The Binding of Porphyrins by Ligandin

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Spectrophotometric and equilibrium-dialysis measurements show that ligandin (glutathione *S*-transferase B, EC 2.5.1.18) binds monomeric porphyrins at a single site with association constants in the range 10^4 – 10^6 litre/mol at pH 7.0. Binding affinities are paralleled by the tendencies of the porphyrins to aggregate, increasing in the order: uroporphyrins I and III < coproporphyrins I and III ~ haematoporphyrin < protoporphyrin IX. From this it is deduced that the hydrophobic effect is the predominant driving-force for binding. The porphyrins can be displaced from their binding site on ligandin by bromosulphophthalein and oestrone sulphate. In enzyme inhibition studies, 50% inhibition was brought about by $8\text{ }\mu\text{M}$ -haematoporphyrin and by $1\text{ }\mu\text{M}$ -protoporphyrin IX. In the analysis of the haematoporphyrin–ligandin system the self-association of haematoporphyrin was studied in detail. It was found to be limited to dimerization in the concentration range 0– $200\text{ }\mu\text{M}$ at pH 7.0, 25°C and a dimerization constant of 1.9×10^5 litre/mol was determined. Coproporphyrin III has a dimerization constant of 5.2×10^5 litre/mol under the same conditions.

The intracellular protein ligandin (glutathione *S*-transferase B, EC 2.5.1.18) binds non-covalently a host of small molecules, the majority of which have hydrophobic moieties (see, e.g., Litwack *et al.*, 1971; Kamisaka *et al.*, 1975; Ketterer *et al.*, 1975; Ketley *et al.*, 1975; Tipping *et al.*, 1976*a,b,c*). The exact physiological consequences of these interactions are unknown, but it is reasonable to suppose that the presence of ligandin in a cell could influence the uptake of ligands by that cell and their intracellular movement and metabolism. The extents of these processes presumably depend upon the stabilities of the protein–ligand complexes and so quantitative descriptions of the interactions are desirable if the non-enzymic physiological function of ligandin is to be determined.

There is considerable information on the binding by ligandin of ferritoporphyrin IX (haem) and its catabolites bilirubin and biliverdin (Meuwissen *et al.*, 1972, 1974, 1975; Kamisaka *et al.*, 1973, 1975; Ketley *et al.*, 1975; Tipping *et al.*, 1976*a,b*; Woolley *et al.*, 1976), but the metal-free porphyrins have received less attention. Koskelo *et al.* (1976) have shown that radioactively labelled coproporphyrin III is bound by a protein of rat liver cytosol that closely resembles ligandin in its physical properties. This follows earlier reports of the binding of haematoporphyrin (Kamisaka *et al.*, 1975) and protoporphyrin IX (Ketterer *et al.*, 1975). Our aim in the present study was to provide more extensive information on ligandin–porphyrin interactions and to

this end we carried out spectrophotometric and equilibrium-dialysis experiments with haematoporphyrin, coproporphyrins I and III, uroporphyrins I and III and protoporphyrin IX. In analysing our results we took into account porphyrin self-association (Gibson, 1964; Pasternack *et al.*, 1972; Gallagher & Elliott, 1973; Brown *et al.*, 1976), the effects of which were studied in detail for the haematoporphyrin–ligandin system.

Experimental

Materials

Uroporphyrins I and III and coproporphyrins I and III were prepared from the porphyrin precursor 5-aminolaevulinic acid by the methods of Koskelo & Toivonen (1966, 1970). Their ^{14}C -labelled counterparts (sp. radioactivity 300–500 Ci/mol) were prepared from 5-amino[4- ^{14}C]laevulinic acid. Samples of coproporphyrin I were also purchased from Porphyrin Products Inc., Logan, UT, U.S.A. Protoporphyrin IX dimethyl ester, haematoporphyrin, bromosulphophthalein and oestrone sulphate were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Protoporphyrin IX was prepared by hydrolysis of the dimethyl ester by the method of Grinstein (1947). Rat ligandin was prepared as described by Tipping *et al.* (1976*a*).

Porphyrins were checked for purity by t.l.c. on silica gel eluted with 2,6-lutidine/water (3:7, v/v) as

described by Koskelo & Toivonen (1966). All moved as single spots with the exception of protoporphyrin IX, which left a faint trail.

