Phycocyanin in Physical–Chemical Studies

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I. Introduction

Biliproteins—phycocyanin, phycoerythrin, phycoerythrocyanin, and allophycocyanin—are light-harvesting and excitation energy transfer pigments that are active in photosynthesis of blue-green algae (cyanobacteria), red algae, and the cryptomonads. They function by absorbing light in regions of low chlorophyll absorption and then participating in highly efficient exciton migration until the energy finally reaches a photochemical reaction center. At the reaction centers, the electronic energy is transduced to chemical energy. Biliproteins are usually directly associated with photosystem II but energy absorbed by biliproteins also reaches photosystem I via spillover.

Biliproteins are oligomeric proteins that use linear tetrapyrrole chromophores (bilins) for their light harvesting. The bilins are covalently attached to the apoprotein by thioether bonds to cysteine residues, and the bilins are not associated with metal ions. These proteins are sometimes referred to as phycobiliproteins to differentiate them from other biliproteins such as phytochrome. Phycocyanobilin when attached to the native apoprotein has an absorption maximum near 615 nm and provides phycocyanin with its blue color (Figure 1). Phycoerythrins are red due to their phycoerythrobilin content. Three less common bilins, which only occur together with one of the major bilins, are cryptoviolin, phycourobilin, and the 697-nm bilin. The biliproteins occur together with chlorophyll *a*, and chlorophyll c_2 is an additional constituent of the cryptomonads.

The intense visible absorption and fluorescence spectra of biliproteins have led to their being a primary material of investigation in a variety of situations that introduced important new technology in the study of biological macromolecules. Biliproteins are among the first molecules investigated by ultracentrifugation, calcium phosphate chromatography, ion-exchange chromatography, and gel filtration. In addition, the growth of blue-green algae in essentially 100% D_2O allowed phycocyanin to be obtained as a totally deuterated protein. It is probably the most carefully studied deuterated protein. These investigations were most often not directly concerned with the biological function of these proteins, but they were interested in models for the study of protein aggregation, protein denaturation, the properties of deuterated proteins, picosecond spectroscopy, and the surface properties of proteins. The focus of this review will be strictly on studies of purified phycocyanin that rely on physical–chemical and biophysical insights together with some discussion of the spectra of allophycocyanin. The targeted research areas are selected because of their general interest in the study of protein structure and function and biological spectroscopy, but are also very important in understanding the biological functions of these proteins. Earlier reviews[1] cover the important findings concerning the structure and function of phycobilisomes, the relationship of biliproteins to photosynthesis, reactivity of bilins, biosynthesis and structure of the biliproteins, evolution, the properties of phycoerythrins and phycoerythrocyanin, biliprotein nomenclature, and some aspects of picosecond fluorescence and should serve to complement this current review.

II. Phycocyanin Aggregation

A. Protein–Protein Aggregation

In blue-green and red algae, biliproteins are found to be assembled into large, distinct granules (phycobili-

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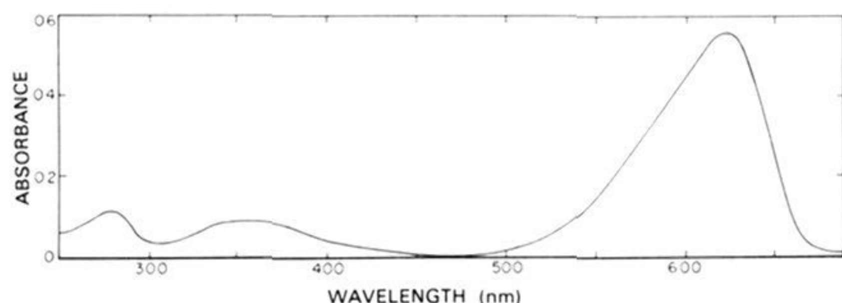

Figure 1. Absorption spectrum of C-phycocyanin. Spectrum is taken in pH 6.0, 0.1 ionic strength, sodium phosphate buffer at room temperature. The source of this protein is the blue-green alga *Phormidium luridum*.

somes) on the outer thylakoid membrane surface. The phycobilisomes are composed of biliproteins and linker proteins. When isolated and purified in low ionic strength buffers, the phycobilisomes partially dissociate into a series of discrete aggregates. The biliproteins are highly water soluble and are composed of two or more subunits.

Svedberg and his colleagues[2-4] purified phycocyanin and phycoerythrin and determined their molecular weights on the then recently developed analytical ultracentrifuge. They chose these proteins because of their absorbance in both the visible and ultraviolet regions of the spectrum and because of the ease with which large quantities of pure proteins could be obtained. The use of the simplified absorption optics on the early ultracentrifuges made the biliproteins ideal material to examine. This absorption methodology allows the examination of protein concentrations of <0.1 mg/mL. The analytical ultracentrifuge permits the determination of molecular weights by two methods, sedimentation equilibrium and sedimentation velocity. With sedimentation equilibrium, phycocyanin at pH 6.8 has a molecular weight of 101 800 and phycoerythrin has a molecular weight of 206 000. With sedimentation velocity, a molecular weight can be obtained from the Svedberg equation

$$M = \frac{sRT}{D(1 - \bar{v}\rho)} \qquad (1)$$

where M is the molecular weight, R the ideal gas constant, T the temperature, D the diffusion coefficient, $\bar{v}$ the partial specific volume of the protein, ρ the solvent density, and s the sedimentation coefficient. Results from this method show that the molecular weights of phycocyanin and phycoerythrin are 105 000 and 227 000, respectively. Purification of phycocyanin by ammonium sulfate precipitation is shown not to change the aggregation state of the protein.[4]

Many years later, sodium dodecyl sulfate gel electrophoresis experiments demonstrated that phycocyanin and phycoerythrin are each composed of two polypeptides (α and β) in equimolar amounts.[1] The polypeptides of both biliproteins are related but have evolved somewhat different amino acid compositions and different chromophore contents. An $\alpha\beta$ unit is called a monomer and monomers have molecular weights of $\sim$38 000. These sedimentation results can now be interpreted to show that a pH 6.8 phycocyanin is a trimer and phycoerythrin is a hexamer. Additional experiments on the analytical ultracentrifuge showed that phycocyanin is hexameric at pH 5.0 and dissociates to trimers at higher pH.[5]

Donald Berns received his Ph.D. degree in physical chemistry from the University of Pennsylvania in 1959. He was a Postdoctoral Fellow at Yale University and worked in the laboratory of R. M. Fuoss on electrolytes and then in the laboratory of S. S. Singer on problems in protein chemistry and immunochemistry. After a year at Argonne National Laboratory as a Resident Research Fellow, he came to the Wadsworth Center for Laboratories and Research of the New York State Department of Health (at that time it was called the Division of Laboratories and Research), progressing, from 1962 to date, from Senior Research Scientist to Head of the Physical Chemistry Laboratory to Chief of the Laboratory of Biophysics to, in 1985, Director of the Clinical Sciences Division. Adjunct Professor appointments during this period were in the Biochemistry Department of Albany Medical School and the Chemistry Department of Rensselaer Polytechnic Institute. At present, he is also a Professor in the School of Public Health of the State University of New York at Albany. In 1972–1973 he was a Visiting Professor at the Hebrew University Hadassah Medical School, and in 1984 he was a Lady Davis Visiting Professor at the Hebrew University in Jerusalem. Since 1961 he has had an active research interest in biliproteins and has published extensively in a variety of research areas related to the physical and immunochemical properties of these proteins and their structure–function relationships. He retains an interest in this field, but has also become increasingly interested and active in application of his research abilities to specific problems of public health interest. This includes laboratory research such as in surfactant properties important in Respiratory Distress Syndrome and analysis of antibody profiles correlating with staging of HIV infection, as well as the impact of science on public health policy analysis.

Robert MacColl was born in Brooklyn, NY, in 1942. He received an undergraduate degree from Queens College, an M.S. in inorganic chemistry from the University of Mississippi, and a Ph.D. in physical chemistry from Adelphi University in 1969. Since obtaining his graduate degree, Dr. MacColl has been a research scientist at the Wadsworth Center for Laboratories and Research, which is part of the New York State Department of Health. His main research interests include the structure and function of proteins and biliproteins and photosynthesis. He has recently written a monograph on the biliproteins. In addition, he has taught classes on various topics at area universities. He is currently a Professor at the State University of New York at Albany in the Department of Biomedical Sciences and the Chief of the Laboratory of Biophysics of the Wadsworth Center.

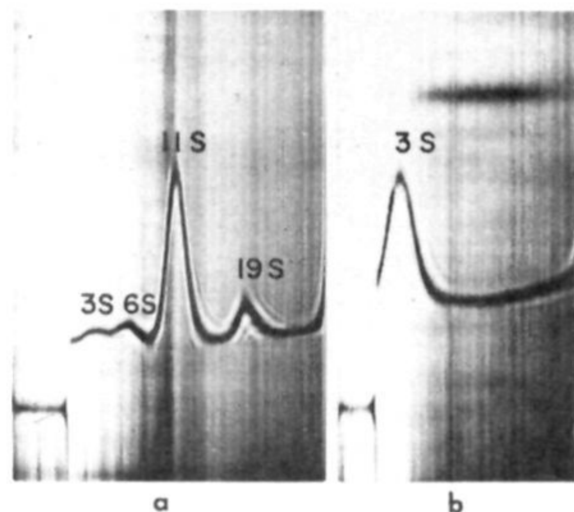

Figure 2. Sedimentation velocity experiments on phycocyanin (*Lyngbya* sp.) using schlieren optics. Schlieren patterns show the change in refractive index with distance, and this is closely related to concentration changes. Sample a is pH 4.7 and 13 mg/mL and sample b is pH 3.9 and 5 mg/mL. Sedimentation is from left to right at 59 780 rpm and 32 min after reaching full speed. Near the isoelectric point, phycocyanin is mainly hexameric (a) with very small amounts of monomer (3S), trimers (6S), and hexamer stacks (19S). At pH 3.9, this phycocyanin is converted totally to monomers (b) but phycocyanins from certain other algae do not show this behavior. Reprinted from ref 9; copyright 1971 Springer-Verlag.

Later, Berns and his colleagues continued these studies by using the analytical ultracentrifuge to examine the aggregation properties of phycocyanin over a wide range of experimental conditions.[6-12] The earliest studies employed schlieren optics and protein concentrations as high as 40 mg/mL, and these sedimentation coefficients are extrapolated to $s^0_{20,w}$ (conditions of zero protein concentration, 20 °C, and water as solvent). The trimer and hexamer of phycocyanin are found to be reversibly interconvertible, as dialysis back and forth between pH 5.0 and 9.0 shows full interconversion. Increasing either the temperature or the ionic strength of the buffer acts to increase the proportion of hexamers over trimers. At pH 5.0, which is near the isoelectric point of phycocyanin, a small amount of a possible monomer is observed. These experiments used the sedimentation velocity method, which yields sedimentation coefficients—the ratio of the velocity of sedimentation to the acceleration. Sedimentation coefficients are then related to the molecular weights of globular proteins, which are assumed to be spherical, by the following:

$$s_1/s_2 = [M_1/M_2]^{2/3} \quad (2)$$

In addition, this work[6] noted the presence of phycocyanin aggregates larger than hexamers in some preparations. Substantial quantities of these larger aggregates are sometimes observed during sedimentation velocity experiments.[8]

Phycocyanin samples at pH 3.9 from certain microbes showed a single 3S boundary in sedimentation velocity experiments (Figure 2). Sedimentation equilibrium studies showed this to be the phycocyanin monomer.[9] Solutions of monomers at pH 3.9 when dialyzed to pH 4.7 show an 85% conversion to hexamers.[10]

Sedimentation equilibrium methodology and the concentration region accessible to sedimentation velocity experiments were greatly improved by the development of absorption methods using a monochromator in conjunction with a photoelectric scanner (Figure 3). Efforts were directed toward studying the

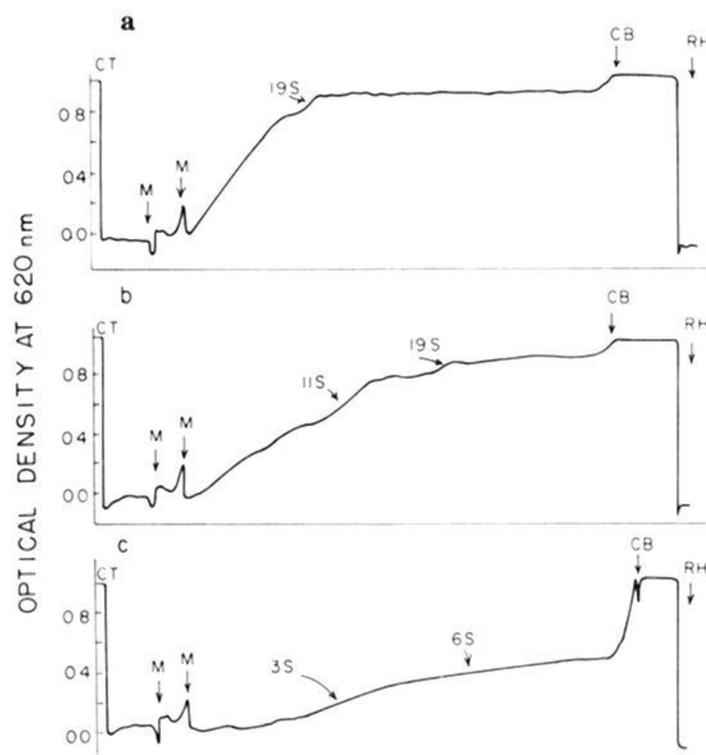

Figure 3. Sedimentation velocity experiment as a function of time for phycocyanin (*Lyngbya* sp.) using the absorption optics system and the photoelectric scanner. Phycocyanin used in Figures 2–7 is purified by ammonium sulfate fractionation only. Light of 620 nm is used and sedimentation is from left to right at 52 640 rpm. Sample is in pH 6.0 buffer at room temperature. M, location of meniscus; CB, cell bottom; RH, outer reference hole; CT, cell top. Times after reaching full speed: (a) 10 min; (b) 27 min; (c) 90 min. Reprinted with permission from *Biochem. J.* **1971**, *122*, 421–426; copyright 1971 The Biochemical Society, London.

