Surface Properties of Phycocyanin

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The change of surface pressure (\(\pi\)) during adsorption and compression of phycocyanin film at the air-water interface indicates that this phycobiliprotein is strongly attracted to the interface. It behaves like a flexible molecule but is not completely unfolded. The steep slope of the curve of \(\pi\) as a function of the bulk concentration (\(C_b\)), when \(C_b < 10^{-3}\) mg/ml, may be attributed to the high number of hydrophobic amino acid residues and other structural features of the protein. At \(C_b > 10^{-3}\) mg/ml the \(\pi\) vs \(C_b\) curve is interpreted as being characteristic of aggregation of the protein. Analysis of the curves of \(\pi\) vs \(C_b\) and \(\pi\) vs molecular area (\(A\)) indicate that the phycocyanin's tetrapyrrole chromophores make only a small contribution to the surface properties of the protein.

INTRODUCTION

Phycocyanin, a biliprotein, is an essential accessory photosynthetic pigment in Photosystem 2 of blue-green and red algae. It is an extrinsic membrane-protein and thus ideally suited for study by adsorption methods. The chromophore in phycocyanin is a linear tetrapyrrole called phycocyanobilin. It is covalently bound to the protein moiety through a thio-ether linkage at the cysteinyl group (1).

Phycocyanin exists in solution as a variety of aggregates (2), and hydrophobic interactions may play an essential role in its aggregation to hexamers (3). There are two non-identical polypeptide chains, which associate to monomers and subsequently, depending on environmental conditions, to larger aggregates. The protein contains a high percentage of \(\alpha\) helix (\(\sim 60\%\)) and \(\beta\) structure (4), as well as a many hydrophobic amino acid residues.

To help elucidate the configuration of this protein at the membrane interface, we have investigated the rate of change and the steady-state surface pressure of phycocyanin during its adsorption at the air-water interface.

Amino acid residues in a protein can have an "all-train" or a "loops and tails" configuration at the air-water interface (5). In the former configuration the residue lies flat at the interface; in the latter some residues of the protein are extended into the subphase. The more amino acid residues are in the train configuration the greater the surface pressure (\(\pi\)). Thus changes in \(\pi\) may provide a probe for the configuration of proteins at the interface. The rate of change in \(\pi\) when protein penetrates into the surface—and thus the configurational rearrangement of adsorbed protein molecules—can be analyzed by the first-order equation (6)

\[
\ln \frac{\pi_{ss} - \pi_t}{\pi_{ss} - \pi_0} = \frac{t}{\tau},
\]

where \(\pi_{ss}\), \(\pi_0\), and \(\pi_t\) are the surface pressure at steady-state conditions, at time zero, and at any time \((t)\), respectively, and where \(\tau\) is the relaxation time, which is the inverse of the rate constant.

During the initial stages of adsorption the arrival of molecules at the interface is presumably controlled only by diffusion, and the resulting adsorption should be irreversible (6, 7). MacRitchie and Alexander (7, 8) have

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proposed a method for analysis of surface adsorption of proteins, which was followed in this investigation. In the absence of convection and desorption the changes in \( \pi \) should follow the expression

\[
n = 2C_b \left( \frac{Dt}{3.142} \right)^{1/2},
\]

where \( n \) is the amount of protein per area, \( t \) is time after injection of protein below the interface, \( C_b \) is the bulk concentration, and \( D \) is the diffusion coefficient.

The rate of adsorption at constant area is obtained from

\[
\left( \frac{dn}{dt} \right)_\pi = \left( \frac{dn}{dt} \right)_\pi \left( \frac{dn}{dt} \right)_\pi,
\]

where \( dn/dt \) is found by drawing tangents to a \( \pi \) vs \( t \) curve, and \( dn/d\pi \) is similarly determined from a \( \pi \) vs \( A \) curve of the spread monolayer. For values of \( \pi \) where there is an energy barrier to adsorption, the adsorbing molecule may be considered to clear a mean area per molecule (\( \Delta A \)) by compressing the molecules already adsorbed at the interface before it is adsorbed itself. This area can be found from the equation

\[
\ln \frac{dn}{dt} = \ln Z - \frac{\pi \Delta A}{kt},
\]

where \( k \) is the Boltzmann constant and \( Z \) is the rate of collision of molecules with the surface. If \( \Delta A \) is assumed to be a constant, a plot of log \( dn/dt \) vs \( \pi \) should be linear, and \( \Delta A \) can be obtained from the slope.

