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Effects of Visible Irradiation of Protoporphyrin IX on the Self Assembly of Tubulin Heterodimers

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Abstract

The formation and the effects of the laser irradiation of the complex formed by protoporphyrin IX (PPIX) and tubulin was investigated. We have used tubulin as a model protein to investigate whether docked photoactive ligands can affect the structure and function of polypeptides upon exposure to visible light. We observed that laser irradiation in the Soret band prompts bleaching of the PPIX which is accompanied by a sharp decrease in the intensity and average fluorescence lifetime of the protein (dominated by the four tryptophan residues of the tubulin monomer). The kinetics indicate non-trivial effects and suggest that the photosensitization of the PPIX bound to tubulin prompts structural alterations of the protein. These modifications were also observed through changes in the energy transfer between Trp residues and PPIX. The result suggest that laser irradiation produces localized partial unfolding of tubulin and that the changes prompt modification of the formation of microtubules *in vitro*. Measurements of singlet oxygen formation were inconclusive in determining whether the changes are prompted by reactive oxygen species or other excited state mechanisms.

Introduction

One of the major paradigms of molecular biophysics and structural biology is the correlation between the conformation of a protein and its function. According to the paradigm, electrostatic and thermodynamic properties (e.g., charge distribution, hydrogen-bond network, entropy) determine the folding of proteins into their stable, yet dynamic, three-dimensional conformation^[1]. According to this view, the ability to modify the conformation of proteins through localized changes in structure would be of interest to probe a more deterministic correlation between specific domains of a protein and its function. Novel methods to achieve this result involve the use of a light activated ligand capable of prompting localized structural changes in a protein through photophysical and/or photochemical mechanisms. Photoisomerization^[2] and photosensitization of singlet oxygen^[2a, 3] have been some of the methods employed. In photoisomerization (which, for instance, forms the operational basis of optogenetics and much of organism photoreception) the rotation of the dye causes conformational effects of the channel protein to which it is bound^[4]. Photosensitization of reactive oxygen species can cause chemical reaction in the protein capable of causing conformational effects^[5]. Other mechanisms, such as photoinduced electron transfer also contribute to induce conformational changes in

proteins [6]. Other photosensitizers can also be used and our group has demonstrated that irradiation of porphyrin ligands at low-irradiance ($< 20\text{mW/cm}^2$) can lead to conformational changes of a small protein such as β -lactoglobulin (BLG) and that these changes are somewhat selective since they occur only at alkaline pH [7]. BLG is a convenient protein model but it has little relevance to widespread applications. Therefore, we have investigated the effects in more biologically impactful proteins including tubulin. Tubulin is a 55 kDa globular protein [8] which typically forms heterodimers that assemble into microtubules (MT) [9]. Microtubules are crucial to a number of important functions such as intracellular transport, morphogenesis, and mitosis [10]. Because of its ubiquity, tubulin has become a prime target in biomedical research [11]. Since native-like MT can be readily formed *in vitro* upon addition of GTP and Mg^{2+} ions at 37°C [12] their formation can be used to probe whether photosensitized mechanisms affect the major function of the tubulin. We demonstrated that structural changes can indeed be prompted on tubulin by a water soluble porphyrin (tetra-phenyl-sulfonato porphyrin, TSPP) [13]. The successful results obtained by the tubulin/TSPP complex prompted us to investigate whether protoporphyrin IX (PPIX), a widely used phototherapeutic drug [14] is also capable of prompting conformational and functional modification of tubulin upon irradiation. Compared to TSPP, PPIX has low solubility in aqueous solution at neutral pH and is likely to dock at a different site on tubulin which would enable us to probe the correlation of the function of the protein with structural modification at a site different than the one probed by TSPP. Moreover, the clinical use of PPIX implies that investigating its effects on tubulin may have a more immediate repercussion in biomedical research. In fact, the photoinduced disruption of MT could be strategic to biomedical applications of photosensitizers. Porphyrins are often used in phototherapy because of their high quantum yields of triplet state [15] that can efficiently sensitize the formation of singlet oxygen; however the possibility of using them to selectively modify protein functions can provide a more direct and controllable path to cell damage [16].

The major hypothesis tested in this work was whether the photosensitization of PPIX bound to tubulin disrupts the ability of the protein to form MT (Scheme 1). In order to test the hypothesis we spectroscopically characterized the binding parameters and reconciled discrepancies which were found in earlier studies [17]. Compared to previous studies the data enabled us to estimate an average between Trp residues and the bound PPIX using FRET. We carried out a more in-depth characterization of the binding using docking simulations. After the binding was characterized, we established whether the irradiation of the bound PPIX produced evidence of photochemical mechanisms that could cause modifications of the structure and function of the protein. Spectroscopically we validated that optical changes were indeed prompted by low irradiance laser irradiation of PPIX and manifested in photobleaching of the irradiated porphyrin ligand that correlated with changes in the emission and emission lifetime of the intrinsic tubulin fluorescence. These changes were also useful as an estimate of conformational modification of the protein through FRET. Finally we established that the photosensitization of PPIX led to changes in the formation of MT both in kinetics and morphology. Also the experimental results obtained by irradiating PPIX docked to tubulin were compared with those obtained with TSPP. Interesting differences emerge that i.) confirm that the effects are non-trivial and dependent on the

porphyrin ligand and ii.) provide a useful initial framework to develop a new methodology for more selective applications of porphyrins in biomedicine.

Results and Discussion

Interaction of PPIX and tubulin

PPIX Fluorescence—Although the absorption spectra are broad and poorly structured in the 350–450 nm region (Figure 1S, Supplemental Information) it is clear that addition of tubulin to aqueous PPIX solutions causes the appearance of a new band centered at ~ 400 nm which is consistent with the absorption of the porphyrin docked to the protein [17]. The broad shoulders to either side of the peak are consistent with the presence of aggregated forms of PPIX which do not interact with the protein [18]. In the same samples the fluorescence of PPIX displays a 12 nm red shift of the emission maximum accompanied by a large increase in the fluorescence intensity (Figure 2S, Supplemental Information). These are spectroscopic features consistent with binding of PPIX to the protein [17, 19]. The changes in the emission intensity of PPIX can be used to generate the B-H plot using equation E1. The plot (Figure 1) is linear, indicating that a single molecule of PPIX binds to tubulin, and yields a binding constant $K_b = 2.4 (\pm 0.7) \times 10^6 \text{ M}^{-1}$ for the formation of the PPIX/tubulin complex. The value of K_b is in agreement with other porphyrin/protein association constants and the linearity of the plot also suggests that contaminants which may remain after the dialysis of tubulin do not substantially interfere with the binding of PPIX.

Tubulin Fluorescence Quenching by PPIX—It had been previously observed [13, 17] that addition of porphyrins to a tubulin aqueous solution decreases its intrinsic fluorescence and produces a blue-shift of its emission maximum from 330 to 326 nm due to quenching of its more exposed Trp residues [13, 17]. Unlike those previous studies, where protein fluorescence was collected with $\lambda_{ex} = 280 \text{ nm}$ (which prompts emission of both Trp and Tyr residues), in the current investigation experiments were conducted with $\lambda_{ex} = 294 \text{ nm}$ where the Trp residues are selectively excited.

