepared as described previously (*23*), equilibrated with the same buffer. PFBP was eluted with a linear gradient consisting of 1 liter of 50 mM phosphate buffer containing 1.2 M ammonium sulfate, pH 7.0, and an equal volume of 50 mM phosphate buffer, pH 7.0. For each of the 18-ml fractions collected, the absorbance at 280 nm and 373 nm was measured because holoPFBP exhibits an absorption maximum of 373 nm (Fig. 5). The fractions containing mainly holoPFBP were pooled and then protein was precipitated by adding ammonium sulfate up to 75% saturation to the pooled fractions. The precipitate was collected by centrifugation and dissolved in 50 mM phosphate buffer containing 1.2 M ammonium sulfate, pH 7.0, and then rechromatographed in the same way as above. The Butyl Cellulofine column chromatography was repeated three times in all.

Detection of PFBP—PFBP was searched for in the fractions eluted from the DEAE-Sepharose column by thin layer chromatographic analysis. Every third fraction was subjected to analysis. An aliquot (1 ml) of each sample was shaken with ammonium sulfate (500 mg) and liquefied phenol (150 μl). The mixture was then centrifuged and a 10 μl aliquot of the separated phenol layer was applied on a silica-gel plate (Merck; silica gel 60 TLC plate) and then developed with a solvent system, *i.e.* isoamyl alcohol-methyl ethyl ketone-acetic acid-water (40:40:7:13). Flavins were detected as to fluorescence under UV light.

Other Assays—The amino acid sequence was determined using a gas-phase amino acid sequencer (Shimadzu, PPSQ-10). A positive ion matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrum was acquired with 256 laser shots in the delayed extraction mode with a Voyager RP-DE mass spectrometer (PerSeptive) using a matrix which was prepared by dissolving 5 mg of 3,5-dimethoxy-4-hydroxysinnamic acid (sinaptic acid) in 1 ml of a water-methanol (9:1, v/v) solution containing 0.1% trifluoroacetic acid. An electro spray ionization (ESI)-TOF mass spectrum was acquired with scanning 95 times in the negative ion mode using a Mariner mass spectrometer (PerSeptive). PFBP was dissolved in 50% methanol and then injected into the mass spectrometer. The absorption spectrum was recorded with a Hitachi spectrophotometer U-3000. SDS-PAGE was performed using gradient polyacrylamide gels (gel concentration, 5 to 20%) as described by Laemmli (*24*). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R.

RESULTS AND DISCUSSION

Purification of PFBP—A DEAE-Sepharose column was used in the first stage of the purification of PFBP. In the case of *P. phosphoreum* IFO13896, two yellow bands of luciferase and FP_{390}, both of which bind P-flavin in the oxidized form, were clearly recognized on this column (*25*). On the other hand, in this study only one faint yellow band was found at a similar position to FP_{390} from *P. phosphoreum*. But even this yellow protein corresponded to thioredoxin reductase, as shown in Fig. 1 (*26*). Then we performed TLC to detect PFBP. Total flavin was extracted with phenol from the fractions eluted from the column and then analyzed by TLC as described under "MATERIALS AND METHODS." As shown in Fig. 1 only one band of protein(s) bound to P-flavin was observed, but the fractions were only slightly colored yellow because the bound flavin was in a reduced form, as described below. The fractions containing PFBP were further purified by gel filtration on Sephadex G-75. At this stage the protein was purified to such an extent that only a single band was detected on SDS-PAGE (Fig. 2). Finally, the protein was purified on a Butyl Cellulofine column, *i.e.* hydrophobic interaction chromatography (*23*), and then the holoprotein was eluted ahead of the apoprotein. On repeating of this purification procedure a further two times, the A_{373}/A_{280} ratio became nearly constant (0.083), and it was estimated that the sample contained mostly pure holoprotein (Fig. 3). This sample was used as PFBP in the following experiment. About 150 mg of holoPFBP was obtained from 1.1 kg of bacterial cell paste. The yield was not good owing to the irreversible binding of the protein to the gel. A large amount of

