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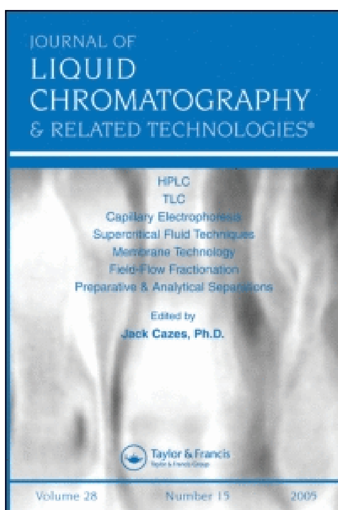

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JOURNAL OF LIQUID CHROMATOGRAPHY, 9(6), 1277-1295 (1986)

CHARACTERIZATION OF ASSOCIATIVE PROPERTIES OF PHYCOBILIPROTEIN PREPARATIONS FROM *ANACYSTIS NIDULANS* R_2 BY DIRECT SCANNING GEL CHROMATOGRAPHY

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ABSTRACT

The effects of salt concentration and pH on biliprotein self-association were examined using polyacrylamide gel electrophoresis and direct scanning gel filtration. Fractions containing different relative concentrations of C-phycocyanin, allophycocyanin, and linker proteins were prepared from Anacystis nidulans R_2. Four linker proteins and three pigmented protein bands were identified by gel electrophoresis. The migration rates of biliproteins were monitored in gel filtration columns using a direct scanning system. High salt concentrations and low pH enhanced the biliprotein migration rates, consistent with an increased self association under these conditions. The increased migration rates were greater in preparations containing larger amounts of linker peptides, indicating a role for these peptides in stabilizing the biliprotein aggregates. The profiles obtained from direct column scanning suggest that a rapid equilibrium exists between monomer and hexamer of C-phycocyanin in the absence of linker proteins.

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INTRODUCTION

Direct scanning gel filtration chromatography is a technique originally developed in Acker's laboratory (1) and is presently being actively used in this laboratory (2-6). Earlier systems permitted scanning of a Sephadex column at only one wavelength. We have modified the scanner to scan at multiple wavelengths; thus far, we use only two at a time. In common with other dual wavelength techniques, this allows one to distinguish spectrally observable states or components in a reacting system. The model system to which we have applied dual wavelength scanning is the association of phycocyanins to form larger aggregates which ultimately yield the phycobilisome (PBS), the light-harvesting pigment complex of cyanobacteria.

The association properties of phycocyanins were studied by Svedberg many years ago (7,8) and have been pursued by a number of groups. Glazer, in a series of recent reviews (9-11) has presented a model for phycobilisome assembly in which specific colorless linker proteins hold complexes of one or more phycocyanins together. The exact nature of the phycocyanins and the structure of the phycobilisomes is species dependent. We have worked with Anacystis nidulans R_2 which is very similar to Glazer's Synechococcus 6301 but which is somewhat better genetically characterized.

The colorless linker proteins were not identified until about a decade ago and most preparations of phycocyanins studied prior to that time were contaminated with linkers. It is clear that linkers are essential for stable assembly of higher order structures (11). The main question that we wish to address is whether there is an obligatory order of assembly of the simplest stable phycocyanin aggregate that had been previously identified, the hexamer.

Glazer (10) has proposed that interaction of a colorless linker protein with monomers of C-phycocyanin (C-PC) mediates the assembly into hexamers, via trimers. We have evidence, briefly

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cited previously (4) that the monomer-hexamer interconversion is a rapid equilibrium process and that the linker protein serves to stabilize the hexamer once formed. This explains the older observations of tight association to hexamers and the ability of Neufield and Riggs (12) to isolate discernible monomer and hexamer peaks by gel filtration of "pure" C-phycocyanin. Lundell et. al. (13) showed that even DEAE chromatography does not remove all of the linker protein unless special care is taken to collect only late-eluting fractions from a shallow gradient. Older preparations frequently used only ammonium sulfate fractionation to purify phycocyanins, and must have contained linker proteins.