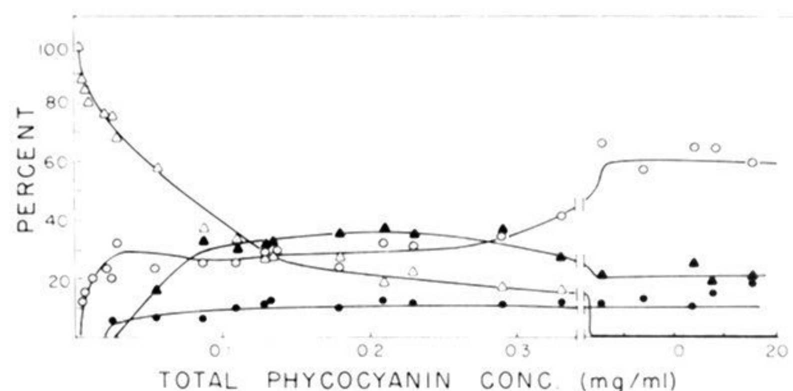

Figure 4. Percentage of each aggregate as determined from sedimentation velocity experiments in the analytical ultracentrifugation experiments as a function of total phycocyanin (*Lyngbya* sp.). Samples are in pH 6.0 buffer and at room temperature. (△) Monomers; (▲) trimers; (○) hexamers; (●) stacks of hexamers. Note that at the lower concentration range only hexamers and monomers are observed. Reprinted with permission from *Biochem. J.* **1971**, *122*, 421–426; copyright 1971 The Biochemical Society, London.

behavior of phycocyanin solutions at low protein concentrations. With the photographic UV absorption optical system, at phycocyanin concentrations as low as 0.2 mg/mL the normally observed aggregates are still present.[7] Much lower protein concentrations can easily be reached by using the absorption optical system together with the photoelectric scanner. In addition, the monochromator allows the specific study of proteins having chromophores. At pH 6.0, phycocyanin aggregation is studied by using varying wavelengths of light to analyze the sedimentation patterns in different concentration regions (Figure 4).[10] The aggregation followed a progression in which the trimers completely dissociate, leaving a solution of hexamers and monomer, and finally at still lower concentrations hexamers completely dissociate to monomers. Using the visible absorption maximum of phycocyanin at 615 nm, one can

observe phycocyanin hexamers down to a protein concentration of 0.008 mg/mL. The equilibrium constants for the phycocyanin aggregation of monomers (M), trimers (T), and hexamers (H) at pH 6.0 are the following:

$$K_{MH} = \frac{[\text{hexamer}]}{[\text{monomer}]^6} = 10^{30}\ (\text{L/mol})^5 \quad (3)$$

$$K_{TH} = \frac{[\text{hexamer}]}{[\text{trimer}]^2} = 4 \times 10^4\ \text{L/mol} \quad (4)$$

$$K_{TM} = \frac{[\text{monomer}]^3}{[\text{trimer}]} = 6 \times 10^{-14}\ (\text{mol/L})^2 \quad (5)$$

A detailed analysis of the sedimentation equilibrium data for a phycocyanin solution at pH 4.8 is carried out to determine the aggregates present and the various equilibrium constants.[12] The weight-average molecular weight ($M_w(r)$) at a position r from the center of rotation is

$$M_w(r) = \frac{2RT}{(1 - \rho\bar{v})\omega^2} \frac{\text{d} \ln c}{\text{d}r^2} \quad (6)$$

where ω is the rotor speed and c is the protein concentration as measured by the absorbance in the photoelectric scanner. A polynomial regression program is used to obtain the best fit of ln c versus r^2. Further mathematical analysis of the data shows that there are monomers, trimers, and hexamers in these solutions. Sedimentation velocity experiments using both boundary and band methods confirm the exclusive presence of these aggregates. In agreement with the sedimentation velocity results, the value of K_{MH} is found to be 1.1×10^{30} (L/mol)^5, from which a Gibbs free energy (ΔG) can be calculated by

$$\Delta G_{MH} = -RT \ln K_{MH} \quad (7)$$

to be −40.3 kcal/mol.

Finally, sedimentation velocity experiments can be used to obtain molecular weights of the aggregates larger than hexamer.[11] Normally, sedimentation velocity experiments are done by following the velocities of the various boundaries. In complex solutions, however, it is easier to obtain more accurate data by measuring the velocities of individual bands of each aggregate using zonal sedimentation methods. This procedure is accomplished via a special centerpiece by layering a small volume of phycocyanin solution onto a more dense liquid column while the rotor is already in motion (Figure 5). The analysis of the data shows that the hexamer has a sedimentation coefficient ($s^0_{20,w}$) of 10.7 S while the two larger aggregates have sedimentation coefficients of 16.8 and 21.4 S. With eq 2, these two larger phycocyanin aggregates are determined to be composed of two hexamers and three hexamers, respectively.

Another group has also undertaken an extensive series of experiments on phycocyanin aggregation mainly relying on sedimentation velocity and sedimentation equilibrium.[13-18] At pH 5.4, their data could be fitted to a monomer–hexamer equilibrium.[17] At 20 °C, the value of K_{MH} is calculated to be 1×10^{29} (L/mol)^5. At pH 6.8, the phycocyanin solutions are analyzed as a combination of monomers, trimers, and tetramers.[16]

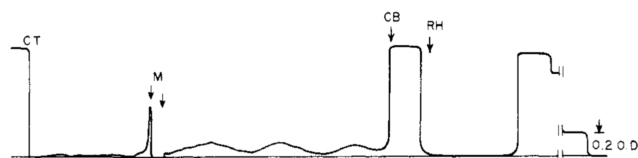

Figure 5. Sedimentation velocity experiment on phycocyanin (*Anabaena variabilis*) using a band-forming centerpiece in the analytical ultracentrifuge and absorption optics. From left to right, the bands are monomers, hexamers, and stacks of hexamers. Experiment is performed at 52 640 rpm and 25 °C. Scans of the cell are made with 620-nm light. Reprinted with permission from *Biochem. J.* 1971, *122*, 421–426; copyright 1971 The Biochemical Society, London. The abbreviations used are as follows: M. Meniscus; CT, cell top; CB, cell bottom; RH, reference hole; OD, optical density.

Tetramers are not generally observed by other physical techniques or in the work reported above by Berns and his colleagues, although there is general agreement in the pH and ionic strength dependence of phycocyanin aggregation between the two groups.

Besides sedimentation methods other physical techniques have been employed in the study of phycocyanin aggregation. Gel filtration experiments on phycocyanins have shown the pH and protein concentration dependence of the aggregation.[19] A major difference between the gel filtration results and those from sedimentation is the finding of a dimer instead of the trimer. It is appropriate to mention here that phycocyanins from a large number of different algae are used in the various studies reported in this review. Therefore, the finding of a dimer instead of a trimer may reflect specific strategies used in the different microbes (see section II.C for a discussion of phycocyanins from various algae). Nonetheless, in most cases we suggest that these variations may stem from the assay methods employed. Osmotic pressure measurements show that the molecular weight of phycocyanin solutions increases with increasing temperature.[20] The diffusion coefficient of phycocyanin is obtained by intensity fluctuation spectroscopy.[21] The diffusion coefficient for a preparation of hexamers is found to be 4.73×10^{-7} cm^2/s. Using eq 1, this diffusion coefficient, and an $s^0_{20,w}$ of 10.2 S yields a molecular weight of 209 000. This is in excellent agreement with the 101 800 that Svedberg and Lewis obtained for the phycocyanin trimer.[2] They have calculated the phycocyanin monomer to be spherical with a radius of 2.2 nm. Direct-scanning gel filtration has been used to study the aggregation of phycocyanin as a function of ionic strength and pH. The aggregation of phycocyanin is found to be enhanced by increased ionic strength and slightly acid pH.[22]

The phycocyanin monomer is composed of two polypeptides in equimolar amounts. In the presence of strong protein denaturing agents, these two subunits can be fully separated from each other. The denaturation of the native phycocyanin structure is accompanied by a loss in visible absorption. In 8 M urea, the spectroscopic properties of phycocyanin suggest extensive denaturation. If the 8 M urea solutions are diluted to 3 M urea and then dialyzed free of urea in an appropriate buffer, there is considerable restoration of the native spectrum.[23] Furthermore, when these subunits are denatured and fully separated by chromatographic methods, their ability to be reassociated can be tested.[24] It is found that the two subunits recombine to form a

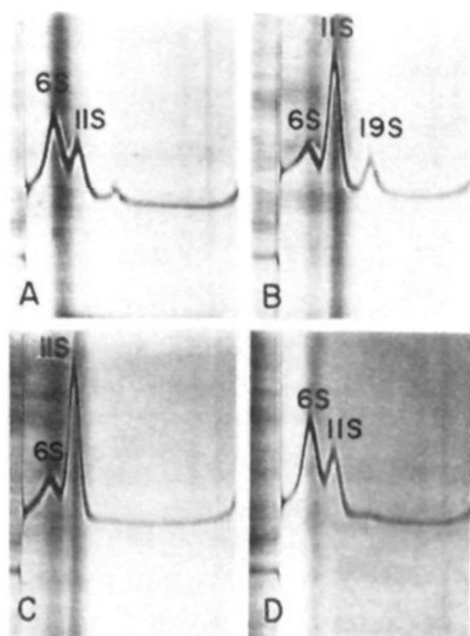

Figure 6. Sedimentation velocity experiments on phycocyanin (*Lyngbya* sp.) in the presence of benzene using schlieren optics. Sedimentation is from left to right at 59 780 rpm, and pictures are taken 16 min after reaching full speed. Sample is at 20 °C and 10 mg/mL. (A) pH 7.0; (B) pH 7.0 saturated with benzene; (C) pH 7.0 and benzene after first disaggregating 19S; (D) benzene removed, pH 7.0. Reprinted with permission from ref 27; copyright 1973 Academic Press Inc.

reconstituted phycocyanin with a 40–60% yield.

B. Interactions with Small Molecules

In the previous section, it is noted that increases in the ionic strength of the solvent produce increases in phycocyanin aggregation. These increases in ionic strength are usually accomplished by adding NaCl to the buffer or increasing the concentration of phosphate salts. Ammonium sulfate may also increase the aggregation of phycocyanin, but several other salts tend to disaggregate the protein.[25] The order of increasing dissociation strength is sodium bromide, nitrate, perchlorate, and thiocyanate. Thiocyanate and perchlorate are called chaotropes because of their ability to disrupt water structure. At 1.0 M, sodium perchlorate and sodium thiocyanate have the ability to dissociate phycocyanin to a homogeneous solution of monomers. The ability of these salts to dissociate phycocyanin to monomers but not to dissociate monomers into the two subunits is a useful trait. A monomer is, of course, the simplest entity that still has all the components of the complete protein. Monomers have proven to be extremely useful in studies centered on understanding the spectroscopic properties of biliproteins.

Allophycocyanin is a biliprotein that, like phycocyanin, has phycocyanobilins for chromophores and is composed of two distinct subunits (the two subunits are called a monomer). Allophycocyanin when purified is found to be a trimer. The trimers have a visible absorption spectrum quite different from that of the phycocyanin aggregates, and the properties of allophycocyanin have been reviewed.[1] Allophycocyanin trimers show the same behavior as phycocyanin aggregates in the presence of sodium thiocyanate and sodium perchlorate.[26] At pH 7.0, $NaClO_4$ (1.0 M) dissociates allophycocyanin completely to monomers as demonstrated by determining the molecular weight from sedimentation equilibrium methods. These monomers can be reassociated to trimers when the chaotrope is removed by dialysis. The equilibrium constant

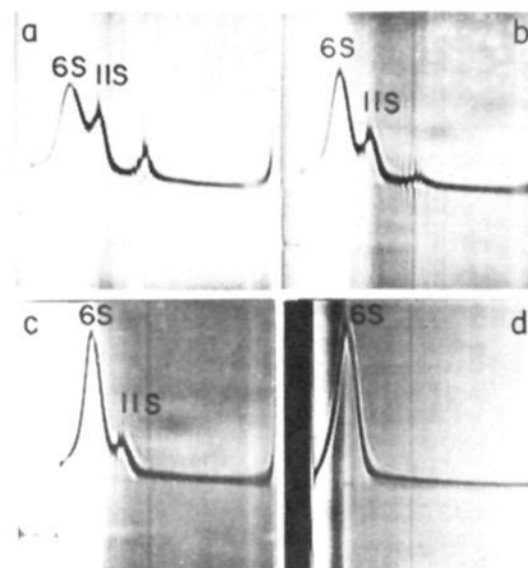

Figure 7. Sedimentation velocity experiments on phycocyanin (*P. luridum*) and various tetraalkylammonium chlorides using schlieren optics. (a) tetramethylammonium chloride; (b) tetraethylammonium chloride; (c) tetrapropylammonium chloride; (d) tetrabutylammonium chloride. Salts are 0.16 M and sedimentation is from left to right at 59 780 rpm, 24 min after reaching full speed, and 23 °C. Reprinted with permission from ref 28; copyright 1974 Academic Press Inc.

for the dissociation of trimers to monomers is calculated to be 6×10^{-16} $(mol/L)^2$.

A completely different type of small molecule, neutral aromatic molecules, causes dramatic increases in phycocyanin aggregation.[27,28] Benzene, phenol, and naphthol produce large increases in hexamers at the expense of trimers (Figure 6). No aggregation effects are found for benzoic acid, benzamide, and benzenesulfonic acid, and they do not impede the effectiveness of phenol.

Other types of small molecules that affect phycocyanin aggregation are the tetraalkylammonium and the azoniaspiroalkane salts.[28-30] Sedimentation velocity experiments are used to study the effect of varying alkyl chain lengths in the tetraalkylammonium cations on phycocyanin aggregation.[28] The longer alkyls—pentyl and butyl—are most effective in producing dissociation of hexamers to trimers (Figure 7). At pH 7.0, 0.16 M tetrabutylammonium bromide produces a solution of homogeneous trimers. Microcalorimetry has been employed to study the interaction of tetrabutylammonium bromide and phycocyanin.[29] It is found that the binding of one cation to a trimer provides inhibition of aggregation. The heat data from the microcalorimeter together with concentration data on hexamers and trimers from sedimentation velocity experiments allow a thermodynamic evaluation of the system. The enthalpy (ΔH) and Gibbs free energy (ΔG) are first obtained, and then the entropy (ΔS) is calculated by

$$\Delta S = (\Delta H - \Delta G)/T \quad (8)$$

The thermodynamic values for the binding of tetrabutylammonium ions to phycocyanin trimers at pH 6.0 are 49 kcal mol^{-1} for ΔH, -1.8 kcal mol^{-1} for ΔG, and 170 eu for ΔS. The thermodynamic parameters for the conversion of phycocyanin trimers to hexamers are found to be 17 kcal mol^{-1} for ΔH, -5.6 kcal mol^{-1} for ΔG, and 76 eu for ΔS. This very large positive entropy is therefore the driving force of the reaction.

