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Chemical Characterization of Lumazine Protein from *Photobacterium leiognathi*: Comparison with Lumazine Protein from *Photobacterium phosphoreum*†

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ABSTRACT: The properties of lumazine proteins purified from the marine bioluminescent bacteria *Photobacterium phosphoreum*, a psychrophile, and *Photobacterium leiognathi*, a relatively thermophilic species, are compared. An accurate 1:1 stoichiometry of binding of the ligand 6,7-dimethyl-8-ribityllumazine to each lumazine protein is established by back-titration of the apoprotein with the authentic ligand, using both fluorescence and absorption measurements. Neither protein contains metal cofactors, organic phosphorus, or carbohydrate. Both proteins are anionic and hydrophilic. They each contain a single Trp residue and have blocked amino terminals but otherwise differ in amino acid composition and other properties (*P. phosphoreum* and *P. leiognathi*, respectively): Met (internal), 1, 2; Cys, 2, 1; Arg, 4, 7; p*I*, 4.78 and 4.83, 4.38 and 4.45; M_r, 19750, 21300. In the *P. phosphoreum* protein both Cys residues are accessible, but in the *P. leiognathi* protein the single Cys is "buried". Modification of this buried Cys and at least one Cys in the *P. phosphoreum* protein prevents binding of the ligand. The UV and visible absorption spectra of both lumazine proteins denatured in 6 M guanidine hydrochloride can be accurately modeled by using the number of equivalents of the lumazine derivative and blocked aromatic amino acid model compounds determined by chemical and spectrophotometric analyses for Trp, Tyr, and Phe.

The purification and biological properties of lumazine proteins (LumPs)[1] from two species of the marine bioluminescent bacteria of the genus *Photobacterium* were described in the preceding paper (O'Kane et al., 1985). These proteins enhance the photon output of bacterial luciferases in vitro (Gast & Lee, 1978; O'Kane et al., 1985) and give rise to spectral parameters of the in vitro reaction that match the in vivo spectra of several strains of *Photobacterium* (O'Kane et al., 1985). The characterization of LumP isolated from *Photobacterium phosphoreum*, a psychrophile, has been reported (Small et al., 1980). This species is found to occur at ambient ocean temperatures of 5 °C (FitzGerald, 1978; FitzGerald & Lee, 1978). *Photobacterium leiognathi* strains have also been shown to have LumP (Lee, 1982; Vervoort et al., 1983; O'Kane et al., 1985). These bacteria are normally grown at 28–30 °C in the laboratory and are found to occur naturally at warm ocean temperatures (Yetinson & Shilo, 1979; Shilo & Yetinson, 1979). Both types of LumP, one from a psychrophile and one from a relatively thermophilic species, have similar spectral and biological properties. In this paper we report the chemical characterization of *P. leiognathi* and *P. phosphoreum* LumPs to point out both the similarities and differences in proteins that perform the same function in bacteria that have evolved in very different environments.

Materials and Methods

Authentic 6,7-dimethyl-8-ribityllumazine (Lum) was generously provided by Prof. H. C. S. Wood (University of Strathclyde). "Sequenal" grade guanidine hydrochloride (Gdn·HCl), [bis(trifluoroacetoxy)iodo]benzene (BTI), and micropolyamide plates (5 × 5 cm) were obtained from Pierce Chemical Co., Rockford, IL. 5-(Dimethylamino)-naphthalene-1-sulfonyl chloride (DNS-Cl), 10% DNS-Cl on Celite, and cycloheptaamylose were obtained from Calbiochem-Behring, San Diego, CA. Tryptophan (Gold Label, >99+%) was purchased from Aldrich Chemical Co., and quinine sulfate dihydrate (standard reference material) was obtained from the National Bureau of Standards, Washington, DC. Trypsinogen (1× crystallized), bovine serum albumin (3× crystallized), *N*-acetyltryptophanamide (NAWA),[2] *N*-acetyltyrosinamide (NAYA), glycylphenylalaninamide acetate salt (GPA), 5,5′-dithiobis(2-nitrobenzoic acid), and DEAE-Sepharose were purchased from Sigma Chemical Co., St. Louis, MO. Chicken egg white lysozyme (6× crystallized) was obtained from Miles Laboratories, Kankakee, IL, while glucagon was the product of the Chemical Dynamics Corp.,

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[1] Abbreviations: LumP(s), lumazine protein(s); Lum, 6,7-dimethyl-8-ribityllumazine; Gdn·HCl, guanidine hydrochloride; BTI, [bis(trifluoroacetoxy)iodo]benzene; DNS-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; BSA, bovine serum albumin; NAWA, *N*-acetyltryptophanamide; NAYA, *N*-acetyltyrosinamide; GPA, glycylphenylalaninamide acetate salt; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HSA, human serum albumin; RNase, ribonuclease; EDTA, ethylenediaminetetraacetate; 2-ME, 2-mercaptoethanol; TNB^-, 5-thio-2-nitrobenzoate; $\bar{\lambda}$, mean wavelength position; Tris, tris(hydroxymethyl)-aminomethane.

[2] Although *N*-acetyltryptophanamide is frequently abbreviated as NATA, single letter abbreviations are used for tryptophan, W, and tyrosine, Y, in the blocked aromatic model compounds to avoid confusion.

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South Plainfield, NJ. Pancreatic ribonuclease (RNase) was obtained from Worthington Biochemicals, Freehold, NJ, and highly purified human serum albumin (HSA) was provided by Dr. James Travis (University of Georgia). Bio-Gel P-2 was the product of Bio-Rad Laboratories.

Protein Purification and Preparation of Apoproteins. LumPs from *P. phosphoreum* strain A13 and *P. leiognathi* strain A2D were purified as described previously (O'Kane et al., 1985). The apoproteins were prepared by two methods. In early experiments, apoprotein was prepared by dialysis of the holoprotein in the dark at 32 °C in 3 M urea in 50 mM phosphate buffer, pH 7.0, containing 3 mM ethylenediaminetetraacetate (EDTA) and 10 mM 2-mercaptoethanol (2-ME). The dialyzed protein samples were then dialyzed against the same buffer minus urea. The dialyzed, urea-free protein samples were then used after centrifugation to remove precipitated protein. In later experiments, the holoproteins were adsorbed to DEAE-Sepharose at room temperature. The column was washed with 2 M urea to remove Lum and then washed with 50 mM phosphate buffer, pH 7.0, containing 3 mM EDTA plus or minus 10 mM 2-ME (as required for the experiment). The apoprotein was eluted from the column with 0.3 M phosphate buffer, pH 7.0, containing 3 mM EDTA plus or minus 2-ME. This procedure was rapid (~45 min), and the apoprotein did not precipitate. The prepared apoproteins were used immediately. The urea used to prepare the apoproteins was made fresh for each experiment and treated with activated charcoal prior to use.