The phycobilisomes (PBS) of Synechococcus 6301 represent a well-studied example of PBS architecture, and contain two biliproteins--C-PC and allo-PC (9). In addition, linker proteins with M_r of 27,000, 30,000, and 33,000 stabilize aggregations of C-PC into disc-like structures (13), while polypeptides at 10,500 and 75,000 M_r serve a similar function in stabilizing allo-PC within the PBS core (14,15). High salt concentrations, low pH, and high biliprotein concentrations have been implicated in maintaining PBS structure by favoring interactions between biliproteins and linkers. The nature of this interaction has not been clearly established, nor have the rates of biliprotein self-association and dissociation been systematically investigated.

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Most recent studies on PBS structure have used supersaturated concentrations of detergents like Triton X-100 to detach PBS from membranes (16). Since detergents are known to bind to chromatographic support matrices and form micelles, these PBS preparations would not be suitable for such analyses. Our strategy, therefore, was to isolate fractions enriched in biliproteins and linker peptides using the differential effects of salts and pH on PBS integrity.

In this report, we characterize a set of biliproteins in preparations reproducibly obtained from A. nidulans R_2. Electrophoretic profiles of these fractions suggest that the PBS of A. nidulans R_2 and Synechococcus 6301 are of similar composition.

These fractions were examined by direct scanning gel filtration. Our results suggest that biliproteins interact in a rapid equilibrium manner, and that salts and pH dramatically influence rates of dissociation.

MATERIALS AND METHODS

Cells of *Anacystis nidulans* R_2 were grown in liquid culture at 33°C under constant illumination (0.4 mW/cm^2) from cool white fluorescent lights (17). Cultures of 15 l were bubbled vigorously with air and reached late log phase in 2-3 days. After harvesting by centrifugation, cells were either used immediately or were stored as pellets at -20°C.

Cell pellets were resuspended in a high salt medium (SPCM) containing 0.5 M sucrose, 0.5 M KH_2PO_4, 0.3 M sodium citrate, and 10 mM $MgCl_2$ at pH 7.5 (16). Mechanical breakage was achieved using glass beads according to the method of Cramer et al. (18) and unresolved cell debris was removed by centrifugation (3,000 x g for 15 min). A pellet, containing the thylakoid membranes, was obtained by centrifugation (27,000 x g for 30 min), and was used to prepare four PC-containing fractions as detailed in Fig. 1 and Results. All of the buffers employed during cell breakage and biliprotein isolation contained 10 μg/ml phenyl methyl sulfonylfluoride, 1 mM benzamidine, and 1 mM norleucine to inhibit protease activity. All PC fractions were extensively dialyzed against a dialysis buffer containing 10 mM Tricine (pH 7.5) plus the protease inhibitors.

Lithium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (LDS-PAGE) was done essentially as described by Guikema and Sherman (17). LDS (2%), 2-mercaptoethanol (10%), and sucrose (6%) were added to samples containing 2-4 mg protein/ml. Sample mixtures were incubated 15 min on ice and were layered atop a 10-20% polyacrylamide gradient gel having a 5% polyacrylamide stacking gel. Gels were prepared using the buffer system of Laemmli (19), having 0.1% LDS in the running buffer. These gels

