Physical Characterization of Lumazine Proteins from *Photobacterium*†

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ABSTRACT: The physicochemical properties of *Photobacterium* lumazine proteins have been investigated. The molecular weights obtained by several physical techniques are in good agreement, and the averages are 2% and 8% higher than the minimum molecular weights from amino acid and ligand content. The average molecular weights, sedimentation coefficients, and molecular radii are respectively the following: *Photobacterium leiognathi* lumazine protein, 21 200 ± 300, 2.18 S, and 22.9 Å; *Photobacterium phosphoreum* lumazine protein, 21 300 ± 500, 2.16 S, and 23.0 Å. The hydrations of the lumazine proteins, estimated in several ways, indicate less hydration for *P. leiognathi* than for *P. phosphoreum*. The frictional ratios corrected for hydration give axial ratios less than 1.3 for both lumazine proteins. These values agree with those obtained by a combination of rotational and translational frictional parameters and elimination of the common hydrated volume terms. There is insufficient area on the exterior surface to accommodate hydration when the lumazine proteins are considered as smooth-surfaced ellipsoids. The required surface area can be accommodated however by surface roughness with a minimum of 30% internal water.

Lumazine proteins (LumPs)[1] have been identified in five strains of bioluminescent bacteria: two strains of *Photobacterium phosphoreum* (Gast & Lee, 1978; Small et al., 1980; Lee, 1982) and three strains of *Photobacterium leiognathi* (Lee, 1982; O'Kane et al., 1985). The LumPs from *P. phosphoreum* strain A13 and *P. leiognathi* strain A2D have been purified in quantity (O'Kane et al., 1985). The two proteins are similar in many respects, such as amino acid composition, but have different isoelectric points (O'Kane & Lee, 1985). Both contain a single residue of tryptophan and 1 mol of the noncovalently bound chromophore 6,7-dimethyl-8-ribityllumazine (Lum). They have only slightly different molecular weights on the basis of amino acid and Lum content (M_r: 21 200, *P. leiognathi*; 19 650, *P. phosphoreum*) (O'Kane & Lee, 1985). In both types of protein the ligand has the same coherent anti-Stokes Raman spectrum (Vervoort et al., 1983) and fluorescence emission spectrum, but the absorption and fluorescence excitation spectra are slightly different (O'Kane et al., 1985; Lee et al., 1985). The fluorescence anisotropy relaxation time (Lee et al., 1985) is also different (17.5 ns, *P. leiognathi*; 19.5 ns, *P. phosphoreum*; both at 2 °C). Visser & Lee (1980, 1982) calculated that this relaxation time corresponds to the rotation of a macromolecule of hydrated M_r around 30 000. However, the directly measured molecular weights for *P. phosphoreum* LumP (Small et al., 1980) ranged from 16 900 (sedimentation equilibrium) to 22 000 (SDS-PAGE). The frictional ratio obtained, 1.3, would lead to a calculated axial ratio of approximately 5 to 1.

Since large quantities of two types of LumP are now available, a systematic investigation of the physical properties of *P. phosphoreum* LumP and a comparison with those of *P. leiognathi* LumP are warranted. The purposes of this study are to provide physical parameters that are reliable and consistent with the observed behavior of LumP in solution and to place limits on the shapes of the two LumPs that are consistent with the physical parameters determined in this study and the rotational behavior of the proteins inferred from anisotropy decay (Visser & Lee, 1980, 1982; Lee et al., 1985).

Materials and Methods

Reagents used to prepare polyacrylamide gels were obtained from Bio-Rad Laboratories, Richmond, CA. Sodium dodecyl sulfate (SDS) was the product of BDH (Gallard-Schlesinger, Carle Place, NY) and was recrystallized from ethanol. Standard proteins[1] used were of the best commercial grades from Sigma Chemical Co. (St. Louis, MO) and Worthington Biochemicals (Freehold, NJ). Guanidine hydrochloride (Gdn·HCl), "Sequenal" grade, was obtained from Pierce Chemical Co., Rockford, IL. *P. phosphoreum* strain A13 and *P. leiognathi* A2D LumPs were purified as previously described (O'Kane et al., 1985).

