Deuteroporphyrin–albumin binding equilibrium

The effects of porphyrin self-aggregation studied for the human and the bovine proteins

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The binding equilibrium of deuteroporphyrin IX to human serum albumin and to bovine serum albumin was studied, by monitoring protein-induced changes in the porphyrin fluorescence and taking into consideration the self-aggregation of the porphyrin. To have control over the latter, the range of porphyrin concentrations was chosen to maker dimers (non-covalent) the dominant aggregate. Each protein was found to have one high-affinity site for deuteroporphyrin IX monomers, the magnitudes of the equilibrium binding constants (25°C, neutral pH, phosphate-buffered saline) being $4.5(\pm 1.5) \times 10^7 \, \mathrm{M^{-1}}$ and $1.7(\pm 0.2) \times 10^6 \, \mathrm{M^{-1}}$ for human serum albumin and for bovine serum albumin respectively. Deuteroporphyrin IX dimers were found to bind directly to the protein, each protein binding one dimer, with high affinity. Two models are proposed for the protein-binding of porphyrin monomers and dimers in a porphyrin system having both species: a competitive model, where each protein molecule has only one binding site, which can be occupied by either a monomer or a dimer; a non-competitive model, where each protein molecule has two binding sites, one for monomers and one for dimers. On testing the fit of the data to the models, an argument can be made to favour the non-competitive model, the equilibrium binding constants of the dimers, for the non-competitive model (25°C, neutral pH, phosphate-buffered saline), being: $8.0(\pm 1.8) \times 10^8 \, \mathrm{M^{-1}}$ and $1.2(\pm 0.6) \times 10^7 \, \mathrm{M^{-1}}$ for human serum albumin and bovine serum albumin respectively.

Porphyrin IX species such as deuteroporphyrin IX, haematoporphyrin IX, haematoporphyrin derivative, mesoporphyrin IX and protoporphyrin IX are photodynamic agents that have been investigated at several levels (including the clinical) for their two potential roles in tumour therapy: localization and regression (see, e.g., Kessel & Dougherty, 1983; Kessel, 1984a,b). These same molecules, when in an aqueous medium, can engage in two types of activities that bear on their therapeutic abilities: (a) self-aggregation, the smallest aggregate being a dimer, the higher-order species being micelle-like polymers (Falk, 1964; Smith, 1975; Brown et al., 1976; Karns et al., 1979; Margalit & Cohen, 1983; Margalit et al., 1983; Margalit & Rotenberg, 1984); (b) binding to serum

proteins, for serum-containing systems, i.e. to albumin, haemopexin and, as suggested more recently, lipoproteins (Morgan et al., 1980; Reddi et al., 1981; Lamola et al., 1981; Smith & Neuschatz, 1983; Moehring et al., 1983; Jori et al., 1984; Grossweiner & Goyal, 1984; Moan & Western, 1984).

The range of porphyrin doses administered in vivo is such that the pre-administered dose is already aggregated. Although dilution on administration might shift the balance among the different aggregates, the magnitudes of the aggregation constants are such that they are not sufficient to drive the aggregates to complete monomerization (Brown et al., 1976; Margalit & Cohen, 1983; Margalit et al., 1983; Margalit & Rotenberg, 1984). With regard to binding of serum proteins, albumin will bind the major share of the dose. Not only is this protein in considerable excess compared with haemopexin, it has also been

Abbreviations used: HSA, human serum albumin; BSA, bovine serum albumin.

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shown to have a higher affinity for porphyrins (Morgan et al., 1980). Hence, at least for the period between porphyrin administration and activation during phototherapy, which is of the order of several hours to 72h (Kessel & Dougherty, 1983), albumin is the endogenous carrier of porphyrin in the circulation. Another clinical situation in which albumin can act as a carrier for porphyrins (endogenous, in this case) is in certain types of porphyrias (Lamola et al., 1981; Lamola, 1982). The activity of albumin as a carrier can have two effects on treatment with porphyrin: detrimental effect, since the carrier can contribute to premature clearance of the drug from the system, and a beneficial effect, since the carrier has been suggested to be involved in the preferential retention of the drug in tumours in vivo (Bugelsky et al., 1981). The relative weights of these effects are not clear yet.