The results are shown in Figure 2 and are consistent with the earlier findings. The blue-shift of the emission spectra (Figure 2) indicates that only a subset of Trp residues are quenched. More exposed residue(s), which typically have a longer emission wavelength [20], are easier to quench than the less accessible ones, that are typically characterized by emission at shorter wavelengths. To the best of our knowledge there has been no attempt to establish the contribution of the individual Trp residues of tubulin to its emission; thus, from the spectral data alone is not possible to suggest which Trp residues are quenched by PPIX. After correction for dilution and inner-filter effects one retrieves linear S-V plots (Figure 3).

As mentioned in the experimental section, linear S-V plots can result from either static or dynamic quenching and one has to resolve the ambiguity. Given the low (μM) concentration of the two components and the observed binding of the porphyrin to tubulin, it appears more likely that the quenching mechanism is static in nature. Nevertheless the static mechanism can be justified in more quantitative terms. Assuming a collisional quenching mechanism where $K_Q = k_q \tau_0$ [21], the substitution of τ_0 with the lifetime of free tubulin (Table 1), one estimates a value of $k_q \sim 3 \cdot 10^{13} \text{ M}^{-1} \text{ s}^{-1}$, which appears unrealistically high. Indeed, the

upper-limit k_q value is related to the diffusion coefficients D_Q and D_T of the quencher and the chromophore (tubulin in this case) through [21]

$$k_q = f_Q k_0 \quad (\text{R1})$$

where

$$k_0 = \gamma (D_T + D_Q) (R_T + R_Q) \quad (\text{R2})$$

In this last relation γ is a constant $\sim 8 \times 10^{21}$ [21], while R_T and R_Q are the radii of the protein and the quencher, respectively, given in meters. From the size of tubulin and PPIX ($R_T + R_Q \sim R_T \sim 1 \times 10^{-8}$ m [22]) and from the diffusion coefficients at room temperature for the two molecules ($D_T + D_Q \sim D_Q \sim 1 \times 10^{-9}$ m²s⁻¹ [23]), one estimates a value $k_q \sim 8 \times 10^5$ M⁻¹s⁻¹ which is eight orders of magnitude smaller than the one obtained from our experiments. The estimated value of k_q would actually be even smaller since the one calculated above considers a quenching efficiency, $f_Q = 1$. Therefore, collisional quenching between PPIX and tubulin can be confidently ruled out. Thus, assuming the quenching to be static, the fitting of the S-V plot with equation E2 yields $K_Q = K_b = 7.5 (\pm 0.2) \times 10^5$ M⁻¹ for PPIX and tubulin. This value is 3-fold smaller than the one retrieved using the B-H method (equation E1). The discrepancy can be explained by the overestimation of the monomeric PPIX concentration. As mentioned earlier, PPIX forms aggregates in aqueous solution [24] that do not bind to proteins [17, 25]. Therefore they may not contribute to static quenching. In addition, however, the docking simulations discussed below indicate that there may only be one binding location in each dimer which would translate into an overestimation of K_b by a factor of two in the B-H method. After the correction the value of K_b from the B-H plot would be $1.2 (\pm 0.5) \times 10^6$ M⁻¹ which is only a factor of 1.6 larger than the one obtained from S-V.

Fluorescence lifetime of tubulin—Along with the quenching of the fluorescence intensity, the addition of PPIX produced also a decrease in the fluorescence lifetime of tubulin. Each tubulin monomer contains 4 Trp residues [26] that are located at different sites within the protein tertiary conformation, hence the decay of tubulin intrinsic fluorescence is best fitted with three components (as expected from the literature [26]) using equation E4 (one would expect a much larger number of components but the instrumental limitation would render meaningless any curve fitting with more than three components). Addition of PPIX to the solution containing tubulin, caused the average fluorescence lifetime of the protein to decrease (Figure 4) by ~ 400 ps.

Closer inspection reveals that the lower average lifetime is due to the decrease in the characteristic decay time of all individual components of the fluorescence decay (Table 1). Interestingly, the sub-nanosecond component (which is near the limit of the time resolution

of the instrument) does not follow the contribution observed in other quenching measurements.

Quenching, even when static in nature, may often increase the relative contribution of the sub-nanosecond component (i.e., α_1 in Table 1) since samples containing proteins and aggregates produce a non-negligible Rayleigh scattering contribution even in the wavelength region of the protein emission. This contribution, because it is elastic, is recorded only in the early channels of the decay in the region overlapped with the profile of the excitation source [27]. Since PPIX quenches the emission of the Trp residues throughout the entire decay window, the combination of the two effects often causes a relative increase of scattering which in turn produces an apparent faster decay. This effect does not appear to occur in our experiments as the value of α_1 for the shortest-lived component remains virtually constant. Conversely, the intermediate component, τ_2 , decreases by 260 ps (~ 5 channels in our experimental settings), while the longer lived component, τ_3 , decreases by 400 ps (~ 8 channels in our experimental settings). As discussed above, the conditions of our experiments are not conducive to collisional quenching, thus, how can one reconcile the static quenching with the decrease in emission lifetime? One possibility is the presence of fluorescence resonance energy transfer (FRET) from Trp to PPIX [28]. The FRET efficiency is defined as [21]

$$E = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle} \quad (\text{R3})$$

where $\langle \tau_D \rangle$ is the average fluorescence lifetime of the FRET donor (the Trp residues of tubulin in this case) in the absence of the acceptor (i.e., PPIX), and $\langle \tau_{DA} \rangle$ is the average fluorescence lifetime of the FRET donor in the presence of PPIX. The equation above implies complete labeling, i.e., that each donor is interacting with one acceptor. However in cases of non-covalent binding it is likely that the labeling is incomplete and that there is a substantial amount of “unlabeled” proteins, i.e., a number of proteins without an acceptor bound to them. In such cases equation R3 is modified to include the fractional labeling

factor, $f_A = \frac{[acceptor]}{[donor]}$, to yield

$$E = \left(1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle} \right) \frac{1}{f_A} \quad (\text{R4})$$

in this case f_A can be calculated from the spectrum of Figure S1 which enables the estimate of the concentration of tubulin (from the OD at 280 nm) and of PPIX (from the OD at 405 nm). Using the values of molar extinction coefficients stated earlier one estimates $f_A \sim 0.12$ which in turn (equation R4) yield $E = 0.66$ when using the values in Table 1. Since the FRET efficiency depends on the distance between donor and acceptor, it is possible to estimate this distance using [21]

$$r=R_0\sqrt[6]{\frac{1-E}{E}} \quad (\text{R5})$$

The Forster's radius, R_0 , between Trp and monomeric PPIX was estimated to be $\sim 25 \text{ \AA}$ (Hu, et al. manuscript in print). In short, the estimate was carried out from the absorption spectrum of PPIX in DMSO. Why? We established previously [7, 17] that electronic absorption and emission spectra of PPIX bound to globular proteins tend to replicate the ones obtained when the protoporphyrin is dissolved in DMSO. Using the estimated $\epsilon_{405 \text{ nm}}^{\text{DMSO}} = 1.5 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [29] we proceeded to calculate R_0 . Taking this value, the average distance between the Trp residues and bound PPIX for the tubulin/PPIX complex is estimated at $\sim 17 \text{ \AA}$. This estimate assumes an additional average quantity represented by the geometric factor between the donor and acceptor transition dipole moments. Although approximate, the donor-acceptor distance that we retrieved can serve as a parameter of reference for docking simulations.