Finally, the interactions of phycocyanin with alkyl alcohols have been studied and thermodynamic parameters obtained.[31] Alcohols produce a dissociation of hexamers to trimers in the following order: cyclo-

hexanol > butanol > propanol > ethanol. The larger alkyl groups produce greater trimer formations. An equation is derived that allows analysis of the data

$$\ln [(K_{app}/K_d)^{1/2} - 1] = m \ln c_x - \ln K \quad (9)$$

where K_{app} is the apparent hexamer dissociation constant in the presence of alcohol, c_x is the concentration of alcohol, K_d is the hexamer dissociation constant, K is the dissociation constant of the trimer–alcohol binding, and m is the molar binding ratio of alcohol and trimer. A plot of $-\ln K_{app}/K_d)^{1/2} - 1]$ versus $-\ln c_x$ gives a straight line, which allows the evaluation of m and K. Since $1/K$ gives the equilibrium constant for binding (K_B) of alcohol to trimer, ΔG_B for the binding can be calculated. Finally, from K and ΔH_B, which is obtained from calorimetry experiments, the value of ΔS_B is calculated. For cyclohexanol, the binding to trimers at pH 6.0 yields 51 kcal mol^{-1} for ΔH_B, -2.2 kcal mol^{-1} for ΔG_B, and 177 eu for ΔS. Again, a large positive entropy is observed. It is interesting that cyclohexanol and phenol have opposite effects on phycocyanin aggregation.

C. Phycocyanin from Various Algae

Blue-green algae grow in a wide diversity of environments, including extremes of temperature, high salinity, marine, and high alkalinity. Red algae are frequently found in marine habitats but one type grows well at pH 2 and fairly high temperatures.

Phycocyanins have been isolated from algal species representing various extremes of habitat and their properties analyzed.[32-38] Particular emphasis is placed in these studies on the sedimentation velocity analysis of the aggregation of phycocyanin isolated from these extreme sources. Phycocyanins from thermophiles, acidophiles, and extremely halo-tolerant sources all possess aggregates that are characteristic of the typical phycocyanin from nonextreme sources. However, the stability of the various aggregates is atypical in each case. For example, phycocyanin isolated from an acidic red alga shows very high quantities of aggregates that are larger than hexamers. These large aggregates are unusually stable.[35]

CD spectroscopy and absorption spectroscopy have been used to study the stabilities of phycocyanins from eight different microbes against urea denaturation.[37] The CD is measured at 222 nm and the absorption at the wavelength of the visible absorption maximum of the proteins. The general course of the change in ellipticity or absorbance is fairly similar—at low urea concentrations there is little or no spectral change, and this is followed by a region of very steep loss of absorbance or ellipticity. It is found that the phycocyanins isolated from thermophilic organisms require higher urea concentrations in order to be denatured. This augmented stability of biliproteins that have been isolated from thermophilic sources could perhaps be used in the future for practical purposes.

D. Linkers

Phycobilisomes are granules, discovered by Gantt and Conti, that are located on the outer thylakoid membrane surface and they contain the biliproteins.[39] Phycobilisomes consist of an allophycocyanin core that is attached to the thylakoid membrane and six rods radiating from the core. The rods contain phycocyanin near the core and, if present, phycoerythrin or phycoerythrocyanin complexes each rod on top of the phycocyanins. Phycocyanin mediates the transfer of excitation energy from the rods to allophycocyanin in the core.[40,41] The rods are composed of stacks of hexamers.[42] The structure of the phycobilisome is maintained by several colorless polypeptides, the linkers.[43] Each linker specifically allows a particular type of hexamer–hexamer assembly. One of the linkers has subsequently been shown to have a chromophore.[1]

Most of the phycocyanin preparations used in aggregation studies probably contain significant amounts of various linkers. The presence or absence of linkers in preparations depends on the type of purification. Only phycocyanin samples that have undergone extensive chromatography are likely to be free of linkers. In many of the experiments on phycocyanin only ammonium sulfate fractionation is used to purify the biliprotein. This method of preparation is perfectly reproducible and is the only method that prepares phycocyanin with the desired aggregation properties. It is a method still used in this field. The various preparations, however, are mixtures having different linkers attached to the same aggregate, and additional study is needed in this area. Other more extensive purification protocols alter the aggregation patterns.

Are any assembly steps possible without linkers? Although not yet studied in rigorous detail, evidence points to self-assembly in the early stages of phycocyanin assembly, and perhaps up to the trimer or, less likely, the hexamer stages the linkers are not mandatory. The molecular weight of hexamers is found to be 209 000.[21] A monomer has a molecular weight of $\sim$37 900 so the calculated molecular weight agrees with the experimental result within experimental error. Since a typical linker has a molecular weight of at least 30 000, it could not be readily included in the 209 000 result. Analysis of direct column scanning during gel filtration of phycocyanin suggests that monomers and hexamers are in equilibrium in the absence of linkers.[22] The re-formation of phycocyanin from purified subunits, which clearly are linker-free, is direct evidence of some undefined level of self-assembly.[24] Other studies suggest that hexamers cannot be formed without linkers, and conclusive evidence is not available on the exact role of linkers in the assembly and stabilization of phycocyanin hexamers. The self-assembly process finally leads to additional assembly that is clearly linker directed.

Linkers may serve another role than assembly and stabilization. From a particular alga, two phycocyanin complexes have been isolated, and these complexes are phycocyanin trimers differing only in that a different linker is attached to each. These complexes do not have the same absorption or fluorescence emission spectra.[44] A similar result showing linker-associated spectral shifts in both absorption and emission of a red algal phycocyanin has been reported.[45] Moreover, picosecond fluorescence kinetics experiments on a phycocyanin trimer with and without a linker show that the linker-associated trimers transfer energy more rapidly within the aggregate.[46] These energy-transfer results are cogent evidence that linkers fine tune the various bilins

in order to increase the efficiency of excitation energy transfer within the biliprotein and phycobilisomes.

The resulting linker–biliprotein complexes are quite stable; in addition, high concentrations of biliproteins are found inside cells. The various studies on the aggregation of phycocyanin demonstrate that at pH and ionic strength conditions in the physiological range, phycocyanin aggregates will show extensive dissociation only at low protein concentrations.[10] In vivo phycobilisomes are localized into specific regions and must occur at very high protein concentrations. It has been shown that these high protein concentrations are important in promoting phycobilisome stability.[47]

III. Deuterium Effects and Hydrophobicity

A. Deuterated Protein

A deuterated protein can be produced and the deuterium isotope effect on a protein's stability studied if a microorganism can be grown in a fully deuterated medium. Blue-green algae were among the first organisms to be grown under these rather unnatural circumstances. Phycocyanin is the major protein component of blue-green algae and is thus the choice to study deuterium effects on a protein.

After 2 years of careful adaptation to higher D_2O concentrations and finally growing in 99.8% D_2O, a blue-green alga is harvested, its water-soluble contents are extracted, and its phycocyanin is purified.[48] It must be remembered that in proteins there are nonexchangeable sites in H_2O (the stable C–D bonds) and exchangeable sites. To conserve D_2O the deuterated protein is purified in H_2O solvents. The deuteriums are subsequently replaced on ionizable groups by exhaustive dialysis with D_2O. Phycocyanin isolated from algae grown in D_2O is called D-phycocyanin whether it is in H_2O or D_2O medium. Two methods are employed to measure the deuterium content on the phycocyanin isolated from D_2O-grown algae and reequilibrated in D_2O. The first method measures the amount of D_2O formed when the protein is burned, and 98.4% of the water obtained is D_2O. Infrared spectroscopy shows that only D–C and N–D bonds are present in D-phycocyanin.

Although fully deuterated, the amino acid composition of D-phycocyanin is, within experimental error, identical with that of normal phycocyanin.[49] Likewise, immunochemical methods also show a close relationship between D- and H-phycocyanin.[50] D-Phycocyanin can be viewed, therefore, as being the same as the hydrogen analogue except for the isotope substitution, and any differences in the stability of the protein structure result from this substitution.

B. Stability of Deuterated Phycocyanin

The thermal stability (primarily the secondary structural stability of the protein) of D-phycocyanin versus H-phycocyanin has been measured by changes in fluorescence,[49] by changes in absorption,[51] and by differential scanning microcalorimetry.[52] In all cases, D-phycocyanin is found to be less stable to temperature increases than H-phycocyanin when both are dissolved in H_2O medium. The fluorescence experiments show that thermal denaturation occurs 5 °C lower for D-

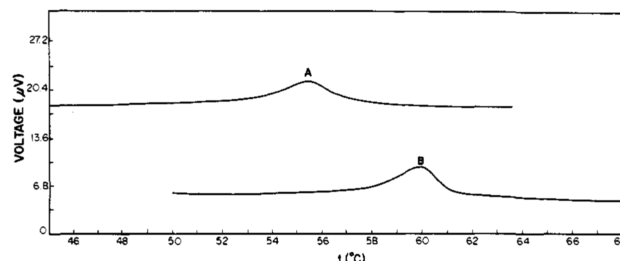

Figure 8. Thermal denaturation of deuterated (A) and normal (B) phycocyanin (*Plectonema calothricoides*) using a microcalorimeter. Reprinted with permission from ref 52; copyright 1983 John Wiley and Sons, Inc.

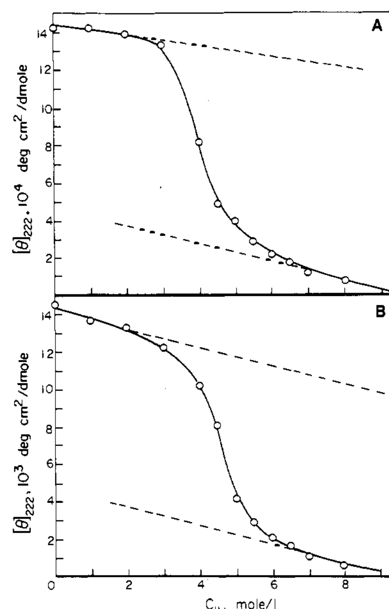

Figure 9. Plot of change in the CD at 222 nm versus urea concentration (C_u) for deuterated phycocyanin (A) and normal (B) phycocyanin (*P. calothricoides*). Reprinted with permission from ref 52; copyright 1983 John Wiley and Sons, Inc.

phycocyanin. Since these proteins differ only in the isotope content of nonexchangeable groups, the contributions of hydrogen bonding and ionic forces may be identical in each. It is, therefore, proposed that hydrophobic forces may be responsible for the lower stability of D-phycocyanin.[49]

The stability of D-phycocyanin has been studied in detail by differential scanning microcalorimetry.[52] As previously found by spectroscopic means, D-phycocyanin is denatured about 5 °C lower than the hydrogen isotope (Figure 8). The differences in enthalpy, entropy, and Gibbs free energy are then calculated for native versus the denatured states. These thermodynamic values are much lower for the D-phycocyanin than H-phycocyanin. These results suggest a lower structural stability for D-phycocyanin, and it is suggested that the structural packing for the D-protein seems more loose.

The relative susceptibilities of D- and H-phycocyanin to urea denaturation in H_2O have been examined by CD spectroscopy.[52] In agreement with the thermal results, D-phycocyanin denatures at a slightly lower urea concentration than H-phycocyanin (Figure 9). The effects of urea are analyzed by assuming a two-state model for denaturation. The dissociation constant is estimated from the CD as follows:[53]

native phycocyanin → denatured phycocyanin (10)

$$K = \frac{[\theta]_N - [\theta]}{[\theta] - [\theta]_D} \quad (11)$$

where $[\theta]$ is the molar ellipticity at 222 nm at any urea concentration, and $[\theta]_N$ and $[\theta]_D$ are the values for native and fully denatured protein at the same conditions. The apparent ΔG of denaturation can then be calculated by eq 7. The apparent ΔG of denaturation at zero urea concentration can be obtained by fitting the apparent ΔG values to an equation linear in urea concentration. This ΔG is between 1.1 and 1.5 kcal mol^{-1} lower for D-phycocyanin than for H-phycocyanin.

C. Aggregation of Deuterated Phycocyanin

Sedimentation velocity studies have been performed on D-phycocyanin.[54,55] The stability of hexamers and larger aggregates in aqueous buffers is greatly reduced for D-phycocyanin compared to H-phycocyanin. At pH 7.0, there is a reduction from 39% of hexamers or larger in the H-phycocyanin case to 11% for the D-phycocyanin.[55] It is proposed that hydrophobic forces contribute to the stability of hexamers and larger aggregates. Trimers appear to be almost entirely unaffected by deuterium substitution and are perhaps more dependent on ionic and hydrogen bonding. It has been suggested that in the deuterated protein the presence of the less polarizable D versus normal H results in a smaller dispersion interaction, which is reflected in the hydrophobic contribution.[55] With eq 7 and 8, the thermodynamic values for the trimer–hexamer equilibrium are calculated at pH 6.0 for D-phycocyanin, and ΔG is −5 kcal mol^{-1}, ΔH is +7 kcal mol^{-1}, and ΔS is +40 eu. The corresponding values for H-phycocyanin under nearly identical conditions are −5.6 kcal mol^{-1} +17 kcal mol^{-1} and +76 eu, respectively. The lower positive entropy is consistent with the proposal of a decrease in hexamer stability of D-phycocyanin being produced by a lessening of hydrophobic interactions.

D. Aggregation in D_2O

The sedimentation velocity technique has been used to study the aggregation of normal phycocyanin in D_2O.[56,57] Under these conditions the ionizable protons are replaced by deuterium. Hexamers are sharply increased in concentration at the expense of trimers. At pH 7.4, 16% of the protein is hexamer in H_2O and 41% is hexamer in D_2O, while in the same sample the amount of trimers changes from 80% in H_2O to 43% in D_2O. Similar results are obtained at pH 6.0 and 8.0. These results suggest the possibility that hydrophobic forces are important in hexamer formation since D_2O is more structured than H_2O. In order to properly make these comparisons, the measurement of deuterium ions (pD) is corrected as follows:

$$\mathrm{pD} = \mathrm{pH(obsd)} + 0.4 \quad (12)$$

Other studies on phycocyanin in D_2O include thermal denaturation monitored by fluorescence,[58] differential scanning calorimetry,[59] CD studies,[59] and spectroscopic studies.[60] All of the aforementioned studies on the perturbation of phycocyanin structure and aggregation indicate that this protein is a good example to study the relative contributions of electrostatic and hydrophobic forces on the secondary, tertiary, and quaternary structures of proteins.