Thus both types of porphyrin equilibria, the self-aggregation and the protein-binding, should co-exist in circulation and be factors in determining the fraction of the dose available at the target, the latter being membranes of the tumour cells as well as intracellular locations. [This holds not only in vivo, but also in vitro, in cell and tissue cultures where effects of porphyrins are studied (see, e.g., Moan & Sommer, 1981; Kessel, 1981, 1982). In such systems the porphyrin is introduced into the aqueous medium, which usually contains several percent serum.]

Sponsored (at least in part) by the clinical implications, the porphyrin–albumin association has been the subject of a fair share of studies for several porphyrin species (Morgan et al., 1980; Reddi et al., 1981; Lamola et al., 1981; Moehring et al., 1983; Smith & Neuschatz, 1983). However, in many of these studies the aggregation state of the porphyrin in the system was not given due consideration. This is encountered in both types of experimental designs regularly employed: titrating a set protein concentration with increasing concentrations of porphyrin, each porphyrin addition constituting a different state of aggregation, or vice versa, i.e. titrating a set porphyrin concentration (aggregated) with protein. This situation, and our previous experience with the self-aggregation of porphyrins (Margalit & Cohen, 1983; Margalit et al., 1983; Margalit & Rotenberg, 1984), have prompted us to investigate anew the binding of porphyrins to serum albumins.

In the first stage of this study, which we report in the present paper, we focused on the monomer/dimer concentration region. As to the choice of experimental technique, the range of porphyrin concentration we employed (up to the micromolar) is too low to make use of the porphyrin absorption spectra. Classical methods such as equilibrium

dialysis, ultrafiltration or gel filtration have limitations, since the porphyrin adsorbs on the membranes and on the gel matrix. Another technique applied previously, monitoring of the quenching of protein fluorescence, might have limited sensitivity for sites removed from the vicinity of the protein fluorophores. We therefore employed the approach of monitoring the changes in porphyrin fluorescence (Lamola et al., 1981), which is most suitable for the range of porphyrin concentrations that we studied, adapting the approach to our specific systems, as detailed below in the Experimental section.

The study reported in the present paper is for albumins of two sources: human (HSA) and bovine (BSA). The porphyrin chosen, to start with, is deuteroporphyrin IX. The rationale for this choice is that data for this species will be relevant for all the porphyrins listed above. Following this, the effect of the peripheral residues constituting the only structural differences among the porphyrins of this series ($-H$, $-CHOHCH_3$, $-C_2H_5$ and $-CH=CH_2$, for deuteroporphyrin IX, haematoporphyrin IX, mesoporphyrin IX and protoporphyrin IX respectively) can be pursued.

Experimental

Materials

Deuteroporphyrin IX was purchased from Porphyrin Products, Logan, UT, U.S.A. HSA and BSA (fractions V) were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Deuteroporphyrin IX stock solutions and phosphate-buffered saline were prepared and all porphyrin solutions were protected from exposure to light as previously described (Margalit & Rotenberg, 1984).

Methods

Reactions were carried out at 25°C. Fluorescence spectra were recorded on a Perkin–Elmer model MPF-44B fluorimeter.

Two types of experimental designs were used. In the first the effort was focused on the contribution of monomers to the binding, and in the second on the contribution of both monomers and dimers. In this section we present the details of the experimental designs and the determination of bound porphyrin. The procedures by which these data were processed in order to obtain porphyrin-protein binding constants are presented together with the data in the Results and discussion section.