Molecular docking—The simulations yielded clusters of energetically favorable sites. The two most energetically favorable binding sites (-10.47 and -8.27 kcal/mol) were at sites that overlap with the reported binding sites for taxol and GTP, respectively. Binding at these sites however contrasts with the experimental evidence which shows that the rate of self-assembly in the presence of GTP or taxol (Figure S3) was not altered by the simultaneous presence of PPIX suggesting that the porphyrin either does not occupy these binding locations or that upon addition of taxol or GTP it is readily displaced by the two ligands. The different kinetics in Figure S3 reflect the different polymerization mechanism of GTP and Taxol. The latter is a strong stabilizer of MT and its effect is reflected on the lack of lag-time within the time of the experiment. Whether PPIX binds or not to the GTP or taxol binding sites it does not explain the quenching of the Trp fluorescence since both sites do not have any Trp residue in their proximity [30]. Therefore it is likely that PPIX binds tubulin at a different location. The next energetically favorable docking site of PPIX is on the β -monomer of the heterodimer (Figures 5).

This site is consistent with the experimental evidence as, i.) it does not overlap with the sites of MT-promoting ligands (e.g. GTP or taxol), ii.) it is $< 20 \text{ \AA}$ from Trp residues (Trp21, Trp103 and Trp407), and iii.) it is characterized by a low binding energy (-7.55 kcal/mol). The combination of experimental and computational results suggest that this site is a likely docking site for PPIX. In addition, this location is not distant from the one we recently established for a water soluble porphyrin [31] and does confirm that PPIX is unlikely to disrupt the formation of MTs.

Photomodification of tubulin

Irradiation at 405 nm of the complex formed by PPIX and tubulin produced effects in several spectroscopic characteristics of the protein. Since the protein does not absorb the laser radiation (405 nm), one can assume that the effects on the protein reported below are mediated by photophysical/photochemical mechanisms initiated by the porphyrin ligand.

Absorption Spectroscopy—Irradiation of the PPIX/tubulin complex produced minor changes in its UV-vis spectrum. One observes a < 10% bleaching of the initial absorption of PPIX accompanied by the appearance of a shoulder ~ 320 nm (Figure 6). Conversely, the photobleaching of PPIX did not produce large changes in the absorption of the protein (< 310 nm). Comparison with these studies reveals that the shoulder at 320 nm is typical of the irradiation of the PPIX complex [7, 32] but is still not clear whether it can be attributed to a photoproduct of PPIX or to a chemical modification of the protein [32a] and mass spectrometry investigations are ongoing to establish the origin of this feature.

The photobleaching of PPIX as a function of the fluence (J/cm^2) (Figure 6, inset) can be fitted using a single exponential decay out of the multi-component decay:

$$\frac{S(E)}{S(E=0)} = \sum_i A_i e^{-k_i E} \quad (\text{R6})$$

where $S(E)$ is the signal recorded at a given fluence E , i.e., absorbance, fluorescence intensity, etc. In this case $S(E)$ is the optical density of the porphyrins. Data analysis yields a

single component decay with $\frac{1}{k_1} = 4.6 \times 10^{-2} \text{ J}/\text{cm}^2 \left(k_1 \sim 22 \frac{\text{cm}^2}{\text{J}} \right)$.

Fluorescence of PPIX—More dramatic changes were observed in the fluorescence spectra. The emission intensity of the PPIX peak decreases to ~ 20% of the initial value while the integrated fluorescence from 590 to 750 nm decreases to < 40% of the initial value (Figure 7).

The relative decrease in PPIX fluorescence is much larger than its photobleaching (Figure 6). In addition, the irradiation of PPIX bound to tubulin yields a complex emission spectrum, with the apparent formation of photoproducts (peak ~ 667 nm) after a fluence of ~ 5 J/cm^2 (Figure 7). The band of the photoproduct decreases with further irradiation (Figure 7). These results are consistent with what had been previously observed for the irradiation of the PPIX/BLG complexes [32a]. The decrease of the fluorescence of PPIX does not follow the same rate as the bleaching of its ground state. The best fitting of the data in the inset of Figure 7 is retrieved using two exponentials (Equation R6) with

$\frac{1}{k_1} = 1.2 \times 10^{-2} \text{ J}/\text{cm}^2 \left(k_1 \sim 83 \frac{\text{cm}^2}{\text{J}} \right)$ and $\frac{1}{k_2} = 2.2 \times 10^{-1} \text{ J}/\text{cm}^2 \left(k_1 \sim 5 \frac{\text{cm}^2}{\text{J}} \right)$. The large discrepancy in the bleaching rates observed between absorption and fluorescence is attributed to two factors: the presence of PPIX aggregates and the formation of fluorescent photoproducts. The aggregated form of PPIX dominates the absorption spectrum (Figure 6 and Figure 1S, Supplementary Information), but does not contribute to the fluorescence. Since PPIX aggregates possess minimal or no photochemical activity [18] they would not photobleach. Thus, the overall photobleaching observed in Figure 6 is small because is only due to the fraction of PPIX that is docked to tubulin (estimated ~ 12% as discussed earlier). Conversely, PPIX aggregates do not contribute to the fluorescence which is instead produced exclusively by the small amount of docked, monomeric PPIX. Thus, emission yields the

actual bleaching produced in the tubulin/PPIX complex. The bleaching of Figure 7 suggests that there is a bimodal mechanism whereby an initial fast decrease is followed by a slower decay. We attribute the bimodal decay to the presence of the fluorescent photoproducts (peak ~ 667 nm) that would create a separate rate of formation/depletion.

Fluorescence of Tubulin—Although the absorption spectrum of tubulin remained unaffected by irradiation (Figure 6), its fluorescence showed a substantial decrease (Figure 8) with increasing irradiation of PPIX. At the end of the irradiation procedure, the emission intensity, due to the Trp contribution, decreased to < 60% of the initial value, whereas the irradiation of tubulin without the porphyrin does not prompt any change in intensity.

The decrease is not accompanied by other spectral changes. Since the laser radiation at 405 nm is exclusively absorbed by PPIX, the decrease of tubulin fluorescence is prompted by the photophysical/photochemical mechanisms produced by the porphyrin ligand. Fitting of the data in the inset of Figure 8 with Equation R6 yields again a bimodal decay with

$\frac{1}{k_1} = 7.5 \times 10^{-2} \text{ J/cm}^2 \left(k_1 \sim 13 \frac{\text{cm}^2}{\text{J}} \right)$ and $\frac{1}{k_2} = 9.2 \times 10^{-1} \text{ J/cm}^2 \left(k_1 \sim 1 \frac{\text{cm}^2}{\text{J}} \right)$. The comparison with the rates discussed previously for PPIX shows that the decrease in tubulin fluorescence is slower than that of the porphyrin. This can be explained, at least in part, by the fact that not all tubulin molecules form a complex with PPIX (we showed earlier that only 12% of all tubulin molecules will be occupied) and the fluorescence contribution of free tubulin is not affected by irradiation causing an apparent slower rate of overall fluorescence decrease.

Tubulin Fluorescence Decay—In order to investigate the effect of PPIX-irradiation on the protein we investigated the fluorescence decay of the tubulin as a function of laser irradiation. Changes in the emission lifetime of Trp residues can be correlated with modifications of their immediate environment [20]. The experiments show that the quenching of the emission of tubulin is accompanied by a decrease of ~ 0.3 ns in its average fluorescence lifetime (Figure 9), similar to that observed in other porphyrin/tubulin complexes [13]. Closer inspection shows that the major effect of the irradiation appears to be on the duration of the longer-lived component (Table 2).

The contribution of the individual components (α_i) to the decay do not change substantially in the irradiated samples. The fact that the sub-nanosecond component is not affected (either in τ or α) is strong evidence that the overall decrease in lifetime is not the result of the relative increase of the scattering component (as discussed before for the quenching data) but is an actual effect on the decay of the Trp chromophores.