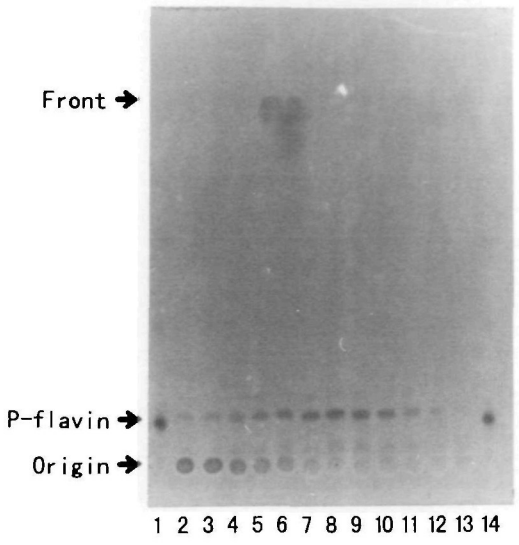

Fig. 1. **Detection of PFBP.** PFBP were searched for as described under "MATERIALS AND METHODS." The TLC-plate was examined by irradiation with UV light and photographed. Lanes 1 and 14, P-flavin extracted from FP_{390}; lanes 2, 3, 4, ... and 13, phenol extracts of fractions No. 73, 76, 79, ... and 106 obtained on DEAE-Sepharose column chromatography, respectively. Only one band of protein(s) binding P-flavin was observed, and fraction Nos. 76 (lane 3) to 100 (lane 11) were collected for further purification. FAD bound to thioredoxin reductase (*19*) was found at the origin in lanes 2 to 6.

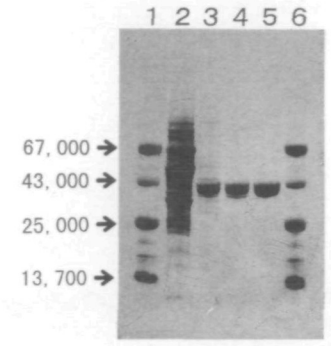

Fig. 2. **SDS-PAGE of PFBP from *V. fischeri* at various stages of purification.** Lanes 1 and 6, molecular weight markers: ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and bovine serum albumin (67,000); lane 2, cell extract; lane 3, pooled eluate from the DEAE-Sepharose column; lane 4, pooled eluate from the Sephadex G-75 column; lane 5, pooled eluate from the third Butyl Cellulofine column.

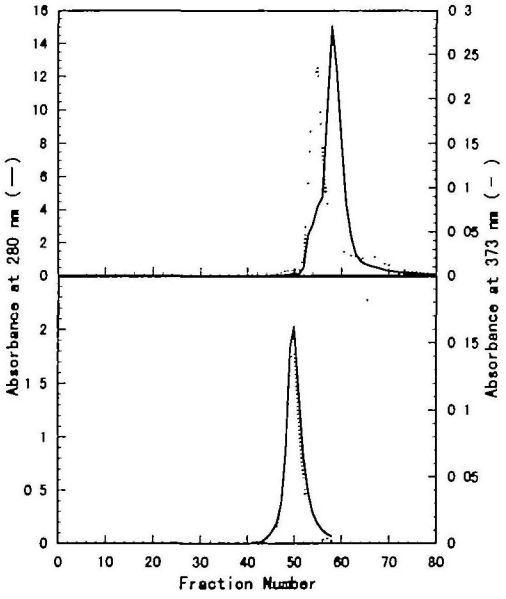

Fig. 3. **Elution profile on the first Butyl Cellulofine column chromatography (upper panel), and that on the third one (lower panel).** To detect holoPFBP, the absorbance at 373 nm as well as that at 280 nm was measured for each fraction. On Butyl Cellulofine chromatography, holoPFBP was eluted ahead of the apoprotein. By performing this chromatography three times in all, a sample containing mostly pure holoPFBP was obtained.

apoprotein seems to be produced in cells because riboflavin synthesis cannot keep up with luciferase synthesis.