Experiment I. For monomers a series of reaction mixtures was prepared in which the porphyrin concentration was constant at 50nM. (For the experimental conditions used, where the ligand,

i.e. the protein, was not in excess, going below this concentration would increase the extent of noise to an undesirable level.) The protein concentration was varied over the $0.1–1.0\,\mu M$ range for BSA and the $5–35\,nM$ range for HSA. The fluorescence emission spectrum for each reaction mixture was recorded over the $600–650\,nm$ range (excitation was at $394\,nm$). In addition, the spectrum of the initial protein-free porphyrin solution was also recorded. Under our experimental conditions peak emission of free aqueous deuteroporphyrin IX is at $612\,nm$ and peak emissions of deuteroporphyrin IX bound to BSA or to HSA are at $616\,nm$ and $625\,nm$ respectively.

$F_{obs.}$, the fluorescence measured for each reaction mixture (at equilibrium) at the wavelength of peak emission for the bound porphyrin, is:

$$F_{obs.} = F_f + F_b \qquad (1)$$

F_f, the emission of the free porphyrin, and F_b, the emission of the bound porphyrin, are related to their respective concentrations as follows:

$$F_f = \alpha[M]; \quad F_b = \varepsilon[S] \qquad (2)$$

where [M] and [S] are the molar concentrations of free and bound (fluorescent) porphyrin respectively, and α and ε are the relevant coefficients relating emission intensity to concentration for a fluorophore.

Denoting the emission of the initial porphyrin solution (before addition of protein) for a given reaction mixture at $616\,nm$ or $625\,nm$ (for BSA or HSA, to be added) as F_0, will give:

$$F_0 = \alpha[T] \qquad (3)$$

where [T] is the total porphyrin concentration in the system, corresponding in this case to the initial total monomer concentration. α and ε were determined by the following procedure. First, the spectrum of a given free porphyrin solution was recorded, and then the porphyrin was titrated with protein to saturation and the spectrum of the fully bound porphyrin was recorded. This procedure was repeated for several solutions, at different days. Although both coefficients were found to vary from one titration to another, we have found their ratio ε/α to have a constant magnitude, 1.25 ± 0.05 for BSA and 2.8 ± 0.2 for HSA. Hence for each experimental system it sufficed to obtain the specific α (eqn. 3) and calculate the specific ε from the known ratio. The desired quantity [S] could be extracted for each reaction mixture from the measured $F_{obs.}$ and the known magnitudes of [T], α and ε.

Experiment II. For porphyrin monomers and dimers a series of reaction mixtures was prepared in which the protein concentration was constant ($0.5\,\mu M$ for HSA, $5\,\mu M$ for BSA) and the porphyrin

concentration was varied over the $0.1–0.6\,\mu M$ range (corresponding to a decrease from 74% to 45% monomers). The upper limit was chosen to avoid systems in which the fraction of aggregates of higher order than dimers is no longer negligible (Margalit *et al.*, 1983; Margalit & Rotenberg, 1984).

Fluorescence spectra were recorded as in Expt. I, and peak emissions of free monomer and bound porphyrin (for HSA and for BSA) were at the wavelengths listed above.

Once the system has both monomers and dimers [the latter non-fluorescent (Margalit *et al.*, 1983; Margalit & Rotenberg, 1984)], eqn. (3) no longer holds, as [T] (which is expressed in terms of mol of porphyrin) and the initial free monomer concentration are no longer the same. Therefore the emission of the reaction mixture at two wavelengths was taken into account:

$$F_{obs.} = \alpha[M] + \varepsilon[S] \qquad (4)$$

$$F_{612} = \alpha'[M] + \varepsilon'[S] \qquad (5)$$

where $F_{obs.}$, α, ε, [M] and [S] are as defined above, F_{612} is the observed emission of the reaction mixture at $612\,nm$, and α' and ε' the corresponding coefficients at that wavelength.