The lifetime of the intermediate component (τ_2) shortens by ~ 100 ps but only after the largest irradiation dose. Conversely, the long-lived contribution to the decay shortens by > 700 ps. As mentioned above the assignment of individual lifetimes to the four Trp residues of each tubulin monomer has not been resolved, thus we cannot attribute the changes unequivocally. Nevertheless, given the location of the binding pocket suggested by the docking simulation and its proximity to the Trp residues (Figure 5), the data could be consistent with changes that involve a rearrangement of the binding site which shortens the

average distance between PPIX and Trp. In fact one can use FRET analysis to estimate the changes. Since there is no spectroscopic evidence that PPIX detaches from tubulin upon irradiation, one can assume that equation R4 still applies. If one factors the value of $\langle \tau_{DA} \rangle$ for the irradiated sample (Table 2) and the photobleaching of PPIX (which would decrease the apparent value of R_0 to 22.5) a new distance $r = 12.6 \text{ \AA}$ is obtained from the Trp-PPIX pair. This change (from the estimated $r = 17 \text{ \AA}$ obtained for the non-irradiated sample) cannot be taken as an absolute value since we cannot rule out, for instance, a change in the value of κ^2 due to a change of the orientation of the porphyrin but also the fact that the presence of the aggregated PPIX prevents more quantitative estimates. Additional experiments will be needed in this regard, however, the data provided validate the probable occurrence of a conformational change in tubulin.

All the spectroscopic data so far are consistent with PPIX-mediated photosensitized modifications of tubulin. The lack of effects in the absorption spectrum of the protein indicates that photosensitization of PPIX does not induce chemical changes in the aromatic residues that would deplete native ground states. Therefore the changes observed in the emission of the protein are due to changes in the environment of one or more Trp residues as shown by the decrease in their emission intensity and of their lifetime (Figure 9). These changes could be created by chemical modifications of other residues (ongoing mass spectrometry investigation aims at establishing this aspect) or prompted by conformational modification of the protein.

Tubulin polymerization—To probe whether the irradiation of PPIX locally affects the structure of the protein, an investigation was undertaken to determine whether the conformational modifications were capable of altering the functionality of tubulin, such as its ability to self-aggregate into MT-like structures *in vitro* [12]. We studied how the irradiation of the PPIX/tubulin complex changes the kinetic of MT formation *in vitro* using the turbidity assay [12] followed by the characterization of the products at the end of the assay using AFM [13].

Solution turbidity—The increase in turbidity due to self-aggregation of tubulin was investigated in non-irradiated samples containing porphyrin/tubulin complexes as well as in samples irradiated with 86.4 J/cm^2 which (Figure 10) is sufficient to produce substantial changes in the fluorescence, thus, in the conformation of tubulin. Figure 10A shows that even at the low tubulin concentration ($\sim 10 \text{ }\mu\text{M}$) used in our experiments, tubulin self-assembles into larger structures in the presence of Mg^{2+} and glycerol. Compared to higher concentrations of the protein, the typical sigmoidal shape of the kinetics of self-assembly [33] has longer lag-time consistent with the a longer time of nucleation due to the fewer tubulin molecules available. Fitting the turbidity data with the simple sigmoidal expression of equation E6 [34] yields a polymerization rate $k = 1.75 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$ for tubulin alone. The presence of PPIX decreases slightly the polymerization rate ($k = 1.03 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$) while irradiation of the PPIX/tubulin complex produces further decrease in the polymerization rate ($k = 0.92 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$) (Figure 10B).

We had previously demonstrated that the irradiation of TSPP bound to tubulin is able to prevent the formation of MT while the porphyrin ligand has little effects on the formation of

MT in the non-irradiated complex [13]. The changes induced by irradiation of PPIX do not appear to affect the formation of MT *in vitro* to the same extent as TSPP [13].

AFM of the microtubules—In analogy with the TSPP/tubulin study, we surveyed our samples with AFM in order to determine the morphology of the self-assembled structures. As expected, in the presence of GTP, tubulin dimers self-assemble into MT (Figure 11A). The tubulin/PPIX complex is also still able to form MT of similar diameter, length and surface density (Figure 11B). Irradiation of the PPIX/tubulin complex, however, has a significant effect on the ability of tubulin to form MT. Figures 11C and 11D show that, after irradiation, assembled structures can still be detected, and although they have diameters similar to the ones before irradiation (~ 20 nm), their surface density and length appear to be greatly reduced. At the same time the topographic images show a larger amount of smaller particles that are fairly regular in structure, larger in size than the tubulin dimers but not organized into any regular self-assembly. This is in contrast with the data obtained from the TSPP/tubulin complex whereby the irradiation of the complex appeared to completely inhibit the formation of MT [13].

The combination of turbidity and AFM data show that, while binding of PPIX does not affect the formation of MT, irradiation of the complex in the region of the Soret band of the porphyrin does prompt changes in the way tubulin self-assembles. The effects are however different from those observed for the irradiation of TSPP. While irradiation of TSPP completely stops any organized self-assembly [13], equivalent irradiation of PPIX appears to reduce the amount of MT but does not stop their formation. As mentioned earlier one explanation may be provided by the small amount of non-aggregated PPIX that actually binds tubulin and therefore the small amount of tubulin/PPIX complexes that are actually affected by the irradiation. If this was true, however, one would expect MT of length similar to the ones seen before irradiation but a sparser distribution caused by the ones that do not participate to self-assembly due to structural damage produced by the irradiation itself. Instead what one observes are shorter MT which appear to indicate a less trivial effect, related to structural changes of tubulin. We are currently investigating whether these changes are related to underlying events such as protein fragmentation induced by the photochemical events started by the irradiation of the bound ligand.

Singlet Oxygen Production—In order to obtain a more complete mechanistic scenario of the laser-induced effects on tubulin we attempted to investigate the photosensitization of $^1\text{O}_2$. The reactivity of $^1\text{O}_2$ can potentially modify certain amino acid residues (e.g., Tyr, Cys, His, etc.) but also modify the amide of the peptide bond, as well as potentially promoting cross-linking within the protein. The sample emission was recorded at several wavelengths ($\lambda = 1110, 1220, 1275$ and 1325 nm) to spectrally separate the $^1\text{O}_2$ emission from other contributions. The fitting of the recorded data using equation E7 yields three components: $\tau_1 \sim 0.8 \mu\text{s}$; $\tau_2 \sim 6 \mu\text{s}$ and $\tau_3 \sim 18 \mu\text{s}$. The time-resolved spectra (Figure 5S) show a maximum at $\lambda = 1275$ nm for the τ_3 component, which confirms the production of $^1\text{O}_2$. However, the signal is too small to be quantified, and only qualitative data can be extracted. The small amplitude of the singlet oxygen signal could be due to the presence of PPIX aggregates in solution or to a much lower production of $^1\text{O}_2$ due to the presence of

competing processes. The data, therefore, is inconclusive and as much as it suggest that $^1\text{O}_2$ could be the agent that drives the conformational changes in tubulin it does not enable us to completely rule out the presence of other events such as photoinduced electron transfer. Spectroscopically changes in the absorption of the aromatic amino acid region did not occur (Figure 1) which leads us to believe that these residues are not affected, non-optically active residues however could still be affected. Studies of the porphyrin-mediated, photosensitized chemical changes in tubulin and other proteins are currently ongoing and they will hopefully shed additional light on the mechanisms that lead to the structural changes of tubulin.

