Fluorimetric studies on the dimerization equilibrium of protoporphyrin IX and its haemato derivative

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The aggregations of protoporphyrin IX and haematoporphyrin IX in aqueous solutions were studied by fluorimetric techniques. Porphyrin concentrations were limited to 0.001–0.1 μM and 0.01–1 μM for protoporphyrin and haematoporphyrin respectively, where dimerization is the dominant aggregation process. The dimerization equilibrium constants (at 25°C, neutral pH, 50 mM-Tris/HCl buffer) were determined to be $3 \times 10^7 \text{ M}$ and $4 \times 10^5 \text{ M}$ for the proto and the haemato derivatives respectively. The fluorescence intensity of a given protoporphyrin solution (within the range indicated above) was markedly decreased by salts in the system, over the salt concentration range 0.1–7 mM at constant ionic strength, in the sequence $\text{CaCl}_2 > \text{MgCl}_2 > \text{KCl} > \text{NaCl}$. The direction of this effect, fluorescence quenching, suggests that these salts promote an increase in aggregation. The differences in the magnitudes of the effect, among different salt species sharing a common anion, at constant ionic strength, imply that the effect is cation-specific. In contrast, the fluorescence intensity of a given solution of haematoporphyrin (within the range indicated above) was unaffected by these salts, under similar concentrations, nor was it sensitive to the total buffer concentration, or to the type of buffer in the system.

Protoporphyrin IX and its deuterio, haemato and meso derivatives tend to aggregate in aqueous solutions, forming dimers and micelle-like polymers, within the nanomolar to the micromolar porphyrin concentration range (Phillips, 1963; Smith, 1975; Brown *et al.*, 1976; Karns *et al.*, 1979; Brown *et al.*, 1980). These aggregations are relevant to biological reactivities of porphyrins, at similar concentrations, such as their photodynamic action (Dougherty *et al.*, 1978; Moan *et al.*, 1980; Kessel & Kohn, 1980; Kessel, 1981; Amagasa, 1981) and binding to serum proteins (Morgan *et al.*, 1980; Lamola *et al.*, 1980).

The aggregations of protoporphyrin IX, deuteroporphyrin IX and haematoporphyrin IX at neutral pH were studied by Brown *et al.* (1976) by using absorption-spectroscopy techniques. They found both dimerization and polymerization to be fast processes. However, the limitations of absorption spectroscopy do not allow a straightforward determination of the dimerization equilibrium constants, only their order of magnitude. Those were determined by Brown *et al.* (1976) to be of the order of

10^8 M for protoporphyrin IX and of the order of 10^6 M for deuteroporphyrin IX and haematoporphyrin IX. Karns *et al.* (1979) have also studied the dimerization of these three porphyrins, together with mesoporphyrin IX, by using absorption spectroscopy, but at alkaline pH (10–20 mM-NaOH). They found the magnitudes of the equilibrium dimerization constants to range from 10^6 M to 10^5 M , in the order protoporphyrin IX > mesoporphyrin IX > deuteroporphyrin IX > haematoporphyrin IX.

Absorption-spectroscopy studies have shown the aggregation of deuteroporphyrin IX at neutral pH to be unaffected by ionic strength at salt concentrations in the range 0.1–0.4 M (Brown *et al.*, 1976). Similar observations, although at lower ionic strengths, have also been made for the aggregation of deuteroporphyrin IX at alkaline pH (Karns *et al.*, 1979). On the other hand, the aggregations of protoporphyrin IX and mesoporphyrin IX at alkaline pH were found to be affected by ionic strength, in the direction of increased aggregation with increasing salt concentrations (Karns *et al.*, 1979). It should be noted that deuterohaemin, at neutral pH, was found to behave in a manner similar

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to the metal-free protoporphyrin IX with respect to salt effects on aggregation (Hatzikostantinou & Brown, 1978; Brown *et al.*, 1980), rather than like its metal-free analogue, deuteroporphyrin IX.