E. Hydrophobicity and X-ray Structure

Solubility studies on amino acids in H_2O versus D_2O suggest that hydrophobic interactions are stronger in D_2O.[61] Adding to this possibility is the finding that in D_2O the critical micelle concentration of certain detergents is smaller than it is in H_2O.[62] However, hydrogen bonding with deuteriums on oxygen and nitrogen atoms is also stronger than with hydrogens. Other solution studies on the formation of hexamers from trimers can also be explained if hydrophobic forces are a major part of hexamer stability. The finding that the dissociation of hexamers increases[31] as the alkyl chain of alcohols becomes longer fits the hydrophobic concept. An explanation of the alcohol effect could be that nonpolar regions on phycocyanin bind the long alkyl chain alcohols. If these nonpolar regions are in the trimer–trimer contact regions in the formation of hexamers, their destabilization effects on hexamers by steric hindrance are understandable. Such nonpolar regions in trimers would, of course, be the basis of the hydrophobic interaction. Likewise, in the hexamer dissociation by tetraalkylammonium salts, the longest alkyls cause the greatest effect.[28-30] The long alkyls would naturally bind more strongly to nonpolar patches on the protein. Once the tetraalkylammonium cation binds to a trimer, if the binding occurs in a nonpolar region of trimer–trimer contact in the hexamer, the positive charge from the quaternary nitrogen will serve to further destabilize hexamers.

Other experimental results that favor the proposal of hydrophobic forces producing a major part of hexamer stability over trimers are the ionic strength effect, the temperature effect, and the thermodynamics of this reaction. When a pH 7.0 solution of phycocyanin is compared to one with 2 M NaCl, the percentage of hexamers increases from 30 to 66% as the ionic strength increases.[6] Since 2 M NaCl may very effectively screen any electrostatic bonding in the formation of hexamers, these data support the possibility of a mechanism like hydrophobic interactions stabilizing the hexamer. This paper[6] also reports on the effect of temperature on the trimer–hexamer equilibrium. At pH 7.0, a temperature increase from 5.4 to 22 °C produces an increase in hexamer (from 43 to 63%) over trimer (from 54 to 27%). Higher temperature is also a factor that will favor hydrophobic interactions. Furthermore, the hexamer is greatly favored at pH values near the isoelectric point. Since the net change on the protein surface will increase away from the isoelectric point (pH 4.6), hexamer stability near this pH may indicate a major contribution from forces other than electrostatic. More support for the hydrophobic concept is found in the thermodynamic data for the trimer–hexamer equilibrium.[29] At 25 °C and pH 6.0, the dissociation of hexamers to trimers shows an equilibrium constant for dissociation of 7.4×10^{-5}, a ΔG of +5.6 kcal mol^{-1} a ΔH of −17 kcal mol^{-1} and a ΔS of −76 eu. The large positive ΔS for the formation of hexamers is consistent with the hydrophobic forces concept. As the nonpolar regions on the trimer surface converge to form the

hexamer, highly structured H_2O at these surfaces is forced out into the bulk water. The resulting increase in disorder from structured to normal water produces the increase in entropy.

The solution properties of phycocyanin exhibit a number of properties that all fit with hexamer formation being favored from trimer via a major contribution from hydrophobic interactions. Naturally the structure of proteins is complex and the hydrophobic concept is not rigorously proven by thermodynamic data, but the general idea seems to fit a wide range of results. Therefore, the appearance of high-resolution X-ray crystallography results on phycocyanin, which seem to argue against a predominance of hydrophobic forces in hexamer formation, is unexpected.

The three-dimensional structure of phycocyanin has been obtained to a resolution of 0.21 nm by X-ray crystallography methods.[63-65] Probably the most exciting aspect of this study is the obtaining of the distances between chromophores and the chromophore orientations (section IV.F). The chromophore distances led these scientists to conclude that the hexamer may be the basic functional unit of the protein. This concept is in line with the conclusions reached from solution studies where the hexamer has been long regarded as a key aggregate form. However, here the forces stabilizing the hexamer are a major concern. The crystal structure shows "Two trimers....are aggregated head-to-head to form the hexamer. Both trimers fit complementarily and are held together by polar and ionic interactions." The surface of a trimer is observed to be hydrophilic and a number of hydrogen bonds are formed in the association to hexamers. Note, however, that in the last paper in this group, refinements are made in the data,[65] but this generalization may still hold. This finding is of interest when compared with the conclusions drawn from solution studies. Several possible and speculative alternatives must be considered:

1. The relationship between the X-ray data and the solution data requires a more detailed analysis, for example, inclusion of linker polypeptide effects or consideration of a complex array of stabilizing forces that include both hydrophobic and electrostatic effects.

2. During crystallization the conformation of phycocyanin changes and new interactions are possibly produced.

3. The trimers observed in the crystals may not be structurally the same trimers that associate to form hexamers in solution. More than one plane can bisect a hexamer to form two trimers and perhaps the plane bisecting the hexamer to form "solution" trimers is perpendicular to that used to define the "crystal" trimer. There is some electron micrographic evidence, however, that indicates there may be a correspondence between the solution and crystal trimers.[66]

IV. Spectra-Aggregation Relationships

A. Phycocyanobilin Conformation

The absorption spectrum of phycocyanin, monomer and all aggregates, exhibits a strong first excited state band at ~615 nm and a much weaker second excited state band at ~360 nm. Both of these bands are due to the properties of phycocyanobilin, a linear tetrapyrrole chromophore. The chemical nature of phycocyanobilin is achieved by its synthesis via heme whose ring is cleaved and certain of its side chains modified to produce the bilin.[67] When the secondary, tertiary, and quaternary structures of the protein are denatured by any number of means—temperature, urea, guanidine hydrochloride, sodium dodecyl sulfate—the visible absorption band will drop in intensity as will the fluorescence and CD intensities. It is also readily observed that as the intensity of the first excited state declines, the intensity of the second excited state (wavelength maximum near 360 nm) will increase (Figure 10). This effect has been studied carefully for the detergent sodium dodecyl sulfate, and the intensities of the two bands are coordinated.[68]

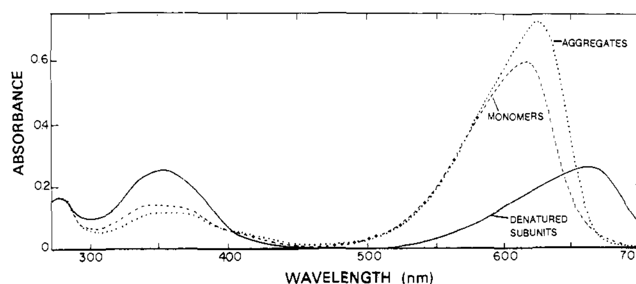

Figure 10. Absorption spectra of phycocyanin (*P. luridum*) as aggregates, monomer, and denatured subunits.

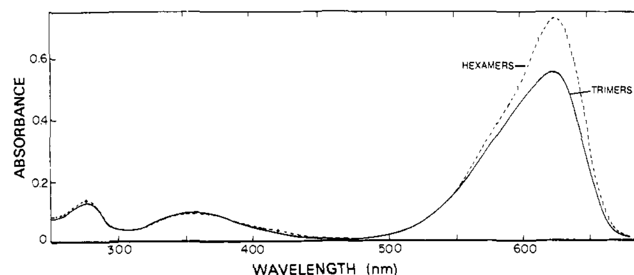

Figure 11. Absorption spectra of phycocyanin (*P. luridum*) as hexamers and trimers.

An explanation for the decrease in the ratio of the visible absorbance to the absorbance in the near-ultraviolet comes from a consideration of various quantum chemical calculations and the spectroscopy of certain model compounds.[69] It is proposed that the conformation of phycocyanobilin is extended in native protein, and when the protein is denatured the influence of apoprotein on the bilin is relaxed and the bilin assumes a more stable cyclic conformation. X-ray crystallography studies on phycocyanin confirm the existence of the extended conformation.[65]

A series of homogeneous phycocyanin aggregates is prepared and the conformation of each aggregate is determined from the ratio of the oscillator strength of the visible and near-ultraviolet bands.[11] The conformation of the phycocyanobilins changes at two stages in the assembly of a phycobilisome. There is a large increase in the ratio when denatured subunits assemble to monomers and a second increase when hexamers are formed (Figures 10 and 11). There are no measurable changes in the ratio when monomers are compared to trimers or when hexamers form either face-to-face stacks or the complete phycobilisome. This again supports the concept of the hexamer being a particularly important step in the aggregation of the biliproteins.

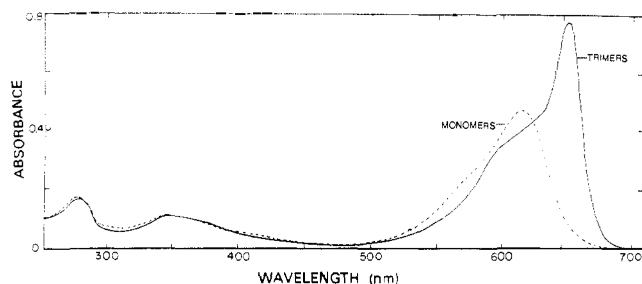

Figure 12. Absorption spectra of allophycocyanin (*P. luridum*) as trimers and monomers. Monomers of allophycocyanin and C-phycocyanin are prepared by the addition of NaSCN or $NaClO_4$ around neutral pH.

B. Photoreversibility

Phycocyanin—isolated from the alga but not treated by any denaturants—shows no photoreversible behavior. However, such activity can be induced by alkaline pH, urea, or guanidine hydrochloride.[70] The concentrations of denaturants that induce this behavior are well below the levels needed for full biliprotein denaturation. A guanidine hydrochloride concentration of 0.4 M at pH 6.0 is an effective agent. In this experiment, light-induced absorbance changes occur with red and green light. The green minus red difference spectrum shows a maximum at 630 nm and a minimum at 570 nm. The absorbance changes are quite reversible when the sample is alternatingly illuminated by red and green light. Photoreversible absorption changes in phycocyanin can also be caused by photobleaching cells prior to isolation[71] and by treating phycocyanin with 75% ethylene glycol.[72,73]

The aggregation of phycocyanin has been investigated under conditions similar to those used in the photoreversibility experiments.[74] Sedimentation velocity experiments have been carried out at three concentrations of guanidine hydrochloride at pH 6.0. At 0.1 M guanidine hydrochloride, there is unexpectedly a very large increase in hexamers at the expense of trimers. At 0.5 M guanidine hydrochloride, there is still a considerable hexamer concentration but also significant disaggregation is evident. At 1.9 M guanidine hydrochloride, the denaturant is acting in the expected way to strongly disaggregate and denature the phycocyanin. These are insufficient data to correlate photoreversibility and the protein's aggregation, but perhaps the photoreversibility is related to the beginning of disaggregation observed at 0.5 M guanidine hydrochloride. It should also be noted that chromophores may be affected by either changes in protein aggregation or changes within a given aggregate.

C. Allophycocyanin

The absorption spectrum of allophycocyanin is saliently different from that of phycocyanin even though they both have only phycocyanobilins for chromophores. Allophycocyanin trimers have a relatively sharp band at 650 nm, while phycocyanin has a maximum at ~615 nm (Figure 12). When allophycocyanin is denatured the 650-nm band is lost and the spectrum resembles that of phycocyanin.[75,76] Complete disaggregation and denaturing of allophycocyanin into subunits by 8 M urea can be reversed and trimers recovered by dialysis into pH 7.0 phosphate buffer.[76] Allophycocyanin monomers also lack the 650-nm band of the trimers and have an absorbance maximum at ~615 nm (Figure 12).[77,78] Monomers also can be readily reconverted to trimers.

The spectroscopic difference between allophycocyanin monomers and trimers can be explained by either of two general concepts—exciton *delocalization* between a strongly coupled pair of bilins[77,79-81] or *conformational* differences between monomer and trimer bilins.[82-86] In addition to offering a model for allophycocyanin's absorption, a viable proposal should also explain the fluorescence (excitation) polarization and CD spectra of the protein since each of these have unique features.

The idea of a conformational change in the bilins producing a spectral shift for allophycocyanin compared to phycocyanin is straightforward and reasonable.[82] The bilins, being linear tetrapyrroles, are flexible and can easily be manipulated by the apoprotein. Furthermore, theoretical calculations have demonstrated that rotation of one of the pyrrole rings can produce the necessary spectral change.[84] In addition, these authors stress that the CD spectrum of their allophycocyanin trimers allows no possibility for the chromophore interaction hypothesis to be correct. The fluorescence polarization of allophycocyanin trimers is quite different from that of phycocyanin trimers in that its polarization is lower and flat across the first excited state. One group presents a twofold explanation for the polarization results within the context of the conformational model: the low polarization is produced by a different angle between the absorption and emission transition dipoles of each bilin for trimers and monomer, and in addition, all the transition dipoles are oriented parallel to each other.[85] The idea that the angle between absorbing and emitting dipoles is variable and controlled by the aggregation state of the biliprotein has not previously been applied to biliprotein spectroscopy. Such a change could result in the lower polarization since the greater the angle, the lower the polarization (p):

$$p = \frac{3 \cos^2 \theta - 1}{\cos^2 \theta + 3} \tag{13}$$

where θ is the angle between absorption and emission dipoles. The value for this angle from several biliprotein examples, where depolarization from excitation energy transfer is excluded, is invariably 23°. To account for the lower polarization of allophycocyanin trimers by the conformational model, this angle must change to 50°. More recently, another quite different suggestion has been advanced that the shape of the fluorescence polarization spectrum can be explained by the conformational model if there is bidirectional excitation energy transfer between bilins on the two subunits.[86] This proposal seems to fail when the large differences between the spectral overlap integrals in the two directions are noted. These overlap integrals can be roughly estimated from their spectral deconvolution. In addition to the difficulty of the conformational model to explain the fluorescence polarization spectrum, CD studies, discussed next, likewise show problems with a purely conformational approach.

When two or more chromophores are close together, they may interact in a way that produces complete

exciton delocalization among them. This process results in spectral splitting of the absorption into high- and low-energy bands. How might this process occur when allophycocyanin monomers aggregate to trimers? If the bilins are located near the surface of the monomers, then the bilin-to-bilin distance between contiguous monomers in the trimer might allow the delocalization event and produce the 650-nm absorption band.[77] A maximum of three pairs of bilins having delocalized excitation may form, and the pairs may also transfer excitons from pair to pair by the mechanism of very weak coupling of dipoles. Since these bilins will be at different angles to each other, this exciton migration among the six bilins of trimeric allophycocyanin would explain the lowering of the fluorescence polarization. A major obstacle in accepting the possibility of the delocalization concept is the CD spectrum. A strongly coupled pair of chromophores, as is proposed here, will have a characteristic CD spectrum. The CD spectrum must have both positive and negative bands and the usual experimental CD spectrum of allophycocyanin has only two, rather weak, positive bands. However, a closer study of the CD spectrum reveals a very important finding as the spectrum can be deconvoluted into four components: two positive and two (or perhaps one) negative.[80] The negative bands are virtually undetectable in many cases in the experimental measurement because of extensive overlap with positive bands. This deconvolution result presents a serious challenge to the conformational model for the allophycocyanin trimer. In order to fit this model to the fluorescence polarization data, all the bilins are assumed to be parallel. This orientation of dipoles would not explain the complex CD spectrum, which is revealed by the deconvolution technique, while the interaction model fits it well.