Preparation of solutions

Except for enzyme assays all experiments were carried out in 0.1 M-KCl/0.025 M-potassium phosphate buffer, pH 7.0, *I* 0.16. Stock solutions of porphyrins were prepared in 1–10 mM-KOH and their concentrations determined from the Soret-region absorptions after dilution with 1 M-HCl. The relevant absorption coefficients are those quoted by Falk (1964) except for haematoporphyrin, for which a value of $\epsilon_{402} = 3.2 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ in 1 M-HCl was determined from weighed samples. Stock solutions of ligandin were made up in buffer and their concentrations determined from $A_{1\text{cm}, 280}^{1\%} = 7.6$, a value based on protein determinations by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Spectrophotometry

Measurements were made with a Pye Unicam SP.1800 instrument in cuvettes of 0.1–4 cm light-path at the temperature of the cell compartment ($25 \pm 2^\circ\text{C}$).

Equilibrium dialysis

Experiments were performed as described by Tipping *et al.* (1976c). Concentrations of uroporphyrins and coproporphyrins were determined from their specific radioactivities after counting for radioactivity in a Packard scintillation spectrometer. Haematoporphyrin concentrations were determined by diluting samples with 1 M-HCl and measuring the A_{402} (see above). The presence of protein did not affect these methods of determining concentrations.

Control experiments in the absence of protein showed that equilibrium was achieved within 40 h for the porphyrins studied at 4°C . It was found that significant adsorption of the porphyrins to the apparatus occurred (up to 60% of the total at low concentrations), but this did not invalidate the results since concentrations of porphyrins in solution on both sides of the dialysis membrane were determined in all cases (i.e. the concentrations of unbound porphyrin and of bound+unbound porphyrin). Porphyrins were checked for purity at the end of the equilibrium-dialysis experiments and no significant decomposition was found.

Enzyme assays

The enzymic activity of ligandin as glutathione S-transferase B (EC 2.5.1.18) was assayed by the

method of Habig *et al.* (1974) with 1-chloro-2,4-dinitrobenzene as substrate.

Results

Haematoporphyrin

Fig. 1 shows spectra in the Soret region of haematoporphyrin in the absence and presence of ligandin. According to several reports (Gibson, 1964; Pasternack *et al.*, 1972; Brown *et al.*, 1976), such spectra reflect the extent of aggregation of porphyrins. Aggregated states are characterized by broad spectral bands of relatively low intensity, whereas the monomeric species give rise to sharp, intense Soret bands with maxima at rather longer wavelengths. Thus in Fig. 1 the spectrum of a $0.1 \mu\text{M}$ -solution refers to almost entirely monomeric haematoporphyrin and the spectrum of a $100 \mu\text{M}$ -solution results from a high degree of aggregation. In the presence of an excess of ligandin, haematoporphyrin gives rise to a spectrum of the monomeric type, although not identical with that of unbound monomer, the maximum absorption coefficient being

Fig. 1. Spectra of haematoporphyrin in the Soret region in KCl/phosphate buffer, pH 7.0 at 25°C

The curves (a)–(d) refer to 0.1, 1.0, 10 and $100 \mu\text{M}$ -solutions of haematoporphyrin respectively. In the presence of $100 \mu\text{M}$ -ligandin, both 0.1 and $1.0 \mu\text{M}$ -haematoporphyrin give rise to the same spectrum (----).

Fig. 2. Variations of absorption coefficients with concentration for haematoporphyrin in the absence and presence of ligandin at pH 7.0 and 25°C

[T], total haematoporphyrin concentration in terms of monomers. Measurements were made at 377.5 nm (○), 395 nm (●) and 402 nm (■). (a) Results with protein-free solutions: the points are experimental results and the curves are calculated for a dimerization constant of 1.9×10^5 litre/mol with the absorption coefficients given in the text. (b) Results in the presence of $10.2 \mu\text{M}$ -ligandin: the points are experimental results and the curves are calculated as in (a), but include an association constant of 5.9×10^5 litre/mol for binding at 1.1 sites on the protein (see Fig. 3) and the extinction coefficients for bound haematoporphyrin from Fig. 1 are used. See the text for details.

somewhat lower and the maximum wavelength somewhat longer. This effect has been observed previously for the ligandin-ferriprotoporphyrin IX system (Tipping *et al.*, 1976b) and is presumably due to differences in the environment of the monomeric chromophore in the bound and free states. At a protein concentration of $100 \mu\text{M}$, the same spectrum was obtained for haematoporphyrin concentrations of $0.1 \mu\text{M}$ and $1.0 \mu\text{M}$, which means that the spectrum represents completely bound ligand.

