Protein induced formation of porphyrin (TPPS₄) nanostructures

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Abstract. The stimulating effect of two cationic proteins lysozyme and serum albumin on TPPS₄ aggregation was studied in aqueous acidic solution (pH 1) by means of absorption and fluorescence spectroscopy. At low TPPS₄ concentrations (<2µM), the presence of both proteins significantly increased formation of TPPS₄ J-aggregates as compared with pure TPPS₄ solution. The absorption intensity of protein-induced J-aggregates was found being dependent on protein nature, TPPS₄ concentration and TPPS₄/protein molar ratio. J-aggregates formed in mixed TPPS₄-protein solutions had a broader absorption band (490 nm), except for TPPS₄:BSA at a ratio 1:10, when it was even narrower and bathochromically shifted. The formation of a new absorption band at 426 nm was also observed for both Lys and BSA at certain molar ratios. The presence of a similar absorption band at 423 nm in the pure highly concentrated TPPS₄ solutions, in which nanotube-like structures were identified on AFM images, suggests that ordered TPPS₄ nanostructures might be also formed in contact with protein molecules.

1. Introduction

Self-assembled J-aggregates of meso-tetra(4-sulphonatophenyl)porfine (TPPS₄) are extensively studied due to its unique ordered structure and nonlinear optical properties, which could further be employed to develop nonlinear optics, design opto-electric conversion devices, model primary energy transfer processes in photosynthetic complexes or produce artificial light harvesting systems.

Protonated TPPS₄ monomers (figure 1B) spontaneously form J-aggregates in aqueous solutions at low pH or high ionic strength [1-3]. J-aggregates formation is indicated by the appearance of a red-shifted sharp excitonic absorption band at 490 nm (the J-band) and a second one at 706 nm. At high concentrations in acid medium TPPS₄ molecules also form H-type aggregates [4]. The absorption band attributed to H-aggregates is blue shifted to about 423 nm [4; 5] in comparison with the Soret band of protonated TPPS₄ monomers. However, despite the extensive spectroscopic studies, the exact

Figure 1. Two ionic forms of TPPS₄ in aqueous solution: A - deprotonated form; B – protonated form.
molecular structure of TPPS₄ aggregate responsible for the absorption band at 423 nm has not been established yet.

Recently, it was shown that in acid, highly concentrated solutions TPPS₄ molecules form complex tube-like nanostructures via a stepwise aggregation mechanism passing several intermediate stages [6-11]. The spectra of TPPS₄ solutions we have measured under such conditions indicated the presence of both J- and H-aggregates absorption bands.

Formation of TPPS₄ J-aggregates as well H-aggregates was reported occurring in mixed solutions too. Different cationic molecules such as surfactants [12-14], proteins [15-17], polypeptides [18; 19], poly(amidoamine) dendrimers [20], cyclodextrins [21], porphyrins [22; 23] could promote aggregation of TPPS₄ in acid medium.

The objective of present work was to elucidate the main factors affecting self-assembling of TPPS₄ aggregates in the presence of two proteins - lysozyme (Lys) and bovine serum albumin (BSA). Studies on formation of highly ordered nanoaggregates in the presence of various organic molecules could serve for both the understanding of fundamental self-organization processes and adaptation of these principles for controllable production of nanostructures.

2. Materials and Methods
TPPS₄ was purchased from Porphyrin Products (USA), BSA from Carl Roth (Germany) and Lys from Fluka (USA).

TPPS₄-protein mixed solutions were prepared by diluting TPPS₄ solution with equal volume of protein solution. The concentration of TPPS₄ was kept constant while protein concentration in mixed porphyrin-protein solutions was varied in each set of experiments.

The pH of all solutions was adjusted to 1.3. The pH value was controlled using a pH–meter IC150 (IQ Scientific Instruments, Inc., USA) with a glass electrode CW711.

Absorption measurements were performed on a fiber optics spectrometer S2000 (Ocean Optics Inc., USA). Fluorescence experiments were carried out using a spectrofluorimeter LS50B (Perkin Elmer, USA).

The samples for AFM measurements were prepared by casting a droplet (20-60 µl) of the porphyrin solution on the glass substrate. The droplet of solution was removed after 30 seconds by blowing it off. Standard glass cover slides were used as substrates. The slides were degreased before the measurements.

AFM images were obtained using an atomic force microscope (Thermomicroscopes Explorer, USA). The surfaces were scanned in the constant force contact mode. Topographic images of 5 µm x 5µm were produced. No further image processing was performed.

3. Results
The spectroscopic results presented below are grouped on the basis of the TPPS₄ concentration considering the initial aggregation state of TPPS₄ in aqueous medium.

At low TPPS₄ concentrations (<2 µM) the absorption spectra of pure aqueous TPPS₄ solutions had no or only an insignificant absorption peak of J-aggregates (figure 2) reflecting the domination of TPPS₄ monomers absorbing at 435 nm. The presence of both proteins significantly increased the absorbance of J-aggregates at the expense of the absorption band of monomers for all used TPPS₄: protein molar ratios (1:0.01-1:10) (figure 2) except for TPPS₄ (1µM):Lys 1:0.01-1:0.1 (spectra not shown).