Ultracentrifugation. Analytical ultracentrifugation was performed with a Beckman Spinco Model E ultracentrifuge. Low-speed sedimentation equilibrium studies were performed with an AN-G rotor operated at approximately 11 200 rpm and with absorption optics at 420 and/or 280 nm at 3-5 °C. The precise conditions for each centrifugation run were employed for calculation of the molecular weight parameters. Samples at several different protein concentrations (9-62 μM) were employed (11 samples, *P. leiognathi* LumP; eight samples, *P. phosphoreum* LumP). The weight-average molecular weight (M_w) was determined from the data by using linear regression analysis, least-squares fitting, and first-derivative approximations, on either a Nova II minicomputer with programs supplied by Dr. J. E. Wampler, Department of Biochemistry, or a Tektronix computer with programs supplied by T. Richardson, Institute of Ecology, both at the University of Georgia.

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[1] Abbreviations: LumP(s), lumazine protein(s); Lum, 6,7-dimethyl-8-ribityllumazine; cyt *c*, cytochrome *c*; Hb, hemoglobin; Mb, myoglobin; SBTI, soybean trypsin inhibitor (Kunitz); CA, carbonic anhydrase B; OA, ovalbumin; BSA, bovine serum albumin; HSA, human serum albumin; G-3-P DH, glyceraldehyde-3-phosphate dehydrogenase; ADH_L, horse liver alcohol dehydrogenase; ADH_Y, yeast alcohol dehydrogenase; PEP, pepsin; Glu DH, glutamate dehydrogenase; RNase, pancreatic ribonuclease; Gdn·HCl, guanidine hydrochloride; 2-ME, 2-mercaptoethanol; HP-SEC, high-performance size-exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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Sedimentation velocity studies were performed at 3–5 °C with an AN-H rotor and absorption optics. Samples of LumP were in the range 9–40 μM and were monitored by absorbance at 420 or 280 nm. The absorption scans were obtained at 4- or 8-min intervals at 60 000 rpm. The obtained sedimentation coefficients (s) were extrapolated to zero protein concentration and were corrected to 20 °C and to the viscosity of water.

All samples for ultracentrifugation were in 50 mM phosphate buffer, pH 7.0, containing 3 mM EDTA, 10 mM 2-mercaptoethanol (2-ME), and 150 mM NaCl and were always blanked vs. this buffer. The buffer densities were determined by weighing the buffer in a calibrated volumetric flask at the temperatures of the centrifugation and at 20 °C. The weights were corrected for the weight of air contained in the volumetric flask and are accurate to approximately 10 μg in 50 g. Both centrifuge rotors were calibrated for the temperatures of the ultracentrifugation runs and were found to be accurate. The partial specific volumes $\bar{v}_2$ of the LumPs were calculated from the amino acid composition of the protein (O'Kane et al., 1985) plus 1 mol of Lum (Cohn & Edsall, 1943). The partial specific volume of Lum was determined to be 0.872 cm^3 g^{-1} by pycnometry, and $\bar{v}_2$ for *P. leiognathi* and *P. phosphoreum* LumPs were calculated respectively as 0.740 and 0.737 cm^3 g^{-1}.

Determination of Molecular Weights by High-Performance Size-Exclusion Chromatography (HP-SEC) in 6 M Gdn·HCl. The molecular weights of the LumPs were determined by HP-SEC in 6 M Gdn·HCl containing 50 mM phosphate buffer, 3 mM EDTA, and 20 mM 2-ME, pH 6.5, on a 0.75 × 7.5 cm precolumn and a 0.75 × 30 cm analytical column of TSK-3000 SW (LKB, Bromma, Sweden) in series. The samples were incubated overnight in the denaturant and heated for 3 min at 50 °C to ensure reduction of all disulfide bonds in the protein standards employed. The void volume of the column V_0 was determined with Blue Dextran ($M_n > 2 \times 10^6$) *or* calf thymus DNA and the pore volume $V_i = V_s - V_0$, where V_s is the elution volume of $CuCl_2$. The column partition coefficients for the standard proteins and the LumPs were determined as follows:

$$K_d = (V_e - V_0)/V_i \quad (1)$$

where V_e is the elution volume of the protein. The cube root of K_d was graphed against $M_s^{0.555}$ (Fish et al., 1969), where M_s is the molecular weight of the standard proteins calculated from their amino acid sequences [see Crofts (1980) for primary references]: chymotrypsin C chain, 10 109; cyt *c*, 12 396; RNase, 13 602; chymotrypsin B chain, 13 999; Hb $(\alpha + \beta)/2$, 15 540; Mb, 16 951; β-lactoglobulin, 18 262; SBTI, 20 095; trypsinogen, 23 968; chymotrypsinogen, 25 719; CA, 28 649; PEP, 34 771; G_T3-P DH, 35 711; ADH_L, 39 801; Glu DH, 55 410; HSA, 66 430. The LumPs were applied at an initial concentration of approximately 45 μM. All elution parameters were determined by absorbance at 278 nm.

Polyacrylamide Gel Electrophoresis. Single dimension denaturing SDS–polyacrylamide gels were prepared as described by Laemmli (1970) using a running gel of 15% acrylamide. The gels were fixed, stained with Coomassie Blue R-250, and destained as described by Bürk et al. (1983). The standard proteins used to determine molecular weight were HSA, Glu DH, OA, ADH_L, PEP, CA, trypsinogen, SBTI, β-lactoglobulin, Mb, RNase, and cyt *c*. The molecular weight of OA was taken as 43 000 (Weber & Osborn, 1969).

Determination of the Hydrated Molecular Radius (r_h). HP-SEC of native LumPs and standard proteins was performed at 23 °C in 50 mM phosphate buffer, pH 7.0, containing 250 mM NaCl, 0.3 mM EDTA, and 10 mM 2-ME on the TSK-3000 SW column system described previously. The values of r_h of the LumPs (applied at 45 μM) were determined from the linear regression of $K_d^{1/3}$ on the r_h of the following standard proteins (Porath, 1963), where r_h was calculated from the diffusion coefficients $D^0_{20,w}$ (10^{-7} cm^2 s^{-1}): cyt *c*, 12.4 (Atlas & Farber, 1954); RNase, 11.7 (Buzzell & Tanford, 1956; Rothen, 1940); Mb, 11.4 (Edmundson & Hirs, 1962); SBTI, 9.5 (Brewer et al., 1974); β-lactoglobulin, 7.8 (Tanford, 1961); OA, 7.1 (Dubin et al., 1967); BSA, 5.8 (Phelps & Putnam, 1960); G-3-P DH, 5.5 (Taylor & Lowry, 1956). The r_h for Hb half-mer ($\alpha\beta$) was taken from Ackers (1964). The r_h for ADH_Y was taken as the average of two divergent values (Hayes & Velick, 1954; Brewer et al., 1974).

Other estimates of the radii of the LumPs were made by assuming rigid anhydrous or hydrated spheres and by using the calculated values of M_r and $\bar{v}_2$, with anisotropy correlation time measurements corrected to 20 °C and the viscosity of water, $\phi^{obsd}_{20,w}$ = 11.0 ns for *P. leiognathi* and 11.8 ns for *P. phosphoreum* LumP, or from an empirical relationship between M_r and s (Pouchon et al., 1978).

Estimation of Hydration (h). The maximum values for hydration of the LumPs h_{max} were estimated (Kuntz, 1971) from the amino acid compositions (O'Kane & Lee, 1985). Assumed hydrations h_{asd} were calculated by multipyling h_{max} by 0.92 to account for incomplete solvation of "buried" residues in the protein. This factor represents the average difference of h measured on native proteins by proton NMR (Kuntz et al., 1969) from h_{max} calculated from the amino acid compositions (Kuntz, 1971).