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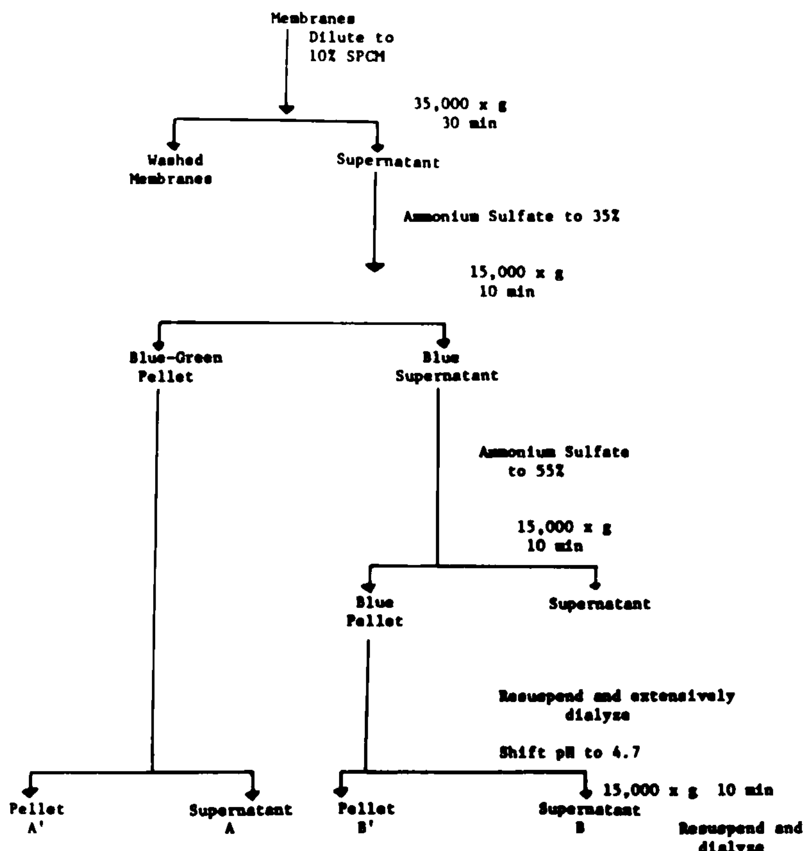

Fig. 1. Isolation protocol and absorption spectra of four biliprotein fractions from Anacystis nidulans R_2. Preparations were resuspended and dialyzed against dialysis buffer. Concentrated fractions were diluted in the same buffer and the absorption spectra were recorded. Each sample showed a full-scale deflection of about 1.0 A, although the tracings have been offset in the figure to facilitate comparisons. (continued)

were run overnight in the cold (4-6°C) at a constant power of 3.5 W. In all instances, gels were examined by staining with Coomassie Blue R-250, followed by counterstaining with silver nitrate (20). The absorption profiles of samples and excised gel pieces after electrophoresis were obtained using an Aminco/SLM DW2-C recording spectrophotometer in dual beam mode.

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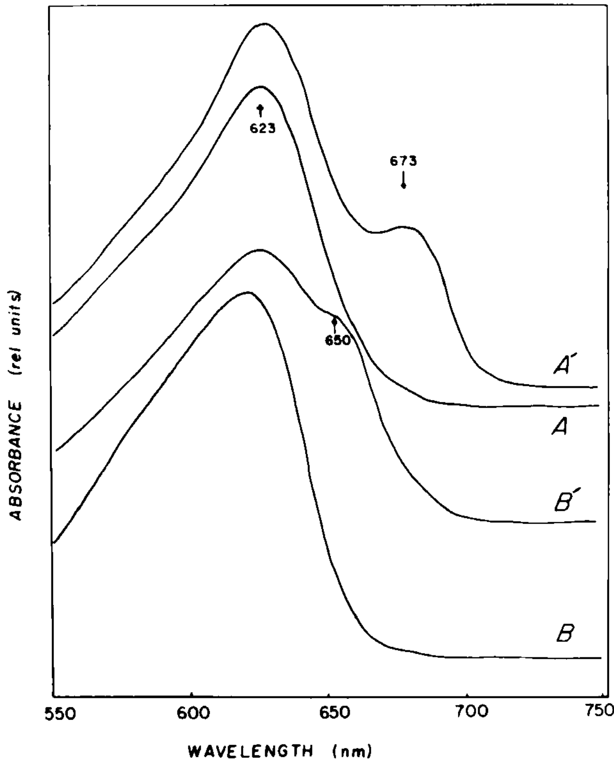

Fig. 1 (continued)

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Our method for direct scanning gel chromatography has been previously described (2-6). In essence it consists of a light source and detector that are moved past a stationary 1 cm diameter column of gel filtration matrix such as Sephadex G200. Flow of buffers and sample application is controlled by a syringe pump. Scans of the absorbance profile of the column at the desired wavelengths are obtained under control of PDP1104 computer. Typically 194 data points are obtained per scan of 20 cm of the column length. Data processing programs allow conversion of raw data to smoothed curves, difference spectra etc.

