Thermodynamics of porphyrin dimerization in aqueous solutions

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The dimerization equilibrium of deuteroporphyrin IX and of mesoporphyrin IX in aqueous solutions were studied by fluorimetric techniques over the 0.01–1 μM concentration range, where dimerization is the dominant aggregation process. Deuteroporphyrin IX was studied at several temperatures over the range 22–37°C, and mesoporphyrin at 25 and 37°C. The magnitudes determined for the dimerization equilibrium constants (25°C, neutral pH, phosphate-buffered saline) are $2.3 \times 10^6 \text{ M}^{-1}$ and $5.4 \times 10^6 \text{ M}^{-1}$ for the deuterio and meso derivatives respectively. The meso, deuterio and haemato species tested show a similar temperature effect, namely dimerization decreasing with increasing temperature, indicating the involvement of a negative enthalpy change. Van't Hoff isochore of the dimerization constants determined for deuteroporphyrin IX was linear within the temperature range of 22–37°C, allowing the calculation of the thermodynamic parameters. For deuteroporphyrin dimerization, those were found to be $\Delta G^0 = -36.4 \text{ kJ} \cdot \text{mol}^{-1}$; $\Delta H^0 = -46.0 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S^0 = -32.2 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ (at neutral pH, 25°C, phosphate-buffered saline), showing the process to be enthalpy-driven. Similar trends have been found for porphyrin species other than those studied here. Our data fit with a hypothesis giving a major role to the solvent in driving porphyrins to aggregate in aqueous solution. The magnitudes and directions of the energetic changes fit better with the expectation of the 'solvophobic force' theory predicting enthalpy-driven association, than with the classic hydrophobic bonding, predicting the association to be entropy-driven.

Protoporphyrin IX and its deuterio, meso, haemato and haemato-derivative analogues known for their photodynamic activities (Dougherty *et al.*, 1979; Moan *et al.*, 1980; Amagasa, 1981; Bugelski *et al.*, 1981; Kessel, 1981; Benson *et al.*, 1982) are also known to aggregate in aqueous solutions (Brown *et al.*, 1976, 1980; Karns *et al.*, 1979; Margalit *et al.*, 1983).

Several investigators have pointed out a correlation between the potencies *in vivo* of these porphyrins as photodynamic agents and their hydrophobicities (Kessel, 1977, 1981, 1982; Moan *et al.*, 1982). Another measure of the apolarities of these porphyrins can be obtained by assessing their tendency to aggregate in aqueous solutions. Furthermore, expressing that tendency to aggregate in quantitative terms should be useful and relevant to investigations of the activities of these porphyrins in biological systems, especially since the porphyrin concentration range where aggregations take place (nanomolar to micromolar) is also the concentration range employed in biological studies.

Karns *et al.* (1979) have indeed sequenced these porphyrins according to their dimerization equilibrium constants. However, that data was obtained at alkaline pH, whereas relevance for biological systems requires neutral pH. We have begun a systematic thermodynamic study of the dimerization of these porphyrins, at neutral pH, employing fluorimetric techniques.

Our findings for protoporphyrin IX and for haematorporphyrin IX have been reported previously (Margalit *et al.*, 1983). Here we report our data for the deuterio and meso, as well as some additional data for the proto and haemato, derivatives. Emphasis has been put on obtaining the dimerization equilibrium constants for conditions relevant to biological activities of these porphyrins, namely, physiological pH and temperature and a suitable buffer/salt system (phosphate-buffered saline). However, to gain more understanding of the aggregation processes at the molecular level, we have extended our studies to other salt and temperature conditions.

Experimental

Deuteroporphyrin IX and mesoporphyrin IX were purchased from Porphyrin Products, Logan, UT, U.S.A. All other reagents were of analytical grade.

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

Deuteroporphyrin IX stock solutions were prepared as previously described (Margalit *et al.*, 1983). Mesoporphyrin stock solutions (0.5–1.0 mM) were made in dimethylformamide. It was verified that the equilibrium constants determined were independent of the conditions of stock preparations. Both stocks were kept at -18°C for periods not exceeding 2 weeks. Fresh solutions of the porphyrins over the range of 1–100 μM were prepared from these stocks daily. Care was taken to protect all porphyrin solutions from light and to minimize their exposure during the recording of the fluorescence spectra.

Three types of buffer were used: (a) 50 mM-Tris/HCl, pH 7.2; (b) phosphate buffer containing 3.3 mM- KH_2PO_4 and 3.3 mM- Na_2HPO_4 , pH 7.2; and (c) phosphate-buffered saline containing (b) above, with the addition of 143 mM- NaCl and 4.7 mM- KCl .