MATERIALS AND METHODS

Phycocyanin was isolated and purified from the blue-green alga Phormidium luridium by a method previously described (9). Bovine serum albumin was obtained from Armour Pharmaceutical Company and was used without further purification. All other chemicals were reagent grade. Phycocyanin and bovine serum albumin concentrations were determined spectrophotometrically, based on \( E_{620}^{1\%} = 62.7 \) and \( E_{280}^{1\%} = 6.9 \), respectively, or by a colorimetric assay developed in this laboratory (10). There was no significant difference in results by the two methods. Protein solutions were prepared in 0.1 \( M \) sodium phosphate buffer, pH 7.0. The pH was adjusted by adding HCl or NaOH. The water was twice distilled in glass.

Measurements of \( \pi \) and \( A \) were taken at 22°C at the air–water interface with a fully automated, continuously recording apparatus previously described (11). The adsorption experiments were done in 28 ml of subphase with a surface area of 40.4 cm\(^2\). Stock protein solution (1.0 to 5.0 ml in volume) was injected into the trough compartment to give the required bulk protein concentration (\( C_b \), subphase concentration before adsorption occurs). The subphase was stirred rapidly for about 1.0 min and then at a considerably slower rate. This procedure does not allow accurate \( \pi \) measurements for the first 2 min of the experiment. Therefore Eqs. [1] to [4] have been used to describe only the ensuing period to the completion of the adsorption process.

The \( \pi \) vs \( A \) isotherms at 22°C were obtained by spreading about 0.02% phycocyanin in 1:1 \( n \)-propanol:0.5 M sodium acetate, pH 7.0, at \( \pi = 0 \).

Adsorption spectra were taken on a Perkin-Elmer Model 320 spectrophotometer.

RESULTS

Kinetics of Adsorption of Phycocyanin at the Air–Water Interface

Injection of \( 8.4 \times 10^{-2} \) mg of phycocyanin into the subphase resulted in a lowering of surface tension at the air–water interface. The effect of phycocyanin on \( \pi \) from pH 2.2 to 7.0 and similar measurements for bovine serum albumin at pH 7.0 are plotted in Fig. 1. The shape of these curves suggests that the absorption process may contain more than one step. Analyzing the curves by Eq. [1] and plotting the data in the form of a log \( [(\pi_{ss} - \pi_t)/(\pi_{ss} - \pi_0)] \) vs \( t \) yields a plot with two distinct slopes (Fig. 1). The discontinuity in
slopes may be characterized by a process suggested by Graham and Phillips (6) that requires two relaxation times $\tau_1$ and $\tau_2$. Values of $\tau_1$ and $\tau_2$ for the adsorption of phycocyanin at pH 7.0 are given in Table I as a function of $C_b$. They become smaller as $C_b$ is reduced from $5 \times 10^{-3}$ to $5 \times 10^{-4}$ mg/ml, and are pH dependent. The values of $\tau_1$ and $\tau_2$ at pH 2.2 for $3.16 \times 10^{-3}$ mg of phycocyanin/ml are 5 and 32 min, respectively. The time ($t_{0.1}$) that it takes for the protein film to produce a change in $\pi$ of 0.1 mN m$^{-1}$ at a $C_b$ of $3.16 \times 10^{-3}$ mg/ml is 5 min at pH 2.2 and 15 min at pH 7.0. Several measurements of the adsorption rate of phycocyanin at various $\pi$ values were obtained by following the change in area at constant $\pi$. A plot of log $A$ vs $t$ is linear and gives a rate of adsorption constant. This constant is $\pi$ dependent (unpublished data). This behavior is consistent with that observed from measuring $\pi$ changes at constant $A$ (Table I and Fig. 1).