The variations with concentration of the absorption coefficients of haematoporphyrin alone and in the presence of $10.2 \mu\text{M}$ -ligandin are shown in Fig. 2. The well-defined isosbestic point at 377.5 nm for the protein-free solutions suggests that the aggregation process is uncomplicated; the simplest situation would be dimerization:

where M and D represent monomer and dimer respectively and square brackets refer to molarities. The absorbance (A), at any wavelength is given by:

$$A = [T]\epsilon_{\text{obs.}} = [M]\epsilon_M + [D]\epsilon_D \quad (2)$$

where

$$[T] = [M] + 2[D] \quad (3)$$

(i.e. [T] represents the total concentration of haematoporphyrin in terms of monomer), $\epsilon_{\text{obs.}}$ is the observed molar extinction coefficient and ϵ_M and ϵ_D are the molar extinction coefficients of monomer and dimer respectively. Rearrangement of eqn. (2) gives:

$$[T] = \frac{[M]\epsilon_M + [D]\epsilon_D}{\epsilon_{\text{obs.}}} \quad (4)$$

As the total porphyrin concentration becomes very small, the concentration of monomer greatly exceeds that of dimer and in the limit $[T] = [M]$, $\epsilon_M = \epsilon_{\text{obs.}}$. Extrapolation of a plot of $1/\epsilon_{\text{obs.}}$ against $[T]$ to $[T] = 0$ thus gives $1/\epsilon_M$ as the intercept on the ordinate. The haematoporphyrin system allows the determination of values of $\epsilon_{\text{obs.}}$ at sufficiently low concentrations for this extrapolation to be made unambiguously at the wavelengths of Fig. 2 and we find $\epsilon_M = 1.76 \times 10^5$ litre·mol⁻¹·cm⁻¹ at 395 nm and $\epsilon_M = 1.19 \times 10^5$ litre·mol⁻¹·cm⁻¹ at 402 nm. Since 377.5 nm is an isosbestic point it is obvious that in this case:

$$\epsilon_M = \frac{1}{2}\epsilon_D = 1.02 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$$

Elimination of [M] from eqns. (1) and (3) gives:

$$[T] = [D]^{\frac{1}{2}}/K_D^{\frac{1}{2}} + 2[D] \quad (5)$$

and elimination of [M] from eqns. (2) and (3) gives:

$$[D] = \frac{[T](\epsilon_M - \epsilon_{\text{obs.}})}{(2\epsilon_M - \epsilon_D)} \quad (6)$$

Combination of eqns. (5) and (6) and rearrangement gives:

$$\left(\frac{[T]}{\epsilon_M - \epsilon_{\text{obs.}}} \right)^{\frac{1}{2}} = \frac{2}{2\epsilon_M - \epsilon_D} \{ [T](\epsilon_M - \epsilon_{\text{obs.}}) \}^{\frac{1}{2}} + \left[\frac{1}{K_D(2\epsilon_M - \epsilon_D)} \right]^{\frac{1}{2}} \quad (7)$$

Thus for dimerization, a plot of $\{ [T]/(\epsilon_M - \epsilon_{\text{obs.}}) \}^{\frac{1}{2}}$ against $\{ [T](\epsilon_M - \epsilon_{\text{obs.}}) \}^{\frac{1}{2}}$ should give a straight line, the intercept and slope of which can be used to calculate K_D and ϵ_D . Our results at 395 nm and 402 nm give excellent linear fits to eqn. (7) ($R > 0.995$ in both cases) and we obtain $\epsilon_D = 1.08 \times 10^5$ litre·mol⁻¹·cm⁻¹ at 395 nm ($K_D = 1.95 \times 10^5$ litre/mol) and $\epsilon_D = 7.0 \times 10^4$ litre·mol⁻¹·cm⁻¹ at 402 nm ($K_D = 1.65 \times 10^5$ litre/mol). The curves of Fig. 2(a) were calculated by using these values for the absorption coefficients and a weighted mean of $K_D = 1.9 \times 10^5$ litre/mol. From this analysis we conclude that the aggregation of haematoporphyrin is confined to dimerization under the conditions of our experiments in the concentration range 0–200 μM .