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RESULTS

Figure 1 presents a protocol used to resolve four biliprotein-containing fractions from a thylakoid membrane fraction of Anacystis nidulans R_2. The thylakoids were isolated in high salt, and maintained high rates of photosystems I and II electron transport (21). Biliproteins were removed from the thylakoids by diluting the high salt buffer, since lowered ionic strength induces PBS dissociation. After the thylakoid fractions were diluted to 10% SPCM, centrifugation yielded a washed membrane preparation and a brilliantly blue supernatant fraction. The supernatant fraction contained a small contaminating amount of chlorophyll.

Ammonium sulfate fractionation at 4°C further resolved the supernatant fraction. A titration showed that 95% of the contaminating chlorophyll pelleted at 30% ammonium sulfate saturation and all of it was removed at 35% saturation. Approximately 65% of the biliprotein was precipitated as well. When the ammonium sulfate was increased to 55% saturation, essentially all of the biliproteins could be pelleted. The two fractions, representing the 0-35% and 35-55% ammonium sulfate cuts, were resuspended and extensively dialyzed against dialysis buffer. These fractions were further partitioned by dialysis against 0.1 M sodium acetate, pH 4.7. This shift in pH yielded a precipitate which was removed by centrifugation. Four fractions, labeled A', A, B' and B, resulted from these treatments. Their absorption profiles are shown in Fig. 1. The chlorophyll (as a marker for residual membrane fragments) was observed in fraction A' as a peak at 673 nm. It is not known if this 673 nm peak also contains allo-PC_B (11). Fraction A' was the only fraction with an appreciable lipid component as monitored by gas chromatography (data not shown). A majority of the allo-PC (at 650 nm) was observed in fractions A' and B', suggesting that pH shifts might be an effective method for enriching in this biliprotein. Small amounts of allo-PC were detected by gel electrophoresis in the

other two fractions as well, with fraction A containing relatively much more than fraction B. The absorption peak for B was shifted several nm to the blue in comparison with A. This shift was probably an expression of the loss of allo-PC from B, and of a lowered organizational state of B owing to a depletion of linkers (11).

The peptide composition of these four fractions was examined by LDS-PAGE (Fig. 2). Both fractions A' and B' showed complex polypeptide profiles, suggesting that the pH shift can precipitate many of the contaminating proteins. Fraction A' contained residual membrane fragments (17), while both A' and B' contained traces of ribulose bisphosphate carboxylase (21). Three colored polypeptides were common to each of the four fractions. These corresponded to the α and β subunits of C-PC and allo-PC at 14,000, 15,500, and 17,000 M_r. We observed colorless peptides with M_r of 26,500, 33,000, 35,000, and 71,000 which correspond to the linker peptides described by Glazer (11) for Synechoccocus 6301. Fractions A and B differed dramatically in their relative content of these linker polypeptides. Fraction B contained only a small amount of the peptide of 35,000 M_r while A contained all four. Because of this difference, we concluded that these preparations would be useful in exploring the role of linkers in protein/protein interactions between biliproteins. Our approach included both non-denaturing gel electrophoresis and direct scanning gel filtration. Details of non-denaturing gel electrophoresis studies will appear elsewhere.