Another spectral probe useful for following the dimerization of porphyrins is offered by their fluorescence. The sensitivity of the fluorescence signal makes it possible to reach extremely low concentrations. Furthermore, only the monomer is assumed to fluoresce (Phillips, 1963; Smith, 1975). This allows, as is shown in the present paper, a straightforward determination of the dimerization equilibrium constants, as well as a re-examination of the salt effects.

By using fluorescence spectroscopy we have, in the present work, studied the dimerization equilibria of protoporphyrin IX and haematoporphyrin IX at neutral pH, and have examined the effects of some common physiological cations on the aggregation processes.

Experimental

Protoporphyrin IX and haematoporphyrin IX were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Fluorescence spectra were recorded on a Perkin-Elmer model MPF-44B fluorescence spectrophotometer.

The following routine was used for the preparation of porphyrin solutions. The desired amount of porphyrin, to give stock solutions of 0.5–1.0 mM, was dissolved in a minimal volume of NaOH (10–50 mM), then diluted by buffer to the final concentration desired, the final ratio of buffer to NaOH being about 5:1. These stocks were kept at -18°C for short periods only. Fresh solutions of 1–100 μM -protoporphyrin were prepared from these stock daily. Care was taken to protect all porphyrin solutions from light and to minimize their exposure during the recording of the fluorescence emission.

The organic phase, in which porphyrins are expected to be monomeric, was octan-1-ol/methanol (1:1, v/v).

Two types of buffers were used: (a) 50 mM-Tris/HCl, pH 7.2; (b) phosphate-buffered saline, containing 3.3 mM- KH_2PO_4 , 3.3 mM- Na_2HPO_4 , 143 mM-NaCl and 4.7 mM-KCl, adjusted to pH 7.2.

Results and discussion

Relationships among porphyrin concentration, porphyrin fluorescence intensity and type of solvent

In order to investigate the aggregation state of a given porphyrin solution, and to determine the equilibrium dimerization constant, we followed the increase of fluorescence intensity with the increase in total porphyrin concentration. Two types of solvents

were used: (a) organic, octanol/methanol (1:1, v/v) solution, (b) aqueous, buffered to neutral pH by 50 mM-Tris/HCl. As expected (Phillips, 1963; Kessel, 1977; Kessel & Rossi, 1982), we found the peak emission to be shifted to the red in the organic medium, compared with the aqueous medium.

With regard to the effects of the increase in total porphyrin concentration on the fluorescence intensity, we found two distinct differences between the organic and the aqueous phases. A typical example is illustrated for protoporphyrin IX in Fig. 1(b): (i)

Fig. 1. Increase in peak fluorescence intensity with increasing porphyrin concentration

(a) Haematoporphyrin IX in aqueous solution, buffered by 50 mM-Tris/HCl, pH 7.2, at 25°C ; excitation was at 394 nm, peak emission at 615 nm. Fluorescence intensity is normalized to 100% at 1.4 μM -porphyrin. (b) Protoporphyrin IX in aqueous (O) and organic (□) solutions. Aqueous solution was buffered by 50 mM-Tris/HCl, pH 7.2, at 25°C ; excitation was at 394 nm, peak emission at 620 nm. Organic solution was octanol/methanol (1:1, v/v) (see the Experimental section for details); excitation was at 403 nm, peak emission at 635 nm. Fluorescence intensity is normalized to 100% at 0.11 μM -protoporphyrin, in the organic phase.

the peak intensity for solutions of similar porphyrin concentrations is enhanced considerably in the organic phase compared with the aqueous one (notice the differences in scales); (ii) the increase of fluorescence intensity with increasing porphyrin concentrations is linear in the organic phase, but non-linear in the aqueous phase. We observed similar behaviour for the other porphyrin studied, haematoporphyrin IX, as exemplified by the data for the aqueous phase illustrated in Fig. 1(a) (although at a higher range of porphyrin concentrations).