In the presence of ligandin, haematoporphyrin exists in more than two forms because of binding to the protein. The simplest situation is that where there is only one class of independent binding sites on the protein, characterized by a single bound spectrum. We may then write for wavelengths λ_1 – λ_3 :

$$A_1 = \epsilon_{11}[B] + \epsilon_{12}[M] + \epsilon_{13}[D] \quad (8)$$

$$A_2 = \epsilon_{21}[B] + \epsilon_{22}[M] + \epsilon_{23}[D] \quad (9)$$

$$A_3 = \epsilon_{31}[B] + \epsilon_{32}[M] + \epsilon_{33}[D] \quad (10)$$

where $[B]$ represents bound haematoporphyrin. If the absorption coefficients ϵ_{11} – ϵ_{33} are known, then measurements of absorbance at the three wavelengths λ_1 – λ_3 allow corresponding concentrations of bound, monomeric and dimeric haematoporphyrin to be determined by solving the sets of simultaneous equations (8)–(10). In principle, the choice of wavelengths is arbitrary. We have chosen $\lambda_1 = 377.5$ nm, $\lambda_2 = 395$ nm and $\lambda_3 = 402$ nm; these are the protein-free isosbestic point, the protein-free $\lambda_{\max}$, and the bound $\lambda_{\max}$, respectively. The values of ϵ_{11} – ϵ_{33} are known from Fig. 1 ($\epsilon_{11} = \epsilon_B(377.5 \text{ nm}) = 6.0 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_{21} = \epsilon_B(395 \text{ nm}) = 1.08 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_{31} = \epsilon_B(402 \text{ nm}) = 1.41 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) and from the analysis of the protein-free solutions of haematoporphyrin. Values of $[B]$, $[M]$ and $[D]$ were calculated from the results

shown in Fig. 2(b). The corresponding values of $\bar{\nu}$ ($= [B]/\text{total protein concentration}$) and $[M]$ were used to construct a Scatchard (1949) plot. This shows that ligandin binds monomeric haematoporphyrin at a single site with an association constant (K_A) of $5.9 \times 10^5 \text{ litre/mol}$. Because the calculation of $[B]$, $[M]$ and $[D]$ by this method does not involve the use of $[T]$, a useful check on the results is to compare the sums $[B] + [M] + 2[D]$ with the values of $[T]$ to which they refer; we find that our results agree to within 5% for each titration point. Another back-check is to compare K_D values determined in the absence and presence of protein; we find a value of $1.8 \times 10^5 \text{ litre/mol}$ from the results of Fig. 2(b), in excellent agreement with that of $1.9 \times 10^5 \text{ litre/mol}$ obtained from the results of Fig. 2(a). In summary therefore we find that under the conditions of our experiments the haematoporphyrin–ligandin system can be described by two equilibria:

where P represents protein. These values, together with the absorption coefficients ϵ_{11} – ϵ_{33} , were used to calculate the curves of Fig. 2(b).

The binding of haematoporphyrin by ligandin was also studied by equilibrium dialysis at 4°C . The results are presented in Fig. 4 where the unbound ligand concentration $[L]$ refers to monomer+dimer (i.e. $[L] = [M] + 2[D]$). The monomer concentrations cannot be extracted from these values of $[L]$ because the dimerization constant for haematoporphyrin at 4°C is not known. However, by assuming that K_D does not vary significantly with temperature we calculated the contributions of monomer and dimer to $[L]$ for the results shown in Fig. 4. A Scatchard plot of $\bar{\nu}/[M]$ against $\bar{\nu}$ then gave $K_A = 4.0 \times 10^5 \text{ litre/mol}$ and n (no. of binding sites) = 1.0. These parameters, together with $K_D = 1.9 \times 10^5 \text{ litre/mol}$, were used to calculate the curve shown in Fig. 4. Admittedly this approach is approximate, since there is no guarantee that K_D is temperature-invariant, but K_D values of 10^5 litre/mol and $4 \times 10^5 \text{ litre/mol}$ give similar binding parameters ($K_A = 4.2 \times 10^5 \text{ litre/mol}$, $n = 0.9$, and $K_A = 3.9 \times 10^5 \text{ litre/mol}$, $n = 1.1$ respectively), indicating that the particular value of K_D chosen does not affect the results critically.