Hydration was also estimated from the molecular radii and sedimentation coefficients determined in this study and from $\phi^{obsd}_{20,w}$, such that

$$h_\phi = \frac{1}{\bar{v}_1}\left(\frac{NkT\phi^{obsd}_{20,w}}{M_r\eta F_S} - \bar{v}_2\right) \quad (2)$$

where it is assumed that the molecule is hydrated and spherical and F_S is a rotational frictional parameter equal to 1.0 for spheres.

Axial Asymmetry. The axial ratios of the LumPs were evaluated from the values of $\phi^{obsd}_{20,w}$, calculated from Lee et al. (1985), and the value of translational frictional coefficients f_{trans} such that [from eq 12-51 and 12-53 of Cantor & Schimmel (1980)]

$$\phi^{obsd}_{20,w} = \frac{\eta M_r}{NkT}(\bar{v}_2 + h\bar{v}_1)F_S \quad (3)$$

and

$$f_{trans}^3 = \frac{3(6\pi\eta)^3}{4\pi N}(\bar{v}_2 + h\bar{v}_1)F_P^3 \quad (4)$$

where F_P ($=f_e/f_0$) is the translational axial asymmetry factor and is related to the axial ratios of prolate and oblate ellipsoids as described elsewhere (Perrin, 1936; Teller et al., 1979). Then

$$F_S/F_P^3 = 162\pi^2\eta^2kT\phi^{obsd}_{20,w}/f_{trans}^3 \quad (5)$$

where F_S is a rotational frictional coefficient of an ellipsoid obeying stick conditions about either axis *a* or axis *b* relative to that of an equivalent sphere [see Cantor & Schimmel (1980), eq 10-20 to 10-22]. Since both F_S and F_P are defined functions of the axial ratios, the axial ratios of the LumPs can be determined by calculating F_S/F_P^3 from the measured quantities (eq 5) and a plot of the theoretical function F_S/F_P^3 [eq 10-20 and -22, Cantor & Schimmel (1980)] vs. axial ratio obtained with the aid of a computer program that calculates

Table I: Molecular Weight of Lumazine Proteins

	LumP	
	P. leiognathi	*P. phosphoreum*
direct methods		
low-speed sedimentation equilibrium	20 470	20 300
sedimentation–diffusion	21 170	20 890
gel filtration, 6 M Gdn·HCl	21 420	22 350
SDS–PAGE	21 640	21 600
av $\bar{M}_r$	21 200[a]	21 300[b]
indirect methods		
amino acid composition and Lum content	21 220	19 650
$s^0_{20,w}\bar{v}_2^{1/3}/(1-\bar{v}_2\rho) \simeq 10^{-2}M^{2/3}$	21 830	21 140
native gel filtration[c]	24 720	25 410

[a] Mean standard deviation = 300. [b] Mean standard deviation = 500. [c] Classified as an indirect method since separation is dependent upon hydrated molecular radius, which may not be directly proportional to molecular weight.

F_P, F_S, and F_S/F_P^3 for input axial ratios. Axial ratios were also evaluated from the frictional ratios $(f/f_0)_{trans}$ (Perrin, 1936), which were factored into F_P and a hydration parameter $(f/f_e)_{trans}$ (Oncley, 1941; Teller et al., 1979).

Results and Discussion

Molecular Weight. The estimates of molecular weight made by various methods are collected in Table I. The sedimentation equilibrium resulted in linear plots of ln c vs. r^2 at all concentrations tested (results not shown). No difference was found whether the concentrations were monitored at 420 or 280 nm. It is concluded that under these conditions both LumPs exist only as monomers and that there is no dissociated free ligand.

Sedimentation velocity measurements result in $s^0_{20,w}$ values of 2.18 (*P. leiognathi*) and 2.16 S (*P. phosphoreum*) from which the sedimentation–diffusion molecular weight in Table I is calculated. Similar values for molecular weight were obtained from an empirical relationship (van Holde, 1975; Teller et al., 1979) and are included under indirect methods in Table I.