Isoelectric Point Focusing (IEF) of Lumazine Proteins. IEF polyacrylamide gels (3-mm diameter; 5% total monomer) were prepared with 2% ampholytes (1.6% pH 4–6, 0.4% pH 3.5–10) in 8 M urea, as described in all other respects by O'Farrell (1975). The gels were run at 400 V for 18 h with top and lower buffers of 0.01 M NaOH and 0.01 M H_3PO_4, respectively. The gels were fixed and stained respectively with the denaturant and IEF fixer/stain of Bürk et al. (1983). The p*I* for *P. leiognathi* LumP was determined by reference to carbonic anhydrase (p*I* = 5.3; Davies, 1961).

Determination of Lum and Protein. The Lum content of native holoproteins was determined by absorption spectrophotometry at the visible absorbance maximum; extinction coefficients of 10 300 M^{-1} cm^{-1} for *P. phosphoreum* LumP at 417 nm and 10 100 M^{-1} cm^{-1} for *P. leiognathi* LumP at 420 nm (O'Kane et al., 1985) were used. The Lum content was also determined from the absorbance at 412 nm of the two LumPs in 6 M guanidine hydrochloride (Gdn·HCl), pH 7.0. Finally, the content of Lum was determined from the extinction difference of Lum in 6 M Gdn·HCl, pH 12.5, and Lum in 6 M Gdn·HCl, pH 7.0, at 412 nm. Protein determination was performed by the method of Goa (1953), as previously described (O'Kane et al., 1985).

Amino Acid Composition. Purified LumPs from *P. phosphoreum* and *P. leiognathi* were dialyzed for 72 h against 10 mM NH_4HCO_3 and were lyophilized in individual ampules (ca. 100 μg of protein each). Norleucine (12.5 nmol) was added as an internal standard. Constant boiling HCl (1 mL) was added to the ampules, which were sealed in vacuo and hydrolyzed at 110 °C for 12, 24, 48, 72, and 96 h. Amino acid analyses were performed in triplicate on a Beckman Model 119CL amino acid analyzer. Cysteine was determined after performic acid oxidation and 24-h hydrolysis (Hirs, 1967). Asparagine and glutamine were estimated by the subtraction procedure of Solby & Johnson (1981) using 4-h modification of the LumPs with BTI at 60 °C and 24-h acid hydrolysis.

Determination of Amino-Terminal Residues. Several procedures were employed: labeling the proteins in phosphate buffer, pH 8.5, with excess DNS-Cl (Zanetta et al., 1970), labeling the proteins with DNS-Cl–cycloheptaamylose complex with or without 8 M urea (Kinoshita et al., 1974), and labeling the proteins with DNS-Cl adsorbed to Celite (Rinderknecht, 1962). The reacted proteins were separated from unreacted DNS-Cl by centrifugation, chromatography on a 1 × 45 cm column of Bio-Gel P-2, and dialysis. The reacted proteins were hydrolyzed for 4–18 h in 6 M HCl, 110 °C (Gros & Labouesse, 1969), and then evaporated to dryness. The residues were dissolved in acetone/glacial acetic acid (3:2 v/v) and spotted on micropolyamide TLC plates. The TLC plates were developed in the first and second dimensions with solvents I and II_M of Lee & Safille (1976). Recrystallized (6×) hen egg white lysozyme, which has an amino-terminal lysine residue (Canfield, 1963; Jolles et al., 1963), was run as a control.

Tryptophan Determination by Fluorescence. The Trp content of the LumPs was estimated by the empirical method of Pajot (1976) using proteins and peptide standards with known Trp contents: RNase with no Trp residues (Hirs et al., 1960), HSA and glucagon with a single Trp residue (Bromer et al., 1957; Bromer, 1971; Meloun et al., 1975), β-lactoglobulin and BSA with 2 Trp residues per monomer (Frank & Braunitzer, 1967; King & Spencer, 1970), and trypsinogen with 4 Trp residues (Mikes et al., 1966). The molar extinction coefficients used to determine the concentrations of these standards were the following (M^{-1} cm^{-1}): Trp, ϵ_{280} = 5800 (Edelhoch, 1967); NAWA, ϵ_{281} = 5690 (Edelhoch, 1967); glucagon, ϵ_{280} = 8010 (Kay & Marsh, 1959); HSA, ϵ_{280} = 38 300 (Schultz & Heremans, 1966); β-lactoglobulin monomer, ϵ_{278} = 17 960 (Baghurst et al., 1972); BSA, ϵ_{280} = 42 000 (King & Spencer, 1970); RNase, ϵ_{278} = 9800 (White, 1961); trypsinogen, ϵ_{280} = 37 000 (Smillie & Kay, 1961).

Spectrophotometric Determination of Trp Residues. Trp residues were determined by the difference in absorbance at 280 and 288 nm of the LumPs in Gdn·HCl, pH 7.0, following removal of Lum since this absorbs in the 280–290-nm region. The absorption spectra of the Lum-free dialyzed proteins in Gdn·HCl were determined, and the molarity of Trp in the protein preparations *M* was determined from the empirical relationship (Edelhoch, 1967):

$$M = (\epsilon_{288}/3103) - (\epsilon_{280}/10318) \quad (1)$$

Spectrophotometric Determination of Tyrosine. The Tyr contents of the LumPs were determined by the spectrophotometric procedure of Edelhoch (1967). Samples of both LumPs in 6 M Gdn·HCl (10^{-4} M Lum) were dialyzed in 6 M Gdn·HCl, pH 7.0, to remove Lum and the absorption spectra determined. The pH of the samples was then raised to pH 12.5 by the addition of a small amount of 10 N NaOH, and the absorption spectra were redetermined. The difference in absorbance at 295 nm was used to determine the number of Tyr residues; the molar difference extinction coefficient of NAYA at this wavelength, $\Delta\epsilon_{295}$ = 2520 M^{-1} cm^{-1} (Edelhoch, 1967), was used.

Inorganic Element Analysis. The LumPs (ca. 20 nmol) were dialyzed for 144 h at 4 °C against 10 mM Tris buffer, pH 7.2 (three changes, 2 L each), to remove the phosphate buffer. The dialysis tubing was prepared by boiling 3 times in $NaHCO_3$ and washing extensively with glass-distilled water and finally with 10 mM Tris buffer, prior to use. The protein samples plus the final dialysis solution blank were examined for the elements Al, Ba, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, and Zn, by plasma analysis using a Jarrel-Ash Model 750 Atom-Comp. The instrument

was calibrated with each set of samples by using two standard solutions containing known concentrations of these elements. The values obtained for the final dialysis buffer were subtracted from those obtained for the protein samples.

Carbohydrate Analysis. Protein-bound carbohydrate was assayed by the phenol/sulfuric acid method (Dubois et al., 1956) as previously done for *P. phosphoreum* LumP (Small et al., 1980), using mannose as the standard.