Next, the interaction model is tested by stopped-flow kinetics studies on the trimer–monomer equilibrium.[81] The dissociation of trimers is monitored by two methods, light scattering, which measures the molecular weight change, and 650-nm absorbance. If the interaction model is valid, the loss of 650-nm absorption must occur at nearly the same rate as the molecular weight change when trimers dissociate to monomers. The kinetics results confirm the linkage of the loss of 650-nm absorption and the change in molecular weight. A second, more slowly occurring, absorbance change, which is independent of molecular weight changes, is also observed and is assigned to a change in chromophore conformation. A proposal is presented that the major absorption at 650 nm occurs for trimers from the chromophore–chromophore interaction and delocalization, but also a smaller contribution comes from a different chromophore conformation (as predicted by ref 82) between trimers and monomers. The kinetics data do not exclude the pure conformational model, but, on the basis of the currently available data, the interaction proposal seems to be more appropriate for the allophycocyanin trimer than a pure conformation model.

D. Physical States for the Bilins

Having presented in the section above evidence that a mixture of strong coupling (delocalization) and very weak coupling may occur for allophycocyanin trimers, we now consider phycocyanin. Very weak dipole coupling is a mechanism of excitation energy transfer in which the spectra of the donor and acceptor molecules are unchanged. After excitation of the donor chromophore and prior to exciton transfer, the higher energy vibrational levels of the first excited state drop toward the lowest energy vibrational level of this excited state.[87] The transfer rate (k_T) between donor and acceptor is inversely proportional to the sixth power of the center-to-center distance (R) between donor and acceptor

$$k_T = \frac{9(\ln 10)\kappa^2\phi_D J}{128\pi^5 n^4 N R^6 \tau_D} \tag{14}$$

where κ^2 is the dipole orientation factor, ϕ_D is the quantum yield of the donor, J is a modified spectral overlap, N is Avogadro's number, and τ_D is the lifetime of the donor in the absence of the acceptor. Models are developed by using the X-ray crystallographic data for chromophore distances and orientations in eq 14.[88] This is the first time exciton transfer rates are calculated from X-ray data for any biliprotein, but as will be discussed, a refinement in the X-ray data has altered some results. It has been usual to assume that exciton transfer occurs by this mechanism for C-phycocyanin and that strong coupling is not a factor, but recent data may suggest a mixture of processes occurs on the trimer level and above. These calculations are performed for a series of phycocyanin aggregates, and very detailed models (see section F for details) are obtained for the flow of excitons among the various chromophore arrangements characteristic of each aggregate.

Other studies have been made to better understand the properties of phycocyanin using X-ray crystallographic and spectroscopic data.[89,90] It has also been shown that chemical changes in the bilin affect the conformation of the protein.[91]

Another important aspect in the spectroscopy of phycocyanin is the conformation of different bilins on the same aggregate. It has been shown by fluorescence polarization spectroscopy that the absorption spectrum of phycocyanin is composed of at least two spectrally distinct types of chromophores, and it is apparent from other experiments that the three bilins of each monomer are all in fact at different energies. Thus, the absorption spectrum of phycocyanin is composed of a combination of phycocyanobilins with different but overlapping spectra. Two functional distinct types of bilins are proposed, sensitizing and fluorescing. The sensitizing chromophores are at a higher energy and transfer excitons very efficiently to the fluorescing chromophores. Fluorescence polarization spectra[92,93] show that there is a difference in orientation between sensitizing and fluorescing chromophores of phycocyanin. Fluorescing chromophores are the fluorescence emitters in the isolated protein, but when part of a large photosynthetic system, they transfer energy to the next pigment in the chain. Similar fluorescence polarization studies have also been applied to the various phycocyanin aggregates.[94] The larger aggregates have lower polarizations due to excitation energy transfer among more chromophores.[94,95] In addition, the studies based on the X-ray crystallographic data have markedly changed the concept of sensitizing and fluorescing chromophores (section IV.F).

High-resolution spectroscopy has been performed on phycocyanin by using photochemical hole burning at temperatures as low as 5.3 K.[96] These experiments suggest the bilins are held in a rigid manner by the apoprotein. Resonance Raman spectra of phycocyanin show that noncovalent interactions with the apoprotein are needed to maintain the extended bilin conformation.[97,98] X-ray crystallographic data indicate which amino acid residues may be involved in this stabilization. Therefore, phycocyanin has chromophores that are held in an extended and rigid manner by the apoprotein, the chromophore conformations may vary with the aggregation state of the protein, the three unique bilins of each monomer have diverse apoprotein interactions and spectra, and excitation energy migration occurs through a series of steps that are specific to the aggregation state of the protein.

E. Picosecond Kinetics

1. Excitation Energy Transfer

It has been observed for some time that not all the chromophores on a biliprotein appear to emit fluorescence and that the apparent nonemitters transfer energy to the fluorescing chromophore. Even biliproteins, like phycocyanin, that structurally have only one type of chromophore have energetically nonidentical bilins and show excitation energy transfer from the highest to lowest energy bilin. Several studies of this energy migration process agree that the efficiency is quite high, occasionally at 99%. Such efficiencies will tend to make direct measurement of exciton transfer difficult, as well as making the transfer times quite fast. Nonetheless, the small amounts of fluorescence that do leak from these relatively nonfluorescent (sensitizing) bilins have now been measured on a time-resolved basis by various mode-locked laser systems.

2. Picosecond Kinetics of Phycocyanin

Biliproteins are fine objects for the development of picosecond laser methods on biological problems. These proteins are very water soluble without the need for detergents, and they are obtainable as discrete, homogeneous oligomers that have definite quantities of covalently attached chromophores. Of course, of most importance for the technique is their absorption spectra that perfectly overlap the wavelengths of the commonly available lasers. Nonetheless, it is the importance of these experiments in understanding the biological functions of the biliproteins that merits our first priority.

The best studied biliprotein by picosecond methods is phycocyanin.[46,89,99–105] All these reports agree on a particularly important facet of biliprotein research that they have measured exciton transfer from high (sensitizing) to low (fluorescing) energy bilins on isolated phycocyanin aggregates. In addition to this fast component (10–80 ps), there will always be a much longer lived decay at $\sim$0.9–2 ns, and this decay corresponds to the normal radiative emission from the fluorescing chromophores. Both these decays have been shown to be heterogeneous, and there have been reports of a third intermediate range of decays at 100–500 ps.[46,89,103,105] Together with measurements of fluorescence emission, picosecond studies have also been performed with time-resolved absorption and fluorescence polarization.

Phycocyanin aggregates (monomers, trimers, and hexamers) have been studied by picosecond methods to determine their relative efficiencies in excitation energy transfer.[89,99] In both studies, the rate of excitation transfer is fastest for hexamers and slowest for monomers. Fluorescence measurements yield lifetimes for sensitizing chromophores of 47–53 (monomers), 35 (trimers), and 10 ps (hexamers).[89] The fluorescence lifetimes for the fluorescing chromophores are 1.3 (monomers), 1.3 (trimers), and 1.8 ns (hexamers). The 10-ps lifetime for excitation energy transfer indicates that hexamers are arranged to be especially efficient in the energy-transfer process, and they possess important chromophore–chromophore pathways that are not found for monomers and trimers.

The phycocyanin trimer has been studied in meticulous detail by picosecond techniques.[46,99–101,103–105] It is found that phycocyanin trimers have a fast decay of 22 ps when they are attached to linker polypeptides, and this decay rises to 36 ps when the linker is removed.[46] This paper further shows that when the emission is examined at long wavelengths, a rise time is observed corresponding in time constant to the decay of the sensitizing chromophores. This is strong evidence supporting that excitation energy transfer from sensitizing to fluorescing chromophores is occurring on the picosecond time scale. Another study of trimeric phycocyanin, which uses polarizing optics, shows isotropic decay times at 120 and 1750 ps and an anisotropic decay at 50 ps.[100] Polarized picosecond fluorescence has also been used to study energy-transfer processes for monomers and trimers of phycocyanins.[101,104] A proposal has been made that a variability in chromophore arrangement can account for different transfer times in these experiments.[103] Slight differences in chromophore geometries can probably account for the different rates that are reported within the three classes of decay.

Monomers and separated subunits of phycocyanin have also been studied by picosecond methods.[102,104,105] With picosecond absorption, a fast component for monomers is found at 57 ps and for trimers it is 27 ps.[105] A kinetics model for excitation energy transfer in the rods and in rod-to-core processes has been developed for phycobilisomes.[106] These detailed calculations show that an excitation transfer time from sensitizing to fluorescing chromophores within phycocyanin hexamers of about 10 ps is consistent with measurements for the entire phycobilisome. Holzwarth has nicely reviewed the applications of picosecond kinetics to phycobilisomes and intact organisms.[1]

3. Picosecond Kinetics and Energy Migration of Cryptomonad Phycocyanins

A caveat should be offered at this point concerning the scope of this presentation. Thus far, the review has been almost totally restricted to a discussion of C-phycocyanin with occasional reference to allophycocyanin. Both these biliproteins are found in the phycobilisomes of blue-green and red algae. There is a third phylum that also contains biliproteins, the cryptomonads. Cryptomonads apparently do not contain phycobilisomes and, when isolated, their biliproteins are found to be aggregated exclusively as dimers. Two cryptomonad phycocyanins, phycocyanin 645 and

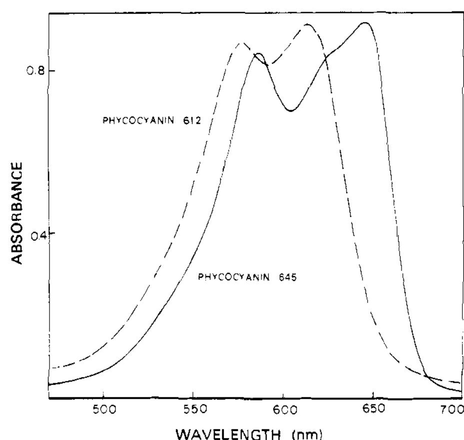

Figure 13. Absorption spectra of phycocyanin 612 (*Hemiselmis virescens*) and phycocyanin 645 (*Chroomonas* sp.). Spectra taken on samples in pH 6.0 buffer at room temperature.

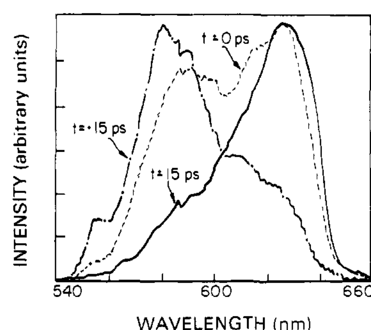

Figure 14. Time-resolved fluorescence spectra of phycocyanin 612 (*H. virescens*). Times are relative to a 30-ps excitation pulse. Samples in pH 6.0 buffer are excited at 532 nm by a mode-locked Nd^{3+}:YAG laser. The band with a maximum at 593 nm transfers energy in 7–10 ps to the fluorescing chromophores at 637 nm. The small band at 555 nm appears to transfer energy in much less than 7 ps to another sensitizing chromophore at 593 nm. Reprinted with permission from ref 109; copyright 1985 Elsevier Science Publishers B.V.

phycocyanin 612 (Figure 13), have been studied by picosecond kinetics. These phycocyanins are more complex than C-phycocyanin in their chromophore content. In addition to phycocyanobilin, phycocyanin 612 has cryptoviolin and phycocyanin 645 has cryptoviolin and the 697-nm bilin as chromophores.[107,108] An important aspect of this bilin content is that 532-nm light will tend to excite cryptoviolins, which have more higher energy absorption than phycocyanobilins. The properties of cryptomonad biliproteins have been reviewed by MacColl and Guard-Friar.[1]

A study on phycocyanin 612 is quite important in understanding the picosecond data for all biliproteins.[109] In these experiments, the actual fluorescence emission spectra of the picosecond transients are obtained instead of examining the decay of single wavelengths (Figure 14). Results show the spectrum of a sensitizing chromophore that decays leading to the appearance of the spectrum of a fluorescing chromophore. The decay time of the sensitizing chromophores corresponds very well with the rise time of the emitter. This result is excellent evidence that sensitizing chromophores transfer excitons to fluorescing chromophores on the picosecond time scale, and in this case the transfer time is 7–11 ps. Further analysis of the picosecond data for phycocyanin 612 is carried out in conjunction with spectral deconvolution of the absorption spectrum.[110] It is established that after excitation of the cryptoviolin, energy is transferred extremely rapidly (much faster than 7 ps) to the highest energy phycocyanobilin, and this phycocyanobilin then transfers this energy to the low-energy, fluorescing phycocyanobilins. The transfer between cryptoviolin and the highest energy phycocyanobilin is the first measurement of excitation energy transfer between two spectrally distinct sensitizing chromophores. The speed of this transfer argues for the utilization of even faster lasers on the femtosecond level.

These picosecond results give us information about three of the eight chromophores attached to a dimer of phycocyanin 612. However, the problem is greatly simplified if the protein consists of two virtually identical halves as its dimeric subunit structure may suggest. This possibility is investigated by preparing a tryptic digest of the protein.[111] Analysis of the digest by chromatography methods shows that there are three phycocyanobilin and one cryptoviolin peptides. The possibility that both halves of the dimer are essentially identical is supported, and the problem of assigning a function to eight chromophores is reduced by half. The picosecond kinetics experiments still leave one chromophore unassigned, and its function is deduced by a study of the CD spectra of the protein as a function of $KMnO_4$ and NaSCN concentrations.[111] These chemicals are selected because of their diverse mode of action in regard to the biliprotein: permanganate may serve to oxidize chromophores and thiocyanate is a chaotrope that may cause protein dissociation and unfolding. The results of these experiments show that the two lowest energy bilins are closely spaced and have excitation energy delocalized between them. One member of the pair is the final chromophore to be assigned. The primary sequence of events in the energy migration scheme after excitation of cryptoviolin is transfer of energy to the highest energy phycocyanobilin and then to the delocalized pair. Fluorescence emission occurs from the pair.