In 6 M Gdn·HCl, $K_d^{1/3}$ is an accurately linear function of $M_s^{0.555}$ for the 16 standard proteins employed: $K_d^{1/3} = -1.23M_s + 1.029$; correlation coefficient = −0.999 (results not shown). The molecular weights of the LumPs by interpolation on this line correspond to the apoproteins since the Lum is completely dissociated in 6 M Gdn·HCl. Therefore, for the listing in Table I, the molecular weight of one Lum has been added.

Both LumPs migrate as single bands on SDS–PAGE (Figure 1). The molecular weights of the LumPs are obtained by use of standard proteins. The standard line (not shown) is accurately linear, and the LumPs are again determined as their apoprotein (six determinations each), so for Table I, 1 mol of Lum is added.

The results for all direct measurements and the first two indirect ones are all in very good agreement, except for *P. phosphoreum* calculated on amino acid content, which is significantly low. When a gel exclusion study under nondenaturing conditions is made (the results are not shown but will be described below) and analyzed from a plot of K_d vs. log M_r, the values for the LumPs (Table I, last line) are considerably higher than for all the other techniques, presumably due to axial asymmetry.

Molecular Radius. The elution parameter $K_d^{1/3}$ is an accurately linear function of the r_h of the native standard proteins (data not shown). The only major deviation from the calculated regression line $K_d^{1/3} = (-8.244 \times 10^{-3})r_h + 1.082$ (correlation coefficient, −0.9995) is the value for the Hb half-mer, which lies outside the 95% confidence interval for the regression line and therefore has not been employed in this calculation.

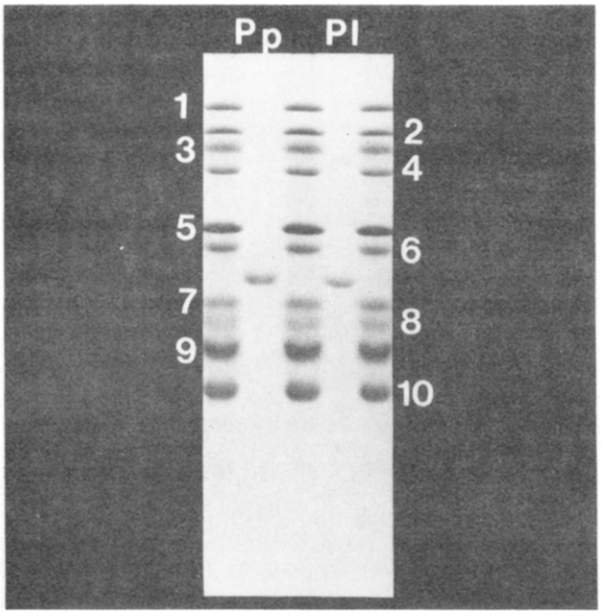

FIGURE 1: Determination of the molecular weight of lumazine proteins by SDS–PAGE. Standard proteins (identified under Materials and Methods) and LumPs were separated by electrophoresis in SDS using a 15% polyacrylamide gel (10 μg of protein for each standard and LumP). The results are described by $R_m = -0.799 \log M_s + 3.892$ (correlation coefficient = −0.994).

Table II: Molecular Radii Determinations for Lumazine Proteins (Å)[a]

	LumP	
method	*P. leiognathi*	*P. phosphoreum*
HP-SEC, native, 22 °C	22.9 ± 0.26	23.0 ● 0.27
empirical relationship[b] r_e	22.7	23.1
anhydrous sphere r_a	18.4	18.4
hydrated sphere[c] r_h	21.5	21.7
anisotropy correlation time[d] r_ϕ	21.9	22.5

[a] Uncertainty is the 95% confidence limit (2.06 × SD). [b] Pouchon et al. (1978). [c] Calculated with h_{asd}, Table III. [d] Calculated with values of $\phi^{obsd}_{20,w}$ (Lee et al., 1985).