Sulfhydryl Analysis. The sulfhydryl contents of the LumPs were determined by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). The proteins were rendered free of 2-ME by chromatography on a microcolumn of DEAE-Sepharose in a Pasteur pipet, washing with 50 mM phosphate buffer containing 3 mM EDTA, and eluting with 0.3 M phosphate buffer, pH 7.2. The Lum and Trp contents of the proteins were determined, as described above. Samples (0.1 mL) were assayed for sulfhydryl content by absorbance at 412 nm in a final volume of 1 mL of 0.1 M phosphate buffer, pH 7.2, containing 5×10^{-4} M DTNB and 3 mM EDTA. In some experiments, the protein was diluted into Gdn·HCl, pH 7.2 (final concentration, 6 M Gdn·HCl). In other experiments, the apoproteins, free of 2-ME and Lum, were prepared and titrated with DTNB. The concentration of sulfhydryl groups was determined from the concentration of the 5-thio-2-nitrobenzoate (TNB^-) produced (ϵ_{412} = 14 150 M^{-1} cm^{-1}, 0.1 M phosphate buffer, pH 7.2; ϵ_{412} = 13 750 M^{-1} cm^{-1}, 6 M Gdn·HCl, pH 7.2; Riddles et al., 1983). The absorbance of Lum at 412 nm was subtracted from the absorbance obtained with DTNB. During the course of the titration, which took as long as 4 h in some cases, the reference and sample cuvettes were not moved to eliminate repositioning errors.

Modeling of Absorption Spectra of Lumazine Proteins in 6 M Gdn·HCl. The absorption spectra of the LumPs were modeled by using the basic procedure of Edelhoch (1967). NAWA and NAYA were respectively used as model compounds for Trp and Tyr residues in the proteins. The spectra of these compounds in 6 M Gdn·HCl, pH 7.0, were obtained at 10^{-4} M for NAWA and 10^{-3} M for NAYA. The concentrations of these two compounds were determined spectrophotometrically; for NAWA, ϵ_{281} = 5690 M^{-1} cm^{-1}, and for NAYA, $\epsilon_{275.5}$ = 1490 M^{-1} cm^{-1} (Edelhoch, 1967). Lum and GPA were included in the modeling experiments. The Lum concentration was determined at 412 nm in 6 M Gdn·HCl (ϵ_{412} = 10 460 M^{-1} cm^{-1}). The concentration of GPA was determined from the extinction coefficient at 258 nm in 6 M Gdn·HCl, which was determined on a thoroughly dried sample to be ϵ_{258} = 202 M^{-1} cm^{-1}.

Once the absorption spectra of the individual components were obtained, these were then adjusted to the same concentration (10^{-4} M) and were added together in various combinations. This is with the exception of Phe residues. Since the absorbance contribution of Phe is small, the number of GPA residues added was held constant for each *P. phosphoreum* LumP's and each *P. leiognathi* LumP's modeled spectrum: 4 residues of GPA per mole of *P. phosphoreum* LumP and 6 residues of GPA for *P. leiognathi* LumP, as determined by amino acid analysis for Phe residues.

Instrumentation and Data Processing. Absorption and fluorescence emission spectra were obtained on a Cary 14 recording spectrophotometer and Spex spectrofluorometer, respectively, interfaced with a Nova II minicomputer as previously described (Wampler, 1978; Mulkerrin & Wampler, 1982). The fluorescence emission spectra are corrected for self-absorbance and for the wavelength-dependent sensitivity of the emission monochromator and photomultiplier tube by referencing the instrumental response with quinine sulfate to the absolute emission spectrum of quinine sulfate (Velapoldi & Mielenz, 1980). The Cary 14 spectrophotometer wavelength position was calibrated by using benzene vapor. The wavelength drive of the emission monochromator of the fluorometer was calibrated by using the lines from a mercury lamp.

The absorbance and fluorescence wavelength maxima were determined from the first derivative of the corresponding spectra and were fitted by using a linear regression program. The overall accuracy of this procedure, including errors in the determination of the original spectrum, is ±0.2 nm standard deviation. The mean wavelength position of a spectrum, $\bar{\lambda}$, the variance about $\bar{\lambda}$, the third and fourth moments U_3 and U_4, and the skewness and kurtosis were determined by moment analysis (Wampler, 1978; Mulkerrin & Wampler, 1982) as previously described (O'Kane et al., 1985). A least-squares matching program was used to analyze differences between the two spectra. The parameters calculated are a scaling factor F plus a wavelength position displacement D, such that

$$\text{spectrum Y} = F(\text{spectrum X}) + D \qquad (2)$$

Perfect agreement between spectra is indicated by values of 1 and 0 for F and D, respectively.

Results

Carbohydrates. No sugars were detected in either *P. leiognathi* or *P. phosphoreum* LumP. This results in a maximum estimate of 0.1 mol of bound carbohydrate per mole of protein.

Inorganic Elements. The dialyzed LumPs were examined for 20 elements, as described under Materials and Methods. The LumPs do not contain any bound phosphorus. With the exception of Na and K, no metal cofactors were detected. A total of 6 mol per mole of protein of monovalent cations was found for *P. leiognathi* LumP despite dialyzing the 1-mL solution for 6 days against a total of 6 L of dilute Tris·HCl buffer. Lesser amounts of monovalent cation (4.2 mol per mole of protein) were obtained with *P. phosphoreum* LumP.

Amino-Terminal Residues. Following treatment with DNS-Cl under a variety of conditions, no N-terminal amino acid was liberated from either of the LumPs (0.2 mg of protein) although ε-DNS-Lys could be detected in the hydrolysates. Chicken egg white lysozyme yielded α,ϵ-$(DNS)_2$-Lys as expected (Canfield, 1963; Jolles et al., 1963).

Isoelectric Point Determinations. Each LumP produced two bands after isoelectric focusing, as observed for *P. phosphoreum* by Small et al. (1980). However, the band with the lower p*I* in each case had only about 10% of the staining intensity of the other and is presumed to arise from cyanate modification of an ε-amino group. The observed p*I*'s for *P. phosphoreum* LumP were 4.78 and 4.83 [cf. p*I* = 4.9 and 5.0, Small et al. (1980)]. *P. leiognathi* LumP is more acidic than *P. phosphoreum* LumP: p*I* = 4.38 and 4.45.

Amino Acid Analysis. The results of the amino acid analysis of the two LumPs are shown in Table I. No amino sugar residues were found. The recoveries of amino acids following analysis exceeded 90% with norleucine as the internal standard. No attempt was made to quantify Trp by amino acid hydrolysis since previous experience with thioglycolate and methanesulfonic acid hydrolysis always resulted in less than half a residue of Trp per mole of protein (Small et al., 1980). Several minor unidentified peaks were observed in the 12- and 24-h hydrolysis time points that disappeared upon further hydrolysis and evidently were small peptides. Amide residues could not be detected by using the BTI subtraction

Table I: Amino Acid Composition of *Photobacterium* Lumazine Proteins

	P. phosphoreum	*P. leiognathi*		*P. phosphoreum*	*P. leiognathi*
Asx[a]	30	30	Ile[d]	18	18
Thr[b]	14	13	Leu	14	20
Ser[b]	11	11	Tyr	3	2
Glx[a]	19	19	Phe	4	6
Pro	3	4	His	3	3
Gly	18	22	Lys	12	7
Ala	4	10	Trp[e]	1	1
$^1/_2$-Cys[c]	2	1	Arg	3	7
Val[d]	19	20			
Met	1	2	total	179	196

[a] As Asp and Glu; may contain a minimum of 1 residue each of Asn and Gln as determined by the procedure of Solby & Johnson (1981). [b] By extrapolation to zero time. [c] After performic acid oxidation as cysteic acid. [d] From 96-h hydrolysis. [e] By fluorescence.

procedure (Solby & Johnson, 1981).