The behaviour observed in the organic medium implies that, at least within the concentration range studied, these porphyrins are in the monomeric state. Similar observations (and interpretations) have been made for porphyrins in organic solutions of other compositions, from measurements of either fluorescence or absorption signals (Brown *et al.*, 1976; Kessel, 1977; Karns *et al.*, 1979; Kessel & Rossi, 1982).

In contrast, we take the behaviour observed in the aqueous phase as an indication that, within the concentration range studied, a progressive change in the system takes place. The direction of this change indicates a decrease of the fraction of the fluorescent species with increasing porphyrin concentration. It should be stressed that the non-linearity of fluorescence with concentration cannot be due to trivial factors, since the absorbance of these porphyrin solutions is extremely low (i.e. within the range where fluorescence should be linear to the concentration of the fluorescent species).

We have used results of the type illustrated in Fig. 1 for aqueous phases to determine the magnitudes of the dimerization equilibrium constants. Arguments that this is the aggregation step that is being observed, as well as the details of processing the data, are given below.

Determination of the dimerization equilibrium constant

Two assumptions are required in order to obtain the dimerization equilibrium constants from data of the type illustrated in Fig. 1 (in aqueous phases). (a) At sufficiently low concentrations, as those used in the present study, dimerization is the dominant aggregation process, the fraction of aggregates of higher order than dimers, of the total porphyrin present, being negligible. (b) Among the porphyrin monomers and dimers present, only the monomers fluoresce.

We show below that the experimental observations of the present study fit with, and therefore support, these two assumptions. First, however, we present several arguments for these assumptions.

Considering the first assumption, there is a body of experimental evidence that shows that the first aggregation step of porphyrins is dimerization, and

that at sufficiently low concentrations this is the dominant aggregation process. Observations from previous studies, not only on protoporphyrin IX but on many of its derivatives and modifications (see, e.g. Das *et al.*, 1970; Pasternack *et al.*, 1972), have been summarized by Falk (1964) and have been updated by Smith (1975). More recent studies by Brown *et al.* (1976, 1980) and by Karns *et al.* (1979) also give experimental evidence in support of this assumption.

We find additional support for this assumption in the experimental observations on the aggregations of fluorescent dyes, such as the cyanines and the thiazines (Rabinowitch & Epstein, 1941; Waggoner, 1979). For these dyes, it has also been shown that the first aggregation step is dimerization, and that at sufficiently low concentrations this is the dominant aggregation state (Rabinowitch & Epstein, 1941; West & Pearce, 1965; Sims *et al.*, 1974; Waggoner, 1979). We suggest that these systems are relevant examples, since, like porphyrins, these dyes are aromatic fluorescent molecules for which hydrophobic interactions have been suggested to be a major force in aggregation (Sims *et al.*, 1974; Brown *et al.*, 1976; Karns *et al.*, 1979).

As to the second assumption, that only monomers fluoresce, this has been accepted and long established as a characteristic of porphyrin fluorescence. Thus this property, when mentioned in reviews, is presented as a statement and not an argument or assumption in need of supportive evidence (see, e.g., Phillips, 1963; Smith, 1975). However, since this assumption is crucial for the determination of equilibrium constants from fluorescence data, we present below some arguments and supportive data.

The phenomenon of a fluorescent monomer that becomes non-fluorescent in the dimeric state, together with shifts in the absorption peaks and changes in the magnitudes of absorption coefficients from the monomer to the dimer, is not restricted to porphyrins. It has been observed for many fluorescent dyes (Rabinowitch & Epstein, 1941; Lavorell, 1957; West & Pierce, 1965; Sims *et al.*, 1974). In fact, the theoretical aspects of the non-radiative pathway of the absorbed energy, in the dimer, goes back to Forrester [see, e.g., the discussion by Lavorell (1957)]. The characteristics required of a fluorescent monomer for the formation of a non-fluorescent dimer can be found in porphyrins also. The absorption band in the Soret region, which is the major band used for excitation, appears both in the monomer and in the dimer, with the peak of the latter species being shifted to the blue (Falk, 1964; Brown *et al.*, 1976). The emission spectrum, in the 600–640 nm region (see, e.g., Fig. 1; also documented in Falk, 1964), overlaps the I absorption band in the 590–640 nm region (Falk, 1964), which also