To check whether the initial adsorption of phycocyanin to the interface is irreversible, $1/C_b$ was plotted as a function of $\Delta t^{1/2}$ (using the data from Table I). The time $t$ was measured for a change in $\pi$ from 0.1 to 1.0 mN m$^{-1}$. A linear curve consists of two slopes was obtained (not shown). By applying Eq. [2] to the two slopes of the curve ($n = 0.74$ and $0.86$ mg/ml at 0.1 and 1.0 mN m$^{-1}$, respectively), two values for the diffusion coefficient were calculated $2.2 \times 10^{-7}$ and $1.5 \times 10^{-6}$ cm$^2$ sec$^{-1}$ for $C_b > 2 \times 10^{-3}$ mg/ml and $C_b < 2 \times 10^{-3}$ mg/ml, respectively.

The surface area ($AA$) that must be cleared by a bulk molecule before it can be adsorbed at the interface at $\pi > 3$ mN m$^{-1}$ was calculated by using Eq. [4]. The $AA$ obtained from the slope of the curve of log $dn/dt$ vs $\pi$, using the data in Table II was $85 \mu^2$ for $C_b = 2.8 \times 10^{-3}$ mg/ml at pH 7.0. For phycocyanin at pH 2.2 the log $dn/dt$ did not vary significantly with $\pi$ up to 10 mN m$^{-1}$ (Table II).

### Surface Pressure Isotherms

The $\pi$ vs $C_b$ isotherms for phycocyanin and bovine serum albumin at the air–water interface are shown in Fig. 2. There is a relatively large increment in $\pi_{ss}$ (up to 15 mN m$^{-1}$) as a function of $C_b$ at low concentrations of phycocyanin ($C_b = 1 \times 10^{-4}$ to $5 \times 10^{-4}$ mg/ml). At $C_b > 0.001$ mg/ml the slope of the $\pi_{ss}$ vs $C_b$ isotherm decreases, but $\pi_{ss}$ continues to increase to about 25 mN m$^{-1}$ (Fig. 2). The $\pi_{ss}$ vs $C_b$ isotherm for bovine serum albumin is quite different from that of phycocyanin. The initial increment in $\pi_{ss}$ is over a relatively larger $C_b$ range ($\Delta C_b$ of $\sim 0.01$ mg/ml compared to $\Delta C_b$ of $\sim 0.0005$ mg/ml for phycocyanin), and $\pi_{ss}$ reaches a plateau at about 16.5 mN m$^{-1}$. The $\pi_{ss}$ vs $C_b$ isotherm for phycocyanin at pH 2.2 also has
The large increase in $\pi_{ss}$ over a narrow range of $C_b$ (as in pH 7.0), but the curve for $C_b > 10^{-3}$ mg/ml is similar to that of bovine serum albumin (i.e., it reaches a plateau).

The $\pi$ vs $A$ isotherm for spread phycocyanin is shown in Fig. 3. Two relatively sharp changes in the slope are observed upon compression, and there is a hysteresis effect on expansion. When the film is continuously compressed up to about 18 mN m$^{-1}$, the isotherm is reproducible. However, compression to higher $\pi$ results in displacement of the curve to a smaller area on further compression-expansion cycles. Some measurements of the $\pi$-$A$ isotherm were performed manually, allowing 2 min for relaxation to occur, the isotherm did not differ significantly from that obtained by continuous slow compression (0.8 cm$^2$/min). After holding a phycocyanin film at constant area and about 24 mN m$^{-1}$ for 2 min, $\pi$ changed by only 0.9 mN m$^{-1}$. Below 24 mN m$^{-1}$ changes in $\pi$ were substantially lower.

The plot of the compressibility coefficient $[(1/A)(dA/d\pi)]$ as a function of $A$ gives a well-defined minimum at 0.83 m$^2$/mg.

The adsorption spectra of phycocyanin at various pH values are shown in Fig. 4.