Conclusions

The study has demonstrated that a clinically-approved photosensitizer (PPIX) can bind an important protein target (tubulin) and photosensitize conformational changes in the protein that inhibit its ability to form full-length MT. We reconciled previous inconsistencies in the calculation of the binding parameters and established a binding constant in the $0.75\text{--}1.2 \times 10^6 \text{ M}^{-1}$. The combination of the experimental data (absorption, fluorescence, FRET) and computational simulations yielded a likely binding site of PPIX located in the β -monomer, $< 20 \text{ \AA}$ away from three of the four Trp residue in the monomer and positioned so that it does not interfere with the formation of MT *in vitro*. We have also demonstrated that PPIX undergoes photosensitization events, as observed from its photobleaching, even at the relatively low irradiance employed in our experiments. The photosensitization of PPIX appears to affect the formation of MT in a way that decreases the amount and length of their formation. We were unable to link these effects to specific conformational changes in the protein, however the fluorescence and emission lifetime of the Trp residues, as well as changes in FRET, suggest that the effects on MT may be prompted by conformational changes of the protein. The generation of $^1\text{O}_2$ appears to be involved in the mechanisms, however, chemical changes were not characterized and the role of PPIX radicals generated by electron transfer cannot be entirely ruled out. Although PPIX is hypothesized to target other proteins in cells (e.g., p53^[35]), demonstrating that protein target can be modified through photosensitized mechanisms may open the possibility of modified application of porphyrin photosensitizers in biomedical applications.

Experimental Section

Chemicals—PPIX (P8293), MgCl_2 (63063) and Guanosine 5'-triphosphate (G8877) were purchased from Sigma-Aldrich (St. Louis, MO), and used as received. TSPP tetrasodium salt dodecahydrate (T40699) was purchased from Frontier Scientific (Logan, UT) while porcine tubulin (T240) was obtained from Cytoskeleton, Inc. (Denver, CO). Dimethylsulfoxide (DMSO) for the preparation of the initial PPIX stock solution, was purchased from Sigma-Aldrich (D8418, St Louis, MO) and used as received.

The concentration of PPIX in aqueous solution was quantified spectroscopically from an equally concentrated solution of the porphyrin in DMSO where one can use $\epsilon_{405} = 1.5 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [29] while the concentration of TSPP was determined using $\epsilon_{413} = 5.1 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [36]. Concentration of tubulin was also determined spectroscopically using $\epsilon_{280} = 1.15 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ($\lambda = 280 \text{ nm}$) [13].

Buffers—All samples were prepared in fresh 10 mM aqueous potassium phosphate monobasic (KH_2PO_4) and 0.1 M sodium fluoride (NaF) buffer which was adjusted to the desired pH by adding small aliquots of NaOH or phosphoric acid. Ionic strength and pH were adjusted to physiological conditions (0.16 M and 7.4 respectively). Ionic strength was adjusted by addition of ~ 150 mM of NaF and. Buffer batches were prepared using deionized water, which was flushed for at least 5 minutes in order to eliminate possible impurities. Buffers were stored at 6 °C and made fresh each week. The buffer used for the self-assembly of MT is modified and is described below in the experimental procedures for turbidity assay measurements.

Sample preparation—Since PPIX does not dissolve in aqueous solutions, an arbitrary amount of the solid porphyrin was first solubilized in a small volume of DMSO (stock solution) and left to equilibrate for at least 12 hours in the dark at room temperature. Under these conditions, stock solutions were stored for up to three weeks. Before each experiments, 100 μL of the PPIX stock solution were diluted into 2 mL of buffer and dialyzed overnight in order to eliminate traces of DMSO. The selective elimination of DMSO from the solution was tested spectroscopically by verifying the absence of DMSO absorption in the region < 300 nm and the simultaneous retention of PPIX absorption in the 350–450 nm region. After dialysis the stock was further diluted in buffer to the desired final concentration of PPIX, which varied for different experiments.

For binding experiments small aliquots (20–40 μL) of the aqueous stock solution were diluted in buffer to yield a final concentration of PPIX of $6.0 \pm 0.3 \mu\text{M}$. Subsequently, 2 ml of this solution were placed in a 1-cm pathlength quartz cuvette for spectroscopic measurements. At this concentration, the optical density remains < 0.15 at 405 nm. A stock solution of tubulin was prepared fresh for each experiment by dissolving 1 mg of solid protein directly into 1 mL of buffer. This solution was dialyzed overnight in order to remove impurities (e.g., glycerol, small peptides, etc.) from the received lyophilized powder (see below). The addition of 740 μL of this stock solution to 2 mL of buffer yields an optical density (OD) < 0.13 at 295 nm where Trp residues can be specifically excited. This ensures the uniform excitation of the sample in solution.

Dialysis—Dialysis was performed using dry tubing (Pur-A-Lyzer™ Maxi Dialysis Kit, Sigma-Aldrich, Co., St. Louis, MO) with two regenerated, EDTA-treated cellulose membranes on the sides. In order to permeabilize the membranes prior to dialysis, the tubing was filled with DI, placed in a floating rack and incubated for 30 minutes in a stirred beaker containing 1 L of fresh aqueous buffer solution.

Dialysis of PPIX aqueous stock solutions containing DMSO were carried out in 1 ml dialysis tubing against a 1L of DI water using 1 kDa cutoff membranes. The same procedure was used for tubulin using a 6 kDa cutoff membrane. Samples were then loaded into the emptied tubes, placed in the floating rack and dialyzed overnight. The exchange buffer was not replaced during dialysis.

Instrumentation and Settings

UV-Vis Spectroscopy—Absorption spectra were recorded with a dual-beam spectrophotometer (Evolution 300 Thermo Fisher Scientific, Waltham, MA) using a 2 nm bandpass and 240 nm/min acquisition speed. Appropriate reference and baseline were used for each scan. Spectra of the porphyrins were collected between 350 and 550 nm in order to detect the region of the Soret band [36–37] while spectra of the aromatic amino acid residues of the proteins were collected between 250 and 350 nm. Spectra in these regions were recorded using 1 cm-pathlength ES quartz cuvettes (NSG Precision Cells, Farmingdale, NY).

Emission Spectroscopy—Steady-state fluorescence spectra were recorded in 1 cm path length, four-clear-sided ES quartz cells (NSG Precision Cells, Farmingdale, NY) using a double-monochromator AMINCO Bowman Series 2 (AB2) Luminescence Spectrometer (Thermo Fisher Scientific, Waltham, MA) with 4 nm bandpass in excitation and emission, acquisition speed of 2 nm/s and voltage at the PMT in a range between 600 and 800 V where the linearity of the response is ensured. All emission spectra were corrected for the instrumental response (using a file provided by the manufacturer) and corrected for inner filter effects as described later. The steady-state fluorescence data were repeated in three separate, identical experiments.

Fluorescence Decay—Fluorescence lifetime was recorded using a time-correlated single-photon-counting (TCSPC) instrument (Fluorocube, Horiba Scientific, Edison, NJ). The details of the method can be found elsewhere [21, 38] and are only summarized in the following: tubulin fluorescence decay was recorded upon excitation with a pulsed LED at 294 nm (NanoLED-295, 1.2 ns pulsewidth, Horiba Scientific), operated at a repetition rate of 1.0 MHz. The instrument response function (prompt) was recorded after each decay curve using a suspension of glycogen (1 mg/mL) in deionized water. All signals were recorded by accumulating 10^4 peak counts at a counting rate below the suggested maximum (2×10^4 cts/s) corresponding to 2% of the repetition rate of the source. The decays shown in Figure 4 and 9 are plots of the logarithm of the number single photons counted as a function of the time delay from the trigger of the pulse of the Nano-LED source.