Phycocyanin association can be conveniently monitored using direct scanning gel filtration. Scanning in a dual wavelength mode aids in discriminating between C-PC and allo-PC. Figure 3 shows a typical migration profile of fraction A under different salt concentrations. Both absolute absorbance and intensity ratios are shown. The absorbance of pure C-PC at 650 nm is half as great as it is at 565 nm while that of allo-PC is five-fold greater at 650 nm than at 565 nm. This relative difference is readily seen in the upper set of scans. Note that these upper

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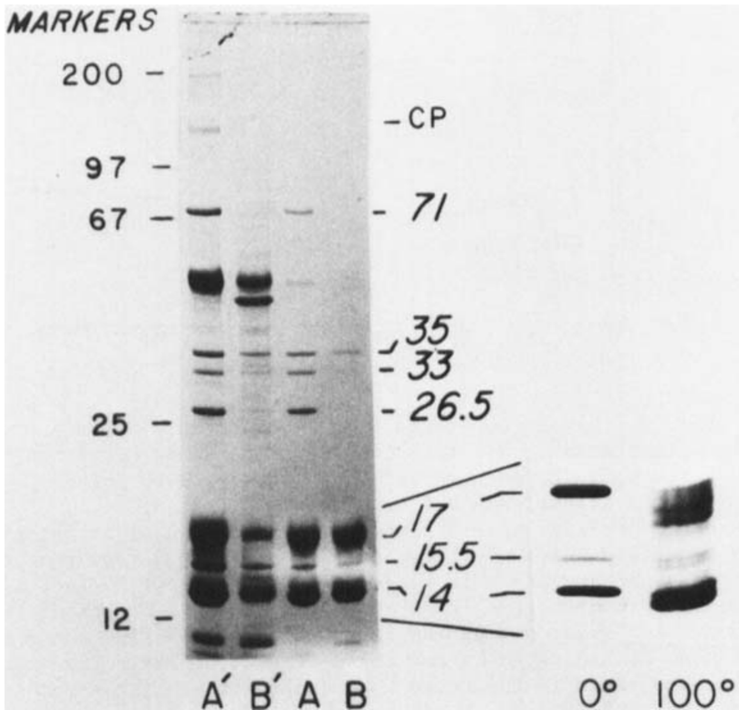

Fig. 2. Polypeptide profiles of fractions described in Fig. 1. The numbers on the left give the position and molecular weights (in $M_r \times 10^{-3}$) for marker proteins run on the same gel. CP denotes a chlorophyll-containing protein band observed in fraction A'. Numbers on the right give the relative molecular weights (in $M_r \times 10^{-3}$) measured for three biliproteins (14-17) and four linker peptides (26.5-71). The inset details the effect which solubilization temperature has on biliprotein migration patterns. Three bands are observed when solubilization is performed on ice (0°). However, each band resolves into several when the samples are boiled for 2 min (100°).

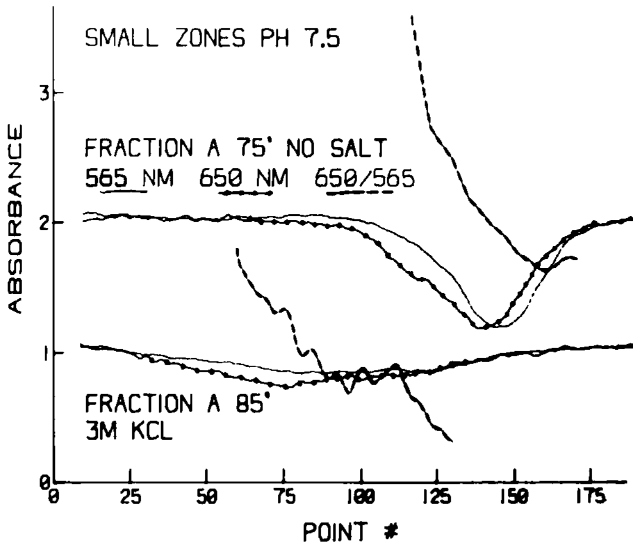

Fig. 3. Migration of fraction A phycocyanin as influenced by salt concentration. The fraction was prepared as described in Fig. 1 and a small zone applied to a Sephadex G200 column equilibrated in dialysis buffer with or without 3 M KCl. Absorbance profiles were obtained at 565 and 650 nm at intervals of 5 minutes. Rates of migration were determined from peak area midpoints and are shown in Table I. Point numbers in the figure correspond to mm column length measured from the bottom of the column. Absorbance at 565 nm is shown by a thin line while that at 650 nm is indicated by the solid line with dots. The absorbance ratio is indicated by a dashed line. The scans shown were obtained at 75 min for the no salt condition and 85 min for the high salt condition.