The difference spectrum that results from the binding of haematoporphyrin by ligandin was decreased considerably by the addition of oestrone sulphate or bromosulphophthalein. These two ligands share a single primary binding site on ligandin (Tipping *et al.*, 1976c) and so it is probable that this site is also shared by haematoporphyrin. Haematoporphyrin also proved an effective inhibitor of the

Fig. 3. Scatchard plot for the binding of haematoporphyrin monomers by ligandin at pH 7.0 and 25°C

The results from Fig. 2(b) were used to determine the concentrations of protein-bound and monomeric unbound haematoporphyrin, $[B]$ and $[M]$ respectively. From these $\bar{\nu}$ ($= [\text{bound porphyrin}]/[\text{protein}]$) and $\bar{\nu}/[M]$ were calculated. The plot thus refers to the equilibrium between bound and monomeric porphyrin.

Fig. 4. The binding by ligandin of haematoporphyrin ($\circ$), coproporphyrin I ($\square$), coproporphyrin III ($\blacksquare$), uroporphyrin I ($\triangle$) and uroporphyrin III ($\blacktriangle$) at pH 7.0 and 4°C

Results are from equilibrium-dialysis measurements with protein concentrations between 5 and 20 μM . [L] is the concentration of unbound ligand (monomers + dimers) and $\bar{v} = [\text{bound porphyrin}]/[\text{protein}]$. The upper curve (haematoporphyrin) is calculated for an association constant of 4.0×10^5 litre/mol, a single binding site and a dimerization constant of 1.9×10^5 litre/mol. The lower curve (coproporphyrins I and III) is calculated for an association constant of 2.6×10^5 litre/mol, a single binding site and a dimerization constant of 4.0×10^5 litre/mol.

glutathione *S*-transferase activity of ligandin: with 1-chloro-2,4-dinitrobenzene as substrate, 50% inhibition was achieved by 8 μM -haematoporphyrin at a protein concentration of 0.04 μM .

Coproporphyrins I and III

Fig. 5 shows the spectra of solutions of coproporphyrin III in the absence and presence of ligandin. These results parallel those for haematoporphyrin and again we conclude that ligandin binds monomers. Closely similar results were obtained with coproporphyrin I. The concentration-dependences of the absorption coefficients at 376 nm and 395 nm for coproporphyrin III are shown in Fig. 6. Again the results parallel those for haematoporphyrin, and are consistent with simple dimerization. We find $\epsilon_M(395 \text{ nm}) = 2.20 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_D(395 \text{ nm}) = 9.2 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, $\epsilon_M(376 \text{ nm}) = \frac{1}{2}\epsilon_D(376 \text{ nm}) = 1.25 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and $K_D = 5.2 \times 10^5 \text{ litre/mol}$ at 25°C.

Fig. 5. Spectra of coproporphyrin III in the Soret region at pH 7.0 and 25°C

Curves (a) and (b) represent 0.25 and 25 μM -coproporphyrin III respectively. The broken line represents 0.25 μM -coproporphyrin III in the presence of 25 μM -ligandin.

Quantitative studies of the interactions of ligandin with coproporphyrins I and III were made by equilibrium dialysis at 4°C. The results are shown as a binding plot in Fig. 4, with the concentration of unbound porphyrin [L] again referring to monomers + aggregates. As these ligands were radioactively labelled, we were able to measure sufficiently low values of ligand to make the total protein concentration essentially equal to the unliganded protein concentration. Bearing in mind that as $\bar{v}$ tends to zero so does the concentration of unbound ligand, and thus $[L] \rightarrow [M]$, then from the Scatchard equation for the binding of monomers at a single site:

$$\frac{\bar{v}}{[M]} = (1 - \bar{v})K_A \quad (11)$$

we may write:

$$\lim_{\bar{v} \rightarrow 0} \left(\frac{\bar{v}}{[L]} \right) = K_A \quad (12)$$

Extrapolation to the ordinate of the binding plots of Fig. 4 thus gives an association constant of 2.6×10^5 litre/mol for both coproporphyrin isomers, if a single binding site is assumed. The curvature of the

Fig. 6. Variations of absorption coefficients with concentration for coproporphyrin III at pH 7.0 and 25°C [T], total coproporphyrin III concentration in terms of monomers. The points are experimental results at 395 nm (●) and 376 nm (○). The curves were calculated for a dimerization constant of 5.2×10^5 litre/mol by using the absorption coefficients given in the text.

binding plot for the two coproporphyrins (Fig. 4) is fitted best by a dimerization constant of 4.0×10^5 litre/mol, which is close to the value of 5.2×10^5 litre/mol obtained at 25°C (Fig. 6).