Turbidity Assay—Turbidity measurements were recorded using the Evolution 300 spectrophotometer fitted with a Peltier thermostatted single cell holder (Thermo Fisher Scientific, Waltham, MA) set at 37°C. At this temperature the self-assembly of MT *in vitro* is promoted [9b, 39]. The absorbance was recorded at 340 nm, where both the protein and the porphyrins have negligible absorption. The signal was recorded every 10 seconds with an integration time of 1 second, for a total acquisition time of 41 minutes.

Laser Irradiation—Irradiation experiments were carried out using a 135 mW, 405 nm, cw solid-state laser (Power Technology Inc., Little Rock, AK). The laser output was expanded and collimated after attenuation with neutral density filters so that the incident power at the sample was kept ~ 18 mW/cm² in all experiments. The beam had a cross section > 1 cm as to fill the front face of the cuvette. Irradiations were carried out in the fluence range 0 – 119 J/cm².

Singlet oxygen ($^1\text{O}_2$) detection—Time-resolved near infrared emission studies were carried out using a customized Fluotime 200 system (PicoQuant, Berlin, Germany) for the direct detection of $^1\text{O}_2$ luminescence. A nanosecond pulsed Nd:YAG laser (FTSS355-Q, Crystal Laser, Berlin, Germany) at 532 nm (10 mW, 1 $\mu\text{J}/\text{pulse}$) was used to excite the sample at a repetition rate of 10 kHz. A 1604 nm rugate notch filter (Edmund Optics, York, U.K.) was placed at the exit port of the laser to remove any residual component of its fundamental emission in the near-IR region. The luminescence exiting from the side of the sample was filtered by long-pass filters (Edmund Optics, York, U.K.). The sample emission was recorded at several wavelengths ($\lambda = 1110, 1220, 1275$ and 1325 nm) and under air, argon and oxygen atmospheres. Spectra were corrected for the filter transmission by dividing the recorded values by a factor of 0.47, 0.66, 1.0 and 0.31 for the 1110, 1220, 1275 and 1325 nm filters, respectively.

Experimental Procedures and Data Analysis

Porphyrin Fluorescence Experiments—Experiments were carried out by adding increasing aliquots (0 – 740 μL) of the 1 mg/mL buffer solution of tubulin to the ~ 3 μM solution of PPIX obtained from the dialyzed aqueous stock solution described above. The accuracy of the recorded data was estimated from at least three separate experiments. Absorption and fluorescence spectroscopy were recorded after each addition of the protein. Fluorescence spectra of PPIX were recorded with $\lambda_{\text{ex}} = 405$ nm (the expected maximum of PPIX absorption when bound to tubulin [19]) and emission in the 580 – 750 nm range. Emission spectra were recorded after each addition of the protein but were not corrected for the instrumental response since the correction provided by the equipment manufacturer has its limit around 600 nm. Fluorescence intensity was calculated as the integral of the spectrum in the recorded range.

Binding constant calculations—The binding constant K_b was estimated using a method adapted from Benesi-Hildebrand (B-H) [40] according to the equation

$$\frac{1}{\Delta F} = \frac{1}{K_b F^* [P]} + \frac{1}{F^*} \quad (\text{E1})$$

where ΔF is the difference in a spectroscopic parameter (in our case the peak fluorescence intensity of PPIX) in the absence or in the presence of a given concentration of tubulin, $[P]$, and F^* is the extrapolated value of F for the pure ligand-host complex. The method can be applied when the initial concentration of the ligand is much larger than the concentration of the protein (as is the case of our experiments) and when the spectroscopic parameter (F) is unambiguously different for the bound vs free ligand (in this case the fluorescence of bound PPIX shifts to longer wavelengths and increases several folds [17]). A linearity of the B-H plots reflects the formation of a 1:1 complex between the protein and the ligand [40a].

Tubulin Quenching Experiments—Quenching experiments were carried out by adding increasing aliquots (0 – 740 μL) of a dialyzed PPIX stock solution to a 5.1 ± 0.1 μM solution of dialyzed tubulin. Stock solutions of dialyzed tubulin and dialyzed PPIX were prepared

fresh for each experiment. Absorption and fluorescence spectroscopy were recorded after each addition of PPIX. The fluorescence of tubulin was recorded with $\lambda_{ex} = 294$ nm, which selectively excites Trp residues, and is consistent with the fluorescence lifetime data (see above). The emission was recorded between 302 and 450 nm. Fluorescence intensity was calculated as the area under the spectrum in the recorded range.

Quenching constant calculation—The quenching constant K_Q between the protein and the dye can be estimated from the Stern-Volmer (S-V) equation [21]:

$$\frac{F_0}{F} = 1 + K_Q [Q] \quad (E2)$$

where, F_0 and F , respectively, are the fluorescence intensities in the absence and presence of a concentration $[Q]$ of quencher. The S-V theory [21] predicts that linearity of the plot occurs with either a purely collisional or with a purely static quenching mechanism, making it difficult to distinguish between them. The physical interpretation of the two mechanisms is however different. In collisional quenching $K_Q = k_q \tau_0$, where k_q represents the bimolecular quenching rate constant [21] and τ_0 is the fluorescence lifetime of the fluorophore when $[Q] = 0$. In static quenching, instead, K_Q represents the binding constant between the host and the ligand [21]. Sometimes deviations from linearity occur (for instance when dynamic and static mechanisms are operating simultaneously) but may also be the results of optical effects such as inner-filter and dilution [21]. These undesired effects can be mitigated by correcting the raw data as follows [41]:

$$F = 10^{\left(\frac{A_{ex} + A_{em}}{2}\right)} F_{raw} \quad (E3)$$

In equation E3 F is calculated from the raw fluorescence intensity by correcting for A_{ex} and A_{em} .

Fluorescence Lifetime—Fluorescence decay of tubulin was recorded at its emission maximum (332 ± 8 nm) on the same samples used for the quenching experiments. Because acquisition time becomes significantly longer as quenching and dilution increases, fluorescence decay experiments were carried out only on selected samples: i.) before the addition of PPIX, ii.) after the addition of 270 μ L of PPIX and iii.) after the last addition of PPIX (740 μ L PPIX added).

Analysis of Fluorescence Decays—Time-resolved fluorescence was analyzed assuming a multi-exponential decay [21]:

$$I(t) = \sum_i \alpha_i e^{-\frac{t}{\tau_i}} \quad (E4)$$

where α_i is the amplitude of the i -th component and τ_i is its time constant. Data were fitted with deconvolution software DAS6.2 (IBH, Glasgow, U.K.). The quality of the fit was determined by the value of the reduced χ^2 , the visual inspection of the residuals and the Durbin-Watson statistical parameter for their correlation [38a]. From equation E4, the average fluorescence lifetime was calculated as

$$\langle\tau\rangle=\sum_i\alpha_i\tau_i \quad (\text{E5})$$

All spectroscopic and fluorescence lifetime experiments were repeated in triplicates.