α' and ε' were determined, using the same procedure for α and ε, and α'/ε' was also verified to be constant, 1.3 ± 0.09 for BSA and 5.4 ± 0.7 for HSA. The specific magnitudes of α, α', ε and ε' for each reaction mixture were determined from the fluorescence of a sample of the porphyrin solution before its addition to the protein, by using also the known [T] for that solution and the magnitude of the dimerization equilibrium constant (Margalit & Rotenberg, 1984). Since α, α', ε, ε' and [T] are known for each reaction mixture, it was sufficient to measure $F_{obs.}$ and F_{612} in order to extract the desired quantity [S].

Results and discussion

Expt. I: binding of deuteroporphyrin IX monomers to HSA and to BSA

We found the increase in bound porphyrin with the increase in total protein to follow a saturating pattern, quite similar to that shown in Fig. 1 (where the data are already processed, as is detailed below).

Assuming a single high-affinity binding site for porphyrin monomers, per protein molecule:

$$M + A \underset{}{\overset{K_1}{\rightleftharpoons}} MA \qquad (6)$$

M representing, as already defined, the free porphyrin monomer, A the free albumin in the system, MA the albumin–porphyrin complex and

K_1 the corresponding equilibrium binding constant.

In the present experimental design the protein is the ligand. Thus processing of the data in accordance with the Langmuir procedure requires knowledge of the concentration of the bound protein. On the basis of the 1:1 stoichiometry, the bound protein (denoted as $[A]_b$) can be replaced by the bound porphyrin:

$$\bar{v} = \frac{[A]_b}{[T]} = \frac{[MA]}{[T]} = \frac{K_1[A]}{1 + K_1[A]} \qquad (7)$$

Typical experimental data are plotted in Fig. 1 for BSA and for HSA (the points), together with the theoretically expected curve according to eqn. (7) for the K_1 magnitudes listed in Table 1. The good fit of the experimental data for each protein with the theoretical expectation over the entire range of protein concentrations employed, for the same (single) magnitudes of K_1, constitutes a confirmation of the model proposed and of the assumption concerning the stoichiometry.

To obtain another, independent, test of the proposed model, we have also processed the data in accordance with the Hill procedure. In this procedure the concentration of the bound macromolecule (porphyrin, in this case) is used directly. For each case, a single linear plot with a slope of 1 was found to fit the data, indicating no co-operativity. The K_1 magnitudes determined are in good agreement with those obtained from the other method of data processing (see Table 1).

To summarize: we have found BSA and HSA each to have a single high-affinity site for deuteroporphyrin monomers, the affinity of HSA being one order of magnitude higher than that of BSA.

Expt. II: binding of porphyrin monomers and dimers to albumin

Typical data showing the increase in bound porphyrin with the increase in the total porphyrin concentration in the system are illustrated in Fig. 2.

In the following, we present several possible models for the binding, process the data in order to obtain the equilibrium constants defined in each model, and then test the data to assess which of these models fits best. Our policy in formulating possible models was to consider simple features (at least to start with). As shown below, acceptable fit of data to model could be obtained without the
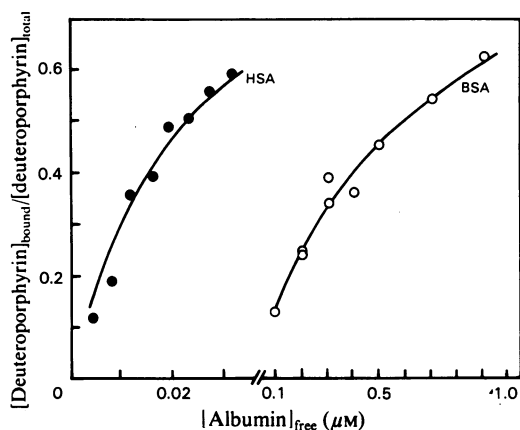

