PROTEIN AGGREGATION IN PHYCOCYANIN - OSMOTIC PRESSURE STUDIES*

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Received October 27, 1969

SUMMARY

The aggregation properties of C-phycocyanin have been investigated by osmotic pressure measurements at pH 6.0 and 7.0 at varying temperatures. Conditions were selected to maximize the amount of 7S and 11S aggregates normally encountered. Corroborating sedimentation velocity experiments are presented. The extrapolated $\pi/\underline{c}$ values were used in the van't Hoff equation to determine the number average molecular weights. The data are consistent with a 7S trimer of 90,000 molecular weight and an 11S hexamer of 180,000. The slope of the $\pi/\underline{c}$ vs. $\underline{c}$ plot, or second virial coefficient, was zero under all conditions. This result indicates little measurable tendency for disaggregation over the concentration range investigated, an observation in agreement with previous sedimentation velocity experiments of Scott and Berns. The lack of disaggregation of the protein is consistent with the suggested micellar behavior of phycocyanin.

The physical-chemical properties of C-phycocyanin have been reported in several recent publications from this laboratory (1-3). We postulated that C-phycocyanin is an aggregating protein with a monomer molecular weight of approximately 30,000 and have verified this finding by several different experimental technics (3). This result is supported by the Sephadex gel filtration work of Neufeld and Riggs (4). The smaller phycocyanin aggregates were postulated to be a trimer of 90,000, a hexamer of 180,000, and a dodecamer of 360,000. These assignments were necessarily tentative since phycocyanin exists as a complex interacting mixture of the above-mentioned aggregates. The relative amount of each species has been reported as a

*This work was supported in part by a grant from the National Science Foundation (GB-7591).

function of pH, ionic strength, temperature, and protein concentration. Sedimentation velocity experiments indicated that the relative amounts of each aggregate present did not vary with concentrations over the range of 4 to 40 mg/ml (1). In addition, at dilutions of 0.2 mg/ml complete dissociation to monomer did not occur and substantial amounts of high aggregates were present (5). Osmometry is an ideal experimental method for checking the concentration dependence or independence of the aggregation and at the same time for estimating the number average molecular weight (M_n) of the aggregates present.

EXPERIMENTAL

The phycocyanin used in this study was isolated from Plectonema calothricoides grown in this laboratory and purified as described previously (1,6). This procedure was used to prepare phycocyanin with the greatest amount of 7S and 11S aggregates in contrast with more recent methods for preparing higher aggregates (7,8). The $OD_{620}:OD_{280}$ ratio was greater than 4.0 for all phycocyanin preparations in this study. The ovalbumin used was from Worthington Biochemical Corporation, 2X crystallized. All buffers were 0.1 ionic strength, sodium phosphate, pH 6.0 or 7.0. Large amounts (about 300 mg) of the protein were exhaustively dialyzed against distilled water. This solution was lyophilized. Individual samples of specific amounts of the lyophilized protein were weighed on an automatic Mettler semi-micro balance. The osmotic pressure measurements of the same protein samples before and after lyophilization were reproducible, indicating no detectable effect of lyophilization on the M_n of the sample. Similar concentrations of protein prepared by dilution from a stock solution or by weighing each sample separately gave identical results. Ovalbumin samples were prepared by weighing the crystalline material and adding the appropriate buffer (pH 6.0, $\mu = 0.1$) by volumetric pipette. Reproducibility of these osmotic pressure measurements was excellent and the agreement of the M_n determined for this sample with the literature value supports the reliability of this method of preparing solutions. Osmotic pres-

sure measurements were made with a (CSM-2) Melabs membrane osmometer. Schleicher and Schuell's B19 membranes were equilibrated overnight in the appropriate buffer. After the membrane was clamped in position, the osmometer cell was allowed to equilibrate for several hours at the temperature of the measurements. After each change in temperature, buffer, or membrane, the osmometer pressure transducer was calibrated by measured hydrostatic pressure. Several of the samples used in the osmotic pressure experiments were examined in the ultracentrifuge at a similar temperature. All sedimentation experiments were performed on a Spinco Model E ultracentrifuge at 59,780 rpm. The photographic plates were analyzed with a Nikon micro comparator (1).