appears in both monomer and dimer, shifted to the blue in the latter (Brown *et al.*, 1980; Kessel & Rossi, 1982). Thus, on the basis of spectral properties, the porphyrin dimer should be non-fluorescent. Furthermore, the linearity of the fluorescence intensity of porphyrins with concentration in organic phases, compared with the non-linearity in aqueous phase as observed in the present study (Fig. 1), fulfils the experimental criterion set by Rabinowitch & Epstein (1941) for self-quenching of dimers.

Turning to the determination of the dimerization equilibrium constants, a simple representation of the process can be given by:

where $[M]$ and $[D]$ represent the concentrations of the monomer and dimer respectively and K_d is the equilibrium constant. By expressing the total porphyrin concentration (as monomers) by $[T]$, and using the first assumption, the following expression can be obtained:

$$[T]/[M] = 2K_d[M] + 1 \quad (2)$$

On denoting the fluorescence intensity at a given wavelength as F , and using the second assumption, the monomer concentration can be replaced by kF , where k is an experimental coefficient, for a given wavelength, under specific experimental and recording conditions. (Since the absorption at the excitation wavelength was lower than 0.08 A unit,

linearity of the fluorescence intensity to the concentration of the fluorescent species can be assumed.) Replacement of the monomer concentration in eqn. (2) by this expression, and rearrangement, gives:

$$[T]/F = 2K_d k^2 F + k \quad (3)$$

Plotting of $[T]/F$ versus F , with care being taken to use only data obtained under identical experimental and recording conditions, should give a linear relationship. The magnitude of K_d can be obtained from the slope and intercept.

Typical examples of this procedure for processing the data are illustrated in Fig. 2 for protoporphyrin IX and haematoporphyrin IX. The experimental results are shown by the points. The straight lines were obtained by linear-regression analysis. The linear relationship observed for haematoporphyrin IX at the concentration range 0.01–1 μM and for protoporphyrin IX in the range 0.001–0.1 μM can be seen to fit with the expected behaviour (eqn. 3), and is typical of the behaviour observed in all the experiments run at these low porphyrin concentrations.

This fit between the experimental data and the theoretical expectation supports the first assumption, that dimerization is the dominant aggregation process, since development of eqn. (1) and eqn. (2) has taken into account monomers and dimers only. We find this strengthened by our observation that at

Fig. 2. Relationship of fluorescence intensity to total porphyrin concentration in aqueous solutions according to eqn. (3). Data of the type illustrated in Fig. 1, for the aqueous phases, were processed in accordance with eqn. (3) in the text, for (a) haematoporphyrin IX and (b) protoporphyrin IX. Symbols show experimental results; the straight lines are calculated by using linear-regression analysis.

porphyrin concentrations exceeding $0.1\ \mu\text{M}$ for protoporphyrin IX and $1\ \mu\text{M}$ for haematoporphyrin IX, where polymers can no longer be neglected (Brown *et al.*, 1976), the fluorescence data no longer fit eqn. (3).

The fit of the experimental data to eqn. (3) also supports the second assumption, that only the monomers fluoresce, which has been utilized in the transformation of eqn. (2) into eqn. (3). We argue that, if the observed fluorescence intensity had been due to both monomers and dimers, the treatment of the data as contributed by the monomers only would not fit the theoretical expectation, nor would the extracted equilibrium constants be of the expected magnitudes (Brown *et al.*, 1976) and be reproducible.