Fig. 1. *Langmuir isotherms of deuteroporphyrin binding to albumins*
Points are experimental: O, BSA; ●, HSA. Continuous curves are theoretical expectations drawn according to eqn. (10) in the text, for the magnitudes of K_1 listed in Table 1.
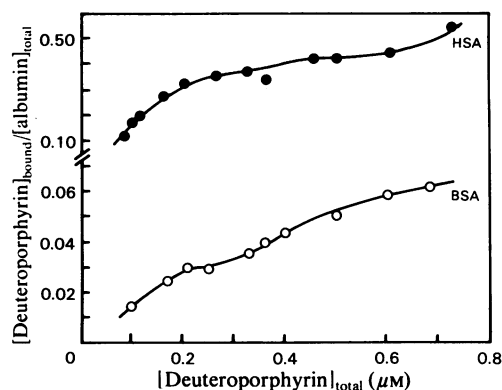

Fig. 2. *Binding of porphyrin to albumin as a function of total porphyrin concentration*
The porphyrin concentration range was within the monomer/dimer equilibrium. Points are experimental: O, BSA; ●, HSA. Curves (non-theoretical) are drawn in order to follow trends in data.

Table 1. *Equilibrium constants of deuteroporphyrin IX binding to serum albumin at 25°C and neutral pH*

Protein	K_1 (M^{-1})		K_2 (M^{-1})	
	Langmuir	Hill	Competitive	Non-competitive
HSA	$4.5(\pm1.5)\times10^7$	6.8×10^7	$6.0(\pm2.5)\times10^8$	$8.0(\pm1.8)\times10^8$
BSA	$1.8(\pm0.2)\times10^6$	2.7×10^6	$1.2(\pm0.5)\times10^7$	$1.2(\pm0.6)\times10^7$

need to resort to models with complex features. Two classes of models were considered, differing in the nature of involvement assumed for the dimers.

Class A: indirect involvement of the dimers in the porphyrin–albumin binding. The basic assumptions are as follows. (1) Only the monomer binds to the protein. Dimers participate through their dissociation into monomers. (2) There is only one high-affinity site for porphyrin monomers, per protein molecule.

Two equilibria suffice to describe the system, the monomer–protein binding (eqn. 6) and the dimerization:

$$2M \overset{K_d}{\rightleftharpoons} D \qquad (8)$$

where D is the dimer. For this model all the equilibrium constants have already been determined.

Class B: direct involvement of the dimers in the porphyrin–albumin binding. Two cases were distinguished: competitive and non-competitive binding.

For case 1 the basic assumptions are as follows: (1) There is only one high-affinity site per protein molecule. (2) Monomer and dimer compete for the same site. (3) Similarly to the experimentally confirmed assumption concerning free dimers (Margalit *et al.*, 1983), the bound dimer remains non-fluorescent.

Three equilibria are needed to describe the system, the monomer–protein binding (eqn. 6), the dimerization (eqn. 8) and the dimer–protein binding:

$$D + A \overset{K_2}{\rightleftharpoons} DA \qquad (9)$$

where DA is the dimer–protein complex and K_2 is the dimer–protein binding constant, taking into consideration eqns. (6), (8) and (9) and expressions for conservation of matter, can yield the following expressions:

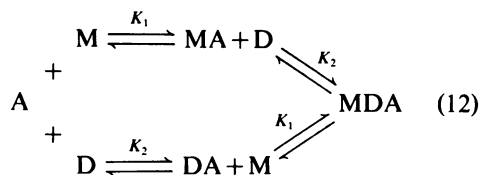$$K_2 = \frac{K_1[M][A]_t - K_1[M][S] - [S]}{K_d[S][M]^2} \qquad (10)$$

$$K_2 = \frac{([T] - [M] - 2K_d[M]^2 - [S])K_1}{2K_d[M][S]} \qquad (11)$$

$[A]_t$ is the total protein concentration and [S] is the concentration of fluorescent porphyrin–protein complexes (excited for porphyrin). K_1 and K_d have been already determined. [T] and $[A]_t$ are known

and [S] is measured for each reaction mixture. Thus eqns. (10) and (11) can be solved to yield K_2. The magnitudes obtained, for BSA and for HSA, are listed in Table 1.