The amino acid analysis reveals a large amount of acidic residues: 27% and 25% of the total amino acid residues, respectively, for *P. phosphoreum* and *P. leiognathi* LumPs while *P. phosphoreum* LumP contains one more basic residue than *P. leiognathi* LumP. The amino acid residues with hydrophilic side chains, Asp, Thr, Ser, Glu, His, Lys, and Arg, account for 46% and 51% of the total residues, respectively, for *P. leiognathi* and *P. phosphoreum* LumPs. The average hydrophobicities of the two LumPs are 919.64 and 917.6 cal/residue for *P. leiognathi* and *P. phosphoreum* LumPs, respectively. This is less than the average value (976.55 ± 339.10 cal/residue) calculated for 620 proteins (Bigelow & Channon, 1979), indicating that both LumPs are relatively hydrophilic. The minimum molecular weights of the LumPs calculated from the amino acid compositions plus 1 mol of the ligand Lum (see below) are the following: *P. leiognathi*, 21 300; *P. phosphoreum*, 19 750.

Tryptophan Determination by Fluorescence. The intensity of intrinsic Trp fluorescence in 6 M Gdn·HCl of standard proteins with defined Trp contents describes a straight line when plotted against the number of Trp residues: intensity = 1.02(Trp) + 0.02 (r = 0.99) (data not shown). When expressed on the basis of either the molarity of the protein (determined from the biuret assay and minimum molecular weight) or the molarity of the Lum ligand (determined below), *P. leiognathi* LumP contains respectively 1.02 and 0.98 Trp residue per mole, while *P. phosphoreum* LumP contains 1.04 and 0.94 Trp residue per mole. The latter is in agreement with the published values of 1.0–1.1 (Small et al., 1980).

Spectrophotometric Determination of Trp Residues. The molarity of Trp residues was determined from the absorbance of the apoproteins at 280 and 288 nm in 6 M Gdn·HCl, using extinction coefficients originally based upon the model compounds NAWA and NAYA to represent Trp and Tyr residues, respectively. This yielded values of 1.16 and 1.10 Trp residues per Lum (Table II) and is consistent with quantification of Trp by fluorescence.

Spectrophotometric Determination of Tyr Residues. The number of Tyr residues in the LumPs was determined from the difference pH-induced hyperchromicity of NAYA in 6 M Gdn·HCl. The absorption spectra of the apoproteins are shown in Figure 1 at pH 7.0 and pH 12.5. The difference spectra of the alkaline minus the neutral spectrum for both proteins show a prominent peak at 295.5 nm, which is indicative of the hyperchromicity of ionized Tyr residues. The absorbance differences indicate 2.99 Tyr residues per Lum for *P. phosphoreum* LumP and 2.16 Tyr residues per Lum for *P. leiognathi* LumP (Table II).

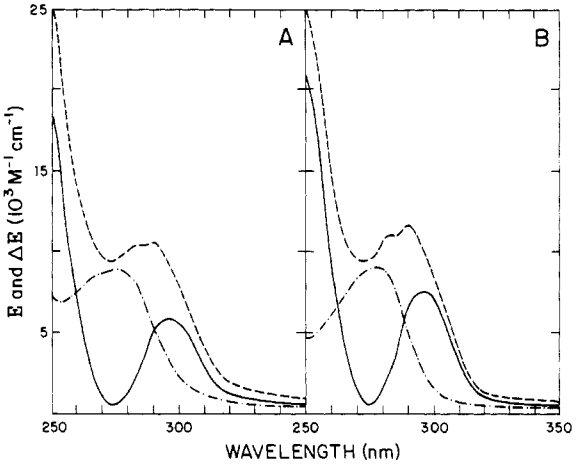

FIGURE 1: Absorption spectra of apoproteins in 6 M guanidine hydrochloride at pH 7.0 and pH 12.5. *P. leiognathi* (panel A) and *P. phosphoreum* (panel B) apolumazine proteins were prepared free of 2-ME. The absorption spectra of the apoproteins were obtained first in 6 M Gdn·HCl, pH 7.0 (–·–), and then at pH 12.5 following the addition of NaOH (––). The difference spectra (pH 12.5 minus pH 7.0) (—) each demonstrate a maximum at 295 nm.

Determination of the Lumazine Derivative Content. The concentration of Lum was determined by three methods on the same *P. leiognathi* and the same *P. phosphoreum* LumP samples. The error in estimating Lum is small regardless of the method employed, with a maximum of 4% difference between techniques (Table III). The most precise estimation of Lum was obtained by the pH-difference absorbance method. The spectrum of Lum in 6 M Gdn·HCl changes markedly upon addition of NaOH with the loss of the visible absorbance maximum and the appearance of a new absorbance maximum at 313 nm (Figure 2A). Similar results are obtained with both LumPs with the exception that the new absorbance maximum is at 301 nm due to the pH-induced hyperchromicity of Tyr residues in the protein (Figure 1). The large absorbance difference at 412 nm however can be used to determine Lum since Rayleigh scattering from the protein is largely canceled in the 350–500-nm region (Figure 2B). The calculated molar difference extinction coefficients for Lum, *P. leiognathi* LumP, and *P. phosphoreum* LumP, respectively, are $\Delta\epsilon_{412}$ = −10 260, −10 120, and −10 310 M^{-1} cm^{-1} from Figure 2. Since the values for $\Delta\epsilon_{412}$ should be identical, the values for the two LumPs represent 1 ± 1% differences from that obtained for Lum.

The stoichiometry of Lum binding to protein was investigated in two ways. The stoichiometry of Lum to protein can be determined from the weight of protein in the sample, the Lum concentration, and the molecular weight of LumP. When the protein content is measured by the biuret procedure, using the minimum molecular weight determined above for both

Table II: Spectrophotometric Determination of Tryptophan and Tyrosine in Lumazine Proteins[a]

				ratio determined		ratio expected	
LumP	[Lum][b]	[Trp]	[Tyr]	Trp/Lum	Tyr/Lum	Trp/Lum	Tyr/Lum
P. phosphoreum	8.15	8.97	24.41	1.10	2.99	1.0	3.0
P. leiognathi	4.52	5.26	9.76	1.16	2.16	1.0	2.0

[a] All concentration units are 10^{-5} M. [b] Concentration of Lum in the protein preparations prior to dialysis.