Competition effects were studied by equilibrium dialysis with [^{14}C]coproporphyrin I as the test ligand and bromosulphophthalein, coproporphyrin III and uroporphyrins I and III as potential competitors. Bromosulphophthalein, which binds strongly to ligandin ($K_A = 1.1 \times 10^7$ litre/mol at pH 7.0 and 4°C; Tipping *et al.*, 1976c) was the most effective competitor, followed by coproporphyrin III and then by the two uroporphyrins. These results suggest that all the ligands share the same binding site.

Uroporphyrins I and III

The spectra of protein-free solutions of uroporphyrin III show that it does not tend to aggregate (Mauzerall, 1965), and we have found that the same applies to uroporphyrin I. Partly because of this, and

Fig. 7. Difference spectra associated with the binding of protoporphyrin IX by ligandin, and the effect of bromosulphophthalein at pH 7.0 and 25°C

The sample cell contained ligandin ($10 \mu\text{M}$), protoporphyrin IX ($10 \mu\text{M}$) and, in one case, bromosulphophthalein ($80 \mu\text{M}$). The corresponding reference cell contained the components of the sample cell except for protein. ----, Difference spectrum in the absence of bromosulphophthalein immediately after mixing; —, same system after 1 h; ····, difference spectrum in the presence of bromosulphophthalein ($80 \mu\text{M}$) 1 h after mixing.

partly because the interactions with ligandin are weak, there is little scope for spectral perturbation by the protein. At a concentration of uroporphyrin I of $1 \mu\text{M}$, we found that the presence of $20 \mu\text{M}$ -ligandin caused a shift in λ_{max} from 399 nm to 401 nm and a small decrease in absorption coefficient from 2.34×10^5 to 2.20×10^5 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$. From equilibrium-dialysis measurements (Fig. 4), and with the argument described for the coproporphyrins, we estimate an association constant of 3×10^4 litre/mol for both uroporphyrins, if, like haematoporphyrin, they bind at a single site.

Protoporphyrin IX

This compound presented considerable experimental difficulties. Under the conditions of our experiments it did not cross the dialysis membrane, thus precluding attempts to measure binding to

ligandin by this method. Spectral studies were hampered by time effects: after preparing an experimental solution by dilution with buffer of a stock solution, the resulting spectrum was not constant with time for at least 1 h. This effect, together with the strong tendency of protoporphyrin IX to adsorb to the silica cuvettes that were used in this work, made reliable measurements at concentrations less than about $5\mu\text{M}$ impossible. At and above this concentration the spectra of protoporphyrin IX indicate that it is highly aggregated, as previously shown by Maehly (1961), Gibson (1964) and Brown *et al.* (1976).

We were able to demonstrate the binding of protoporphyrin IX by ligandin by using difference spectroscopy (Fig. 7), but for reasons given above quantitative measurements were not attempted. Bromosulphophthalein brought about a slight diminution in the difference-spectrum (Fig. 7), but was far less effective than when competing for haematoporphyrin at the same concentrations of protein and ligands. Protoporphyrin IX was a more effective inhibitor of the glutathione *S*-transferase activity of ligandin than haematoporphyrin, 50% inhibition being achieved by $1\mu\text{M}$ -protoporphyrin under conditions where $8\mu\text{M}$ -haematoporphyrin was required (see above).