Phycocyanin 645 has also been investigated by picosecond methods.[99,112] Both of these studies report a fast-decaying component that is assigned to excitation energy transfer between sensitizing (high energy) and fluorescing (low energy) chromophores. This decay time is measured at 8 ps (absorption methods) and 15 ps (fluorescence methods).[112] A most important finding is that the 15-ps decay component depends on the excitation and emission wavelengths. When 569-nm excitation is used, the relative yield of this fast component drops from 54% at 585-nm emission to 2% at 620-nm emission. Excitation at 617 nm shows no 15-ps decay.[112] This is good support of the hypothesis that the higher energy bilins transfer excitons on the picosecond time scale to the fluorescing bilins. The lifetime of the fluorescent chromophore is 1.44 ns.[112] The efficiency of exciton transfer from the directly excited, high-energy bilin to the emitter is about 99%, assuming the same lifetime for the excited bilin in the absence of energy transfer. The 1.44-ns lifetime is found to be constant over the entire emission band. A third intermediate component of 360–680 ps is also found in the fluorescence study.

In discussing phycocyanin 645, it is necessary to point out that some of its chromophores appear to be strongly coupled and exhibit exciton delocalization within cer-

tain pairs of bilins.[113] Further analysis of this finding in conjunction with a Gaussian–Lorentzian spectral deconvolution of the absorption has led to a model for exciton flow through this protein.[114] This model incorporates both very weak coupling of dipoles between some bilins and delocalization of excitation between certain bilin pairs, and, in this regard, it resembles the model developed above for allophycocyanin trimers and phycocyanin 612 dimers. Excitation of cryptoviolins would lead to exciton transfer to the delocalized pair of bilins. In addition, there is another isolated bilin that will also, after direct excitation, transfer excitons to the delocalized pair. Fluorescence emission is viewed as emitting from these pairs of bilins.[114] An alternative viewpoint for the emitter is there is a group of dissimilar fluorescing chromophores that do not transfer energy among themselves.[112] With the assumption that the eight chromophores are, like those of phycocyanin 612, distributed in two essentially identical halves of the protein, the proposed energy migration scheme for the four chromophores covers the function of all the eight chromophores of the protein.[114]

F. Exciton Migration in Phycocyanin Aggregates

Each monomer of phycocyanin has two subunits and three chromophores. The α subunit has one chromophore covalently attached to a cysteine, which is residue 84 from the N terminus, and the β subunit has two chromophores attached to cysteines at positions 84 and 155.[115–117] The X-ray crystallographic results for phycocyanin provide excellent data on the distances and orientations between the chromophores within a monomer, trimer, and hexamer.[63–65] Since the rate of exciton transfer is extremely sensitive to distance (eq 14), careful analysis of each aggregation state and their varying of bilin-to-bilin distances is essential to understand the flow of excitons through the complex bilin matrix of a phycobilisome.

From the refined crystallographic data, the important center-to-center distances between bilins can be obtained.[65] Within the same monomer, these distances are as follows: α84 to β84, 5.0 nm; α84 to β155, 4.8 nm; β155 to β84, 3.4 nm. Within a trimer, a significantly closer approach is obtained for β84 and α84 on contiguous monomers of 2.1 nm. Within a hexamer, several routes for intertrimer transfer are opened. Examples of possible channels for exciton flow between trimers are as follows: β155 to β155, 2.9 nm; α84 to α84, 2.6 nm. This paper also gives the orientation factors for the various chromophore pairs. These authors point out that the flow of excitons between trimers may readily compete with the flow within a trimer, and they point to this as demonstrating the functional importance of the hexameric unit. In addition, this work establishes the geometry of the bilins, which are maintained by interaction between the nitrogens on the two central pyrroles and oxygen from an aspartate amino acid residue. These results show that the phycocyanins isolated from *Mastigocladus laminosus* and *Agmenellum quadruplicatum* are very similar.

The α84 chromophore is found to be intermediate in energy to the two chromophores on the β subunit. There is general agreement[65,88–90] that the β84 bilin may be the lowest energy chromophore and the β155 bilin the highest energy chromophore in phycocyanin. The

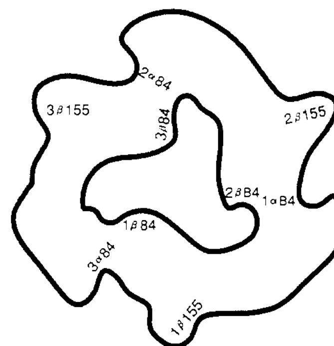

Figure 15. Schematic drawing of a phycocyanin trimer (*Mastigocladus laminosus*) and the various phycocyanobilins as observed by X-ray crystallography. 1α84 is the chromophore attached to the α polypeptide at residue 84 from the N terminus and is on monomer number 1. Note the close approach of α84 and β84 chromophores on contiguous monomers of the trimer.

initial basis for this assignment is the comparison of the flow of the excitation energy with the positions of the bilins in the three-dimensional structure obtained from the X-ray crystallographic data.[90] A method to assign a particular chromophore in the amino acid sequence to a particular spectrum from chemical modification data has also been introduced.[118] They note that only one cysteine residue of phycocyanin is free and not attached to a bilin. This cysteine is in the β subunit and from crystallographic data is located very close (0.4 nm) to β84 and much more distal to the other bilins. It is easy to accept that covalently binding *p*-(chloromercuri)benzenesulfonate to this single reactive cysteine will tend to affect only the spectrum of the neighboring bilin at β84. They perform this experiment on phycocyanin and both its subunits. The spectra are observed to change in the region of 620 nm upon binding of the probe. This is the spectral region of the fluorescing chromophore and establishes the assignment of β84 as this bilin. The assignment of the two remaining bilins proceeds straight away from the spectra of the subunits. The other β-subunit bilin (β155) absorbs at ~600 nm and is clearly the highest energy or sensitizing bilin, and the α84 bilin is, therefore, at 616–618 nm the intermediate-energy bilin of phycocyanin (see also section V.B.3). As we will develop later in this section, these assignments are correct for isolated monomers, but for trimers and larger aggregates the situation of α84 and β84 may change markedly. The resonance-enhanced coherent anti-Stokes Raman scattering spectra of treated trimers confirm that only one bilin is affected by *p*-(chloromercuri)benzenesulfonate.[119]

For monomers, only three chromophores are present and the routes of exciton transfer are best established. It is agreed that the β155 chromophore efficiently transfers energy to the β84 chromophore.[88,90] The transfer from β155 to α84 is much slower. They suggest that the fluorescence emission from monomers is a mixture of contributions from the β84 and α84 chromophores since little energy transfer seems to occur between chromophores on different subunits.[88]

For trimers, an important mode of exciton transfer is established between the α84 chromophore on one monomer and the β84 chromophore on a contiguous monomer (Figure 15). Two general categories of proposals have been put forward to explain exciton migration in trimers. The most commonly expressed idea is that very weak coupling of dipoles is the only mech-

anism in use. Later in this section, another idea is discussed wherein the β84 and α84 chromophores on contiguous monomers in the trimer are linked in a different way.

On going from trimers to hexamers, X-ray crystallographic data show that new avenues for exciton transfer open, and the rate of exciton migration between the two trimers of a hexamer should be quite fast. Picosecond kinetics studies have found these concepts to be correct, and the excitation transfer rates increase as phycocyanin aggregation increases. Using eq 14 and spectroscopic and X-ray crystallographic data, it is possible to calculate the rates for excitation energy transfer between any pair of bilins.[88,120] From these calculations, models for exciton migration through each phycocyanin aggregate are constructed. These models demonstrate that pathways using both sensitizing and fluorescing chromophores are established for exciton migration as the aggregation state is increased. On formation of hexamers, routes providing very fast exciton flow are opened between trimers. Bilins, which are sensitizing in the trimer, serve to transfer excitons to bilins in the contiguous trimer of a hexamer as do those that are fluorescing chromophores in trimers. This is true for the sensitizing β155 chromophores that in the hexamer very efficiently transfer excitons to either another β155 or to an α84 chromophore across the trimer-to-trimer contacts.

Originally, calculations were based on X-ray data that have since been corrected, and reevaluation of chromophore-to-chromophore interactions using the new positions and orientations of the bilins produces quite interesting proposals.[103,120] The new calculations show a much better interaction of α84 and β84 chromophores on adjacent monomers in trimers. In addition, Sauer and Scheer[120] have found that a better choice of refractive index also produces a significant increase in their calculated rate constants over their previous report. Using Förster theory, this closely spaced pair of bilins has the fastest transfer and should transfer excitons in <1 ps. These calculations show quite a large exciton interaction energy, 56 cm^{-1}, between the two bilins of this pair. A very important conclusion is, therefore, reached that the chromophores at α84 and β84 on adjacent monomers may share delocalized excitation. These authors[120] discuss data supporting this intriguing concept, and other experimental support for this idea comes from CD spectroscopy.[121] The lack of coincidence between the maximum in the CD spectrum (632 nm) and the absorption maximum (620 mm) together with urea denaturation data suggests a band-splitting contribution to the CD spectrum is quite reasonable. The chromophore at β155 would transfer excitons to the delocalized pair by very weak coupling of dipoles.[120] The transfer of energy between sensitizing and fluorescing chromophores of trimeric phycocyanin that is measured at 10–80 ps would be this transition from the bilin at β155 and not that between α84 and β84 chromophores. These latter chromophores would together form the fluorescing chromophores. This new concept of chromophore interaction for phycocyanin should be very carefully studied.

The variability in picosecond lifetimes is dependent on the excitation wavelength, possibly owing to microheterogeneity in chromophore position (section IV.E.2). Likewise, the long lifetime decay is variable due to the chromophore microheterogeneity. In addition to chromophore heterogeneity for a given aggregate, we suggest other possible causes of variable results in these experiments could be mixtures of aggregation states and heterogeneous populations of linkers for a given aggregation state. Trimers, for example, with different linkers attached will exhibit diverse properties. Nonetheless, since it has been found that the heterogeneity is confined to monomers and trimers and is almost abolished in hexamers,[89] it is probable that heterogeneity is not an important factor in energy-transfer processes in the phycobilisome.

A recent symposium volume has many excellent presentations on the energy-transfer processes in biliproteins.[125] Contributions on phycocyanin trimers support the ideas that the fastest of the three transitions measured by picosecond kinetics is the transfer of excitons from β155 to β84 and α84.[126,127] Another concept has also been projected in which the 10–80-ps measurement is not transfer between chromophores but perhaps is the relaxation between the upper and lower energy levels of the delocalized pair.[120] Some discussion has been given for the cause of the intermediate picosecond lifetime.[105]

Recently, a new method to study the bilins has begun.[122-124] Apophycocyanin is reacted with bilins and the products are investigated. This approach may provide excellent opportunities to address many major questions concerning biliproteins.

The hypothesis that excitation energy transfer occurs by two distinct methods—delocalization and very weak coupling of dipoles—in C-phycocyanin trimers is an important basis for further study of this biliprotein. In addition, the proposal that pairs of chromophores form a delocalized state is very similar to previous proposals for allophycocyanin (section IV.C) and cryptomonad phycocyanins (section IV.E.3). The spectral differences between C-phycocyanin and allophycocyanin may then be primarily a matter of the orientation and distance between chromophores in these delocalization pairs.

V. Surface Properties and Oriented Samples

A. Surface Studies

The study of biliproteins on surfaces can be grouped into three fields: black lipid membranes (BLM), biliproteins coated onto metal electrodes, and monolayer surface measurements. Two types of information are obtained from these experiments that are not commonly gleaned from other experiments. It is found that biliproteins have interesting photoelectric behaviors and that biliproteins have well-developed affinities for concentrating on lipid surfaces. In addition, some of these experiments are designed to study the properties of pigments in artificial membrane systems that may mimic certain aspects of the biological situation in the thylakoid membrane. The close association of the biliproteins with the thylakoid membrane, which contain the chlorophylls, makes the lipid studies quite relevant.

1. Black Lipid Membranes

The BLM system consists of a planar lipid bilayer that is coated over a small orifice in a Teflon cup. This

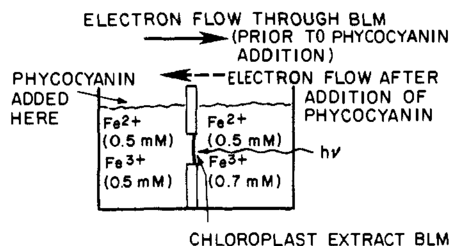

Figure 16. Schematic drawing of BLM experiment using chloroplast extract and phycocyanin. Reprinted with permission from ref 128; copyright 1976 Pergamon Press.

technique provides important advantages since very precise electrical measurements can be readily performed on membrane processes. The content of the solutions on either side of the bilayer can be varied and, most valuable for photobiology, irradiation light can be shined on the orifice. A review covering the major technical details of forming the bilayer and performing the electrical measurements is available,[128] and this presentation will center on the activity of biliproteins in this system.

BLM are constructed either with chloroplast extracts or with specific lipids, carotenoids, and chlorophyll *a*. A redox potential is established across this bilayer by asymmetric concentrations of such ions as ferrous and ferric or cerous and ceric. The effectiveness of phycocyanin to modify the electron flow across these BLM has been studied.[129-133] The activity of phycocyanin in these artificial membranes is probably caused by its association with the lipid surface. The phycocyanin is added to the aqueous solution on the side of the membrane opposite from which the light will be shining (Figure 16). The photoelectric spectrum of the BLM reflects the absorption spectrum of the phycocyanins. In addition, a specific interaction between ferric ion and phycocyanin is demonstrated by fluorescence measurements, and this complex formation may influence the protein's participation in BLM processes.[134]

The activity of phycocyanin in the BLM is divided into two properties, a modification of intensity and an alteration in the direction of electron flow.[130,131] In the absence of phycocyanin, the chlorophyll-containing BLM show a photovoltage across the membrane when the membrane is irradiated. The effect of phycocyanin is to increase the photovoltage (Figure 17). A typical experiment might yield a doubling of the photoresponse upon phycocyanin addition. In order to observe the second phycocyanin-induced activity, a change in the direction of electron flow, careful attention must be given to experimental detail. A ferric/ferrous concentration cell is established and the side to which phycocyanin is to be added must be minimally oxidizing. Before the biliprotein is added, the appropriate BLM will have photovoltages of 2 mV or less, and electrons will flow from the side on which phycocyanin will be placed. Phycocyanin is then added. In many cases, the flow of electrons is observed to reverse. It is proposed that phycocyanin modifies the energy barrier on its side of the BLM so that the movement of an electron over that barrier is made more probable. Prior to the addition of phycocyanin, the electron flow acts to dissipate a preexisting redox gradient. This is no longer the case, and the new process resembles the storage of energy that is characteristic of photosynthesis.