Table III: Determination of Lumazine Content of Lumazine Proteins

method	[Lum][a]	
	P. leiognathi	*P. phosphoreum*
vis absorbance max[b]	47.9	65.6
A_{412nm} in Gdn·HCl[c]	45.2	63.2
Gdn·HCl difference spectrum[d]	46.9	64.7
av stoichiometry[e]	0.97	0.98

[a] All micromolar concentrations. The lumazine protein is 47.9 (*P. leiognathi*) and 65.6 μM (*P. phosphoreum*). [b] $\epsilon_{418} = 10300\ M^{-1}\ cm^{-1}$, *P. phosphoreum*; $\epsilon_{420} = 10100\ M^{-1}\ cm^{-1}$, *P. leiognathi* (O'Kane et al., 1985). [c] $\epsilon_{412} = 10460\ M^{-1}\ cm^{-1}$, Lum, determined this study. [d] $\Delta\epsilon_{412} = -10260\ M^{-1}\ cm^{-1}$, Lum, determined this study. [e] Standard deviations 0.03 and 0.01, respectively.

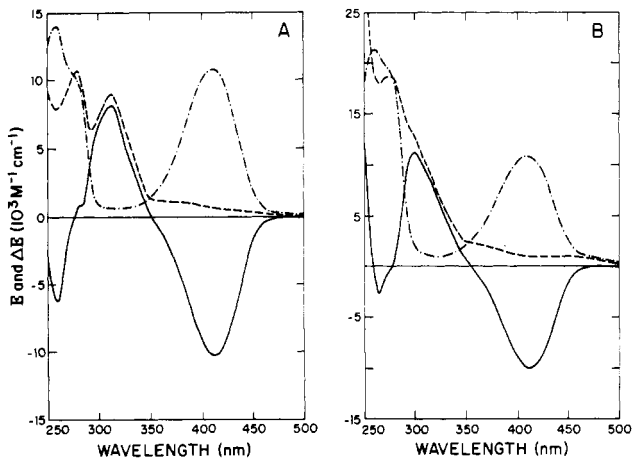

FIGURE 2: Absorption spectra of 6,7-dimethyl-8-ribityllumazine and lumazine proteins in 6 M guanidine hydrochloride at pH 7.0 and pH 12.5. The absorption spectra of Lum (panel A) and *P. leiognathi* LumP (panel B) were first obtained in 6 M Gdn·HCl, pH 7.0 (–·–), and then at pH 12.5 following the addition of NaOH (––). Both difference spectra (pH 12.5 minus pH 7.0) (—) show prominent minima at 262 and 412 nm; panel A shows a maximum at 313 nm (Lum), while panel B shows a maximum at 301 nm (LumP). The spectrum of *P. phosphoreum* LumP (not shown) is virtually identical with that of *P. leiognathi* LumP in the 320–500-nm region and is not shown for clarity.

LumPs, a 1:1 stoichiometry of Lum to protein is found (Table III). Put in another way, the amount of protein, as assayed in the biuret reaction, can be predicted within 2–6% error on the basis of the Lum concentration and the minimum molecular weight of the LumPs.

The stoichiometry of Lum binding was also examined by back-titration of the apoproteins with authentic Lum. Samples of both apoproteins (no 2-ME) were titrated with DTNB to determine the sulfhydryl concentration of the apoprotein preparations (see below). The apoproteins were diluted to the same protein concentration and were immediately titrated with authentic Lum at 2 °C. Since tight binding of the ligand is observed with the holoproteins under these conditions, $K_d \sim 10^{-8}$ M for both (Lee et al., 1985), the position of the absorbance and fluorescence maxima should not change until the apoproteins have been fully titrated. This is the case as seen in Figure 3. Both *P. leiognathi* and *P. phosphoreum* apoproteins were present at 31 μM protein concentration on the basis of sulfhydryl titration (dashed vertical line). The absorbance and fluorescence emission maxima for the *P. leiognathi* preparation do not change (419.5 and 474.0 nm, respectively) until the added concentration of Lum exceeds approximately 30.5 μM (98% recovery). The intrinsic apoprotein fluorescence from Trp was noted to be quenched upon the addition of Lum. Quenching was completed at 29.7 μM added Lum (95% recovery). No change in the intensity of

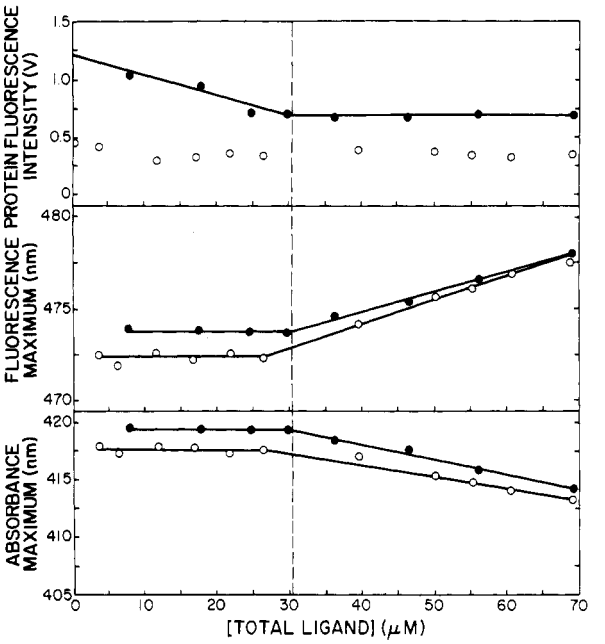

FIGURE 3: Titration of apolumazine proteins with 6,7-dimethyl-8-ribityllumazine. The apoproteins (●, *P. leiognathi*; ○, *P. phosphoreum*) were prepared free of 2-ME and were back-titrated with Lum. The visible absorbance maximum, the wavelength of the fluorescence emission maximum, and the intensity of Trp fluorescence were determined on corrected spectra obtained at 2 °C in 300 mM phosphate buffer, pH 7.0, with a 1-cm absorbance path and a 3-mm emission path. The concentration of both apoproteins was initially 31 μM.

Trp fluorescence was observed at higher concentrations of added Lum. These results directly indicate a 1:1 stoichiometry of Lum binding to apoprotein.

Similar results are obtained with *P. phosphoreum* apoprotein. The absorbance and fluorescence maxima do not change during the titration until the added Lum concentration exceeds 26.7 μM (85.5% recovery). Above this concentration, these maxima change linearly. Unlike *P. leiognathi* apoprotein, however, no relationship could be drawn between the intensity of Trp fluorescence and Lum concentration. The recovery of reconstituted holoprotein results in a binding stoichiometry of 1 Lum per 1.17 protein. This low recovery is possibly due to the greater reactivity of the sulfhydryl groups in *P. phosphoreum* LumP and their oxidation during the course of titration in the absence of 2-ME (see below).

Spectral Modeling of LumPs. The UV absorbance and visible absorbance of the two LumPs in 6 M Gdn·HCl have been spectrally modeled by using NAWA (1–2 residues), NAYA (0–6 residues), Lum (1–2 residues), and GPA (4 residues for *P. phosphoreum* and 6 residues for *P. leiognathi* LumP). Cystine is not included since DTNB titration of both LumPs indicates that no disulfide bonds are present. The results of the spectral modeling are shown in Figure 4.