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spectra are arbitrarily offset on the scale of absorbance ratio by 1.0, and that absolute absorbance increases in the downward direction. The lower set of spectra was obtained in the same buffer in the presence of 3 M KCl. Again, the ratio change indicates that the allo-PC is selectively associated with rapidly moving material. The rate of migration is much more rapid in the presence of high salt and the small zone is dissociating and/or heterogeneous. Electrophoresis, on LDS gels, of fractions collected at early, middle and late regions of the column eluate,

showed the presence of several linker proteins associated with the larger aggregates.

When fraction B was similarly chromatographed, high salt induced a specific aggregation to a form with a size consistent with a hexamer (180,000 M_r) relative to catalase (Fig. 4). Only a small amount of one colorless linker protein of about 35,000 M_r on SDS gels was observed to be associated with this fraction of essentially pure C-PC. The column profiles were not broadly dispersed like those of fraction A, indicating a relatively homogenous tight association of the protein. The small zone profile was symmetrical and the same width in either high or low salt indicating a rapid equilibrium association; with no evidence for separable aggregates (12). Table I summarizes the migration rates for fractions A and B as a function of salt and pH. Broad zone profiles revealed asymmetry between leading and trailing edges as expected for an associating system. The absence of discrete peaks in the difference profile suggests a monomer-trimer-hexamer association pathway rather than a simple monomer-hexamer association (22,23,25).

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DISCUSSION

In this report, we have presented preliminary evidence using direct scanning gel filtration as a useful tool for examining PBS structure. The direct scanning results of fraction A, shown in Fig. 3, were particularly interesting. High ionic strength induced an association of peptides within fraction A which resulted in an extremely swift migration of this material through the gel matrix. This salt-induced aggregate contained both C-PC and allo-PC, and possessed all four non-colored linker polypeptides observed in fraction A. A similar aggregation was not observed at low ionic strengths. We have not yet examined intermediate conditions, to determine the salt dependency of aggregation.

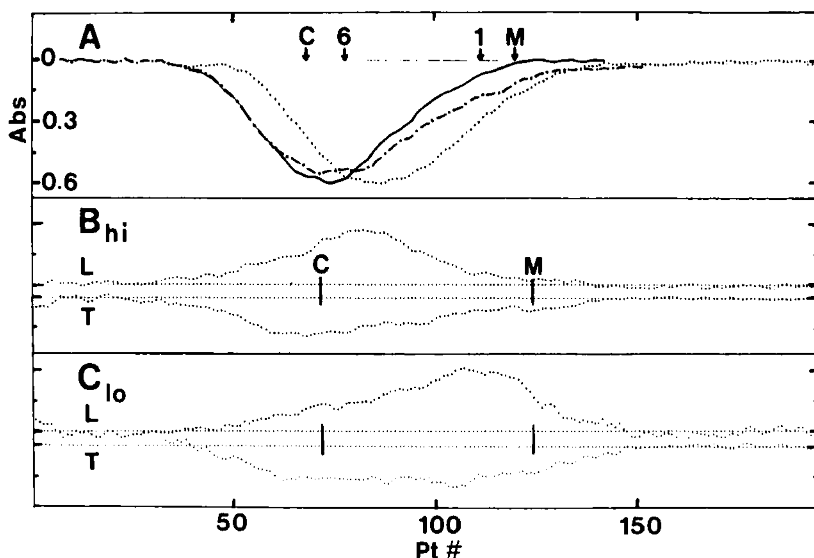

Fig. 4. Effect of salt on gel filtration of C-phycocyanin (fraction B). Part A shows small zones of phycocyanin observed at 550 nm and run with or without 3M KCl for 90 min. These are indicated by dashed and dotted lines respectively. The solid line indicates catalase run 90' in 3M KCl for comparison of dispersion properties (offset horizontally to the right by 9 mm to align it with C-phycocyanin on the leading edge). Expected peak positions for a spherical monomer protein of 30,000(1) and hexamer of 180,000(6) M_r are indicated by numbered arrows. Actual migration rates for catalase and myoglobin are shown as C and M. Absorbance scales are the same for all three parts. Relative position is indicated by the point (1 mm) in the column, counting from the bottom of the column. The phycocyanin peak is broader than that of catalase, suggestive of dissociation during the course of the run.