Results and discussion

Dimerization equilibrium of deuterio- and mesoporphyrin IX

Within the micromolar concentration range we have found the increase in fluorescence intensity of

deuterio- and meso-porphyrin IX with increasing porphyrin concentrations to be non-linear in aqueous solutions and linear in organic and detergent solutions. Typical results are illustrated in Figs. 1(a) and 2(a) for deuteroporphyrin IX and for mesoporphyrin IX respectively.

This pattern is similar in its general features to that previously reported by us for the haemato and proto derivatives (Margalit *et al.*, 1983), as well as by others for the fluorescence (Kessel & Rossi, 1982) and absorption (Brown *et al.*, 1976; Karns *et al.*, 1979) spectra of porphyrin IX species.

The fluorescence data obtained for the aqueous solutions, indicative of a decrease in the fraction of the fluorescent species with the increase in total porphyrin concentration, can be processed to yield the dimerization equilibrium constant (Margalit *et al.*, 1983). This is the first step in the aggregation process, and the dominant, over the porphyrin concentration range investigated. [In our previous paper (Margalit *et al.*, 1983) we discussed this issue, and that of non-fluorescent as against fluorescent porphyrin species. Therefore, to avoid redundancy, the reader is referred to that publication.]

K_D , the dimerization equilibrium constant, is defined by:

$$K_D = [D]/[M]^2 \quad (1)$$

where $[M]$ and $[D]$ represent the concentrations of the monomer and the dimer respectively. Taking into consideration conservation of matter, and expressing the monomer concentration through F , the fluorescence intensity of a porphyrin aqueous

Fig. 1. Relationship between fluorescence intensity and total deuteroporphyrin IX concentration at 25°C (a) Deuteroporphyrin in (○) aqueous (phosphate-buffered saline) and (□) organic (Triton X-100) solutions (as described in the Experimental section). (b) Determination of dimerization equilibrium constant by plot of $[T]/F$ against F , according to eqn. (2). Excitation was at 394 nm and peak emission at 612 nm. The symbols indicate the experimental results; the straight line was calculated by linear-regression analysis.

Fig. 2. Relationship between fluorescence intensity and total mesoporphyrin IX concentration at 25°C (a) Mesoporphyrin in (○) aqueous and (□) organic solutions (as described in Fig. 1 and in the Experimental section). (b) Determination of dimerization equilibrium constant according to eqn. (2). Excitation was at 390 nm and peak emission at 615 nm. The symbols show experimental results; the straight line was calculated by linear-regression analysis.

Table 1. Dimerization equilibrium constants of porphyrins, at neutral pH

References: (a) Margalit *et al.*, 1983; (b) the present work; (c) Margalit & Cohen, 1983; (d) R. Margalit & S. Cohen, unpublished work.

Temperature	Buffer/ salt	$K_D(\text{M}^{-1})$			
		25°C		37°C	
Porphyrin		Tris/HCl(50mM)	Phosphate(6.6mM)	Phosphate-buffered saline	Phosphate-buffered saline
Haemato		$4.0(\pm 0.4) \times 10^5(a)$	—	$4.0(\pm 0.4) \times 10^5(a)$	$2.8(\pm 0.4) \times 10^5(c)$
Deutero		$1.6(\pm 0.4) \times 10^6(b)$	$6.5(\pm 0.8) \times 10^5(b)$	$2.3(\pm 0.8) \times 10^6(b)$	$1.1(\pm 0.45) \times 10^6(b)$
Haemato derivative		—	—	$2.3(\pm 0.7) \times 10^6(c)$	$1.9(\pm 0.9) \times 10^6(c)$
Meso		—	$1.3(\pm 0.7) \times 10^6(b)$	$5.4(\pm 0.8) \times 10^6(b)$	$1.7(\pm 0.5) \times 10^6(b)$
Proto		$3.0(\pm 0.4) \times 10^7(a)$	—	$1.9(\pm 0.4) \times 10^7(d)$	—

solution at a given wavelength, the following expression is obtained (Margalit *et al.*, 1983).

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

where $[T]$ is the total porphyrin concentration (as monomers) and k is an experimental coefficient relating the monomer concentration linearly to F , at a given wavelength for specific experimental and recording conditions.

Replotting data of the type illustrated in Figs. 1(a) and 2(a) for the aqueous phases according to eqn. (2), should give a linear relationship of F against $[T]/F$, from which the magnitude of K_D can be extracted. This, indeed, was found to be the case, as illustrated by the results presented in Figs. 1(b) and 2(b) for the deutero and meso derivatives respectively.