When graphed against the number of NAYA residues employed, the mean absorbance wavelength, $\bar{\lambda}$, for each class of spectra (i.e., the model spectra obtained with constant numbers of residues of NAWA, Lum, and GPA and variable numbers of NAYA residues) describes slightly curved lines. The $\bar{\lambda}$ values for *P. phosphoreum* and *P. leiognathi* LumP are respectively 331.3 and 333.0 nm. Those spectra with 2 NAWA residues, 1 Lum residue, and varying NAYA reisdues all have $\bar{\lambda}$ values less than those obtained for the authentic LumPs. This indicates that it is impossible for either LumP to contain 2 Trp residues per Lum residue. Similarly, it is possible to eliminate those spectra created by using 2 residues of Lum and 1 or 2 residues of NAWA since those spectra converge

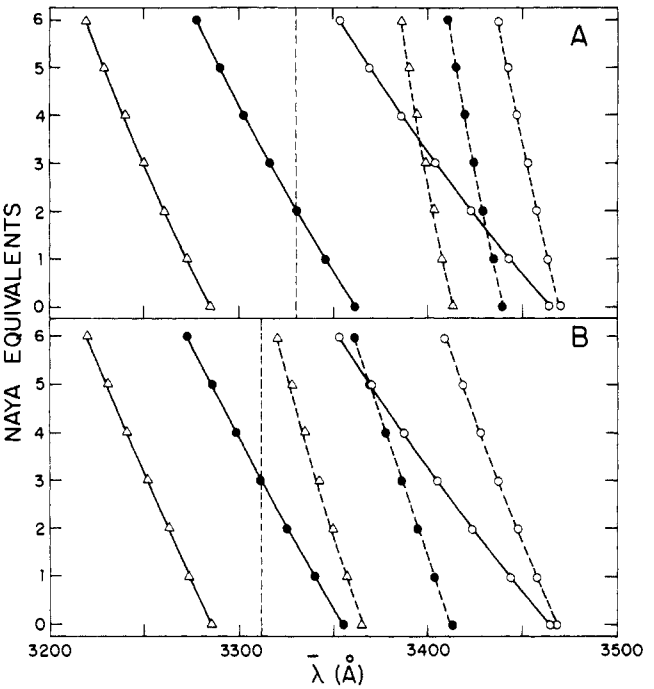

FIGURE 4: Spectral modeling of lumazine proteins. The mean absorbance wavelengths, $\bar{\lambda}$, of spectra representing proteins containing 1 Lum residue (—) or 2 Lum residues (---) and 0 Trp residue (○), 1 Trp residue (●), or 2 Trp residues (△) with increasing numbers of Tyr residues were determined from the absorption spectra of Lum, NAWA, and NAYA in 6 M Gdn·HCl. Those spectra modeled for *P. leiognathi* LumP (panel A) each contain GPA representing 6 residues of Phe while the *P. phosphoreum* LumP spectra (panel B) contain GPA representing 4 residues of Phe. The actual values of $\bar{\lambda}$ for authentic LumPs in 6 M Gdn·HCl are indicated by the dashed vertical lines.

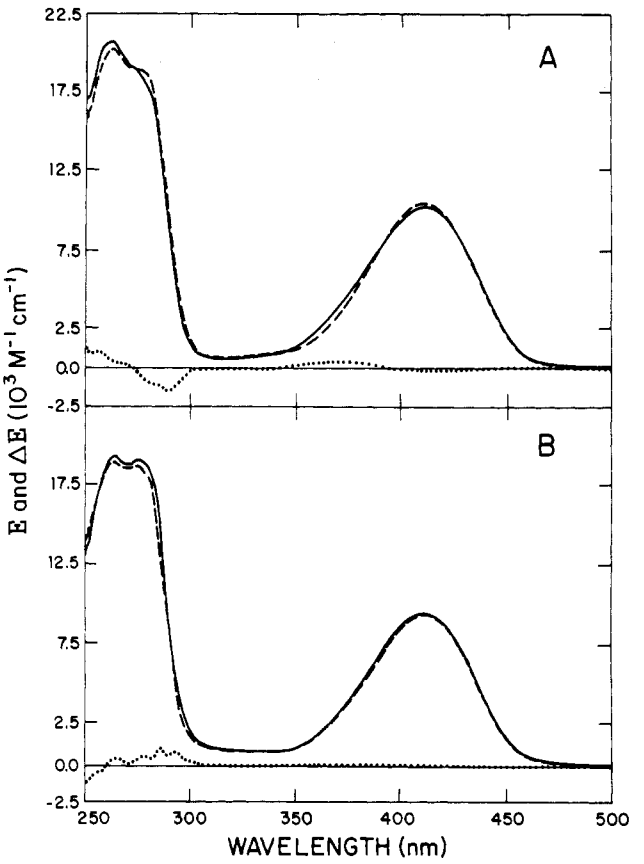

FIGURE 5: Absorption spectra of lumazine proteins in 6 M Gdn·HCl and computed absorption spectra obtained with model compounds. The absorption spectra of *P. leiognathi* LumP (panel A) and *P. phosphoreum* LumP (panel B) were obtained in 6 M Gdn·HCl, pH 7.0, at approximately 10^{-4} M each (—). The computed absorption spectra (--) were obtained with 1 equiv each of Lum and NAWA, 2 equiv of NAYA, and 6 equiv of GPA for *P. leiognathi* (panel A) and 1 equiv each of Lum and NAWA, 3 equiv of NAYA, and 4 equiv of GPA for *P. phosphoreum* (panel B). The difference spectra (LumP minus computed) are indicated (···).

on the $\bar{\lambda}$ values of the authentic LumPs only at values for NAYA far greater than what can be expected on the basis of the spectrophotometric determination of, and the amino acid analysis for, Tyr residues. This indicates that both LumPs have the basic stoichiometry of 1:1 Lum:Trp. The lines described by those spectra containing 1 Lum and 1 NAWA residue both intersect with the lines for the mean wavelength position of the authentic LumPs at 2.05 residues of NAYA for *P. leiognathi* and 2.97 residues of NAYA for *P. phosphoreum*. These values agree with those obtained from amino acid analysis and spectrophotometric titrations for Tyr. The absorption spectra of the authentic LumPs in 6 M Gdn·HCl, pH 7.0, and the modeled absorption spectra of the LumPs are shown in Figure 5. The major discrepancy between the authentic spectra and the modeled spectra lies in the 250–260-nm region and between 290 and 295 nm.

The parameters of the authentic LumP spectra and their modeled absorption spectra are summarized in Table IV. Excellent matching is evident for all parameters. When a least-squares matching is performed on the authentic LumPs' spectra and the respective modeled absorption spectra, scaling factors (F and D, Table IV) very close to 1 and 0 are obtained, indicating near-perfect matching. All other modeled spectra yield poorer fitting for all of the parameters in Table IV (not shown). It is concluded that the UV and visible absorption spectra of the LumPs can be completely accounted for with 1 Lum, 1 Trp residue, and 2 Tyr residues (*P. leiognathi*) and with 3 Tyr residues (*P. phosphoreum*).