For case 2 the basic assumptions are as follows. (1) Each protein molecule has one high-affinity site for monomers and one (different) high-affinity site for dimers. (2) There is no co-operativity between sites. (3) Assumption (3) of case 1 is also adopted here.

The equilibria required to describe this system are the dimerization (eqn. 8) and the following:

$$\begin{array}{c} M \overset{K_1}{\rightleftharpoons} MA + D \\ + \qquad \qquad \searrow^{K_2} \\ A \qquad \qquad \qquad MDA \qquad (12) \\ + \qquad \nearrow^{K_1} \\ D \overset{K_2}{\rightleftharpoons} DA + M \end{array}$$

where MDA is the protein species with both sites occupied.

Taking into consideration eqns. (8) and (12) together with expressions for conservation of matter, the following expression can be derived:

$$K_2 = \frac{([T] - 2K_d[M]^2 - [M] - 3[S]) + 2K_1[M]([A]_t - [S])}{K_d[M]^2(2[A]_t + [S] + [M] + 2K_d[M]^2 - [T])} \qquad (13)$$

[S] is, for this case, the sum of concentrations of fluorescent porphyrin–protein complexes (excited for porphyrin). Eqn. (13) can be solved to yield the magnitude of K_2 for each reaction mixture. The magnitudes obtained, for both proteins, are also listed in Table 1.

In order to test the fit of each model to the data, as well as the fit of both K_1 and K_2, we have gone through the following procedure. Starting with the measurable quantity [S], the known total protein concentration and the now fully known magnitudes of K_1, K_2 and K_d, we have calculated from the specific expressions of each model the total porphyrin concentration (denoted $[T]_{calc.}$) that should be in a given reaction mixture. We then compared these with the known total porphyrin concentration introduced into that system (denoted here as $[T]_{exp.}$). Plotting $[T]_{exp.}$ versus $[T]_{calc.}$ should give a slope of 1, going through the origin, for an ideal fit. Thus the closer the actual magnitudes are to the expectation the better is the fit of the model. The plots for HSA and BSA, represented in Figs. 3 and 4, clearly allow us to rule out the model of indirect dimer involvement, for both proteins. As to the models for direct involvement, differentiating the competitive from the non-competitive cases is not simple. For BSA they give the same fit and an acceptable one (therefore only one case is presented). For HSA the non-
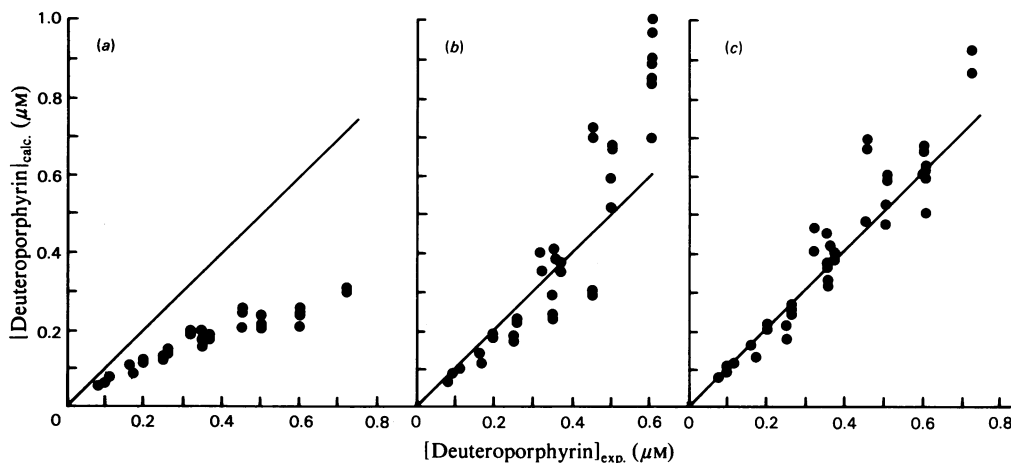
Fig. 3. *Test of the fit of the models proposed for the binding of deuteroporphyrin monomers and dimers to HSA*
(*a*) Indirect involvement of dimers. (*b*) Direct involvement of dimers, competitive model. (*c*) Direct involvement of dimers, non-competitive model. The linear plot is the expectation for ideal fit.
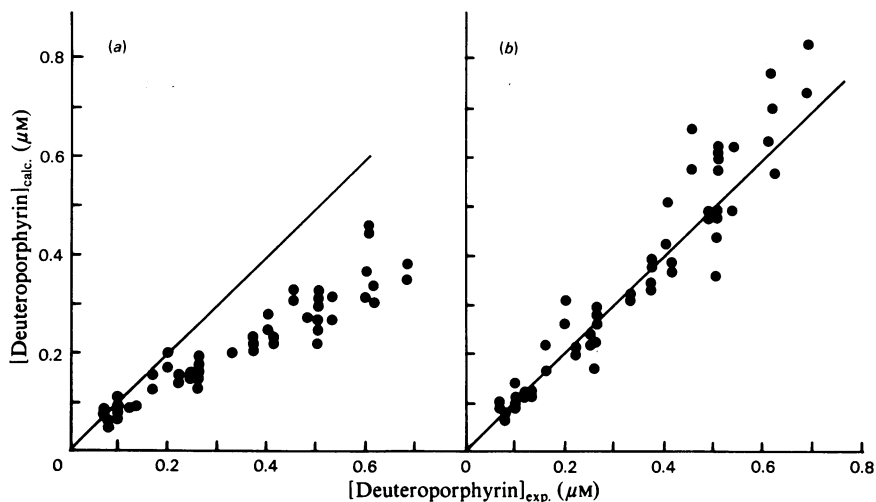