The magnitudes of K_d were found to be $3 (\pm 0.4) \times 10^7\ \text{M}$ and $4 (\pm 0.4) \times 10^5\ \text{M}$ (means $\pm$ S.D.) for protoporphyrin IX and haematoporphyrin IX respectively. The increased stability of the protoporphyrin compared with the haemato derivative, which corresponds to a free-energy difference of $12.5\ \text{kJ/mol}$ ($3\ \text{kcal/mol}$), is similar to that found for the proto and the haemato derivatives of ferrihaems (Brown *et al.*, 1980), implying that the increased stability might be due to the vinyl residues of the proto molecules.

The magnitudes of the dimerization constants, determined in the present study, at neutral pH, are each one order of magnitude higher than those determined by Karns *et al.* (1979) at alkaline pH. The decrease in the stability of the dimer with increasing pH, implied by the differences, fits with the effects of pH on dimerization observed by Brown *et al.* (1976).

Salt effects

We also have followed the effects of low concentrations of salts on the fluorescence intensity of protoporphyrin IX and haematoporphyrin IX. The salts studied were NaCl, KCl, MgCl_2 and CaCl_2 , over the concentration range $0.1\text{--}7\ \text{mM}$ -salt, for porphyrin concentrations of $0.001\text{--}0.1\ \mu\text{M}$ -protoporphyrin IX and $0.01\text{--}1\ \mu\text{M}$ -haematoporphyrin IX. Typical results are illustrated in Fig. 3.

With regard to protoporphyrin IX, these salts show a progressive and considerable effect on its fluorescence intensity, in the sequence $\text{CaCl}_2 > \text{MgCl}_2 > \text{KCl} > \text{NaCl}$. The effects seem to be salt-specific (actually cation-specific, since all salts share a common anion), rather than a general ionic-strength effect. The major part of the fluorescence quenching is seen at the lower salt concentrations, where the ionic strength of the system (composed mainly of the buffer) is constant. For example, $0.5\ \text{mM}$ - CaCl_2 is sufficient to cause 50% of the maximum effect seen for this salt. Another indication that the effect is salt-specific is furnished by

Fig. 3. Salt effects on the fluorescence intensity of (a) haematoporphyrin IX and (b) protoporphyrin IX solutions within the region of monomer-dimer equilibrium

Initial 'salt-free' solutions were buffered by $50\ \text{mM}$ -Tris/HCl, pH 7.2, at 25°C . Measurements were made in solutions containing the indicated final concentrations of the following salts: □, NaCl; ▽, KCl; ○, MgCl_2 ; △, CaCl_2 . Excitation of haematoporphyrin IX was at $394\ \text{nm}$, peak emission at $615\ \text{nm}$. Excitation of protoporphyrin IX was at $394\ \text{nm}$ also, peak emission at $620\ \text{nm}$. For each experiment fluorescence intensity is normalized to 100% at the initial 'salt-free' state.

the differences in the magnitude of the response observed for similar concentrations of the different salt species. The direction of the salt effect, quenching of the fluorescence, seems to indicate a decrease in the monomer fraction, and hence an increase in the aggregation, of the given porphyrin solution. This would fit with the salt effects with haemin dimers (Hatzikostantinou & Brown, 1978) and with protoporphyrin IX at alkaline pH (Karns *et al.*, 1979), observed by changes in the absorption spectra.

In marked contrast with protoporphyrin IX, the effects of these salts on the fluorescence intensity of the haematoporphyrin IX are negligible. [In the interest of clarity, only the data points for NaCl and CaCl_2 are presented in Fig. 3(a); those of the other two salts lie within the range of the drawn points.]

An implication of the lack of effect of these salts on the fluorescence intensity of haematoporphyrin IX, which is in agreement with the absorption studies by Brown *et al.* (1976) and Karns *et al.* (1979), is that the magnitude of the dimerization constant of this porphyrin, determined for a specific ionic composition of the porphyrin solutions (for details see the legend to Fig. 1), should apply to a wide range of solution compositions. In fact, we determined (at 25°C and neutral pH) the same magnitude of dimerization constant, $4 \times 10^5 \text{ M}$, for haematoporphyrin IX in Tris/HCl buffers of concentrations in the range 10–100 mM and in phosphate-buffered saline.