Determination of Sulfhydryl Groups. Amino acid analysis indicates the presence of 2 Cys residues in *P. phosphoreum* LumP (Table I) but only 1 Cys residue in the *P. leiognathi* protein. This was confirmed by sulfhydryl titration with

Table IV: Spectral Parameters of Lumazine Proteins and Their Modeled Spectra

	P. leiognathi		*P. phosphoreum*	
parameter[a]	LumP	model	LumP	model
λ_{max}, UV (Å)	2627.1	2635.2	2648.4, 2762.8	2644.9, 2749.8
λ_{max}, vis (Å)	4117.4	4118.7	4117.1	4118.5
$\bar{\lambda}$ (Å)	3330.3	3330.6	3312.7	3312.2
integral	1.459	1.458	1.540	1.514
U_2 (σ^2)	4.881	4.873	4.795	4.792
U_3	9.599	10.127	11.070	11.742
U_4	3.240	3.242	3.209	3.234
skewness	0.282	0.298	0.333	0.354
kurtosis[b]	−1.640	−1.656	−1.604	−1.592
F	0.999		1.009	
D	9.29×10^{-4}		5.01×10^{-3}	

[a] Units for integral and moments U_2, U_3, and U_4 are respectively 10^3, 10^5, 10^7, and 10^{11} and are dimensionless. Their magnitude depends upon the absolute dimensions of the absorbance and wavelength axes. For definitions see Lee et al. (1985). [b] Relative to the normal distribution.

DTNB (Figure 6). When the proteins were adjusted to the same Lum concentrations (5.3 μM), the 2:1 ratio of sulfhydryl groups in *P. phosphoreum* LumP to those in *P. leiognathi* LumP is observed as expected, confirming the results of the amino acid analyses and also eliminating the possibility of disulfide bridges. Differences are noted, however, in the rates of reactivity of sulfhydryl groups between *P. phosphoreum* and *P. leiognathi* LumPs. There is very little difference in the rate

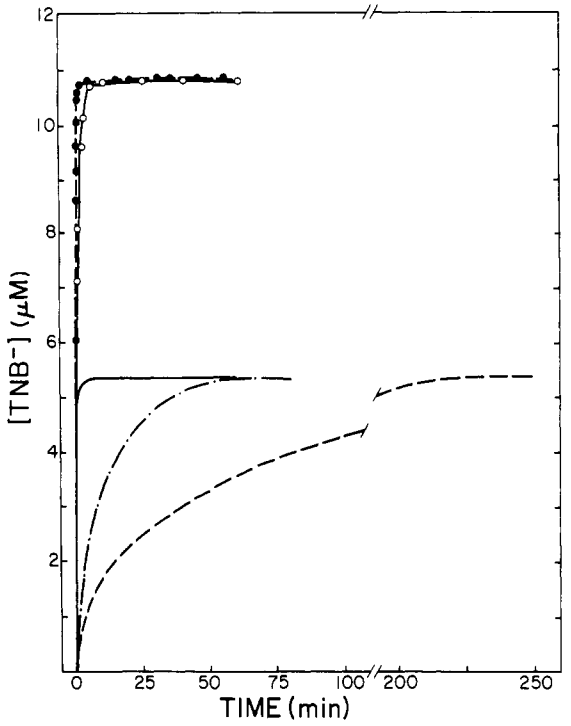

FIGURE 6: Titration of lumazine proteins with DTNB. Equal concentrations (5.3 μM) of native *P. leiognathi* and native *P. phosphoreum* LumP or apoprotein were titrated with excess DTNB at pH 7.2 in phosphate buffer or 6 M Gdn·HCl, and the increase in TNB^- concentration was monitored over time. *P. phosphoreum* LumP holoprotein (○) and holoprotein in Gdn·HCl (●); *P. leiognathi* LumP holoprotein (---) and holoprotein in Gdn·HCl (—); apoprotein (-·-).

at which the sulfhydryl groups are modified in *P. phosphoreum* LumP when the native holoprotein is compared with the holoprotein denatured in 6 M Gdn·HCl. A major difference in the rates of DTNB modification is found for the *P. leiognathi* LumP. The denatured holoprotein reacts rapidly (completion takes about 5 min), whereas the complete modification of the native holoprotein requires approximately 3.5 h at 23 °C. This suggests a "buried" sulfhydryl in *P. leiognathi* LumP. The *P. leiognathi* apoprotein is modified more rapidly than the holoprotein, but much more slowly than the denatured holoprotein.

After complete modification of the holoproteins with DTNB, Lum is quantitatively dissociated from the proteins as was originally reported for *P. phosphoreum* LumP (Small et al., 1980). The fluorescence emission maximum of both ultrafiltrates (YM-2 membrane, M_w cutoff 1000) is 489 nm, the same as the fluorescence emission maximum of free Lum. Complete removal of Lum from both DTNB-treated proteins can be achieved in this way. If excess 2-ME is added to the fully DTNB-titrated proteins prior to ultrafiltration, free Lum is not recovered in the ultrafiltrate but is found bound to the protein (~85% recoveries) with the fluorescence emission maximum at 475 nm. The DTNB-treated apoproteins are incapable of rebinding Lum. When a sample of *P. leiognathi* apoprotein (5.25 nmol) was titrated with excess DTNB and then titrated with Lum, no Lum binding could be detected as assayed from the position of the fluorescence emission maximum. A control sample of apoprotein was subsequently titrated with Lum and was found to quantitatively bind the ligand.

DISCUSSION

The two *Photobacterium* LumPs are quite similar on the basis of spectral properties (O'Kane et al., 1985) and on the basis of chemical characterization. They contain no detectable protein-bound carbohydrates and no metal cofactors and have blocked amino-terminal residues. The minimum molecular weights from the amino acid analyses plus 1 mol of Lum per mole of LumP are 21 300 and 19 750 for *P. leiognathi* and *P. phosphoreum*, respectively. These values are within 2% and 7%, respectively, of values for molecular weights obtained by direct physical measurements (O'Kane & Lee, 1985). Both LumPs are highly acidic and hydrophilic proteins and have the same average hydrophobicities.

Table V: Relatedness of Lumazine Proteins from Amino Acid Compositions[a]

index	calcd value	critical value[b]
DI	7.48	10.1
D	5.97×10^{-2}	6.48×10^{-2}
$S\Delta Q$	35.66	42
$S\Delta n$[c]	68.5	82.3

[a] Hypothesis: the two lumazine proteins are closely related. [b] Reject hypothesis if calculated values > critical values. Critical values are determined for *P. leiognathi* LumP with 196 amino acid residues. [c] A value of 1 has been subtracted from $S\Delta n$ to correct for the size difference between *P. leiognathi* and *P. phosphoreum* LumPs.

The overall similarity of the amino acid composition data (Table I) suggests that these two proteins are closely related. Although it is best to compare proteins of known primary sequences, some measure of the degree of relatedness can be obtained from the amino acid compositions. Various tests of the relatedness of proteins employing amino acid compositions have been employed: the difference index DI (Metzger et al., 1968), composition divergence D (Harris et al., 1969), $S\Delta Q$ (Marchalonis & Weltman, 1971), and $S\Delta n$ (Cornish-Bowden, 1983). Each test of relatedness involves summations of the differences between the two amino acid compositions or of the squares of the differences [see Cornish-Bowden (1983) for a comparison of the various tests].