Irradiation—The porphyrin/tubulin complex was prepared under different conditions depending on the probing experiments. Samples for the study of the effects of porphyrin irradiation on the protein structure were prepared with two different porphyrins, PPIX and the cationic TSPP using an ~ 1:1 porphyrin:tubulin ratio, and studied using fluorescence decay under the following conditions: i.) the concentration of tubulin was ~ 6 μM , which ensured formation of MT *in vitro* [12], and ii.) the absorbance of the complex at the irradiation wavelength (405 nm) was < 0.3 in order to ensure uniform energy deposition of the laser light during irradiation. The dialyzed protein solution was freshly prepared before each experiment. For experiments with PPIX, after dialysis, 200 μL of the porphyrin stock solution were diluted in 1.3 mL of buffer and added to the cuvette containing the protein to yield final concentrations of $6.0 \pm 0.1 \mu\text{M}$ and $6.0 \pm 0.2 \mu\text{M}$ for tubulin and PPIX respectively. The same final ratio was obtained for the tubulin/TSPP sample without the dialysis of the porphyrin which is water soluble.

Samples were irradiated in increasing time intervals corresponding to a fluence range of 0 – 119 J/cm^2 . The samples prepared absorbed between 0.1 and 0.15 at the laser wavelength, corresponding to a 21–29% intensity actually absorbed by the sample. Thus the maximum fluence absorbed by the sample ranges between 25 and 35 J/cm^2 . The irradiance used (~ 20 mW/cm^2) is almost an order of magnitude less than what is typically employed in photodynamic therapy and it was set, specifically, to probe whether lower irradiance could be adopted to cause biological damage, in this case to a biomolecule such as a protein. In addition, at such low irradiance, thermal effects can be neglected [13, 42]. Absorption and fluorescence spectra were recorded after each irradiation whereas the kinetic of self-assembly (i.e., turbidity assay, see below) was recorded at the end of the total irradiation period. Identical control samples were kept in the dark for the same length of time as the irradiated samples.

Turbidity experiments—The optimal experimental conditions for self-assembly of tubulin *in vitro* require the presence of Mg^{2+} , GTP and glycerol, as well as a temperature $T \approx 37^\circ\text{C}$ [12]. Thus, for each turbidity experiments the following protocol was used for the solution containing tubulin, tubulin/porphyrin complexes or irradiated complexes. Samples were placed in a 1 cm-pathlength micro plastic cell (BrandTech Scientific Inc., Essex, CT). The micro-cuvette allowed us to maintain the same pathlength as the other spectroscopic

measurements while using a smaller total volume (0.7 ml). Following a 10 minutes equilibration, 100 μ L of glycerol, 100 μ L of the $MgCl_2$ solution and 100 μ L of the GTP solution were added. After an additional 30 s equilibration the turbidity experiments were carried out as described earlier.

Analysis of the Turbidity Data—The quantitative analysis of the increase in scattering due to the formation of MT *in vitro* has been the subject of detailed investigations [12, 43]. However, the detailed models for the formation of MT *in vitro* cannot be used in our case because they require the variation of tubulin over a large range of concentrations whereas in our experiments the concentration is fixed because of the irradiation conditions. Therefore, we adopted a simpler approach that, although failing to provide all the details of the polymerization, is still able to yield the rate of assembly of the MT *in vitro*. At relatively low tubulin concentration the formation of MT follows a sigmoidal kinetic with a longer lag-time than at higher concentration of the protein [12, 43]. This trend has been observed before and can be characterized by the simple logarithmic relationship [34]

$$\ln(OD_{\infty} - OD(t)) = -k_{app}t \quad (E6)$$

where OD_{∞} is the optical density of the solution ($\lambda = 340 \text{ nm}$) at the end of the polymerization process, $OD(t)$ is the optical density of the solution (at the same wavelength) at time t and k_{app} is the apparent assembly rate constant of MT.

Singlet Oxygen Experiments—Samples for singlet oxygen experiments were prepared as described for the irradiation experiments in 2 ml volumes. Reference samples of buffer, TSPP, as well as control samples of PPIX and tubulin were prepared as follows: i.) the TSPP reference sample was prepared by first dissolving an arbitrary amount of solid TSPP in 2 mL of fresh aqueous phosphate buffer and its absorbance at 532 nm, where the irradiation is carried out, was adjusted to match the absorbance of the tubulin/PPIX sample; ii.) the buffer reference solution consisted of 2 mL of fresh aqueous phosphate buffer; iii.) the PPIX control sample was prepared by adding PPIX from a stock solution to 1.5 mL of freshly prepared aqueous phosphate buffer and its absorption at 532 nm was adjusted to match the absorbance of the sample containing the porphyrin/tubulin complex; iv.) the tubulin control sample was prepared by dissolving 1 mg of dialyzed tubulin in 2 mL buffer. Additional control samples were generated purging with argon or oxygen.

Analysis of Singlet Oxygen Experiments—Near-infrared luminescence at 1275 nm in biological environments often provides evidence for the formation of 1O_2 . Formation and decay of singlet oxygen is a bi-exponential process in which two species are involved: the triplet state of the photosensitizer and O_2 . Consequently, from the detected signal at 1275 nm, $S(t)$, it is possible to isolate the singlet oxygen lifetime by fitting the following equation:

$$S_{\Delta}(t)=S_{\Delta}(0)\frac{\tau_{\Delta}}{\tau_{\Delta}-\tau_T}\left(e^{-\frac{t}{\tau_{\Delta}}}-e^{-\frac{t}{\tau_T}}\right) \quad (\text{E7})$$

where $S(0)$ is a quantity proportional to the quantum yield of $^1\text{O}_2$ production, while τ and τ_T are the lifetimes of $^1\text{O}_2$ and of the triplet state of the photosensitizer, respectively.

Molecular Docking Simulation—The structure of tubulin α,β -dimer was downloaded from the Protein Data Bank (file PDB 1TUB). Kollman charges were added to the protein using AutoDock Tools 1.5.6 (The Scripps Research Institute, La Jolla, CA) [44]. The PPIX molecule was built using ChemBioDraw Ultra 12.0 (Cambridge Soft, PerkinElmer Group, Waltham, MA). Molecular simulations of the docking of PPIX to tubulin were carried out using AutoDockTools 1.5.6. Gasteiger partial charges were assigned to the porphyrin, and all non-polar hydrogen atoms were merged (i.e., deleted with their charges added to their closest bonded non-hydrogen atom) in AutoDock Tools. All rotatable bonds of the ligand were allowed to rotate during docking while the structure of tubulin remained rigid. Grid maps were generated with AutoGrid which recalculates atomic affinity, electrostatic, and desolvation potentials for each atom type in the ligand molecule being docked. The energy of interaction of each ligand atom with the protein is assigned to a point in a grid box which was typically $126 \times 126 \times 126 \text{ \AA}$. This box size is too small to contain the tubulin dimer. Therefore, different simulations were carried out by placing the largest possible box at three separate locations in order to probe the entire protein. The locations were chosen with sufficient overlap ($\sim 1/2$ box) to ensure that all regions of the protein were properly explored. This approach was practically achieved by placing the center of the grid box alternatively i.) on the β -monomer, ii.) at the center of the intra-dimeric interface, and iii.) on the α -monomer.

For each box location 10 independent docking runs were carried out by placing PPIX at random initial coordinates within the box (see below), and exploring interactions and conformations until the best set of binding configurations was found. Results from each grid box were re-clustered, yielding a histogram, containing the frequencies of conformations with similar binding energies. Re-clustering was carried out at $\text{RMSD} = 2.0$, meaning that the variation of the binding energy among the configurations is less than 2.0. Resulting complexes were analyzed separately and were graphically represented using the software PyMOL™ Molecular Graphics System, Version 1.5.0.4 (Schrödinger, LLC).

