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Aggregation of Tetrakis(4-methylpyridyl)porphyrin and Tetrakis(4-sulphonatophenyl)porphyrin in Water

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Abstract. The homo- and heteroaggregation of two oppositely charged water soluble porphyrins, tetrakis(4-methylpyridyl)porphyrin, TMPyP and tetrakis(4-sulphonatophenyl) porphyrin, TSPP has been studied by means of absorption and fluorescence spectroscopy in phosphate buffered saline. All experimental results on TSPP are consistent with a monomer-dimer equilibrium (ΔH= –19.6 and ΔS=19.3 kJ mole^{-1}). The absorption spectrum of the TSPP dimer was obtained from a global fit of a set of spectra to the monomer dimer model. TMPyP does not aggregate at concentrations up to 10^{-4}M. Changes in the fluorescence spectrum of TMPyP upon dilution are due to adsorption phenomena. 1:1 heteroagregates of both porphyrins cannot be characterized in water solution due to extreme small solubility. A 2:1 heteroaggregate is detectable in solution. It is prpoosed to exploit the extreme small solubility of heteroaggregated oppositely charged porphyrins to increase the selective retention of porphyrins provided there is an initial selectivity of ether component.

The chemical and physical properties of porphyrins and their aggregates are of considerable interest because of the vital role these compounds play in biological processes. Particularly, porphyrin aggregates serve as models for the initial photoelectron transfer reaction in the special pair, cf. *e.g.* references [1, 2]. An outstanding application of porphyrins is the photodynamic cancer therapy. Tetrakis(sulphonatophenyl)porphyrin (TSPP) is known to localize in certain tumors to a high absolute concentration. The direct interaction and strong binding of TSPP with tubulin is involved in the biochemical mechanism for selective retention [3]. Several derivatives of tetrakis(4-methylpyridyl) porphyrin (TMPyP) have also been tested as sensitizers for photodynamic therapy in various biological media [4]. The more or less selective retention is a crucial criterion for the applicability of a particular sensitizer. The basic question underlying this study is whether it would be possible to improve selective retention by successive administration of two oppositely charged porphyrins. In this regard, it is important to examine the aggregation properties of these compounds, in particular the interaction of TSPP with TMPyP.

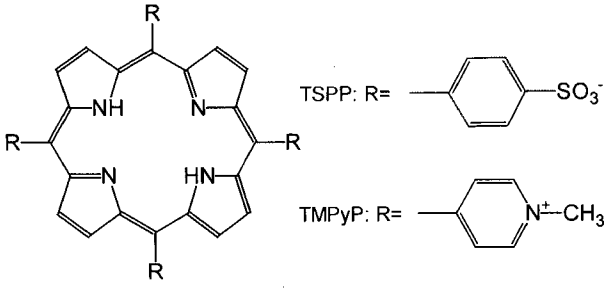

Fig. 1 Structural formula of the porphyrins investigated

The anionic TSPP and its dimerization has been extensively investigated [5–8]. A spectrum of the TSPP dimer has not been published nor has there been any published study dealing with temperature dependence of the dimerization constant derived from optical absorption spectra. This is done in the present paper which reports thermodynamic parameters determined from this dependence.

Until now, there have been proposed two different models which try to explain the nature of aggregation of TMPyP. Our detailed analysis of absorption and flu-

orescence spectra of TMPyP should bring some new facts to the contradictory discussion of Pasternack's monomer model [9–12] and Kano's dimerisation model [13–17].

Finally, heterogeneous aggregation of the two oppositely charged compounds was observed. The literature contains very few studies on porphyrin – phthalocyanine heterodimers [18–23]. Shimidzu and Iyoda [24] were the first who studied the heteroaggregation of oppositely charged porphyrins: TMPyP and tetrakis(4-carboxyphenyl)porphyrin (TXPP). As far as we know, there are no reports about heteroaggregation of TSPP and TMPyP. We tried to identify heteroaggregates of that porphyrins. Whereas a 1:1 complex cannot be detected in aqueous solution higher aggregates do exist.

Experimental

TMPyP (tetratosylate) and *TSPP* (dihydrochloride) were purchased from Porphyrin Products, Inc., Logan, Utah and were used as delivered. Absorption spectra were recorded on a Perkin Elmer PE Lambda 16 UV/VIS spectrophotometer, in part also with the help of a M400 UV/VIS spectrophotometer (Carl Zeiss, Jena). The solvent used was phosphate buffered saline, PBS (1.33 g $Na_2HPO_4 \cdot 2H_2O$, 0.44 g $NaH_2PO_4 \cdot H_2O$, and 8.50 g NaCl per 1L, pH=7.4). The fluorescence spectra were determined on a Perkin Elmer PE LS 50 spectrofluorimeter. For fluorescence studies, very dilute solutions ($<10^{-7}$ M) were used to avoid spectral distortions due the inner filter effect and emission re-absorption. Merely to detect the dimer fluorescence of TSPP higher concentrations were used. TMPyP has a marked tendency to adhere on the cell walls. Before measuring fluorescence spectra, the cells were first cleaned with chromosulfuric acid. Temperature measurements were performed in sealed cuvettes controlling the temperature within 0.1 °C.

Calculations of the various parameters of monomer dimer equilibrium of TSPP were carried out by a global fitting procedure using a modified version of the ALAU program [25].

Results and Discussion

TSPP

TSPP tends to spontaneous stacking in aqueous solution. The positively charged counterions existing in the solution partially shield the negatively charged sulphonate groups of the porphyrine. In this way electrostatic repulsion is reduced and aggregation through van derWaals attraction between hydrophobic porphyrin macrocycles is possible. We assume that only dimerization takes place

$$M + M = D \quad K_D = \frac{[D]}{[M]^2}$$

in the concentration range of 1×10^{-6} to 8×10^{-5}M where M and D represent the monomer and the dimer of TSPP, resp., and K_D the dimerization constant. We could observe by increasing the concentration a red shift of the Q-bands and a blue shift of the Soret band. A theoretical analysis predicts a negligible effect of a side-by-side dimerization on the absorption spectrum [25]. Gouterman determined an exciton interaction which is proportional to R^{-3}, where R is the separation distance between the ring centers. Our measured shifts of the Soret and Q-bands are consistent with a face-to-face geometry of the porphyrin dimer. The dimerization constant is found to be $K_D = (3.48 \pm 0.26) \times 10^4$ M^{-1} at $T = 298$ K which is consistent with literature data [8] and also argues for the hypothesis that only dimerization takes place. Figure 2 shows the monomer and dimer spectra of TSPP together with that of TMPyP for comparison. There is a red shift of the Q-bands of the dimer relative to those of the monomer and a minor blue shift of the soret band combined with an increase of intensity. The small differences between the two spectra indicate that the aggregation of the porphyrin macrocycles does not strongly perturb their electronic structures. The exciton model requires the oscillator strength of the dimer to be twice as large as that of the monomer. We have determined that ratio from the integrated absorptivities of the soret bands as 1.97 (cf. Figure 2). The exciton model does not account for the opposite spectral shifts observed with the Q- and soret bands, however, an appreciable charge transfer character of the Q-band transition can explain this discrepancy [26].

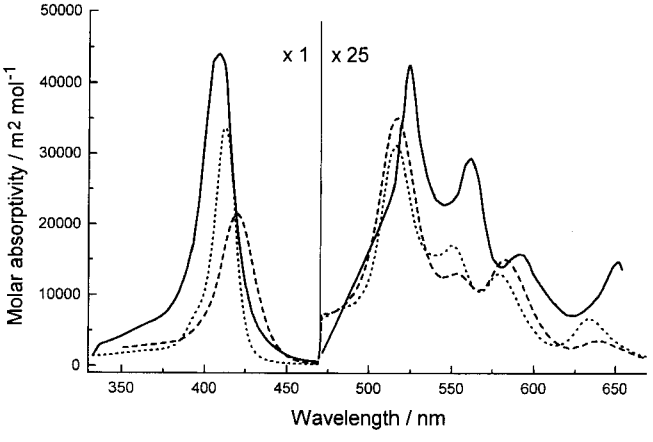

Fig. 2 Molecular absorptivity of the TSPP-dimer (solid line) obtained from a fit of a set of absorption spectra to the monomer-dimer aggregation model. The spectra for the fitting procedure were recorded in the concentration range from to 1×10^{-6} to 8×10^{-5}M. For comparison the molar absorptivities of the monomers TSPP (dotted), and TMPyP (dashed) are also given. Monomer data were derived from 8.0×10^{-7}M and 2.0×10^{-6}M, solutions, respectively.

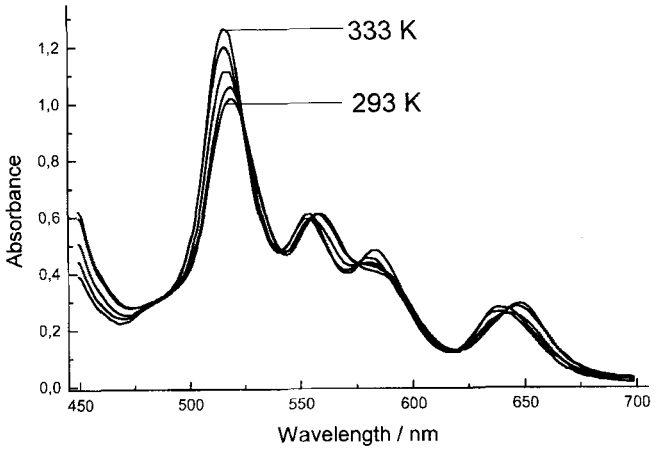

Fig. 3 Temperature dependence of the TSPP absorption spectrum showing the deaggregation with increasing temperature (T= 293, 303, 313, and 333 K), 1×10^{-4} M in PBS, corrected for the density change

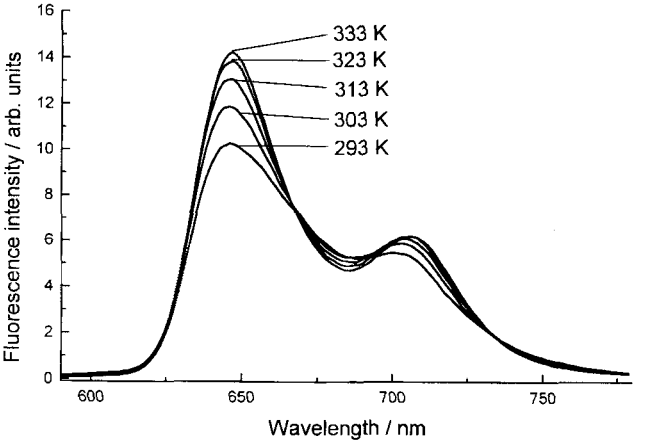

Fig. 4 Fluorescence spectrum of TSPP (8×10^{-6}M) as a function of temperature indicating the intensity increase with increasing temperature; excitation wavelength 517 nm.

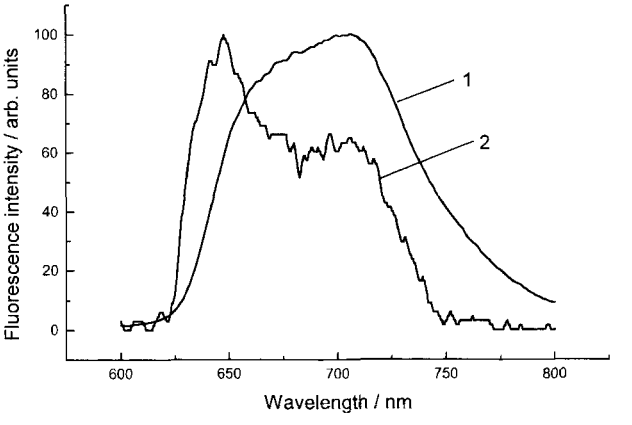

Fig. 5 Fluorescence spectrum of TMPyP in 1×10^{-8}M, 1: immediately after preparation (mainly fluorescence from the dissolved TMPyP) and 2: after 90 min (essentially fluorescence from adsorbed TMPyP)

Kemnitz *et al.* [27] determined the dimerization enthalpy and entropy derived from fluorescence decay analysis (($\Delta H = -18.0 \pm 2.4$) kJ/mol, $\Delta S = (12 \pm 2)$ J/mol K). In order to determine the thermodynamic parameters of the monomer-dimer equilibrium of TSPP we measured the spectra of a set of solutions with concentrations ranging from 2×10^{-5} to 2×10^{-6}M in the temperature range from 293 to 333 K. Figure 3 shows the transformation of the spectra of mixed monomer and dimer to that of pure monomer with increasing temperature as an example.

The enthalpy ΔH and entropy ΔS of dimerization are obtained from the slope and intercept of the linearized van't Hoff plot

$$\ln(K_D) = -\Delta H/R \times 1/T + \Delta S/R\}$$

to be -19.56 ± 0.06 kJ/mol and 19.27 ± 0.07 J/mol K, respectively. Whereas the enthalpy fully agrees within the estimated error with Kemnitz' value [27] the entropy does not. (In Kemnitz' procedure for the determination of the equilibrium constants equal absorptivities of both the monomer and the dimer was assumed which might not be fully justified.)

The temperature and concentration dependence of emission spectra support the interpretation of the absorption data. Figure 4 shows the temperature dependence of a 8×10^{-6}M TSPP solution. Principal component analysis reveals two significant principal components which represent 99.93% of the total variance. These components can be attributed to the monomer and dimer of TSPP. This proves again the validity of our assumption that the highest aggregate up to a concentration of 2×10^{-5}M is the dimer. Another conclusion is that the TSPP dimer is weakly or non fluorescent.

TMPyP

The dispute in the literature concerning the state of aggregation of TMPyP in aqueous solution lasts for more than ten years. Pasternack [9–12] pursues the monomer model which states that TMPyP does not aggregate in the concentration range of 10^{-4} up to 10^{-7}M. No deviations from Lambert–Beer law upon dilution could be detected by this author. Also n.m.r. experiments have shown a very small value of an autoassociation constant upon varying the concentration of the solutions from 0.1 mM to 50 mM. Kano [13–17], the advocate of the dimer model, observed changes in the fluorescence spectrum of TMPyP upon decreasing the concentrations down to 10^{-8}M. The almost structureless broad fluorescence spectrum (cf. Figure 5) turnes at this concentration into a typical porphyrin fluorescence spectrum with two well-resolved vibronic bands. According to Kano TMPyP exists as a monomer below 10^{-7}M and above

this concentration exclusively as a dimer. Both authors also considered the spectra of TMPyP in the presence of SDS (sodium dodecyl sulphate). While Kano reports the total conversion of the dimeric TMPyP in the presence of 0.1 M SDS, Pasternack has concluded that TMPyP is monomeric from a series of stopped flow experiments where 10^{-6}–10^{-5}M TMPyP solutions were mixed with SDS solutions of varied concentration. Pasternack [12] also proves the monomeric nature of TMPyP by the absence of perturbations on the porphyrin dimerization equilibrium while interacting with nucleotides. Finally, Kemnitz [27] based on fluorescence dynamics found that dimerization seems to be entropy driven by positive entropy. The latest investigations on this field were done by Vergeldt *et al.* [28]. These authors support the monomer model. They could not detect any changes in the fluorescence spectrum upon dilution using ultrapure water. With water of less purity Kano's fluorescence spectra could be reproduced. Vegeldt has shown that the controversial results could be explained by adsorption of TMPyP onto solid surfaces or impurities. The changes observed in the fluorescence spectrum of TMPyP were interpreted in terms of vibronic mixing of the S_1 state with an energetically nearby CT state. The visible spectrum of TMPyP is shown in Figure 2. No changes could be detected upon dilution down to 10^{-7}M or with increasing temperature (20–60 °C). We also observed the dramatic changes in the fluorescence spectrum at concentrations below 10^{-7}M. The following result strongly supports Vergeldt's interpretation of the spectral changes in terms of porphyrin adsorption both to impurities and to the cell walls. To prove the latter a carefully cleaned cuvette was filled with a 2×10^{-8}M TMPyP solution and the variation of the fluorescence spectra with time was recorded. Figure 6 shows the temporal evolution of the emission at 650 nm and 750 nm excited at 420 nm. The decrease of the fluorescence intensity taken from the center of the cuvette clearly reflects the concentration depletion due to adsorption of TMPyP on the cell walls. Additionally, a variation of the band shape is observed, that means an evolution from the typical TMPyP fluorescence spectrum to a well resolved two bands spectrum (see Figure 5). This results fit to Vergeldt's findings. The fluorescence spectrum can be regarded at any moment as a sum of two components: One component which is time independent shows well resolved Q(0,0) and Q(0,1) bands. This can be attributed to a small amount of TMPyP which might be adsorbed at particles in solution and which Kano presumed to be the monomer spectrum. The second component giving rise to the „normal" fluorescence decreases in time due to the adsorption at the cell walls.

Heteroaggregates

We observed heteroaggregation by spontaneous association of the cationic and anionic porphyrins. In this case, both coulombic attraction between charged substituents and hydrophobic interaction of the aromatic macrocycles concur in holding the individual constituents together. Thus very stable complexes are formed with a close face-to-face geometry.

Upon mixing the two types of porphyrins in aqueous solution, precipitates are formed. In the case of 1:1 solution the entire amount of reactants precipitates. After filtering off the solid and thoroughly washing with PBS we tried to resolve the aggregate in PBS. Neither absorption nor fluorescence could be detected in the supernatant solution. From the detection limit we can estimate the solubility of the 1:1 aggregate to be smaller than 10^{-9}M.

In order to reveal whether there do exist heteroaggregates with a composition other than 1:1 we mixed solutions TMPyP and TSPP under a variation of the

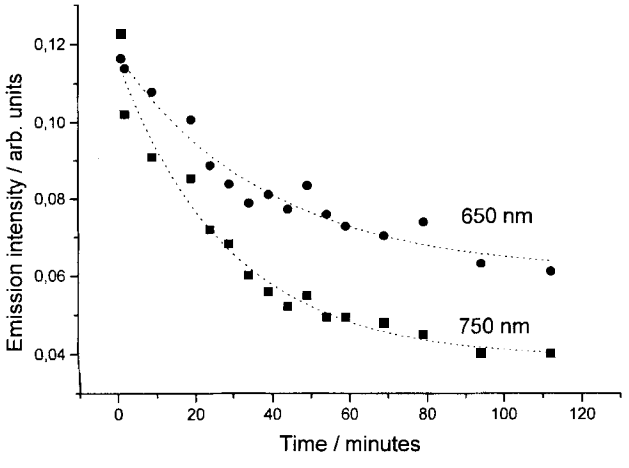

Fig. 6 Temporal evolution of the fluorescence intensity of a 2×10^{-8} M TMPyP solution in PBS at two wavelengths

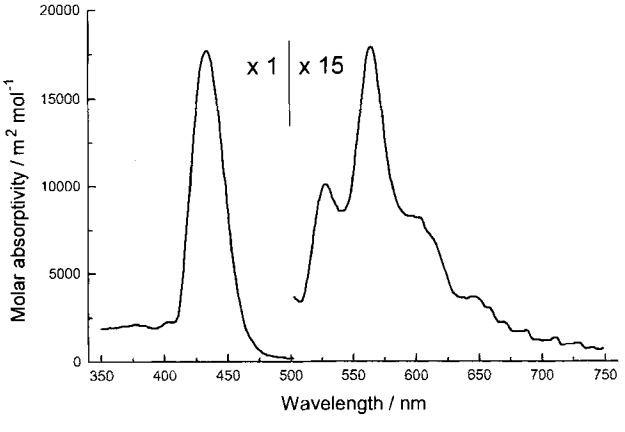

Fig. 7 Spectrum (molar absorptivity) of the $(TMPyP)_2 \times TSPP$ aggregate; left and right ordinate axis correspond to the Soret and the Q-band spectral region, respectively.

Table 1 Positions (in nm) and molar absorptivities (given as log ε in parenthesis, where ε is expressed in $m^2\ mol^{-1}$) of the absorption maxima of the monomeric species and the aggregates investigated

	Soret	$Q_y(0-1)$	$Q_y(0-0)$	$Q_x(0-1)$	$Q_x(0-0)$
TSPP	412 (4.53)	516 (3.10)	550 (2.83)	580 (2.72)	634 (2.43)
TSPP×TSPP	409 (4.33)	524 (3.19)	562 (2.72)	590 (2.78)	652 (2.16)
TMPyP	420 (4.65)	516 (3.24)	552 (3.07)	583 (2.80)	641 (2.78)
TMPyP×TSPP×TMPyP	433 (4.25)	524 (2.80)	562 (3.08)	602 (2.70)	645 (2.22)

concentration ratio from 1:1 up to 1:10. We measured the absorption spectra of the supernatant solution. Interesting changes in the absorption spectra of TSPP/TMPyP solutions of stoichiometry 1:1.2 up to 1:1.8 range could be observed. The resulting spectra differ markedly from the sum of those of the reactants. They could always be fitted to a linear combination of the spectra of TMPyP and another one which we presume to be that of the complex. This spectrum is shown in Figure 7. In Table 1 we listed the absorption maxima of all types of porphyrins and their aggregates studied. It is obvious that the Q-band of the heteroaggregate is red shifted whereas the Soret band undergoes a minor blue shift. These shifts in the absorption bands of the complex and the reduced solubility support the conclusion of strong intermolecular interaction, meaning marked orbital overlap. Shimidzu and Iyoda [24] have also observed a red-shifted Q-band and a blue-shifted Soret band with TMPyP/TCPP heteoaggregate without changes in the UV region. They conclude that there is no π-π interaction between the *meso*-substituents other than a coulombic one.

If we suppose that a 1:1 ratio is maintained in the precipitate we can conclude that apart from uncomplexed TMPyP triple decker sandwich-type heterotrimers are present in the supernatant solution containing two cationic porphyrins separated by one anionic species. Note that an excess of TMPyP is required to form the 2:1 aggregate.

Aggregation in the presence of BSA

Investigations into the interaction of porphyrins with proteins have been published nearly exclusively with metalloporphyrins [28–29] where axial binding has been found important. We expect that also in case of the metal-free porphyrin bases the aggregation may considerably be influenced by the interaction with proteins. As a preliminary result of an investigation into the aggregation of highly charged porphyrins in the presence of bovine serum albumine (BSA) we found a dimerization constant for TSPP of $K_D = 1.11 \pm 0.18\ M^{-1}$ at room temperature (in PBS containing 1.6 g/l BSA) which is one order of magnitude smaller in comparison to the pure PBS solution. The shift of the monomer dimer equilibrium can also be followed with solutions of 5×10^{-4}M TSPP with increasing concentrations of BSA.

The quantitative investigation of the heteroaggregation equilibria in the presence of proteins is considerably more complicated because of very slow equilibration rates. Agglomeration renders the solutions turbid; precipitation is hindered by the protein.This will be the subject of a forthcoming study.

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