Both LumPs are strongly related on the basis of amino acid compositions. The calculated values for the various tests, as well as the critical values for rejection of the hypothesis of strong relatedness, are listed in Table V. The value for $S\Delta n$ (Cornish-Bowden, 1983) is informative since it predicts the number of sequence differences between two proteins on the basis of amino acid compositions such that

$$S\Delta n = M(1 \pm 0.38) \quad (3)$$

where M is the actual number of insertions, deletions, and amino acid substitutions. Consequently, it can be predicted that the number of sequence differences in the primary structures of the two LumPs should be $50 < S\Delta n < 110$.

Both LumPs also have blocked N-terminal residues, indicated by the failure to obtain N^{α}-DNS derivatives by using a variety of different procedures, while lysozyme readily yielded the correct N-terminal residue. Preliminary primary structure work indicates that the blocked amino acids are neither fMet nor pyroglutamyl residues (O'Kane, unpublished results). The Met residues are known to be internally located in the proteins from CNBr cleavage experiments. The α-amino groups of the two LumPs could be formylated, acetylated, or methylated.

The binding stoichiometry of Lum to either protein is accurately determined to be 1:1. The results of back-titration of the apoproteins agree well with the determination of the Lum content of the purified LumP preparations. The highest precision of Lum quantification is obtained from the difference absorbance method at pH 12.5 and pH 7.0 in 6 M Gdn·HCl since the contribution of Rayleigh scattering to the apparent

absorbance in the spectral regions to either side of the visible absorbance maximum (412 nm) is fortuitously canceled by this technique. The near-UV maximum for Lum at 313 nm (pH 12.5) cannot be employed to quantify Lum in the presence of protein due to the pH-induced hyperchromicity of Tyr residues. The Tyr absorbance combines with the Lum absorbance to produce a new spectral maximum at 301 nm (Figure 1). Although the difference absorbance method is the most precise method for determining Lum content, only 2–4% maximum error can be introduced when the published extinction coefficients for the native LumPs are used to estimate the Lum content (O'Kane et al., 1985). Consequently, if the positions of the absorbance maxima for the LumPs are determined and are at the correct wavelengths, the Lum content can be readily estimated and it can be determined that the LumPs are fully saturated with Lum. Visible absorbance maxima less than 419.5 and 417.5 nm for *P. leiognathi* LumP and *P. phosphoreum* LumP, respectively, indicate less than complete saturation of the proteins with Lum due to partial ligand dissociation (Small et al., 1980). A rapid check on the degree of Lum saturation can be performed by measuring the 280-nm to 419.5- or 417.5-nm ratios for *P. leiognathi* and *P. phosphoreum* LumPs, respectively. These ratios, from experimental measurements and spectral modeling, should both be 2.18 and agree with the limiting ratios obtained during purification of the LumPs (O'Kane et al., 1985).

The spectral modeling study leaves little doubt that the absorbance spectra of both LumPs in 6 M Gdn·HCl can be attributed to a single Lum and Trp residue and the contributions to absorbance by the Tyr plus Phe residues (Figures 4 and 5). Although this analysis does not eliminate the possibility of oligomer structures for LumPs in nondenaturing solution, it will be shown in a following paper (O'Kane & Lee, 1985), by direct physical measurement of molecular weights, that both LumPs are monomeric proteins. Since they can be easily purified in large quantities (O'Kane et al., 1985), and since they have few His residues and a single Trp residue (Tables I and II), these proteins may become attractive models for use in NMR studies and for studying rapid (i.e., picosecond) oscillations in proteins (Visser et al., 1985).

The rates of reactions of the sulfhydryl residues in native *P. phosphoreum* LumP are nearly equal to those observed with the holoprotein denatured in 6 M Gdn·HCl, which suggests that both –SH groups are relatively exposed in the native holoprotein. Very different results are obtained with *P. leiognathi* LumP. The native holoprotein reacts at a very slow rate compared to the denatured holoprotein and is consistent with the Cys residue being buried. Removal of the Lum ligand increases the rate of reaction of this Cys residue with DTNB, however not to the same extent as does denaturation with 6 M Gdn·HCl. These results are consistent with three models for the slow reaction of the single Cys in *P. leiognathi* LumP. First, DTNB reacts with the conjugate base $R–S^-$, not with the un-ionized thiol (Riddles et al., 1983). Consequently, if the pK_a for the single Cys residue in *P. leiognathi* LumP is substantially higher than that for either sulfhydryl in *P. phosphoreum* LumP, and if the localized ionic environment is acidic, then the rate of reaction with DTNB would be expected to be slow. Removal of the Lum residue would have to result in an increase in the pH of the localized environment of the –SH group or a decrease in the pK_a, or both, to account for the increased rate of reactivity of the –SH with DTNB. This proposal cannot be excluded since the pK_a of the –SH groups is not known and the pH of the environment surrounding the –SH group cannot presently be determined. Second, since DTNB is anionic at pH 7.0, electrostatic repulsion could explain the low reactivity of this sulfhydryl group. Both LumPs are anionic at pH 7.0, and the amino acid composition data (Table I) indicate that at least 25% of the total amino acid residues are Asp + Glu. Simple electrostatic repulsion of DTNB by the protein is not favored as an explanation for the slow reactivity of the *P. leiognathi* LumP since both proteins are highly acidic and both Cys residues in *P. phosphoreum* LumP react rapidly. Electrostatic repulsion, however, may play a role in determining the rate of reaction, but not a crucial role. Lastly, the Lum residue may confer protection to the Cys residue. If the thiol group of *P. leiognathi* LumP was present in the Lum binding site and was directly shielded from DTNB by Lum, then its rate of reaction would be low in the holoprotein and higher in the apoprotein. It is not impossible to have the Cys residue located at a distance from the Lum binding site and buried in the holoprotein but to have the Cys residue become "exposed" in the apoprotein through a conformation change. Several experiments are consistent with the presence of the Lum residue affecting the accessibility of the single Cys residue in *P. leiognathi* LumP. First, neither LumP will bind Lum if the Cys residues are completely modified by DTNB treatment, while removal of the TNB^- groups with excess 2-ME permits rebinding of Lum to the apoproteins. Second, in the case of *P. leiognathi* LumP, the intrinsic Trp fluorescence of the apoprotein is quenched by addition of Lum and a relationship could be seen between the degree of Trp quenching and Lum addition (Figure 2). No such relationship could be detected with *P. phosphoreum* apoprotein. This could be interpreted, although not definitively, as evidence for a conformation change in *P. leiognathi* LumP upon binding Lum. Experiments to distinguish between these various explanations for the relationship between Lum binding and Cys modification have been initiated.

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Registry No. NAWA, 2382-79-8; NAYA, 1948-71-6; Lum, 5118-16-1; Cys, 52-90-4; Trp, 73-22-3; Tyr, 60-18-4; Phe, 63-91-2; glycylphenylalaninamide, 1510-04-9.

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