Absorption of new-formed J-aggregates in the presence of BSA was more intense as compared with Lys at the same TPPS₄:protein molar ratios. The highest absorbance of J-aggregates was observed at intermediate (1:0.1-1:1) TPPS₄:BSA ratios and at the highest (1:10) TPPS₄:Lys ratio (figure 2). In addition, formation of a new absorption band at 426 nm together with the most pronounced broadening of the J-band was observed for Lys and for BSA at the highest (1:1-1:10) and intermediate (1:0.1-1:1) porphyrin:protein ratios, respectively.
Furthermore, the spectra of J-aggregates registered in the presence of proteins differed from the spectra of those having the same absorbance intensity in pure aqueous solutions (at higher TPPS₄ concentrations). The J-band was broader at all measured TPPS₄:protein molar ratios, except for TPPS₄:BSA 1:10, when it narrowed and underwent a bathochromic shift (figure 2). Moreover, only for BSA at this ratio the Soret band split into two peaks at 440 nm and 423 nm with simultaneous appearance of Q bands. These spectral changes could be explained by the formation of several TPPS₄–BSA complexes involving both protonated and deprotonated forms of TPPS₄ monomers [17] (figure 2B).

**Figure 2.** Absorption of TPPS₄ (2µM) in the presence of Lys (A) and BSA (B) at different TPPS₄:protein molar ratios (pH 1).

Pure aqueous acid solutions of TPPS₄ at higher concentrations (≥5µM) in addition to the intense J-band also possess an absorption band at 423 nm typical for a highly aggregated state (figure 3). In contrast to the J-band at 490 nm, this band is usually ascribed to TPPS₄ H-type aggregates due to its hypsochromic shift in comparison with the Soret band of TPPS₄ monomers (at 435 nm) [4].

Our experiments showed that starting from 5µM porphyrin concentration the appearance of the band at 423 nm in the specimens coincides with the detection of TPPS₄ nanotubes by means of AFM (figures 3, 4). The relative amount of nanotubes observed in AFM images at higher TPPS₄ concentrations correlated with the increase in absorbance at 423 nm. (figures 4, 3)

Comparison between spectroscopic data and AFM images implies that the absorption band at 423 nm and the J-band at 490 nm together reflect the formation of the ordered structure - TPPS₄ tube-like aggregates.

**Figure 3.** Normalized absorption spectra of TPPS₄ in aqueous HCl solution (pH 1) at various concentrations.

**Figure 4.** AFM images of TPPS₄ samples on silica prepared from 50 µM (A) and 5 µM (B) TPPS₄ aqueous acid solutions (pH 1).
In the presence of protein at TPPS₄ concentrations equal or higher than 5 µM, formation of green coprecipitate was observed at intermediate (1:0.1-1:1) TPPS₄:Lys molar ratios while in the case of BSA precipitation occurred only at ratios lower than 1:0.01. The absorption spectrum of the fraction of Lys coprecipitates resembled the spectrum of highly concentrated pure TPPS₄ solution (figure 5). The spectrum of the TPPS₄-BSA solution containing the coprecipitated fraction differed from the latter in both the spectral width of the Soret band and its intensity ratio to the J-band (figure 5). In contrast to Lys, coprecipitates of BSA could not be homogenized by intensive stirring of the solution.

4. Discussion
Various TPPS₄ species present in the pure TPPS₄ solutions interacted with protein in a different way and affected the final TPPS₄-protein equilibrium state depending on the initial TPPS₄ concentration as well as the type of the protein and the porphyrin:protein ratio.

Both proteins present in solutions at low TPPS₄ concentration demonstrated the ability to induce the formation of J-aggregates from the monomeric species. The positively charged proteins could serve as centers of condensation due to partial neutralization of approaching TPPS₄ molecules thus helping them to come into closer contact with each other.

The different size and structure of proteins might be responsible for the differences between the spectra of J-aggregates measured in mixed solutions at the same ratios of Lys and BSA. Smaller Lys molecules seem to be less efficient in production of J-aggregates therefore the higher amounts of Lys are required to get the same absorption intensity of J-aggregates.

The broadening of J-band as well as resemblance of TPPS₄ absorption band at 426 nm in mixed solutions at intermediate BSA and high Lys ratios to the absorption band at 423 nm appearing in pure TPPS₄ solutions suggest the formation of the two-dimensional aggregated structures on the surface of protein. The larger surface area of BSA enabling the stronger retention of formed aggregates could be among the factors affecting the intensity and spectral width of its absorption bands. On the other hand, supraaggregates of TPPS₄ (nanotubes) existing at higher TPPS₄ concentrations seem to interact with proteins retaining their spectral identity and preserving the structure.

The coprecipitation, which was observed only at TPPS₄ concentrations above 5µM, required significantly lower amounts of BSA to occur. The main factors regulating the intensity of this process, probably, include the size and the total charge of protein. This could affect the strength of the protein interaction with aggregated porphyrin species, which seem to be higher in the case of BSA.

The spectroscopic evidence of the monomeric TPPS₄ species observed at the highest BSA ratios, implies that only the structure of BSA can act on TPPS₄ molecules as a shield from the aqueous environment.

5. Conclusions
The net positive charge and resulting electrostatic attraction between proteins and negatively charged monomeric species of TPPS₄ facilitate formation of J-aggregates as well as its retention on the surface of protein. The more effective formation of J-aggregates in the presence of BSA at the corresponding
TPPS₄:protein molar ratios could be related to the greater size of the protein resulting in the higher local concentration of TPPS₄ monomers in the vicinity of protein and providing them with the wider area for interaction.

Thus the possible reasons of the spectral differences observed between J-aggregates present in aqueous solution and those formed in the presence of proteins could be related to the protein-assisted assembling and subsequent superficial localization of size-limited aggregated structures.

The necessary conditions for the coprecipitation seem to require the presence of TPPS₄ monomers (presumably initiating the superficial neutralization of protein) as well as highly aggregated species serving as a substrate finalizing the cohesion.

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