Part B shows time difference spectra (90-80 min.) for leading and trailing edges of a broad zone of phycocyanin. The wavelength of observation was 550 nm and protein concentration was about 1.6 mg/ml.

Part C shows the time difference spectra for leading and trailing edges of a broad zone of phycocyanin run under the same conditions as B but diluted 6.6-fold to give about 0.25 mg/ml and observed at 625 nm. The phycocyanin used for all these experiments had all been passed through a G200 Sephadex column previously and reprecipitated with 66% saturated ammonium sulfate.

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TABLE 1

Migration Rates of PC Fractions During Gel Filtration as a Function of Salt Concentration and pH.

Fraction	Buffer System	Migration Rate (mm/min)	Measuring Wavelength (mm)
Experiment I -- pH 7.5			
A	dialysis buffer[1]	0.88	565
		0.99	650
B	dialysis buffer	0.98	565
A	dialysis buffer + 3 M KCl	1.42	565
		1.60	650
B	dialysis buffer + 3 M KCl	1.28[2]	565
Experiment II -- pH 5.5[3]			
A	0.2 M acetate	1.79	565
B	0.2 M acetate	1.08	565
A	0.2 M acetate + 3 M KCl	1.97	565
B	0.2 M acetate + 3 M KCl	1.37	565

[1]Relative rates of migration for ferritin, catalase, and potassium chromate were 1.5, 1.3, and 0.7 mm/min on this column.

[2]This measurement was the average of two broad zones, leading and trailing edges.

[3]Relative rates of migration for ferritin, catalase, and myoglobin were 1.8, 1.5, and 1.0 mm/min on this column.

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The polypeptide composition of fraction A was similar to the composition of intact PBS from Anacystis nidulans observed by Grossman and Brand (26). There is a marked similarity between fraction A and the PBS composition of a closely related strain, Synechoccocus 6301 (15,27).

This is the first application of direct scanning gel filtration to the study of biliprotein association. Figs. 3 and 4 and Table I demonstrate the utility of this technique and show the associative characteristics of PC at different salt and pH conditions. Two points should be noted. First, fraction A can be resolved during gel filtration into an allo-PC-enriched and a C-PC-enriched region using a dual-wavelength monitoring system. Table I shows that the migration of the allo-PC region slightly exceeds that for C-PC when the gel has been equilibrated with the low ionic strength dialysis buffer. Increasing the salt concentration alters the migration rates of both C-PC and allo-PC. These increases were observed with both fractions A and B. However, fraction A contained an enhanced complement of linker peptides, and this was reflected in a more dramatic effect of high salt. The leading edge of a gel filtration experiment (Fig. 3), run in the presence of high salt, contained a majority of the linker proteins. This finding of association in small zones in the presence of salt shows clearly that the association is a rather tight one with a relatively slow dissociation. Earlier studies in which discrete peaks of different size were obtained provided evidence suggesting a tight association of C-PC itself (12). However these experiments were almost certainly done with a significant amount of contaminating linker protein and allo-PC, and our experiments show that no distinct multiple peaks are resolved if preparations free, or nearly free, of linkers are used for comparable experiments (such as fraction B). Because the protein concentration is constantly varying during a chromatography run when a small zone is applied, no reliable estimate of association constants can be obtained with small zones (22). Quantitation of the association process to yield reliable values

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of association constants requires the use of broad zone experiments, as shown in Fig. 4, with computer simulations, which are in progress.