Fig. 4. *Test of the fit of the models proposed for the binding of deuteroporphyrin monomers and dimers to BSA*
(*a*) Indirect involvement of dimers. (*b*) Direct involvement of dimers, non-competitive model. The linear plot is the expectation for ideal fit.

competitive case gives a somewhat better fit over the entire range of porphyrin concentrations.

Conclusions

In this study we have re-examined the association of porphyrins with serum albumins, taking into account the self-aggregation of the porphyrin. Emphasis was on the porphyrin concentration range where the fraction of aggregates of higher order than dimers is negligible.

The results obtained clearly show that albumin can bind both porphyrin monomers and dimers, with one high-affinity site for each. The data do not allow a clear-cut distinction betwen competitive and non-competitive binding. However, since the latter model gave a better fit in the case of HSA, especially at the higher end of porphyrin concentrations employed, we are inclined to favour this model.

We stress the issue of high-affinity site(s), since there have been reports indicating that an albumin molecule has additional porphyrin-binding sites of

lower affinity and lower specificity (Morgan *et al.*, 1980; Lamola *et al.*, 1981; Reddi *et al.*, 1981; Moan & Western, 1984). The ranges of porphyrin and of protein concentrations that we employed in this study were sufficiently low so that only the high-affinity sites come into expression. It should also be noted that additional sites that come into expression with sufficient increase in the total porphyrin concentration, and therefore interpreted to have lower affinity, might not necessarily be so. Rather, they might be binding sites (of higher or of lower affinity) for aggregated porphyrin, expressed only when the total porphyrin concentration has reached a level where these aggregates can be formed in detectable amounts.

Returning to the issue of competitive versus non-competitive binding, we propose that, regardless of the actual model prevailing, the data allow us to draw several conclusions, some of them with biological relevance.

(1) With the increase in total porphyrin concentration of a given protein system, the balance of porphyrin–protein complexes should shift from monomer (only)-containing to dimer-containing protein species. This situation will occur, not only in a soluble 'test-tube' system, but also in serum-containing biological systems, *in vivo* and *in vitro*, with the increase in porphyrin dose.