RESULTS

The osmotic pressure data are shown in Fig. 1, plotted as $\pi/\underline{c}$ vs. $\underline{c}$. Included in these data is a test run with commercial ovalbumin. All the data were analyzed by a simple least squares analysis of the form $\pi/\underline{c} = (\pi/\underline{c})_{\underline{c} \longrightarrow 0} + B\underline{c}$. The greatest uncertainty in these measurements, as in all osmotic pressure measurements, is probably in the determination of

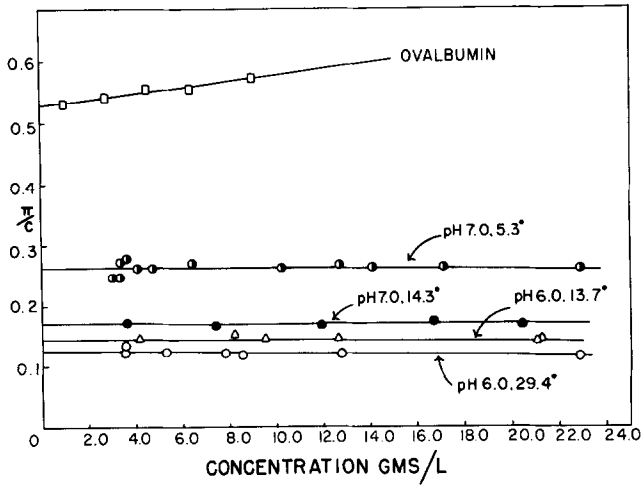

Fig. 1. The C-phycocyanin osmotic pressure data are plotted as $\pi/\underline{c}$ vs. $\underline{c}$. π is in cm of H_2O. Included are osmotic pressure data for ovalbumin experiments, performed in pH 6.0, μ = 0.1, buffer at 14°. All buffers are 0.1 ionic strength and temperature and pH are listed on the appropriate line. Sedimentation velocity studies on aliquots of samples from the osmotic pressure studies are presented in Fig. 2 and 3.

protein concentration (9). The accuracy of the protein concentration is probably no better than ~3%, although the reproducibility is likely to be much better. The data from all experiments gave an extrapolated value of $\pi/\underline{c}$ with a standard error not greater than 2%.

The molecular weights were calculated by substituting in the van't Hoff equation $\pi = (\underline{c}/M)(RT)$. The appropriate limiting value $(\pi/\underline{c})$ as $\underline{c} \longrightarrow 0$ and $M_{\underline{n}}$ are listed in Table I. Analysis of the data by any of the other methods suggested by Rowe and Abrams (10) resulted in $M_{\underline{n}}$ values in agreement with those obtained by the $\pi/\underline{c}$ vs. $\underline{c}$ method. The slope of the $\pi/\underline{c}$ vs. $\underline{c}$ plot (B) is often interpreted in terms of the second virial coefficient (11) and more complex interaction parameters (9). However, for all temperature and buffer systems under which C-phycocyanin was analyzed, the magnitude of the slope was sufficiently small and had a sufficiently large error so that we were forced to conclude the B value was zero. The $(\pi/\underline{c})_{\underline{c} \longrightarrow 0}$ value for the ovalbumin result was 0.530 ± 0.002 and $M_{\underline{n}}$ = 46,000 ± 200. The literature value for ovalbumin is $M_{\underline{n}}$ = 45,000 (12).

Extensive sedimentation velocity studies of C-phycocyanin from P. calothricoides have been reported (1). This report includes estimates of the relative amounts of each aggregate present from the relative areas under

TABLE I

Number Average Molecular Weights from Osmotic Pressure Data

pH	Temperature	$(\pi/\underline{c})_{\underline{c} \longrightarrow 0}$	$M_{\underline{n}}$ [a]
6.0	13.7°	0.149 ± 0.002	164,000 ± 2,000
6.0	29.4°	0.129 ± 0.003	198,000 ± 5,000
7.0	14.3°	0.172 ± 0.001	142,000 ± 1,000
7.0	5.3°	0.263 ± 0.002	89,400 ± 700

[a] By use of van't Hoff equation.