Processing of Luciferase—PFBP seemed to be composed of a single polypeptide chain because it gave only one band at nearly the same position as the β-subunit of luciferase on SDS-PAGE (Fig. 2). The amino acid sequence was determined up to 30 residues from the N-terminus. The following sequence was obtained. Here, the two amino acids in parenthesis, respectively, indicate that nearly equal amounts of these amino acids were found at each stage, M-K-F-G-(N,L)-(I,F)-F*-(F,L)-(S,N)-(Y,F)-Q-(P,K)-(P,D)-G-(E,I)-T-(H,S)-(K,E)-(Q,E)-(V,T)-(M,L)-D-(R,N)-(F,M)-V-(R,K)-(L,T)-(G,V)-(I,T)-(A,L). These sequences completely coincide with those of the α- and β-subunits of the luciferase (the first and second amino acids in parenthesis, respectively) (*11*) with one exception; cysteine, which is the seventh amino acid residue of the α-subunit, was not found, but this amino acid is often not found on sequencing using a sequencer. These results indicate that the obtained PFBP is composed of two polypeptide chains: one is the intact β-subunit of luciferase and the other is its α-subunit with a deletion at the C-terminus. To determine the extent of deletion of the α-subunit, a MALDI-TOF mass spectrum of PFBP was obtained. As shown in Fig. 4a, a high peak was observed in the region of m/z^+ values of 37,100-37,900 and a low peak at a m/z^+ value of about 75,000, but no peak was present at a m/z^+ value of around 40,000. These results indicate that about 25 amino acid residues are deleted from the C-terminus of the α-subunit of luciferase in PFBP since the molecular weight of the α- and β-subunits are 40,310 and 37,209, respectively (*11*).

Baldwin *et al.* and Holzman and Baldwin reported that the α-subunits of the luciferases from *V. harveyi*, *V. fischeri*, and *P. phosphoreum* undergo proteolytic hydrolysis under nondenaturing conditions but that their β-subunits are resistant to proteases (*27, 28*). However, they did not explain why the α-subunits are sensitive to proteases. On the other hand, we isolated the P-flavin-bound β-subunit of the luciferase from *P. phosphoreum*, which can be explained as follows (*23*): P-flavin is produced by luciferase but it binds tightly to the enzyme, so the α-subunit is hydrolyzed to produce the P-flavin-bound β-subunit to facilitate the release of P-flavin from the P-flavin-luciferase complex and transfer of the flavin to $apoFP_{390}$. In *Vibrio*, only a single band of protein(s) bound to the reduced P-flavin was observed on DEAE-chromatography, as described above. We concluded that the P-flavin-bound β-

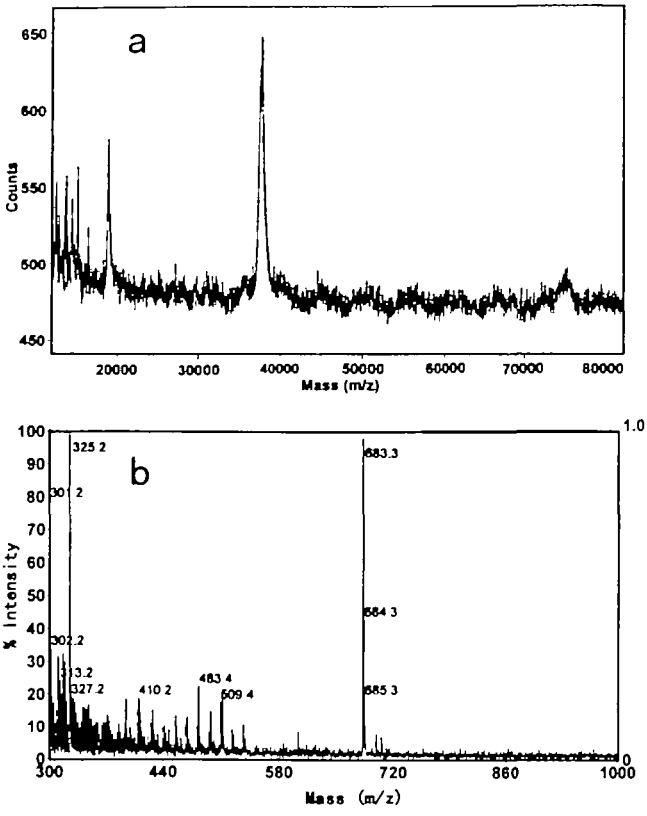

Fig. 4. **Mass spectra of PFBP.** The spectra were measured as described under "MATERIALS AND METHODS." a, positive ion MALDI-TOF mass spectrum of PFBP; b, ESI-TOF mass spectrum of PFBP. Unfortunately, protein ions were not observed for unknown reasons.