**DISCUSSION**

The large increase in $\pi$ for phycocyanin relative to bovine serum albumin in Fig. 1 is an indication that phycocyanin has a strong attraction to the air-water interface. Phycocyanin contains a relatively large number of hydrophobic amino acid residues, no known disulfide linkage, and a very high percentage of helix structure. These factors may contribute to its high surface activity.

When the adsorption of phycocyanin is measured at pH 2.2 rather than at pH 7.0, dramatic differences are observed (Table II and Figs. 1 and 2). The $\pi_{ss}$ of $3 \times 10^{-3}$ mg/ml phycocyanin is larger, the lag time for the initial observation of a rise in $\pi$ is shorter, and the rate of change in $\pi$ is greater. The spectrum of phycocyanin at pH 2.2 (Fig. 4) is characteristic of a protein in a completely

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**TABLE II**

Log of the Rate of Adsorption of Phycocyanin (3.16 $\times 10^{-3}$ mg/ml) as a Function of Surface Pressure ($\pi$) at pH 7.0 and 22°C

<table>
<thead>
<tr>
<th>$\log \frac{dn}{dt}$ (mg m$^{-2}$ min$^{-1}$)</th>
<th>pH 7.0</th>
<th>pH 2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\pi$ (mN m$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.52</td>
<td>1.00</td>
<td>3</td>
</tr>
<tr>
<td>1.80</td>
<td>0.96</td>
<td>6</td>
</tr>
<tr>
<td>2.12</td>
<td>0.96</td>
<td>10</td>
</tr>
<tr>
<td>2.34</td>
<td>1.25</td>
<td>12</td>
</tr>
</tbody>
</table>

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**FIG. 2.** Steady-state surface pressure ($\pi_{ss}$) vs bulk protein concentration ($C_b$) at 22°C for (Q) bovine serum albumin, pH 7.0; (●) phycocyanin, pH 7.0; (O) phycocyanin, pH 2.2.

**FIG. 3.** Surface pressure ($\pi$) vs molecular area ($A$), compression-expansion cycle, for phycocyanin at 22°C, pH 7.0 (---), and compressibility coefficient $[(1/A)(dA/d\pi)]$ vs $A$ plot for phycocyanin (---).
unfolded configuration (1). Apparently unfolding exposes more hydrophobic amino acid residues, which then may exist in the train conformation at the air–water interface.

The close similarity of the spectra of phycocyanin at pH 3.9 and 7.0 suggests that at pH 3.9 the subunits are still associated into various aggregation states (Fig. 4). Analytical centrifugation data indicate that in the presence of 0.1 M phosphate buffer at pH 3.9 the protein is about 60% monomer and 20% hexamer (Robert MacColl's personal communication). At higher pH (up to pH 7.0) the percentage of hexamers would be expected to increase (2). The \( \pi_{ss} \) of phycocyanin solution at pH 3.9 is still somewhat higher than at pH 7.0 but lower than at pH 2.2 (Fig. 1).

The lowest number of amino acid residues in train conformation seems to be at pH 5.5 to 7.0. This suggests that at pH 7.0 the protein may form loops which are partially extended into the subphase, so that the protein is not completely unfolded at the air–water interface. This type of behavior could be relevant to the biological activity of phycocyanin, whose native environment is an interface.

The values of \( \tau_1 \) and \( \tau_2 \) are dependent on the phycocyanin subphase concentration (Table I). This behavior is similar to that of \( \beta \)-casein but in contrast to that of bovine serum albumin and lysozyme, for which \( \tau_1 \) was constant over this concentration range (6). This suggests that the distribution at the interface of phycocyanin segments in trains or loops follows the behavior of \( \beta \)-casein more closely than that of bovine serum albumin or lysozyme. \( \beta \)-Casein has a relatively large fraction of residues in the train configuration (5). Both proteins lack known disulfide linkages and have many hydrophobic amino acid residues, which may explain some similarities in their surface properties.

The smaller values for \( \tau_2 \) obtained at pH 2.2 are presumably due to phycocyanin's greater flexibility in this condition. The molecule apparently can adjust relatively quickly to environmental constraints.