In the refined docking simulations the grid points were separated by $0.186 \text{ \AA}$. AutoGrid uses the distance-dependent dielectric of Mehler and Solmajer [45]. During the AutoDock calculation, the energetics of a particular ligand configuration is evaluated from the values of the grid using the Lamarckian genetic algorithm for minimization with default parameters that include random starting positions, orientations, and torsions that were generated before each run. The standard docking protocol for rigid and flexible ligand docking consisted of 10 independent runs per ligand, using a population of 150 randomly placed individuals, with 2.5×10^7 energy evaluations, a maximum number of 2.5×10^6 generations, a mutation rate of 0.02, a crossover rate of 0.80, and an elitism value of 1. The probability of performing a

local search on an individual in the population was 0.06, using a maximum of 300 iterations per local search. The possible binding configurations were analyzed by visual inspection and were compared to other known tubulin binding sites. [9a, 46] The selection of the binding configurations was carried out according to the following criteria: i.) the location of the bound porphyrin is in proximity of at least one Trp residues in agreement with the fluorescence quenching results observed in this and previous investigations [13, 17], ii.) the overlap with the sites of other known tubulin ligands (such as GTP and taxol) is minimal since these ligands do not appear to compete with the binding of PPIX (see below) and, iii.) among the sites that satisfy i.) and ii.) the docked configuration corresponds to the lowest binding energy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Benesi-Hildebrand plot of the fluorescence of PPIX upon addition of tubulin. The experiments were carried out with excitation at 405 nm and at temperature $T = 23\text{ }^{\circ}\text{C}$.

Figure 2. Emission spectra of tubulin in buffer solution (pH = 7.4) as a function of the concentration of PPIX (0 – 6 μM). The arrows indicate the decrease of the fluorescence intensity and the shift of the emission peak as a function of the increasing PPIX concentration.

Figure 3. Representative Stern-Volmer plot of the quenching of the fluorescence of tubulin upon addition of PPIX, corrected for dilution and inner filter effect. $\lambda_{ex} = 294 \text{ nm}$. The experiments were carried out at temperature $T = 23 \text{ }^\circ\text{C}$.

Figure 4.

Log-plot of the fluorescence decay of tubulin ($\lambda_{ex} = 294$ nm, $\lambda_{em} = 330 \pm 8$ nm) as a function of concentration of PPIX. Red = 0 M [PPIX]; Blue = 3.5 μ M [PPIX]; Pink = 6.5 μ M [PPIX]. The plot shows that the average lifetime of the emission of tubulin (due to the 4 Trp residues) decreases with increasing concentration of PPIX.

Figure 5.

A.) Binding site characterized by the lowest binding energy. Only β -monomer of tubulin is represented. The closest Trp residues (Trp21, Trp103 and Trp 407) are represented in blue. It can be noticed how in this docked configuration PPIX is within 20 Å of three Trp residues, thus within the estimated Förster's radius for FRET. B) Space fill representation of the binding site with PPIX in orange and the Trp residues in magenta.

Figure 6.

Representative absorption spectra of PPIX/tubulin complex upon irradiation. The numbers of the legend of the diagram indicate the total time, in minutes, that the sample has been exposed (0 to 110 min). (Inset) Relative decrease of the absorption of PPIX as a function of the total irradiation fluence at 405 nm. The arrows indicate the direction of the changes of the spectra as irradiation is increased from 0 to 119 J/cm².

Figure 7. Representative emission spectra of PPIX in the PPIX/tubulin complex upon irradiation. (inset) Relative decrease of the emission of PPIX as a function of the total irradiation fluence at 405 nm. The black arrow indicates the decrease in PPIX fluorescence for increasing laser irradiation (from 0 to 119 J/cm²); the crimson arrow indicates the emergence of a photoproduct at ~ 670 nm.

Figure 8.

Emission spectra of tubulin as a function of the total amount of irradiation received (0 to 119 J/cm²). The vertical arrow indicates the decrease of fluorescence intensity with increasing laser irradiation from 0 to 119 J/cm². (Inset) Relative decrease of the emission of Tubulin as a function of the total irradiation fluence at 405 nm.

Figure 9.

Lifetime decay of the fluorescence of tubulin upon excitation with the 294 nm pulsed source: (black) = decay of the non-irradiated complex, (red) = decay of the complex irradiated with 43.2 J/cm² at 405 nm, (blue) = decay of the complex irradiated with 119 J/cm² at 405 nm. The change in the average decay lifetime suggests changes in the conformation of the tubulin.

Figure 10.

A) Turbidity of tubulin (6 μ M) alone (black), tubulin (6 μ M) with added PPIX (6 μ M) (red) and after irradiation of the PPIX/tubulin complex (blue). B) Turbidity of tubulin (6 μ M) (black) with added TSPP (5 μ M) (red) and after irradiation of the TSPP/tubulin complex (blue). All experiments were carried out in the presence of 100 mM GTP, 5% gl

Figure 11.

Representative AFM images of tubulin and MT self-assembled in solution in assembly buffer at 37 °C. (A) MT formed after 60 minutes incubation by a 10 μM solution of tubulin. (B) MT formed after 60 minutes incubation of tubulin solution irradiated with 119 J/cm^2 . No difference is noticed in either the surface density or the length of the MT. (C) MT formed after 60 minutes incubation of tubulin/PPIX complexes. One notices that the surface density of the MT is smaller and their length shorter. (D) Image formed after 60 minutes incubation of tubulin/PPIX complexes that had been irradiated with 119 J/cm^2 .

Scheme 1.

Schematic representation of the hypothesis. 1. The porphyrin binds to the α - β tubulin dimer; 2. The porphyrin is irradiated with a laser at 405 nm; 3. Photosensitization promoted by the porphyrin alters the ability of tubulin to form microtubules in vitro.

Table 1

Decay parameters of tubulin fluorescence without and with an increasing PPIX concentration ($\lambda_{ex}=293$ nm). Concentration of PPIX in Samples 1, 2 and 3 are 0, 8.72 $\pm$ 0.2 and 19.2 $\pm$ 0.4 μ M, respectively.

[PPIX] μ M	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)
0	4.07 $\pm$ 0.4	0.05 $\pm$ 0.01	0.42 $\pm$ 0.10
(270 μ L PPIX)	3.84 $\pm$ 0.1	0.06 $\pm$ 0.01	0.32 $\pm$ 0.01
(740 μ L PPIX)	3.62 $\pm$ 0.1	0.07 $\pm$ 0.01	0.31 $\pm$ 0.14

Table 2
Decay parameters of tubulin fluorescence with increasing irradiation of the PPIX ligand at 405 nm.

Irradiation (J/cm ²)	$\langle \tau \rangle$ (ns)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)
0	2.22 ± 0.4	0.33 ± 0.03	0.45 ± 0.04	0.38 ± 0.01	1.54 ± 0.06	0.29 ± 0.01	5.12 ± 0.09
32	2.07 ± 0.3	0.31 ± 0.02	0.44 ± 0.01	0.41 ± 0.01	1.54 ± 0.03	0.28 ± 0.06	4.66 ± 0.05
64	2.00 ± 0.4	0.31 ± 0.01	0.42 ± 0.02	0.43 ± 0.01	1.55 ± 0.02	0.27 ± 0.05	4.45 ± 0.04
96	1.92 ± 0.4	0.28 ± 0.03	0.38 ± 0.02	0.45 ± 0.03	1.45 ± 0.02	0.27 ± 0.03	4.30 ± 0.04