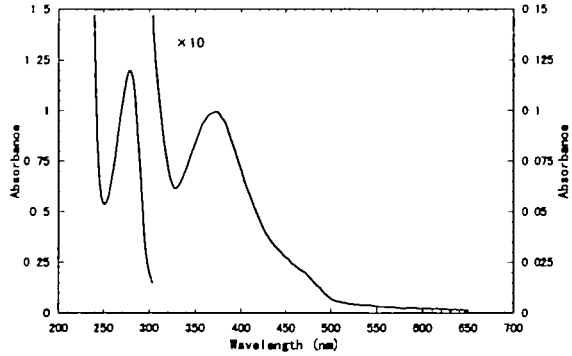

Fig. 5. **Absorption spectrum of PFBP.** The spectrum was recorded in 50 mM phosphate buffer, pH 7.0.

subunit is not present in these P-flavin containing fractions, as explained below. Because the prosthetic group of such a β-subunit is in an oxidized form in *Photobacterium* (*23*), the corresponding β-subunit, if present, should also be in an oxidized form even in *Vibrio*. Even if in a small amount, the presence of oxidized flavin in the reduced form can be easily recognized because the absorbance of the oxidized flavin in the near UV and visible regions is much stronger than that of the reduced form. An intact P-flavin-bound luciferase was not found in *Vibrio* but the C-terminus of the α-subunit of the luciferase was slightly deleted, as described above, and the deletion point seemed to be located on the surface of the luciferase, as suggested by the 3D structure of the luciferase from *Vibrio harveyi* (*29*). PFBP should not be a simple enzyme-product complex or a half degraded product but a protein which has a physiological function, as discussed below. For these reasons, we concluded that the sensitivity as to proteases of the α-subunit is a property designed for the following purposes: in *Photobacterium* species, removal of the α-subunit from P-flavin-bound luciferase to produce the P-flavin-bound β-subunit, and in *Vibrio*, removal of the peptide from the C-terminus of the α-subunit to produce the mature protein from such a luciferase.

Properties of the Prosthetic Group Bound to PFBP—The absorption spectrum of the purified PFBP is shown in Fig. 5. Only one peak (absorption maximum, 373 nm) was observed in the near UV and visible regions at every stage of purification, although two peaks (absorption maxima, 390 and 442 nm) were observed in the case of FP_{390} (*25*). Since this spectrum resembles that of reduced flavin, reduced P-flavin is the most probable candidate for the prosthetic group of PFBP. To further confirm the structure, an ESI-TOF mass spectrum of PFBP was obtained in the negative ion mode. As shown in Fig. 4b, an outstanding $(M-H)^-$ peak was observed at a m/z^- value of 683.3, which corresponds exactly to that of the (reduced P-flavin$-H)^-$ ion, but no peak was observed at a m/z^- value of 470.1 $(FMN-H)^-$, 472.1 $(FMNH_2-H)^-$, or 681.3 (P-flavin$-H)^-$. This indicates that the prosthetic group of PFBP is uniquely the reduced P-flavin.

Mitchell and Hastings first obtained a photoexcitable luciferase from *V. harveyi* MAV and *V. fischeri* ATCC 7744 (*30*). Tu and Hastings described the properties of that from *V. harveyi* (*31*). The reported absorption spectrum of this protein is quite similar to that of PFBP (*31*). Because they did not present the SDS-PAGE pattern of this protein we can not conclude that PFBP is the same protein as the photoexcitable luciferase. But it is highly probable that the two proteins are identical. They attempted to isolate the prosthetic group from this luciferase and obtained at least three components, a chromophore designated as B, FMN, and a flavin in the oxidized form, and concluded that chromophore B is 4a-substituted $FMNH_2$, citing many reasons (*31*). But chromophore B seems to be the reduced P-flavin because the prosthetic group of PFBP is just so as described above. FMN seems to be formed from the reduced P-flavin during the isolation process, probably photochemically. An oxidized flavin, which they obtained but did not study further, seems to be P-flavin in the oxidized form. It is note worthy that the reduced P-flavin is reoxidized smoothly on shaking of the solution, although more slowly than usual flavins (riboflavin, FMN, and FAD). They hypothesized that chromophore B is a false intermediate of the bacterial bioluminescence reaction (*31*). In the meantime, we concluded that the reduced P-flavin bound to PFBP is a cofactor of the protein and has a physiological function, as described below.