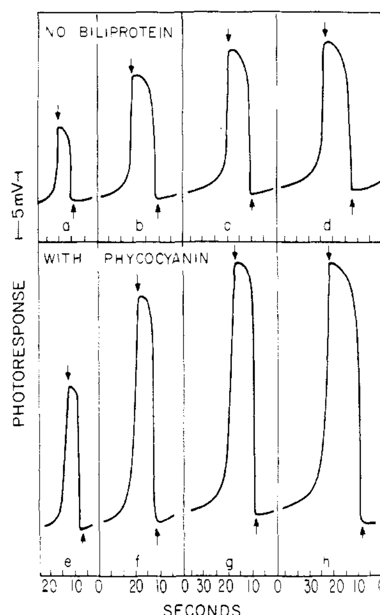

Figure 17. Selection of chlorophyll-containing BLM experiments stimulating a photovoltage with and without phycocyanin (*P. calothricoides*). The arrow indicates light on (↑) or off (↓). Panels a–d are various membranes arranged in order of increasing dark resistances and, therefore, larger stimulated photovoltages; panels e–h show the corresponding enhancement of the photovoltages of each by the action of phycocyanin.

Additional experiments on BLM and phycocyanin have been carried out by using other components of the biological system.[132,133] It is found that BLM composed of a specific mixture of lecithin/chlorophyll *a*/carotene show the same phycocyanin sensitivity as membranes that are formed from chloroplast extract. The copper-containing protein, plastocyanin, is also found to effect the photoresponse of BLM in the opposite sense as does phycocyanin.[132] Plastocyanin, therefore, facilitates electron uptake from the aqueous medium into the membrane while phycocyanin does the reverse. Following the phycocyanin situation, a variety of oxidants and reductants have been tested in similar BLM situations and found to effect the photovoltage.[133] It is, perhaps, a possibility that phycocyanin may function biologically using this electron-directing capability.

2. Biliproteins Coated on Metal Electrodes

Platinum electrodes can be coated by a layer of a biliprotein.[135] These coated electrodes are submerged in certain electrolyte solutions and irradiated. During irradiation a negative potential exists that disappears in the dark. These experiments, like those in the section immediately above, could be implicating phycocyanin in a not yet discovered biological function.

Another approach in this area is to place a layer of biliprotein solution between two electrodes, one a semiconductor and the other metallic.[136] The semiconductor is a transparent electrode of SnO_2, which allows irradiation, and the metallic electrode is gold. A photoresponse is observed during irradiation of the sample that is reversible in the dark. Quite different results for the potentials are obtained for biliproteins depending on whether they are dissolved in H_2O or D_2O. The effects of D_2O on phycocyanin aggregation have been discussed earlier (section III.D).

3. *Phycocyanin and Monolayers*

Surface pressure measurements have been made on solutions of phycocyanin in both the presence and absence of a lipid monolayer.[137,138] Changes in surface pressure indicate that phycocyanin is strongly attracted to an air–water interface. If the surface is covered with a monolayer of lipid, the surface activity of phycocyanin is increased over that at an air–water interface. The lipids used are dipalmitoylphosphatidylcholine and monogalactosyldiglycerol, and the interaction with the latter is preferred. These monolayer experiments together with the BLM results develop the concept that phycocyanin has an affinity for lipid membranes.

B. Oriented Samples

1. *Polyvinyl Films*

Biliproteins have been incorporated into poly(vinyl alcohol) films and a variety of spectra recorded.[139–145] Particular emphasis in these studies is placed on the photoacoustic spectroscopy of relaxed and stretched films. Mixtures of phycoerythrins and phycocyanins have been studied.[139–141] Photoacoustic spectroscopy has been used in these systems to study excitation energy transfer. For phycoerythrins, different yields of energy transfer are found for the different chromophores on an aggregate.

In addition, these techniques have been used to study allophycocyanin and phycocyanin–allophycocyanin mixtures.[145] When the poly(vinyl alcohol) films are stretched, the spectral changes are different for the solution of both allophycocyanin and phycocyanin than for the individual biliproteins. They propose that aggregates of the two biliproteins are formed and that they can be better oriented in the film than either biliprotein alone.

2. *Electric Field and Gel-Squeezing Orientation*

Linear dichroism studies have been performed on biliprotein samples oriented by either pulsed electric fields or gel-squeezing methods.[146,147] The pulsed electric field method relies on the tendency of particles to orient with their largest dimension parallel to the applied electric field. Conversely, when polyacrylamide gels are squeezed the largest dimension of a particle will tend to align perpendicular to the applied forces. Linear dichroism is used to study these oriented samples and is described by

$$\Delta A = A_{\parallel} - A_{\perp} \tag{15}$$

where $A_{\parallel}$ is the absorbance when the light beam is parallel to the direction of the applied electric field or parallel to the applied force during the squeezing of the gels, and $A_{\perp}$ is when the light beam is perpendicular. When these techniques are applied to a mixture of phycoerythrin and phycocyanin, the 620-nm absorbance, which corresponds to a phycocyanin transition, tends to be oriented within the planes of the disks.[146] Linear dichroism studies on a phycocyanin aggregate in 10% gelatin gels show a minimum at 598 nm, a weaker minimum at 560 nm, and a positive band at 631 nm. The 631-nm band is assigned to the lower energy bilin on the β subunit of the trimer, and the 598-nm band is assigned to the β155 bilin.[147] These linear dichroism results have been discussed as evidence for exciton delocalization in phycocyanin.[120]

3. *Spectroscopy on Crystals*

Thin transparent single crystals of phycocyanin have been studied by spectroscopic methods.[148] The crystals are dichroic as the position of the absorption maximum shifts between 612 and 626 nm depending on whether the light is parallel or perpendicular. The fluorescence polarization spectrum of the crystals has also been obtained. The β84 chromophore is found to be the lower energy absorber on the β subunit (section IV.F).

The absorption spectra of phycocyanin in solution and in crystals are similar, but some differences appear in the high-energy side of the band. Some analysis should be given to whether or not this small change in absorbance reflects on differences in relative chromophore positions or orientations in crystals versus solution.

VI. Epilogue

For phycocyanin, its ability to form discrete aggregation states has dominated the ways research has been applied. From the development of the analytical ultracentrifuge to the applications of the most advanced laser systems, the key to phycocyanin lies in its aggregation, a process that is well exploited and well studied, but still only partially understood. A number of diverse experiments point to the particular importance of the hexameric aggregation state. Recently, two additional techniques—small-angle neutron scattering and high-pressure size-exclusion liquid chromatography—have been applied in a very detailed study of phycocyanin aggregation. These experiments show phycocyanin solutions can be prepared as mixtures of monomer, trimer, and hexamer aggregates that exist in a reversible equilibrium. An increase in protein concentration produces an increase in aggregation. No evidence for a dimer is obtained. At pH 5.7 and a protein concentration of 1–10 mg/mL, phycocyanin is predominantly a hexamer. At pH 8.0 and a protein concentration of 5 mg/mL, phycocyanin is mostly trimeric. Monomers are produced at low protein concentration. In addition to protein concentration, pH, and ionic strength, the composition of the buffer is found to alter the distribution of aggregates.[149] These results should be compared to those discussed previously (section II.A).

In this review, two topics of high current interest are covered. The first involves efforts to understand the photobiology of phycocyanin from the X-ray crystallographic results showing the positions and geometries of the bilins. Efforts to interpret these findings to better explore the excitation energy transfer properties of the pigment are yielding important new understandings. As the high-resolution crystal structures of other biliproteins are forthcoming, this process will continue. The crystal structure of allophycocyanin may reveal very important aspects of the excitation energy migration processes. It is not possible to estimate how far in the future such revelations may be. For the present, the X-ray crystallographic results on phycocyanin can be used with caution as a model for other biliproteins. For example, a comparison of the amino acid sequence of the cryptomonad phycocyanin 645 to this crystal structure has suggested a rationale for the

quaternary structural differences between the two proteins.[150]

A second field of strong current interest is fast kinetics. With the use of mainly fluorescence and some absorbance methods, a steady level of progress has been established over the past several years. The actual measurement of exciton transfer between two bilins of known energy is now possible. This field of research is linked with technological advances in lasers.

VII. Literature Citations

(1) MacColl, R.; Guard-Friar, G. *Phycobiliproteins*; CRC Press: Boca Raton, FL, 1987. Scheer, H. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 241–261. Zilinskas, B. A.; Greenwald, L. S. *Photosynth. Res.* **1986**, *10*, 7–35. Holzwarth, A. R. *Photochem. Photobiol.* **1986**, *43*, 707–725. Zuber, H. *Photochem. Photobiol.* **1985**, *42*, 821–844.
(2) Svedberg, T.; Lewis, N. B. *J. Am. Chem. Soc.* **1928**, *50*, 525–536.
(3) Svedberg, T.; Katsurai, T. *J. Am. Chem. Soc.* **1929**, *51*, 3573–3583.
(4) Svedberg, T.; Eriksson, I.-B. *J. Am. Chem. Soc.* **1932**, *54*, 3998–4010.
(5) Eriksson-Quensel, I.-B. *Biochem. J.* **1938**, *32*, 585–589.
(6) Scott, E.; Berns, D. S. *Biochemistry* **1965**, *4*, 2597–2605.
(7) Berns, D. S.; Morgenstern, A. *Biochemistry* **1966**, *5*, 2985–2990.
(8) Lee, J. J.; Berns, D. S. *Biochem. J.* **1968**, *110*, 457–464.
(9) Kao, O.; Berns, D. S.; MacColl, R. *Eur. J. Biochem.* **1971**, *19*, 595–599.
(10) MacColl, R.; Lee, J. J.; Berns, D. S. *Biochem. J.* **1971**, *122*, 421–426.
(11) MacColl, R.; Berns, D. S. *Isr. J. Chem.* **1981**, *21*, 296–300.
(12) Huang, C.; Berns, D. S. *Biochemistry* **1981**, *20*, 7016–7021.
(13) Saito, T.; Iso, N.; Mizuno, H. *Bull. Chem. Soc. Jpn.* **1974**, *47*, 1375–1379.
(14) Kotera, A.; Saito, T.; Iso, N.; Mizuno, H.; Taki, N. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 1176–1179.
(15) Mizuno, H.; Saito, T.; Iso, N. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 3496–3499.
(16) Iso, N.; Mizuno, H.; Saito, T.; Nitta, N.; Yoshizaki, K. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2892–2895.
(17) Saito, T.; Iso, N.; Mizuno, H.; Kitamura, I. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 3471–3474.
(18) Saito, T.; Iso, N.; Mizuno, H.; Ozeki, F.; Matsui, Y. *Bull. Chem. Soc. Jpn.* **1980**, *53*, 599–602.
(19) Neufeld, G. J; Riggs, A. F. *Biochim. Biophys. Acta* **1969**, *181*, 234–243.
(20) Berns, D. S. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 65–73.
(21) Kato, M.; Lee, W. I.; Eichinger, B. E.; Schurr, J. M. *Biopolymers* **1974**, *13*, 2293–2304.
(22) Davis, L. C.; Radke, G. A.; Guikema, J. A. *J. Liq. Chromatogr.* **1986**, *9*, 1277–1295.
(23) Murphy, R. F.; O'Carra, P. *Biochim. Biophys. Acta* **1970**, *214*, 371–373.
(24) Glazer, A. N.; Fang, S. *J. Biol. Chem.* **1973**, *248*, 663–671.
(25) MacColl, R.; Berns, D. S.; Koven, N. L. *Arch. Biochem. Biophys.* **1971**, *146*, 477–482.
(26) MacColl, R. *Arch. Biochem. Biophys.* **1983**, *223*, 24–32.
(27) MacColl, R.; Berns, D. S. *Arch. Biochem. Biophys.* **1973**, *156*, 161–167.
(28) Chen, C.-H.; MacColl, R.; Berns, D. S. *Arch. Biochem. Biophys.* **1974**, *165*, 554–559.
(29) Chen, C.-H.; Berns, D. S. *J. Phys. Chem.* **1977**, *81*, 125–129.
(30) Chen, C.-H.; Berns, D. S. *J. Phys. Chem.* **1978**, *82*, 2781–2786.
(31) Chen, C.-H. *J. Phys. Chem.* **1980**, *84*, 2050–2053.
(32) Berns, D. S.; Scott, E. *Biochemistry* **1966**, *5*, 1528–1533.
(33) Kao, O. H. W.; Berns, D. S.; Town, W. R. *Biochem. J.* **1973**, *131*, 39–50.
(34) MacColl, R.; Edwards, M. R.; Mulks, M. H.; Berns, D. S. *Biochem. J.* **1974**, *141*, 419–425.
(35) Kao, O. H. W.; Edwards, M. R.; Berns, D. S. *Biochem. J.* **1975**, *147*, 63–70.
(36) Kao, O. H. W.; Berns, D. S. *Can. J. Microbiol.* **1977**, *23*, 510–517.
(37) Chen, C.-H.; Berns, D. S. *Biophys. Chem.* **1978**, *8*, 203–213.
(38) Adams, S. M.; Kao, O. H. W.; Berns, D. S. *Plant Physiol.* **1979**, *64*, 525–527.
(39) Gantt, E.; Conti, S. F. *Brookhaven Symp. Biol.* **1966**, *19*, 393–405.
(40) Gantt, E.; Lipschultz, C. A. *Biochim. Biophys. Acta* **1973**, *292*, 858–861.
(41) Gantt, E.; Lipschultz, C. A.; Zilinskas, B. *Biochim. Biophys. Acta* **1976**, *430*, 375–388.
(42) Gantt, E. *Annu. Rev. Plant Physiol.* **1981**, *32*, 327–347.
(43) Tandeau de Marsac, N.; Cohen-Bazire, G. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1635–1639.
(44) Yu, M.-H.; Glazer, A. N.; Williams, R. C. *J. Biol. Chem.* **1981**, *256*, 13130–13136.
(45) Watson, B. A.; Waaland, S. D.; Waaland, J. R. *Biochemistry* **1986**, *25*, 4583–4587.
(46) Wendler, J.; John, W.; Scheer, H.; Holzwarth, A. R. *Photochem. Photobiol.* **1986**, *44*, 79–85.
(47) Kume, N.; Isono, T.; Katoh, T. *Photobiochem. Photobiophys.* **1982**, *4*, 25–37.
(48) Berns, D. S.; Crespi, H. L.; Katz, J. J. *J. Am. Chem. Soc.* **1962**, *84*, 496–497.
(49) Berns, D. S.; Crespi, H. L.; Katz, J. J. *J. Am. Chem. Soc.* **1963**, *85*, 8–14.
(50) Berns, D. S. *J. Am. Chem. Soc.* **1963**, *85*, 1676–1678.
(51) Hattori, A.; Crespi, H. L.; Katz, J. J. *Biochemistry* **1965**, *4*, 1213–1225.
(52) Chen, C.-H.; Liu, I.-W.; MacColl, R.; Berns, D. S. *Biopolymers* **1983**, *22*, 1223–1233.
(53) Chen, C.-H.; Kao, O. H. W.; Berns, D. S. *Biophys. Chem.* **1977**, *7*, 81–86.
(54) Hattori, A.; Crespi, H. L.; Katz, J. J. *Biochemistry* **1965**, *4*, 1225–1238.
(55) Scott, E.; Berns, D. S. *Biochemistry* **1967**, *6*, 1327–1334.
(56) Lee, J. J.; Berns, D. S. *Biochem. J.* **1968**, *110*, 465–470.
(57) Berns, D. S.; Lee, J. J.; Scott, E. *Adv. Chem. Ser.* **1968**, *84*, 21–30.
(58) Berns, D. S. *Biochemistry* **1963**, *2*, 1377–1380.
(59) Chen, C.-H.; Tow, F.; Berns, D. S. *Biopolymers* **1984**, *23*, 887–896.
(60) Bialek-Bylka, G. E. *Stud. Biophys.* **1985**, *105*, 129–133.
(61) Kresheck, G. C.; Schneider, H.; Scheraga, H. A. *J. Phys. Chem.* **1965**, *69*, 3132–3144.
(62) Emerson, M. F.; Holtzer, A. *J. Phys. Chem.* **1967**, *71*, 3320–3330.
(63) Schirmer, T.; Bode, W.; Huber, R.; Sidler, W.; Zuber, H. *J. Mol. Biol.* **1985**, *184*, 257–277.
(64) Schirmer, T.; Huber, R.; Schneider, M.; Bode, W.; Miller, M.; Hackert, M. L. *J. Mol. Biol.* **1986**, *188*, 651–676.
(65) Schirmer, T.; Bode, W.; Huber, R. *J. Mol. Biol.* **1987**, *196*, 677–695.
(66) Mörschel, E.; Koller, K.-P.; Wehrmeyer, W. *Arch. Microbiol.* **1980**, *125*, 43–51.
(67) Brown, S. B.; Holroyd, J. A.; Troxler, R. F.; Offner, G. D. *Biochem. J.* **1981**, *194*, 137–147.
(68) Rosenberg, R. M.; Crespi, H. L.; Katz, J. J. *Biochim. Biophys. Acta* **1969**, *175*, 31–40.
(69) Scheer, H.; Kufer, W. *Z. Naturforsch.* **1977**, *32c*, 513–519.
(70) Ohki, K.; Fujita, Y. *Plant Cell Physiol.* **1979**, *20*, 483–490.
(71) Ohki, K.; Fujita, Y. *Plant Cell Physiol.* **1979**, *20*, 1341–1347.
(72) De Kok, J.; Braslavsky, S. E.; Spruit, C. J. P. *Photochem. Photobiol.* **1981**, *34*, 705–710.
(73) De Kok, J. *Photochem. Photobiol.* **1982**, *35*, 849–851.
(74) Berns, D. S.; Morgenstern, A. *Arch. Biochem. Biophys.* **1968**, *123*, 640–642.
(75) Erokhina, L. G.; Krasnovskii, A. A. *Mol. Biol.* **1974**, *8*, 651–659.
(76) Brown, A. S.; Foster, J. A.; Voynow, P. V.; Franzblau, C.; Toxler, R. F. *Biochemistry* **1975**, *14*, 3581–3588.
(77) MacColl, R.; Csatorday, K.; Berns, D. S.; Traeger, E. *Biochemistry* **1980**, *19*, 2817–2820.
(78) Ohad, I.; Schneider, H.-J. A. W.; Gendel, S.; Bogorad, L. *Plant Physiol.* **1980**, *65*, 6–12.
(79) MacColl, R.; Csatorday, K.; Berns, D. S.; Traeger, E. *Arch. Biochem. Biophys.* **1981**, *208*, 42–48.
(80) Csatorday, K.; MacColl, R.; Csizmadia, V.; Grabowski, J.; Bagyinka, C. *Biochemistry* **1984**, *23*, 6466–6470.
(81) Huang, C.; Berns, D. S.; MacColl, R. *Biochemistry* **1987**, *26*, 243–245.
(82) Murakami, A.; Mimuro, M.; Ohki, K.; Fujita, Y. *J. Biochem.* **1981**, *89*, 79–86.
(83) Mimuro, M.; Murakami, A.; Fujita, Y. *Arch. Biochem. Biophys.* **1982**, *215*, 266–273.
(84) Sugimoto, T.; Kikushima, M.; Saito, M.; Suzuki, H. *J. Phys. Soc. Jpn.* **1984**, *53*, 874–881.
(85) Yeh, S. W.; Glazer, A. N.; Clark, J. H. *J. Phys. Chem.* **1986**, *90*, 4578–4580.
(86) Füglistaller, P.; Mimuro, M.; Suter, F.; Zuber, H. *Biol. Chem. Hoppe-Seyler* **1987**, *368*, 353–367.
(87) Förster, T. *Discuss. Faraday Soc.* **1959**, *27*, 7–17.
(88) Sauer, K.; Scheer, H.; Sauer, P. *Photochem. Photobiol.* **1987**, *46*, 427–440.
(89) Holzwarth, A. R.; Wendler, J.; Suter, G. W. *Biophys. J.* **1987**, *51*, 1–12.
(90) Mimuro, M.; Füglistaller, P.; Rümbeli, R.; Zuber, H. *Biochim. Biophys. Acta* **1986**, *848*, 155–166.
(91) Mimuro, M.; Rümbeli, R.; Füglistaller, P.; Zuber, H. *Biochim. Biophys. Acta* **1986**, *851*, 447–456.