Further studies of these salt effects are required in order to understand why, at the concentration regions where dimers are the dominant aggregates, protoporphyrin is affected by the salts, whereas haematoporphyrin IX is not affected at all. Further studies are also needed to locate the aggregation step(s) affected (i.e. dimerization, polymerization, or both). Nevertheless, we considered it appropriate to present the salt effects observed at the regions of dimerization constants from fluorimetric data, since this phenomenon in itself is noteworthy.

The results obtained in the present study suggest that if one employs haematoporphyrin IX in studies of biological reactivities of porphyrins, with the use of porphyrin fluorescence as a probe, the observed changes in fluorescence can be attributed exclusively to the biological reactivity. Also, the fraction of dimers present, which can be calculated from the known salt-independent equilibrium constant, would not be affected by the conditions of the medium. In contrast, with protoporphyrin IX the aggregation state could change. Also, the observed changes in the fluorescence intensity could be due, in part, to the effects of the salts in the medium on the porphyrin aggregation state, with no relation to the biological phenomena being studied.

References

- Amagasa, J. (1981) *Photochem. Photobiol.* **33**, 947–955
 Brown, S. B., Shillcock, M. & Johns, P. (1976) *Biochem. J.* **153**, 279–285
 Brown, S. B., Hatzikonstantinou, H. & Herries, D. G. (1980) *Int. J. Biochem.* **12**, 701–707
 Das, R. R., Pasternack, R. F. & Plane, R. A. (1970) *J. Am. Chem. Soc.* **92**, 3312–3316
 Dougherty, T. J., Kaufman, J. E., Goldfarb, A. & Weishaupt, K. R. (1978) *Cancer Res.* **38**, 2628–2635
 Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, pp. 85–87, 232–246, Elsevier, Amsterdam
 Hatzikonstantinou, H. & Brown, S. B. (1978) *Biochem. J.* **174**, 893–900
 Karns, G. A., Gallagher, W. A. & Elliot, W. (1979) *Bioorg. Chem.* **8**, 69–81
 Kessel, D. (1977) *Biochemistry* **16**, 3443–3449
 Kessel, D. (1981) *Cancer Res.* **41**, 1318–1323
 Kessel, D. & Kohn, K. (1980) *Cancer Res.* **40**, 303–307
 Kessel, D. & Rossi, E. (1982) *Photochem. Photobiol.* **35**, 37–41
 Lamola, A., Asher, I., Muller-Eberhard, U. & Poh-Fitzpatrick, M. (1980) *Biochem. J.* **196**, 693–698
 Lavorell, J. (1957) *J. Phys. Chem.* **61**, 1600–1605
 Moan, J., Wakvik, H. & Christensen, T. (1980) *Cancer Res.* **40**, 2915–2918
 Morgan, W. T., Smith, A. & Koskelo, P. (1980) *Biochim. Biophys. Acta* **624**, 271–285
 Pasternack, R. F., Huber, P., Boyd, P., Engasser, G., Francesconi, L., Gibbs, E., Fassela, P., Venturo, G. C. & Hinds, L. (1972) *J. Am. Chem. Soc.* **94**, 4511–4517
 Phillips, J. N. (1963) *Compr. Biochem.* **9**, 34–73
 Rabinowitch, E. & Epstein, L. F. (1941) *J. Am. Chem. Soc.* **63**, 69–78
 Sims, P. J., Waggoner, A. S., Wang, C.-H. & Hoffman, J. F. (1974) *Biochemistry* **13**, 3315–3330
 Smith, K. M. (1975) *Porphyrins and Metalloporphyrins*, pp. 265–268, Elsevier, Amsterdam
 Waggoner, A. (1979) *Annu. Rev. Biophys. Bioeng.* **8**, 47–68
 West, W. & Pearce, S. (1965) *J. Phys. Chem.* **69**, 1894–1903