The magnitudes of the dimerization equilibrium constant thus obtained are $2.3(\pm 0.8) \times 10^6 \text{ M}^{-1}$ and $5.4(\pm 0.8) \times 10^6 \text{ M}^{-1}$ for deuteroporphyrin IX and mesoporphyrin IX respectively at neutral pH, 25°C, in phosphate-buffered saline.

Comparison of dimerization equilibrium constants among vinyl-substituted protoporphyrin IX derivatives

Having determined the dimerization constants for the deutero- and meso-porphyrin IX molecules, we can now compare the dimerizations of protoporphyrin IX and a series of its derivatives. Listed in Table 1 are the dimerization equilibrium constants for the haemato, deutero, meso and proto derivatives as well as for the preparation haemato-

porphyrin derivative (Lipson *et al.*, 1961), under several salt conditions and at two temperatures.

Concentrating first on the data at 25°C, although there are some differences in the magnitudes, depending on the salt conditions, these do not detract from the following conclusions: The process of dimerization is highly favourable in the direction of dimer formation, ranging from $\Delta G^0 = -31.8 \text{ kJ} \cdot \text{mol}^{-1}$ for haematoporphyrin to $\Delta G^0 = -41.4 \text{ kJ} \cdot \text{mol}^{-1}$ for protoporphyrin (at 25°C in phosphate-buffered saline).

As to the effects of buffer type and/or concentration on the magnitudes of the dimerization constants, the increase in the magnitude of the constant with increasing ionic strength, for example, with deuteroporphyrin from phosphate (10.013), to Tris/HCl (10.05) to phosphate-buffered saline (10.16) and with mesoporphyrin from phosphate to phosphate-buffered saline, is in the direction expected for the association of species carrying a net change of the same sign. On the other hand, the differences observed for protoporphyrin between Tris/HCl and phosphate-buffered saline seem to indicate a specific salt effect for this derivative, as has been observed before (Margalit *et al.*, 1983).

Temperature effects

To assess the general trend of the effect of temperature on the dimerization equilibrium, we first determined the dimerization constants for four porphyrin species at another temperature. We chose 37°C, reasoning that data at this temperature have relevance on their own. We have listed the dimerization constants obtained at 37°C in Table 1 along those obtained at 25°C under similar salt conditions. The trend is immediately obvious: for all species tested, the dimerization constants decrease with increasing temperature, indicating a negative enthalpy change.

We have not attempted to extract from this data the magnitude of the enthalpy change. To do so would require the assumption that ΔH^0 is temperature-independent within the 25–37°C range. At this stage we had no data to support this assumption. To obtain such support, and thereafter obtain the enthalpy and entropy changes, we have focused on deuteroporphyrin, determining its dimerization constant at additional temperatures within the 22–37°C range. We concentrated on this porphyrin specifically because peripheral effects of the substituents at the variant vinyl position would be minimal in this case. Thus the data obtained should give us insight into the major forces driving the dimerization, forces common to all porphyrin species tested.

We plotted the data for deuteroporphyrin as a Van't Hoff isochore, as shown in Fig. 3. The

Fig. 3. Relationship between K_D and temperature according to Van't Hoff

Symbols represent experimental data; the straight line was calculated by linear-regression analysis. The solution used was phosphate-buffered saline.

dependence of K_D on $1/T$ is linear within the range studied, given $\Delta H^0 = -46.0 \text{ kJ} \cdot \text{mol}^{-1}$. Since ΔG^0 at 25°C was found to be $-36.4 \text{ kJ} \cdot \text{mol}^{-1}$, this gives an entropy change of $\Delta S^0 = -32.2 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ (at 25°C). The magnitudes and directions of these thermodynamic parameters show the aggregation to be enthalpy-driven. Thermodynamic studies of other porphyrins have shown a similar trend, as can be seen from the results given in Table 2. The similarities (first four rows of the Table) argue that the major forces operating are not due to specific conditions such as alkaline pH (Karns *et al.*, 1979) nor to the nature of the porphyrin modification (White & Plane, 1974), but are intrinsic properties of the porphyrin core.

Considering the forces driving porphyrins to aggregate, π - π interactions between the aromatic moieties have been shown to operate for porphyrin esters in organic solutions (Abraham *et al.*, 1966; Janson & Katz, 1972). It should also be noted that dimerization in those cases was detected at high concentrations, over the 0.001–0.10 M range (Abraham *et al.*, 1966; Janson & Katz, 1972). Qualitatively, interactions of this type are independent of the nature of the medium in which the dimerization takes place (Abraham *et al.*, 1976) and are therefore also expected to be a driving force in the dimerization of porphyrins in aqueous solutions (Brown *et al.*, 1980).

Table 2. *Thermodynamic parameters for molecular associations in aqueous solutions*

Molecule	Process	ΔG^0 (kJ·mol ⁻¹)	ΔH^0 (kJ·mol ⁻¹)	ΔS^0 (J·K ⁻¹ ·mol ⁻¹)	Reference
Deuteroporphyrin	Dimerization (neutral pH)	-36.4	-46.0	-32.2	The present work
Haematoporphyrin	Dimerization (alkaline pH)	-28.8	-25.5	+11.9	Karns <i>et al.</i> (1979)
Ethylenediamine protoporphyrin dimethyl ester	Dimerization	-34.3	-43.1	-29.3	White & Plane (1974)
Putrescine protoporphyrin dimethyl ester	Dimerization	-32.2	-34.7	-12.5	White & Plane (1974)
Actinomycin	Dimerization	-15.5	-62.7	-158.8	Crothers <i>et al.</i> (1968)
Actinomycin- deoxyguanosine	Complexation	-18.4	-43.1	-82.8	Crothers & Ratner (1968)
Methylene Blue	Dimerization	-19.2	-24.7	-18.8	Mukerjee & Gosh (1970)
Purine	Dimerization	-1.8	-17.6	-54.3	Ts'o (1970)
Deoxyadenosine	Dimerization	-4.2	-27.2	-75.2	Ts'o (1970)
Cytidine	Dimerization	+0.3	-11.3	-41.8	Ts'o (1970)
Ribosylpurine	Dimerization	-1.6	-10.5	-29.3	Ts'o (1970)

However, several arguments indicate these π - π interactions are not enough to account for the present case. Those types of interactions, between metal-free porphyrins, are rather weak (Abraham *et al.*, 1976). Also, at the concentration range we have studied, dimerization is found, experimentally, to be restricted to pure aqueous solutions (recall Figs. 1a and 2a here; also Margalit & Cohen, 1983; Margalit *et al.*, 1983; Moan *et al.*, 1982; Kessel & Rossi, 1982). An additional driving force is, therefore, required, and we postulate it to involve the water.

Involvement of water in molecular associations is accounted for in the theory of the hydrophobic effect. According to this theory, which has been tested and verified experimentally for non-polar hydrocarbons and non-polar residues of amino acids, such associations are entropy-driven, with little or no enthalpic effect (Kauzmann, 1959; Nemethy & Scheraga, 1962; Tanford, 1973; Paterson *et al.*, 1981).

On the other hand, enthalpy-driven solvent-involving associations in the aqueous phase, as we have found, have been observed for other systems as well: systems which have considerably greater structural similarities to porphyrins than hydrocarbons have. Several examples of such systems are also listed in Table 2. A theoretical approach, which can account for enthalpy-driven, solvent-involving associations has been developed by Sinanoglu and co-workers (Sinanoglu & Abdulnur, 1965; Sinanoglu, 1968, 1981) and named the 'solvophobic effect'. The results given in Table 2 constitute experimental support for the theory.

A detailed discussion of the solvophobic effect is beyond the scope of the present paper. However, we find it desirable to discuss, in brief, the major factor contributing favourably to dimerization and requiring involvement of the solvent. When a molecule is dissolved, a cavity has to be created in the solvent to accommodate the molecule. The formation of the cavity is an increase in the surface area of the solvent and therefore requires input of energy, mainly enthalpic, to work against the surface tension of the solvent. When two such molecules, already dissolved, associate, two cavities are joined into one, reducing, therefore, the surface area of the solvent and releasing some of the energy invested in creating the individual cavities. This enthalpic driving force has been termed the 'solvophobic force'. It should be noted that this force is not restricted to aqueous solutions, but is expected to be strongest there, since the surface tension of water is exceptionally high compared with that of other common solvents. Thus this theory predicts a decrease in the association (in the binding constant) from water to pure solvents of lower surface tension, and at the extreme, no detectable association. This fits with our findings that porphyrins do not dimerize in organic solutions under the same conditions in which they dimerize in aqueous systems (Margalit *et al.*, 1983). Also, the addition of detergent to aqueous systems, decreasing the surface tension, causes monomerization (recall Figs. 1 and 2 here; also Margalit & Cohen, 1983).

In conclusion, the thermodynamic parameters found for the dimerization of porphyrins in

aqueous solution can be understood in terms of the solvophobic theory better than in terms of the classical hydrophobic theory.

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