(92) Dale, R. E.; Teale, F. W. J. *Photochem. Photobiol.* **1970**, *12*, 99–117.
(93) Teale, F. W. J.; Dale, R. E. *Biochem. J.* **1970**, *116*, 161–169.
(94) Vernotte, C. *Photochem. Photobiol.* **1971**, *14*, 163–173.
(95) Goedheer, J. C.; Birnie, F. *Biochim. Biophys. Acta* **1965**, *94*, 579–581.
(96) Friedrich, J.; Scheer, H.; Zickendraht-Wendelstadt, B.; Haarer, D. *J. Am. Chem. Soc.* **1981**, *103*, 1030–1035.
(97) Szalontai, B.; Gombos, Z.; Csizmadia, V. *Biochem. Biophys. Res. Commun.* **1985**, *130*, 358–363.
(98) Szalontai, B.; Gombos, Z.; Csizmadia, V.; Lutz, M. *Biochim. Biophys. Acta* **1987**, *893*, 296–304.
(99) Kobayashi, T.; Degenkolb, E. O.; Bersohn, R.; Rentzepis, P. M.; MacColl, R.; Berns, D. S. *Biochemistry* **1979**, *18*, 5073–5078.
(100) Hefferle, P.; Nies, M.; Wehrmeyer, W.; Schneider, S. *Photobiochem. Photobiophys.* **1983**, *5*, 325–334.
(101) Hefferle, P.; John, W.; Scheer, H.; Schneider, S. *Photochem. Photobiol.* **1984**, *39*, 221–232.
(102) Switalski, S. C.; Sauer, K. *Photochem. Photobiol.* **1984**, *40*, 423–427.
(103) Schneider, S.; Geiselhart, P.; Siebzehnrübl, S.; Fischer, R.; Scheer, H. *Z. Naturforsch.* **1988**, *43c*, 55–62.
(104) Hefferle, P.; Geiselhart, P.; Mendl, T.; Schneider, S.; John, W.; Scheer, H. *Z. Naturforsch.* **1984**, *39c*, 606–616.
(105) Sandström, A.; Gillbro, T.; Sundström, V.; Fischer, R.; Scheer, H. *Biochim. Biophys. Acta* **1988**, *933*, 42–53.
(106) Suter, G. W.; Holzwarth, A. R. *Biophys. J.* **1987**, *52*, 673–683.
(107) MacColl, R.; Guard-Friar, D. *Biochemistry* **1983**, *22*, 5568–5572.
(108) MacColl, R.; Guard-Friar, D. *J. Biol. Chem.* **1983**, *258*, 14327–14329.
(109) Hanzlik, C. A.; Hancock, L. E.; Knox, R. S.; Guard-Friar, D.; MacColl, R. *J. Lumin.* **1985**, *34*, 99–106.
(110) Csatorday, K.; MacColl, R.; Guard-Friar, D.; Hanzlik, C. A. *Photochem. Photobiol.* **1987**, *45*, 845–848.
(111) MacColl, R.; Guard-Friar, D.; Ryan, T. J.; Csatorday, K.; Wu, P. *Biochim. Biophys. Acta* **1988**, *934*, 275–281.
(112) Holzwarth, A. R.; Wendler, J.; Wehrmeyer, W. *Biochim. Biophys. Acta* **1983**, *724*, 388–395.
(113) Jung, J. J.; Song, P.-S.; Paxton, R. J.; Edelstein, M. S.; Swanson, R.; Hazen, E. E., Jr. *Biochemistry* **1980**, *19*, 24–32.
(114) Csatorday, K.; Guard-Friar, D.; MacColl, R.; Berns, D. S. *Photochem. Photobiol.* **1988**, *47*, 285–291.
(115) Frank, G.; Sidler, W.; Widmer, H.; Zuber, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 1491–1507.
(116) Offner, G. D.; Brown-Mason, A. S.; Ehrhardt, M. M.; Troxler, R. F. *J. Biol. Chem.* **1981**, *256*, 12167–12175.
(117) Troxler, R. F.; Ehrhardt, M. M.; Brown-Mason, A. S.; Offner, G. D. *J. Biol. Chem.* **1981**, *256*, 12176–12184.
(118) Siebzehnrübl, S.; Fischer, R.; Scheer, H. *Z. Naturforsch.* **1987**, *42c*, 258–262.
(119) Schneider, S.; Baumann, F.; Klüter, U. *Z. Naturforsch.* **1987**, *42c*, 1269–1274.
(120) Sauer, K.; Scheer, H. *Biochim. Biophys. Acta* **1988**, *936*, 157–170.
(121) Lehner, H.; Scheer, H. *Z. Naturforsch.* **1983**, *38c*, 353–358.
(122) Arciero, D. M.; Bryant, D. A.; Glazer, A. N. *J. Biol. Chem.* **1988**, *263*, 18343–18349.
(123) Arciero, D. M.; Dallas, J. L.; Glazer, A. N. *J. Biol. Chem.* **1988**, *263*, 18350–18357.
(124) Arciero, D. M.; Dallas, J. L.; Glazer, A. N. *J. Biol. Chem.* **1988**, *263*, 18358–18363.
(125) Scheer, H.; Schneider, S., Ed. *Photosynthetic Light-Harvesting Systems, Organization and Function*; Walter de Gruyter: Berlin, 1988.
(126) Gillbro, T.; Sandström, A.; Sundström, V.; Fischer, R.; Scheer, H. *Photosynthetic Light-Harvesting Systems, Organization and Function*; Walter de Gruyter: Berlin, 1988; pp 457–467.
(127) Sauer, K.; Scheer, H. *Photosynthetic Light-Harvesting Systems, Organization and Function*; Walter de Gruyter: Berlin, 1988; pp 507–511.
(128) Berns, D. S. *Photochem. Photobiol.* **1976**, *24*, 117–139.
(129) Ilani, A.; Berns, D. S. *J. Membr. Biol.* **1972**, *8*, 333–356.
(130) Ilani, A.; Berns, D. S. *Biophysik* **1973**, *9*, 209–224.
(131) Chen, C.-H.; Berns, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3407–3411.
(132) Chen, S. S.; Berns, D. S. *J. Membr. Biol.* **1979**, *47*, 113–127.
(133) Alexandrowicz, G.; Berns, D. S. *Photobiochem. Photobiophys.* **1980**, *1*, 353–360.
(134) Ilani, A.; Berns, D. S. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 1423–1430.
(135) Evstigneev, V. B.; Bekasova, O. D. *Biofizika* **1970**, *15*, 807–815.
(136) Frackowiak, D.; Erokhina, L. G.; Jadzyn, Cz.; Shubin, L. M.; Shkuropatov, A. Ya. *Photosynthetica* **1981**, *15*, 36–48.
(137) Almog, R.; Berns, D. S. *J. Colloid Interface Sci.* **1983**, *91*, 448–453.
(138) Almog, R.; Marsilio, F.; Berns, D. S. *Arch. Biochem. Biophys.* **1988**, *260*, 28–36.
(139) Frackowiak, D.; Hotchandani, S.; Fiksinski, K.; Leblanc, R. M. *Photosynthetica* **1983**, *17*, 456–459.
(140) Frackowiak, D.; Hotchandani, S.; Leblanc, R. M. *Photochem. Photobiol.* **1985**, *42*, 559–565.
(141) Frackowiak, D.; Hotchandani, S.; Bialek-Bylka, G.; Leblanc, R. M. *Photochem. Photobiol.* **1985**, *42*, 567–572.
(142) Frackowiak, D.; Erokhina, L. G.; Balter, A.; Lorrain, L.; Szurkowski, J.; Szych, B. *Biochim. Biophys. Acta* **1986**, *851*, 173–180.
(143) Frackowiak, D.; Gantt, E.; Hotchandani, S.; Lipschultz, C. A.; Leblanc, R. M. *Photochem. Photobiol.* **1986**, *43*, 335–337.
(144) Frackowiak, D.; Erokhina, L. G.; Picard, G.; Leblanc, R. M. *Photochem. Photobiol.* **1987**, *46*, 227–285.
(145) Romanowski, M.; Erokhina, L. G.; Frackowiak, D.; Leblanc, R. M. *Photosynthetica* **1987**, *21*, 71–75.
(146) Gagliano, A. G.; Hoarau, J.; Breton, J.; Geacintov, N. E. *Biochim. Biophys. Acta* **1985**, *808*, 455–463.
(147) Juszczak, L.; Geacintov, N. E.; Zilinskas, B. A.; Brenton, J. *Photosynthetic Light-Harvesting Systems, Organization and Function*; Walter de Gruyter: Berlin, 1988; pp 281–292.
(148) Schirmer, T.; Vincent, M. G. *Biochim. Biophys. Acta* **1987**, *893*, 379–385.
(149) Saxena, A. M. *J. Mol. Biol.* **1988**, *200*, 579–591.
(150) Sidler, W.; Zuber, H. *Photosynthetic Light-Harvesting Systems, Organization and Function*; Walter de Gruyter, Berlin, 1988; pp 49–60.