Function of PFBP—P-flavin was first found as a prosthetic group of the luciferase from *P. phosphoreum* by Matsuda and Nakamura (*5*). Later it was found that FP_{390} binds a similar flavin, which was designated tentatively as Q-flavin since it was not clear whether these two flavins are identical or not (*6*). Q-flavin extracted from FP_{390} com-

prises a mixture of two components, the structure of the major one being determined to be 6-(3″-myristic acid)-FMN and that of the minor one to be 6-(4″-myristic acid)-FMN (*6*). The former structure was confirmed by X-ray crystallography (*32-34*). Now P-flavin seems to be the same compound as Q-flavin for many reasons (*23*). In this study the prosthetic group of PFBP was identified as P-flavin on TLC and mass spectrometry, and so it could not be judged if the prosthetic group is a mixture like the flavin from *P. phosphoreum*. However, it was confirmed that P-flavin is the product of a luciferase even in *Vibrio* species. P-flavin bound to PFBP is in the fully reduced form, as shown above. P-flavin seems to be produced in the oxidized form on the luciferase, followed by reduction because of the following reasons. (i) The reaction catalyzed by luciferase is an oxidative one. (ii) P-flavin bound to luciferase is in the oxidized form in *Photobacterium* species because P-flavin-bound luciferase is just an enzyme-product complex and has no additional activity (*25*). PFBP is an unusual flavoprotein because flavoproteins are usually in the oxidized form under aerobic conditions. However, a few flavoproteins are exceptionally in a fully or half reduced form even under aerobic conditions. For example, various DNA photolyases bind fully reduced FAD to split the cyclobutane ring of the pyrimidine dimer through a radical mechanism (*35, 36*). Also, the flavodoxins from various sources usually occur in the half reduced semiquinone form under aerobic conditions (*37*). Flavodoxins act as strong single electron reductants in both anaerobes and aerobes. In the latter case, one of the flavodoxins' functions is the reductive reactivation of such oxidatively inactivated proteins as cobalamin-dependent methionine synthase (*14-19*), because the semiquinone form is not oxidized so quickly even under aerobic conditions. Thus, the evidence that P-flavin bound to PFBP is in the reduced form indicates that PFBP is not a simple half degraded luciferase-P-flavin complex. Furthermore, this evidence strongly supports our proposal that PFBP has a flavodoxin-like function.

On the following basis we proposed in the previous reports that FP_{390} has a flavodoxin-like function. (i) One of two P-flavins that bind to FP_{390} is on the surface of the protein and the structure resembles that of flavodoxins (*32-34*). (ii) *P. phosphoreum* utilizes cobalamin-dependent methionine synthase but not the cobalamin-independent one, and so needs a flavodoxin under aerobic conditions (*20*). (iii) The biosynthesis of FP_{390} is induced at relatively high ionic strength because in such an environment, the association of the flavodoxin and methionine synthase is interfered with (*18*) or the usual flavodoxin seems to be inactivated (*20*). (iv) The *lux* operon is induced by acyl derivatives of homoserine lactone (*3, 4*) and a hydrolyzate of these compounds is a precursor of methionine. On the other hand, if FP_{390} has a flavodoxin-like function the prosthetic group should be in a fully reduced form or a half reduced form, as described above, but we could not obtain evidence of this. This lack of success was mainly caused by the fact that a monomer of FP_{390} binds two P-flavins, one at the active site and the other at a position for accommodation of a by-product in the reaction catalyzed by luciferase, 6-(4″-myristic acid)-FMN (*33*), and so the absorption spectrum of FP_{390} comprises those of these two flavins: the absorption spectrum of FP_{390} appears to be that of oxidized flavin even if the flavin bound to the active site is in the reduced form because the other flavin, which binds to the position for accommodation of the by-product, should be in the oxidized form. In this study, we isolated a FP_{390} equivalent protein (PFBP) from *Vibrio* and showed that the prosthetic group of PFBP is in the reduced form under aerobic conditions, so that it is highly probable that the flavin bound to the active site of FP_{390} is in the fully reduced form like that bound to PFBP, although this remains to be confirmed.

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