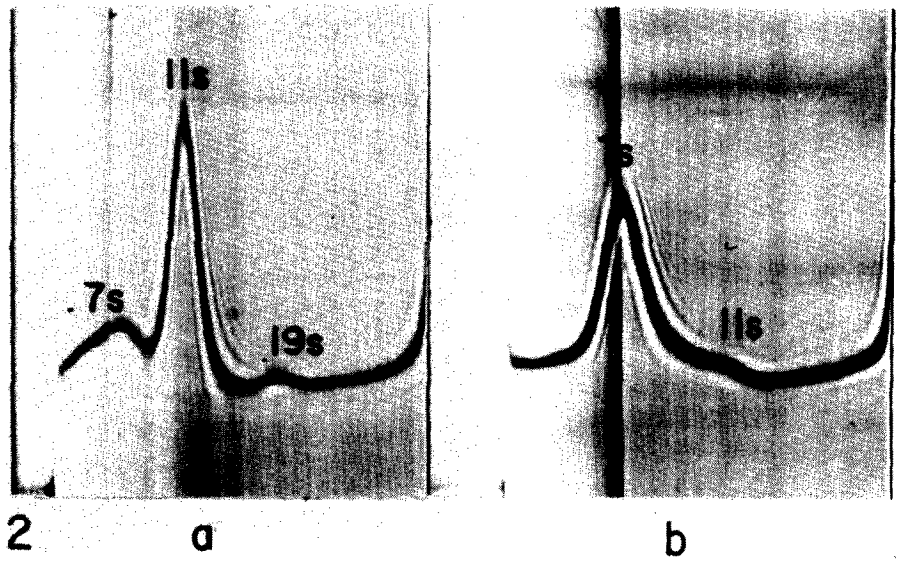

Fig. 2. Sedimentation velocity studies performed at 59,780 rpm. Note that the designation 7S and 11S is convenient notation for trimer and hexamer; the actual S values may be as low as 5.5S or 9.5S.
a) pH 6.0, 29.4°, 24 min; b) pH 7.0, 5°, 78 min.

the schlieren peaks. The several studies now reported (Figs. 2 and 3) were performed on aliquots of the samples used for osmotic pressure measurements. These results, including area measurements under the schlieren peaks, are in complete agreement with the work of Scott and Berns (1), which should be consulted for additional data.

DISCUSSION

The M_n obtained from the osmotic pressure data should be correlated with the sedimentation data. It is seen from Fig. 2b that at pH 7.0, $\mu = 0.1$, and a temperature of $\sim 5°$, a mixture of 7S ($\sim$92%) and 11S ($\sim$8%) is present. The M_n from osmometry is 89,400. If we use the suggested molecular weight for the 7S trimer of 90,000 and for the hexamer of 180,000, we calculate from the distribution of aggregates estimated from the schlieren studies an M_n of 93,400 which is in good agreement with the observed results. The suggested molecular weight of 140,000 for 7S (13) and 268,000 for 11S (14,15) would