Gilbert, (23) some years ago, treated the case of a self-associating system forming a series of _n_-mers, describing idealized boundary shapes for broad zone mass transport. In the instance of a monomer-hexamer equilibrium, an accentuation of the hexamer leads to a bimodal peak across the boundary. This bimodality can give the appearance of resolution of monomer from _n_-mer(s) if only the trailing boundary is considered, as in centrifugation. However, for free-boundary electrophoresis (and gel filtration chromatography) the leading and trailing boundaries will be non-enantiographic. In the case of β lactoglobulin cited by Gilbert, (23) a monomer-tetramer gives a sharp leading edge but a broad, apparently resolving trailing edge. A second diagnostic criterion of an equilibrating as opposed to a resolving system is that, for the equilibrating system, increasing the concentration in experiments will enhance the size of only the higher aggregate form while in the resolving system there will be no change in relative peak size. Examining the peak profiles of parts B and C of Figure 4 it is clear that a high concentration gives much more of the faster moving component. This is especially obvious in the leading edge where the peak position shifts from near hexamer to near monomer on dilution. In the trailing edge the relative amount of material in faster and slower moving regions also changes with concentration in the same manner. There is no good evidence for resolvable peaks at either concentration (i.e. no inflection in the smooth curve between monomer and hexamer positions). The simulations of Zimmerman (24) show that in time difference profiles such an inflection should be observable if the larger _n_-mer is accentuated. He examined a monomer-octamer system but Gilbert (23) showed that _n_-mers of 6-12 had very similar relative profiles, so the results of Zimmerman (24) are applicable. Simulation work by Zimmerman et al (25) showed that for a monomer-tetramer system, gel filtration chromatography could give

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somewhat varied results depending on the porosity of the gel, bead size, flow rate and time of observation, as well as depending on the tightness of the association process. However, we know that Neufield and Riggs (12) were able to resolve on Sephadex G200, monomer and hexamer forms of C-phycocyanin, when the hexamer was presumably stabilized by linker protein. In their experiments the linear buffer velocity was somewhat greater than ours (1.5 - 2.5 times) so that dispersion effects would have been greater. Thus we may conclude that our failure to even partially resolve monomer from hexamer as inflections in the boundary shape of a broad zone is indicative of the presence of intermediates between monomer and hexamer and not a simple chromatographic problem. That is, the hexamer is not strongly accentuated in the equilibrium, until the linker protein is present. As shown in Figure 4 the interconversion of monomer to hexamer is not directly dependent on the presence of the linker protein. It seems reasonable, therefore, to conclude that the linker protein serves to stabilize the hexamer once it is formed, not that it causes its formation.

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Some estimates of the rate of exchange of PCs into the aggregates that ultimately constitute the PBS can be obtained from studies of the exchange of specifically labeled proteins. We have done preliminary studies with ^{35}S-labeled PCs and found that the half time for association is in the range of minutes at low salt and in the range of several days in 3 M salt. There is thus a very dramatic shift in association properties as a function of salt concentration. Further experiments to quantitate these effects are in progress.

According to recent models (11,16), phycobilisomes contain biliproteins and linker polypeptides. The integrity of the PBS depends upon two factors: (i) the associative characteristics of biliproteins to assemble into stable hexameric discs, and (ii) the stabilization and stacking of these discs into rods through association with linker proteins. Two distinct assembly mechanisms may be visualized: either (a) an initiation of aggregation by a linker plus a monomer of PC, or (b) an aggregation of PC

monomers followed by the binding of a linker. Glazer (10) prefers model a, based on work with Anabaena variabilis. Lundell et al. (13) studying Synechococcus 6301 failed to identify a trimer of $(PC)_3$-X which would be a necessary intermediate for Glazer's model a. Our work with A. nidulans R_2 is consistent with Lundell, et. al. (13). There may be significant species differences in the stability of these smaller aggregates, so that the choice between models a and b is species dependent. Direct scanning gel filtration of purified PC free of linker can yield information about the order of assembly of the PCs into specific aggregates. In addition, experiments with exchange of labeled proteins may be used to examine the interaction with linker proteins by monitoring the dissociation of these specific aggregates.

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