Table III: Hydration of Lumazine Proteins[a]

		LumP	
method		*P. leiognathi*	*P. phosphoreum*
amino acid composition	h_{max}	0.48	0.52
and Lum content	h_{asd}	0.44	0.48
anisotropy decay[b,c]	h_ϕ	0.51	0.61
sedimentation[c]	h_s	0.45	0.53
diffusion[c,d]	h_D	0.69	0.71

[a] Units are grams of H_2O per gram of protein. [b] Values of $\phi^{obsd}_{20,w}$ are from Lee et al. (1985) and are corrected to 20 °C. [c] Calculated for spherical molecules. [d] Using values of r_h from HP-SEC (Table II).

Table II collects the results for molecular radii obtained for direct and indirect methods. The HP-SEC value agrees well with the empirically obtained r_e, but the others, r_h and r_ϕ, calculated on the spherical assumption, are slightly lower. As expected, the value r_a calculated for a rigid anhydrous sphere is a severe underestimate.

Hydration. Table III compares the results for the several methods of estimating hydration. The estimates are in good internal agreement except for h_D. It is apparent that the

Table IV: Frictional Properties and Axial Ratios of Lumazine Proteins[a]

	LumP	
	P. leiognathi	*P. phosphoreum*
translational frictional coefficients[b]		
Svedberg relationship	4.09	4.18
Einstein–Sutherland relationship[c]	4.07	4.12
$(f_0)_{trans}$	3.48	3.49
translational frictional ratios		
$(f/f_0)_{trans}$	1.169	1.199
$(f/f_e)_{trans}$[d]	1.166	1.182
F_P	1.003	1.015
F_P[e]	1.004, 1.000	1.004, 1.001
axial ratios, a/b		
no hydration	3.8, 4.1	4.1, 4.3
h_{asd}[d]	1.20, 1.21	1.50, 1.51
Figure 2[e]	1.23, 1.23	1.24, 1.18

[a] All calculations are made for 20 °C. [b] Units are 10^{-8} g cm^{-1} s^{-1}. [c] Assuming h_{asd}. [d] Calculated by assuming h_{asd} according to Oncley (1941). [e] From Figure 2 with F_S = 1.062 (*P. leiognathi*) or 1.069 (*P. phosphoreum*).

hydration of *P. phosphoreum* LumP is higher than that of *P. leiognathi* LumP.

Axial Asymmetry. The LumPs have small axial ratios as indicated from combined rotational and translational frictional parameters (Figure 2). The intersections of the lines for the calculated values of F_S/F_P^3 with the curves for the theoretical values for ellipsoids indicate axial ratios of <1.25 regardless of the type of ellipsoid. These results agree with those obtained from translational frictional parameters (Table IV). Ignoring hydration, limiting values for the maximum axial ratios are 3.8–4.3. However, the frictional ratios are almost fully accounted for by the hydration parameter $(f/f_e)_{trans}$, leaving axial asymmetry parameters slightly in excess of unity indicating axial ratios ≤1.5.

CONCLUSIONS

In summary, we can conclude that the LumPs isolated from the two species of *Photobacterium* behave in solution as nearly spherical, highly hydrated molecules. While many of their other properties are different, they exhibit virtually identical hydrodynamic behavior and have the same molecular weight. The molecular weights determined by the different methods are generally in excellent agreement. The significantly high molecular weight obtained for *P. phosphoreum* LumP by HP-SEC in Gdn·HCl could be explained by partial aggregation. Alternatively, as pointed out by Fish et al. (1969), $M_s^{0.555}$ is based upon the radius of gyration, which is dependent upon the average length of the peptide bonds in the protein, which in turn is determined by the primary sequence of amino acids. The two LumPs could have different elution behaviors simply due to sequence differences, although they are closely related on the basis of amino acid composition.