(2) Our findings imply that the covalent dimer present in the haematoporphyrin derivative, and proposed to be the biologically active component there (Kessel, 1984*b*), can also be bound by albumin, and therefore carried by it *in vivo*. Experimental evidence for such binding has been reported by Grossweiner & Goyal (1984), although their ranges of porphyrin and protein concentrations and the abundance of binding sites that they determined suggest that in their case it is the lower-affinity sites that are dominantly expressed.

(3) The dimerization equilibrium will still be a factor in determining the porphyrin dose concentration at the target, even if the total dose administered is such that it contains no monomers (practically). This argument is based on two findings: (*a*) that dimers bind directly to albumin (present work); (*b*) that dimers do not bind directly to membranes, but indirectly through their dissociation to monomers (Margalit & Cohen, 1983).

In a situation where dimers are carried by albumin to the vicinity of the target cell, the transfer of porphyrin, off the protein and on to the cell membrane, will be mediated by the dimeriza-tion equilibrium:

$$DA \rightleftharpoons A + D \rightleftharpoons 2M \overset{+L}{\rightleftharpoons} ML \quad (14)$$
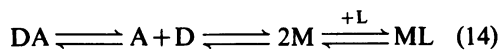
where L represents a lipid region of a membrane and ML the porphyrin monomer bound to it.

References

Brown, S. B., Shillcock, M. & Johns, P. (1976) *Biochem. J.* **153**, 279–285

Bugelsky, P. J., Porter, C. W. & Dougherty, T. J. (1981) *Cancer Res.* **41**, 4606–4612

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam

Grossweiner, L. I. & Goyal, G. C. (1984) *Photochem. Photobiol.* **40**, 1–5

Jori, G., Reddi, E., Salvato, B., Tomio, L. & Gozzani, I. (1984) *Abstr. Porphyrin Photosensitization Workshop*

Karns, G. A., Gallagher, W. A. & Elliot, W. B. (1979) *Bioorg. Chem.* **8**, 69–81

Kessel, D. (1981) *Cancer Res.* **41**, 1318–1323

Kessel, D. (1982) *Cancer Res.* **42**, 1703–1706

Kessel, D. (1984*a*) *Biochem. Pharmacol.* **33**, 1389–1393

Kessel, D. (1984*b*) *Photochem. Photobiol.* **39**, 851–859

Kessel, D. & Dougherty, T. J. (1983) *Porphyrin Photosensitization*, pp. 3–25, Plenum Press, New York

Lamola, A. A. (1982) *Acta Derm.-Venereol. Suppl.* **100**, 57–66

Lamola, A. A., Asher, I., Muller-Eberhard, U. & Poh-Fitzpatrick, M. (1981) *Biochem. J.* **196**, 693–698

Margalit, R. & Cohen, S. (1983) *Biochim. Biophys. Acta* **736**, 163–170

Margalit, R. & Rotenberg, M. (1984) *Biochem. J.* **219**, 445–450

Margalit, R., Shaklai, N. & Cohen, S. (1983) *Biochem. J.* **209**, 547–552

Moan, J. & Sommer, S. (1981) *Photobiochem. Photobiophys.* **3**, 93–103

Moan, J. & Western, A. (1984) *Abstr. Porphyrin Photosensitization Workshop*

Moehring, G. A., Chu, A. H., Kulansik, L. & Williams, T. J. (1983) *Biochemistry* **22**, 3381–3386

Morgan, W. T., Smith, A. & Koskelo, P. (1980) *Biochim. Biophys. Acta* **624**, 271–285

Reddi, E., Ricchelli, F. & Jori, G. (1981) *Int. J. Pept. Protein Res.* **18**, 402–408

Smith, A. & Neuschatz, T. (1983) *Biochem. J.* **214**, 503–509

Smith, K. E. (1975) *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam