Biophysical Chemistry 25 (1986) 99–104
Elsevier



BPC 01094

# THE PHYSICOCHEMICAL STATE OF PROTOPORPHYRIN IX IN AQUEOUS SOLUTION INVESTIGATED BY FLUORESCENCE AND LIGHT SCATTERING

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Received 18th March 1986
Revised manuscript received 27th August 1986
Accepted 27th August 1986

*Key words: Protoporphyrin IX; Aqueous solution; Fluorescence spectroscopy; Light scattering; Micelle; Physicochemical state*

Fluorescence spectroscopy and light scattering have been used to investigate the physicochemical behaviour of protoporphyrin IX in aqueous solutions. In the alkaline range large micelles are formed with a hydrodynamic radius of 130 nm and a molecular mass of $5.0\times10^7$ Da. The micelles are fluorescent with an emission maximum at 620 nm. A pH lowering caused quenching of the micelle fluorescence. On a collision encounter these micelles will disintegrate and they are reformed by nucleation of collision fragments. From measurements of the fluorescence intensity of the micelles versus total concentration an equilibrium constant of $4.0\times10^6$ $M^{-1}$ was found for this collision-nucleation process. In the pH range between 6 and 3 another micelle type of twice the size of those in the alkaline range was stable with respect to the solute. These micelles have free base porphyrin fluorescence with an emission maximum at 634 nm. A lowering of the pH below unity causes disintegration of these micelles and monomer fluorescence from the protoporphyrin dication was observed.

## 1. Introduction

Protoporphyrin (PP) is widespread in nature and is, for instance, the metabolic precursor for both chlorophylls and cytochromes. The functional properties of a molecule are related to its structure, and since biological systems are essentially aqueous, the physicochemical properties of PP in water are of great importance. The structure of porphyrins in water has also been questioned by researchers in cancer therapy, where porphyrins are used as photosensitizing agents [1].

Experiments concerning the structure of porphyrins in aqueous solutions have been given different interpretations. The changes in shape of the Soret absorption band of PP with respect to increasing concentrations were attributed to dimerization by Karns et al. [2]. In simple monomer-dimer equilibrium reactions, a higher ratio of dimeric PP to monomeric PP molecules is expected with increasing total concentration. On the other hand, Brown et al. [3] suggested that micellization of many porphyrins occurred in aqueous solutions when the concentration exceeded a certain level. This conclusion was based on the observations of sudden shifts in the positions of Soret absorption peaks for several porphyrins to increments in concentrations above a critical point. Below the critical concentration for micelle formation a simple monomer-dimer equilibrium distribution of the prophyrins was valid. PP, however, appeared to be aggregated for all concentrations [3].

In order to determine particle size, light scattering is superior to absorption measurements. In this paper fluorescence spectroscopy and light scattering will be used to investigate the physicochemical properties of PP in water, with particular emphasis to settle the question of whether or not PP aggregation occurs beyond dimerization. The state of PP will be investigated as a function of both the pH of the solution and the total concentration of PP.

## 2. Materials and methods

PP was bought from Porphyrin Products, Utah. A stock solution was made by dissolving 6.0 mg PP into a mixture of 5 ml of 90% ethanol and 0.01 M NaOH. The concentration of this stock solution, referred to PP in monomeric form, was therefore $10^{-3}$ M. As in the work of Karns et al., the solvent was 0.02 M NaOH in distilled water giving a pH of the solution of 11.5. In the pH titration experiments the pH of the solution was changed by minute additions of HCl, in order to avoid any changes in PP concentrations.

The fluorescence measurements were made using a Spex Fluorolog 222 (Spex Industries). The 90° light scattering experiments were performed with the same instrument by simply making the emission wavelength equal to the excitation wavelength, which always was 397 nm. The pH values of the solutions were determined by a Philips PW 9409 digital pH-meter with a miniature C 14/02 electrode, which could be inserted into the cuvette. The dynamic light scattering equipment is described elsewhere [4].

## 3. Results

### 3.1. pH titration measurements

Four different fluorescence emission spectra, each of them present in different pH ranges, were observed during the pH titration of PP in aqueous solution. In these experiments the concentration of PP is kept constant while the pH of the solution is gradually changed from pH 11.5 to 0.8. These four spectra are shown in fig. 1. When the pH of the solution was between 11.5 and 7, the spectrum with the emission maximum at 620 nm was present. In the pH range 6–3 a second spectrum with a peak at 634 nm, characteristic of the free base porphyrin in hydrophobic environments, was dominant. When the pH was between 2 and 1 the third characteristic spectrum, with an emission maximum at 603 nm, appeared. The fourth spectrum, which is the ordinary dication spectrum (two hydrogens and two protons in the porphyrin center), has an emission maximum at 606 nm and

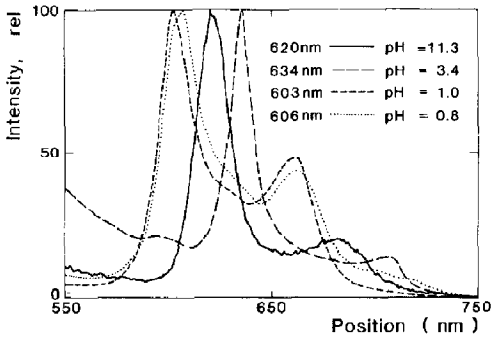


Fig. 1. Fluorescence emission spectra from four $10^{-8}$ M protoporphyrin IX aqueous solutions of different pH values. The excitation wavelength was 397 nm.

appears very abruptly when the pH drops below 0.8. The 603 nm spectrum is quite similar in shape to that of the 606 nm spectrum. The differences between the third and fourth spectrum are small, but a distinction between them seems appropriate.

In fig. 2 the intensities at the emission maxima

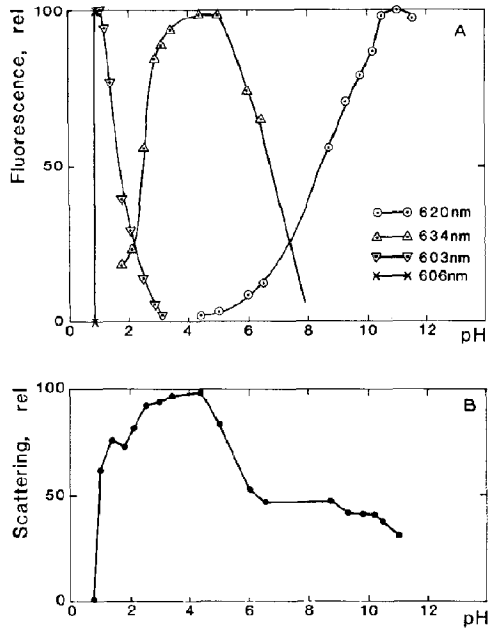


Fig. 2. (A) Relative intensities of the four characteristic spectra given in fig. 1 vs. pH of the solution. The pH was changed by dropwise addition of HCl to a $10^{-8}$ M protoporphyrin aqueous alkali solution ($10^{-2}$ M NaOH). (B) relative intensity of the 90° scattered light at 397 nm from the same solutions.

of the four different spectra are plotted as a function of pH in the solution. The total PP concentration was $10^{-8}$ M. It is seen that the different spectra were present in different pH ranges. In the same figure the light intensity scattered at 90° with respect to the exciting beam from the solution is also plotted vs. pH. Above pH 6, when the first spectrum was present, the degree of scattering was fairly constant. Below pH 6, the scattering efficiency was double compared to that above pH 6. Below pH 0.8, there was a substantial reduction in the scattering efficiency from the solution which indicated that PP entered into a monomeric state. The changes in scattering efficiency from the solution occur over the same pH intervals where the fluorescence spectra change from one type to another. These pH-dependent changes in fluorescence and scattering properties of the system were reversible.

### *3.2. Fluorescence and 90° scattering versus concentration*

In fig. 3A the fluorescence intensity for the 620 nm emission spectrum present at pH 11 (0.02 M NaOH), where these measurements were made, is plotted vs. the total PP concentration. In the concentration range $10^{-10}$–$10^{-8}$ M the relation between fluorescence intensity and total concentration is linear, since the slope in the double-logarithmic plot is unity. At higher concentrations the intensity increases approximately to the square root with respect to the total concentration, since the slope is close to one half (fig. 3A).

The intensity of the light scattered at 90° with respect to the exciting beam is also plotted vs. concentration in fig. 3A. Also, the scattering efficiency of the solution increases less rapidly than the total concentration.

In fig. 3B the fluorescence intensity from the solution divided by the total concentration, which is indicative of the relative yield of fluorescence, is plotted vs. the concentration. In the low concentration range the relative yield is constant while at higher concentrations the yield decreases.

### *3.3. Light scattering measurements*

The simple 90° scattering measurements connected to the fluorescence measurements can only give relative changes in the particle size vs. concentration or pH. In order to determine the absolute particle size both dynamic light scattering as well as measurements of scattered light intensity as a function of scattering angle were performed on calibrated equipment. Dynamic light scattering can be used to measure the diffusion coefficient of translation, and in fig. 4A the auto-

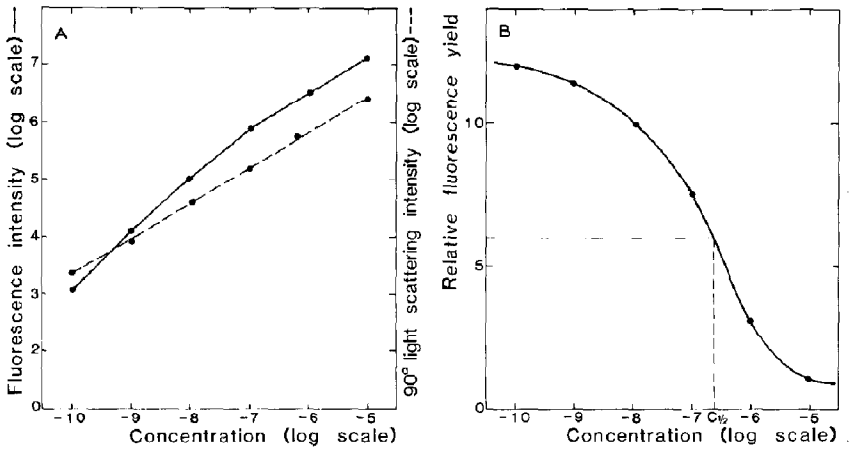


Fig. 3. (A) Fluorescence intensity at 620 nm (solid line) and the intensity of the 90° scattered light (broken line) vs. protoporphyrin concentration. The pH of the solution was 10.5 and the excitation wavelength 397 nm. (B) Relative fluorescence yield, or fluorescence intensity divided by the total concentration, vs. protoporphyrin concentration.

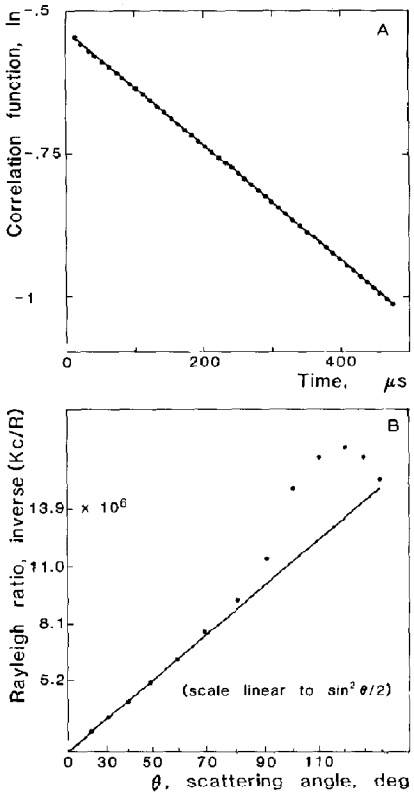


Fig. 4. (A) Autocorrelation function for the intensity fluctuations in the 90° scattered light from a $10^{-5}$ M PP solution (phosphate-buffered saline, pH 6.7). (B) Form factor of the scattered light intensity from the same solution vs. sine squared of half the scattering angle.

correlation function for the intensity fluctuations in the light scattered at 90° with respect to the exciting beam from a $10^{-5}$ M PP solution of pH 6.7 is shown. The autocorrelation function is close to exponential, the diffusion coefficient derived from the slope being $1.83 \times 10^{-12}$ $m^2$ $s^{-1}$. Furthermore, the variation of the diffusion coefficient is less than 10% of the diffusion coefficient itself, which means that the particle size is quite homogeneous. If Stokes' law is applied a hydrodynamic particle radius of 130 nm is derived.

In fig. 4B the reciprocal of the intensity of the scattered light is plotted vs. sine squared of the half scattering angle. In the angular range 30–100° a straight line can be fitted to the experimental relation, and from the slope of this line a radius of gyration for the particles of 230 nm is found, which indicates, since their hydrodynamic radius is 130 nm, that they have a somewhat elongated form.

The extrapolation of the straight line in fig. 4B back to zero scattering angle gives a $Kc/R$ value, which equals the inverse of the molecular mass of the scattering particles, of $2.0 \times 10^{-8}$. $c$ is the concentration of scattering particles and $K$ a constant that depends upon the refractive index increment, which is estimated in this case to be 1 ml $g^{-1}$ at 515 nm. $R$ is the Rayleigh ratio [5]. This gives a molecular mass of $5.0 \times 10^7$ Da of the scattering particles. The number of PP units in each aggregate is therefore $9 \times 10^4$, since each unit has a molecular mass of 562 Da.

## 4. Discussion

Three independent sets of observations strongly favour the idea of Brown et al. that large micelles or aggregates are formed when PP is dispersed in water. Firstly, dynamic light scattering gives a hydrodynamic radius of 130 nm of these aggregates, which in addition are quite monodisperse, and, secondly, angular measurements of static light scattering indicate that the radius of gyration for the particles is 230 nm and that the molecular mass is $5 \times 10^7$ Da. Finally, the tremendous, drop in intensity of the scattered light at 90° from the system when the pH drops below unity, where PP is known to be in a monomeric state, indicates that above pH 1, PP is in an aggregated configuration. A crude estimation shows that the ratio of scattering intensities above and below this pH value giving the transition between aggregates and monomers is equal to the number of PP molecules in each aggregate.

Even if the particles are rather homogeneous in size, since the autocorrelation function derived from the dynamic light scattering is close to exponential and thus allows only a small range of diffusional coefficients, the measurements of fluorescence and 90° scattering intensity vs. concentration indicate that the PP aggregates are subjected to a concentration-dependent equilibrium

reaction. It can be seen from fig. 3A that both scattering and fluorescence intensities behave similarly and nonlinearly with respect to changes in the total concentration. It is therefore likely that the particles contributing to scattering are also fluorescent and the following model, which predicts the number of such particles, is put forward: PP exists either as aggregates (A) or as fragmented aggregates ($A_f$), which are smaller particles, in aqueous solution. On a collision encounter the aggregates will disintegrate into fragments, which in turn will condense by, for instance, nucleation into an aggregate of the original type. The equation for these reactions may be written:

$$A + A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} xA_f \tag{1}$$

where $x$ is the number of fragments created by the collision. The rate of formation of fragments is $k_1 A^2$ and it is further assumed that the rate of creation of aggregates is in proportion to the concentration of fragments. This may be the case when one of the steps in the nucleation process is rate-limiting. At equilibrium these formation rates are equal, so:

$$k_1 |A|^2 = k_{-1} |A_f| \tag{2}$$

The same basic equation was used by Karns et al., however, their A was a monomer and $A_f$ was a dimer.

The solution of this equation, when it is taken into consideration that the amount of PP in the solution exists as either large aggregates or smaller fragments, is:

$$|A| = \left(-1 + \sqrt{1 + 8K_0C}\right)/4K_0 \tag{3}$$

which in the two limiting cases will be:

$$|A| = C \quad \text{when} \quad 8K_0C \ll 1 \tag{4}$$

$$|A| = \sqrt{C/2K_0} \quad \text{when} \quad 8K_0C \gg 1 \tag{5}$$

where $C$ is total PP concentration and $K_0 = k_1/k_{-1}$. The measurement of fluorescence intensities vs. concentration is well described by this equation. At low PP concentrations the fluorescence intensity is linearly related to total concentration, while at higher concentrations a square root dependence seems appropriate (see fig. 3a). In fig. 3B the relative fluorescence yield, defined as the fluorescence intensity at a certain PP concentration divided by this concentration, is plotted. According to the above equation for the amount of fluorescent substance ($A$) vs. total concentration ($C$), the concentration of PP ($C_{1/2}$) at which the fluorescence yield is reduced to one half of its maximum value is related to the equilibrium constant by the following simple relation (see the appendix):

$$1 = K_0 C_{1/2}, \tag{6}$$

so from fig. 3B it is derived that $K_0 = 4.0 \times 10^6$, which is a value in close agreement with that obtained by Karns et al. ($K_0 = 3.1 \times 10^6$) in the absorption measurements.

The scattering intensity from this system is expected to be:

$$I_s \propto |A_f| M_f^2 + |A| M_A^2 \tag{7}$$

where $M$ is the molecular mass of the fragments and aggregates, respectively. The contribution from the fragments can explain the small deviation from the square root dependence of scattered intensity from the concentration expected for the aggregates (see fig. 3A).

From the pH titration experiments it is seen that the aggregate fluorescence is high at pH values between 12 and 10 and approaches zero at about pH 6 (see fig. 2). At high pH values the aggregates are deprotonated and fluorescent while the protonated form is nonfluorescent. When the logarithm of the concentration ratio between fluorescent to nonfluorescent aggregates, obtained from fig. 2, is plotted vs. pH in the alkaline range a straight line is obtained with a slope of 0.47. If the binding sites for protons, which probably are the carboxyl side groups of PP, on the aggregate were identical and independent the slope would be unity. A slope smaller than unity is in accordance with the existence of two or more binding sites with different binding constants [6].

The binding of protons obviously destabilizes the aggregates because in the pH range 6–3 another aggregate type of larger size is stable. This aggregate may be formed by a fusion of two aggregates

stable in the alkaline pH range because the scattering efficiency has increased by a factor 2. These aggregates contain free base PP in hydrophobic compartments, since fluorescence of this type is observed.

In a very narrow pH range between 2 and 1 the free base porphyrins in these largest aggregates becomes protonated and fluorescence from the dication is observed. At about pH 0.8 there is a sudden shift in the emission maximum of the dication fluorescence from 602 to 606 nm which may indicate a complete disintegration of the aggregates, in line with the abrupt changes in scattering, into PP in a monomeric state.

## Appendix

The relation $K_0C_{1/2} = 1$ (eq. 6), used in order to determine the equilibrium constant ($K_0$) for the second-order reaction introduced in the discussion (eq. 1), will be derived. $C_{1/2}$ is the value of the total concentration at which the relative quantum yield is reduced to one half of its value in the initial case of low concentrations. The fluorescence intensity is proportional to the concentration of fluorescent substance, which for the system under investigation is given by the square root expression given in eq. 3. The relative quantum yield ($\eta_{rel}$), or the fluorescence intensity divided by the total concentration ($C$) of porphyrin, will therefore have the following dependency on $C$ in this case:

$$\eta_{rel} = \frac{\left(-1 + \sqrt{1 + 8K_0C}\right)/4K_0}{C}$$

From the graph of the relative quantum yield vs. the total concentration the particular concentration at which the yield is reduced to one half of the initial value ($C_{1/2}$) can be easily obtained. For this case one has according to the above expression:

$$\frac{1}{2} = \frac{\left(-1 + \sqrt{1 + 8K_0C_{1/2}}\right)/4K_0}{C_{1/2}},$$

which further gives:

$$\sqrt{1 + 8K_0C_{1/2}} = 2K_0C_{1/2} + 1$$

When both sides of the equation are squared one obtains:

$$1 + 8K_0C_{1/2} = 1 + 4K_0C_{1/2} + 4K_0^2C_{1/2}^2,$$

which finally gives:

$$1 = K_0C_{1/2} \quad \text{or} \quad K_0 = C_{1/2}^{-1},$$

which is a simple and exact expression for the equilibrium constant.

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