The diffusion coefficient of \( 2.2 \times 10^{-7} \) cm\(^2\) sec\(^{-1}\) for phycocyanin at \( C_b > 2 \times 10^{-3} \) mg/ml (calculated from the data in Table I) is of the same magnitude as that obtained from immunodiffusion studies, \( 4 \times 10^{-7} \) cm\(^2\) sec\(^{-1}\) (9). This may indicate that the factors taken into consideration in deriving Eq. [2], such as irreversible adsorption, were appropriate. At \( C_b < 2 \times 10^{-3} \) mg/ml convection apparently contributes to a higher diffusion coefficient (7).

A rather small \( \Delta A \) of 85 Å\(^2\) was calculated for phycocyanin (using the results from Table II). MacRitchie and Alexander (7) calculated for several proteins values between 100 and 175 Å\(^2\). Apparently fewer amino acid residues are needed to penetrate the interface before a molecule of phycocyanin is adsorbed—a property consistent with the preferential presence of this molecule at interfaces (12). The lack of change in \( \log d n/d t \) with increase in \( \pi \) (up to \( \pi = 10 \) mN m\(^{-1}\)) for the adsorption at pH 2.2 (Table II) may be an indication that at this \( \pi \) range, relatively little work is performed by the unfolded, penetrating phycocyanin molecules. The unfolded protein is very flexible, and the molecules at the interface can probably alter their conformation in response to only slight change in \( \pi \).

The relatively greater slope of the \( \pi \) vs \( C_b \) curve (Fig. 2) for phycocyanin than for bovine albumin may be explained, as in the case of apolipoprotein (13), by the tendency of phycocyanin to form aggregates. However, unfolded phycocyanin at pH 2.2 behaves...
similarly (Fig. 2). Since at pH 2.2 the protein cannot form aggregates, the large slope may be rather a consequence of the high number of hydrophobic amino acid residues and the conformation of the protein. The continuous rise in $\pi$ of the $\pi$ vs $C_b$ curve for high phycocyanin concentration at pH 7.0 is interesting (Fig. 2). At pH 2.2 (Fig. 2), where aggregation does not occur, phycocyanin does not exhibit this behavior, suggesting that the increase may result from formation of aggregates. As aggregates are formed at the surface, new molecules may be able to penetrate the interface, causing an increase in $\pi$. Apparently aggregate formation is due to an increase in surface concentration and not due to high surface pressure. If high $\pi$ were responsible, the $\pi$ vs $t$ curves at high $C_b$ should not reach a plateau, but they do (Fig. 1 and unpublished results).

The areas of 0.95 m²/mg at $\pi = 5$ mN m⁻¹ and 0.83 m²/mg for the minimum compressibility coefficient (Fig. 3) are characteristic for dilute films of proteins (14). The reproducible irregularities observed in the $\pi$ vs $A$ curves and the bimodal patterns of $[(1/A)(dA/ d\pi)]$ vs $A$ (due to these irregularities) are similar to those observed for $\beta$-casein (15) and are consistent with other evidence that these proteins behave in a similar way at the air-water interface.

Apparently the chromophores do not contribute greatly to the surface properties of the spread film of phycocyanin, since the $\pi$ vs $A$ curve (0.95 m²/mg at $\pi = 5$ mN m⁻¹) is in agreement with that expected from a spread protein (14). Linear tetrapyrrole molecules at the interface may be expected to contribute to the molecular area or compressibility of the protein. However, in partially unfolded phycocyanin the chromophore may change its conformation from a linear to a cyclic state (1), which may be partially extended into the subphase. In this configuration it should not make a large contribution to the $\pi$ vs $A$ of phycocyanin.

The behavior of unfolded phycocyanin at pH 2.2 is consistent with this conclusion. A change in conformation of the chromophore from linear to cyclic tetrapyrrole should cause a reduction in $\pi_{ss}$ instead of the observed increase (Fig. 1). Therefore it is reasonable to assume that when the protein unfolds, the chromophore makes only a small contribution to the apparent $\pi$.

REFERENCES