Discussion

In common with other workers (Gibson, 1964; Gallagher & Elliott, 1973; Brown *et al.*, 1976) we have found that, as a result of aggregation processes, the spectra of protoporphyrin IX, haematoporphyrin and coproporphyrins I and III are dependent on concentration. For haematoporphyrin and coproporphyrin III we find that the concentration-dependences can be ascribed to simple dimerization. Dimerization was also considered sufficient to explain the aggregations of protoporphyrin IX, mesoporphyrin and haematoporphyrin at pH 9.1 (Gibson, 1964), of haematoporphyrin in 20 mM-NaOH (Gallagher & Elliott, 1973), and of several *meso*-substituted synthetic porphyrins at pH 7.5 (Pasternack *et al.*, 1972). On the other hand Brown *et al.* (1976) considered that at pH 6.98 haematoporphyrin, coproporphyrin III and deuteroporphyrin undergo more complex aggregation processes, with micellization superseding dimerization at approximately $4\mu\text{M}$ in all three cases. However, for haematoporphyrin and coproporphyrin III these conclusions were based on only a few results and were made largely by analogy with results for deuteroporphyrin. Our own more extensive results indicate that these analogies are invalid and so in analysing the protein-porphyrin binding results, our treatment of the unbound porphyrins in terms only of monomer and dimers is justified.

The hydrophobicities of the porphyrin side chains

are apparently correlated with both binding affinity and the tendency of the porphyrins towards self-association. The two uroporphyrins have four acetate side chains, show little tendency to aggregate (Mauzerall, 1965, and the present work) and bind weakly to ligandin ($K_A = 3 \times 10^4$ litre/mol at 4°C). Coproporphyrins I and III, which have four propionate and four methyl side chains, bind more strongly ($K_A = 2.6 \times 10^5$ litre/mol at 4°C) and aggregate significantly ($K_D = 5.2 \times 10^5$ litre/mol for coproporphyrin III at 25°C). Haematoporphyrin, which possesses two propionate, two methyl and two 1-hydroxyethyl side chains, has a binding affinity ($K_A = 5.9 \times 10^5$ litre/mol at 25°C) marginally greater than that of the coproporphyrins and a dimerization constant of 1.9×10^5 litre/mol at 25°C . The most hydrophobic set of side chains, two propionate, four methyl and two vinyl, is possessed by protoporphyrin IX, which aggregates strongly (Brown *et al.*, 1976, and the present work). Although technical difficulties precluded the accurate measurement of the binding of this porphyrin by ligandin, our results suggest that it binds more strongly than haematoporphyrin, since at equal concentrations the latter is more easily displaced from ligandin by bromosulphophthalein than is protoporphyrin IX. Furthermore, protoporphyrin IX is a more effective inhibitor of the glutathione *S*-transferase activity of ligandin than is haematoporphyrin. A high binding affinity for protoporphyrin is also suggested by our study of the ferriprotoporphyrin IX-ligandin system (Tipping *et al.*, 1976b) in which it was shown that monomeric ferriprotoporphyrin IX binds to ligandin with an association constant of 1×10^7 – 2×10^7 litre/mol with binding probably not involving the central iron atom.

Our competition results indicate that the porphyrins studied bind at the same site on ligandin as oestrone sulphate and bromosulphophthalein. All evidence presently available suggests that this site is common to all ligands, with the exception of glutathione (Kamisaka *et al.*, 1975; Ketterer *et al.*, 1975; Tipping *et al.*, 1976a,b,c) and that the major driving force for binding is the hydrophobic effect, as shown in the present work by the increase in affinity with the increase in hydrophobicity of the porphyrin side chains (see above). A minor participation of electrostatic interactions is apparently involved in the binding of steroid conjugates (Ketterer *et al.*, 1975; Tipping *et al.*, 1976c) and so it is conceivable that interactions involving the propionate side chains in positions 6 and 7 common to all six porphyrins studied make a contribution to the free energy of binding.

Much of the foregoing discussion may also apply to the other non-specific intracellular binding protein, aminoazodye-binding protein A (Ketterer *et al.*, 1976), which is probably identical with Z-protein (Levi *et al.*, 1969). The range of ligands bound by this protein parallels that of ligandin rather closely

(Ketterer *et al.*, 1976; Tipping *et al.*, 1976a,b). Spectrophotometric measurements indicate that protein A binds the monomeric forms of haemato-porphyrin and coproporphyrin I (E. Tipping, B. Ketterer & P. Koskelo, unpublished observations), and the gel-filtration experiments of Koskelo *et al.* (1976) revealed the presence of a porphyrin-binding protein with a lower molecular weight than ligandin, which possibly corresponds to protein A.

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