Despite some minor inconsistencies, the two LumPs are best modeled by prolate ellipsoids with axial ratios only slightly above unity and with high hydrations. Although the small calculated axial ratios might seem surprising, these are not substantially different from those calculated for hemoglobin and carboxypeptidase in crystals (Kuntz & Kauzman, 1974). The hydrations are comparable to Hb (0.43 g/g) and gelatin (0.5 g/g) (Kuntz & Kauzman, 1974). The calculated hydrated molecular weights for the prolate model would be 30 300 and 31 500 for *P. leiognathi* and *P. phosphoreum* LumP, respectively, in agreement with the value of 30 000 estimated by Visser & Lee (1980). However, the surface area

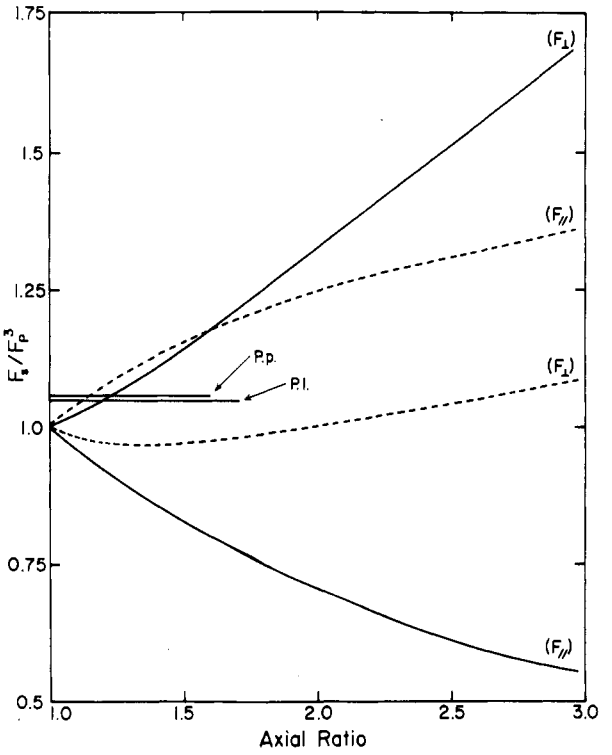

FIGURE 2: Determination of the axial ratios of lumazine proteins. The relationship between F_S/F_P^3 and the axial ratio for prolate (solid lines) and oblate (dashed lines) ellipsoids is plotted for rotation about the long axis a, $F_{\|}$, and short axis b, $F_{\perp}$, for axial ratios (a/b) from 1 to 3. The solid horizontal lines labeled *P.l.* and *P.p.*, respectively, indicate the calculated values of F_S/F_P^3 for *P. leiognathi* and *P. phosphoreum* LumPs from eq 5. The symbols in parentheses ($F_{\|}$, $F_{\perp}$) indicate the relationship between F_S and rotation relative to the unique axis of ellipsoids.

required to bind this water exceeds that which is calculated for a smooth ellipsoid [for mensuration formulas see *Handbook of Chemistry and Physics* (1961)]. Consequently, a minimum of 30% of the interacting water must be located internally. This "internal" surface area must be considered to be in frictional contact with bulk solvent in order to satisfy the calculated hydrodynamic properties of the LumPs.

Teller et al. (1979) have introduced the concept of surface rugosity (roughness) and provide an empirical formula for estimating it. Rugosity has been ignored in calculating F_P and h. Any rough surface exposed to solvent and obeying stick boundary conditions contributes to the frictional interaction of the protein with solvent. Therefore, the axial ratio of the LumPs assuming a smooth rigid structure may be overestimated. A more nearly spherical structure with enough surface rugosity to accommodate hydration would be consistent with all the data. Only the three-dimensional structure determined by X-ray diffraction will solve this problem. Assuming that the LumPs are similar to other globular proteins where the solvent-accessible surface area has been calculated from X-ray diffraction studies (Teller et al., 1979), the estimated accessible surface area provided by rugosity is sufficient to account for hydration.

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