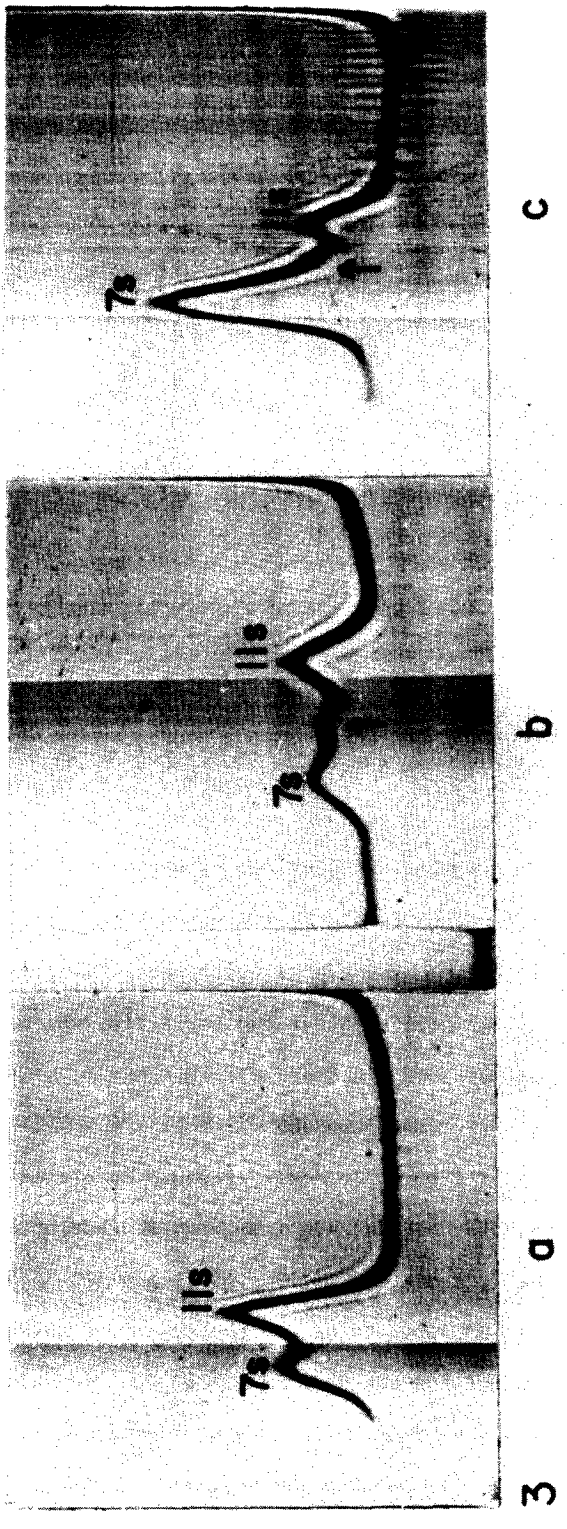

Fig. 3. Sedimentation velocity studies performed at 59,780 rpm. Note that the designation 7S and 11S is a convenient notation for trimer and hexamer; the actual S values may be as low as 5.5S or 9.5S.
a) pH 6.0, 10.4°, 32 min; b) pH 6.0, 10.4°, 72 min; c) pH 7.0, 14°, 48 min. Arrows indicate the probable presence of species intermediate between 7S and 11S.

give an M_n of 134,000 in clear disagreement with the osmotic pressure measurements. The same sort of calculations may be carried out with the other data. Another interesting calculation is that for pH 6.0, μ = 0.1, 29.4° phycocyanin. The sedimentation data at 29.4° (Fig. 2a) indicate a large percentage of 11S species. From these results and the temperature studies by Scott and Berns on the sedimentation properties, we estimate that at 29.4°, the percent of 11S material is close to 80; the 7S, about 17; and the 19S, 3. The calculated M_n using 180,000 for the 11S hexamer, etc., would be 157,000 while the experimental value is $\sim$198,000. The use of a value of 268,000 for the 11S hexamer, etc., would lead to a calculated M_n = 236,000.

The designation of molecular weights of the aggregates most consistent with the osmotic pressure data seems to be the proposal of a trimer of 90,000 and a hexamer of 180,000. Estimates of M_n from the relative area under the schlieren patterns are at best an order of magnitude calculation. These calculations are most meaningful where a single aggregate is overwhelmingly predominant (Fig. 2a,b). This situation is most closely approximated at pH 7.0, T = 5.3°. The assumption of a trimer-hexamer equilibrium is an oversimplification, and intermediates between trimer and hexamer may well exist (1). Larger deviations between M_n calculated from sedimentation data and M_n determined by osmotic pressure measurements may be the result of the presence of substantial amounts of intermediate between trimer and hexamer, which become evident on prolonged sedimentation (Fig. 3b,c). The existence of intermediates has been previously suggested (1,16) and must be considered if we are to analyze the system in a quantitative manner.

We should also consider the difficulty and pitfalls in attempting to analyze complex sedimentation patterns (17,18). We are analyzing a boundary separation method with complex interactions (1). To assume each boundary is a simple representation of a species is a drastic oversimplification. There is, however, corroborating evidence for the existence of each species; namely, immunodiffusion, electron microscopy (2,19), sucrose density gradients

(7,8), and Sephadex gel filtration experiments (4,20). When the trimer species is present individually as 90% or more of the total material, the osmotic pressure data fit rather well with the proposed simplified equilibrium in which we assume a hexamer of 180,000 and a trimer of 90,000 molecular weight.

Perhaps more interesting than the correlation between the estimated M_n from schlieren patterns and M_n from osmometry is the slope of the π/c vs. c plots. The most that can be stated about the magnitude of all of the slopes is that they are very small and probably negative. The negative value would be consistent with the presence of an aggregating system. The magnitude of the slope, however, which is extremely small and essentially zero, is quite difficult to explain, if, as we decrease the protein concentration, there is any substantial disaggregation. We have reported previously that analysis of the areas under the sedimentation patterns in the range of concentrations of 4 to 40 mg/ml indicates there is little if any detectable change in the relative concentration of species present (1). The osmotic pressure data under all conditions would appear to confirm this lack of disaggregation.

Physical studies of the phycocyanin system have contributed substantial evidence that this protein aggregation phenomenon may be similar to micellization. Very low concentrations must be used to disaggregate the system completely. Neufeld and Riggs (4), in work with phycocyanin from Anacystis nidulans, find they must go to concentrations of $\sim$0.02 mg/ml to resolve monomer completely by Sephadex gel filtration. Using the scanner system in the Model E ultracentrifuge, Dr. J.J. Lee has demonstrated in our laboratory that phycocyanin aggregates exist at protein concentrations close to 0.02 mg/ml. The existence of proteins that behave as micelles and the possibility of applying the principles of micellization can be of considerable importance in attempting to elucidate the properties of structural proteins and protein assembly in membranes.

ACKNOWLEDGMENT

We wish to thank J. Vaughn for his excellent technical assistance

with the sedimentation velocity measurements and Dr. D. Treble for assistance with computer analysis of the osmotic pressure data.

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