Non-Covalent Interactions with Proteins Modify the Physicochemical Properties of a Molecular Switch

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Abstract: We report our findings that spiropyran – a widely investigated molecular photoswitch – can be stabilized in aqueous environments in the presence of a variety of proteins, including human serum albumin, insulin fibrils, lysozyme, and glucose oxidase. The optical properties of the complexed photoswitch are protein-dependent, with human serum albumin providing spiropyran with unique emission features previously observed for the photoswitch confined in media of high viscosity. Despite the binding to the protein molecules, spiropyran can undergo a ring-opening reaction upon exposure to UV light. This photoisomerization process can affect the properties of the proteins: here, we show that the electrical conduction via human serum albumin to which spiropyran is bound increases following the ring-opening reaction.

Bacteriorhodopsin has long impressed scientists with the elegance with which it employs the energy of sunlight to guide the motion of protons across cell membranes. This function is enabled by the photoswitchable retinal moiety strategically positioned within the protein structure; light-induced photoisomerization of retinal triggers a cascade of reactions, resulting in proton transfer against a pH gradient.[1, 2] Inspired by this and other examples, chemists have developed different strategies to covalently immobilize photoswitchable molecules within various biomacromolecules,[3-9] with the primary goal of controlling their structures and/or functions. Attractive and diverse applications have become possible, including photoswitchable enzymatic catalysis,[10, 11] and light-activated ion transport through a channel protein.[12]

Here, we considered a simpler approach, which takes advantage of non-covalent interactions[13, 14] between a molecular switch (in our case, spiropyran[15-19]) and the target proteins. We found that a variety of proteins, including human serum albumin (HSA), insulin, glucose oxidase, and lysozyme can provide spiropyran with good water solubility, indicating the formation of protein-spiropyran complexes. Within these complexes, spiropyran remained photosensitive; however, its optical properties depended on which protein "host" it was bound to. We also found that photosomerization of spiropyran bound to the serum albumin results in markedly increased electrical conduction through the protein.

We worked with the most widely investigated spiropyran – that is, 1,3-dihydro-1,3,3-trimethyl-6-nitrosopyrrole(2H-1-benzoazole-2,2'- (2H)-indole) (1 in Fig. 1a; Section S1 in the Supporting Information). This compound is practically insoluble in water, as evidenced by an experiment in which we injected 8.6 mL of a 3.5 mM solution of 1 in DMSO to 1 mL of a phosphate buffered saline (PBS) solution (5 mM phosphate buffer; 70 mM NaCl; pH = 8). The characteristic optical response of 1 disappeared within 4 hours, indicating quantitative precipitation (Fig. 1b). However, when the same amount of 1 was injected into the buffer solution containing HSA at 30 μM (an equimolar ratio of 1 to the protein), spiropyran remained soluble indefinitely (Fig. 1d; the orange trace shows the UV-Vis spectrum after 24 hours).

To further confirm the binding of 1 to HSA, we used fluorescence spectroscopy to follow the quenching of the fluorescence of HSA’s tryptophan (Trp) residue (λem = 350 nm) by the photoswitch. As shown in Fig. 1c, titrating a solution of HSA in the PBS buffer with 1 gradually quenches the intrinsic fluorescence of Trp, with ~77% quenching achieved upon the addition of one equivalent of spiropyran with respect to the protein. Quantitative analysis[20] of these results gave a Stern-Volmer quenching constant of 8·10^5 M^-1 (see Section S2 in the Supporting Information), which is similar to quenching constants of serum albumins with other small molecules.[21-24] Exposure of the resulting solution of 1-HSA to UV light (we used a 4-W hand-held 365 nm UV lamp with a light intensity of ~0.7 mW·cm^-2) turned it pink owing to an absorption band centered at ~539 nm, which can be attributed to the open-ring form of 1 (merocyanine, MC; 1oc) (Fig. 1d, purple trace) (notably, a weak band in this region can also be seen prior to UV irradiation, suggesting the presence of a small fraction of 1 in the open-ring form upon binding to HSA). To gain additional insight into the nature of binding of 1 to HSA, we took advantage of the solvatochromic properties of 1oc, whose absorption band in the visible region depends on the polarity of the medium, with low-polarity solvents promoting a bathochromic shift[17, 25-27] (e.g., λmax = 526 nm in methanol and ~606 nm in toluene; see Section S3 in the Supporting Information). The fact that the band is centered at ~539 nm indicates that 1 is bound in a relatively polar microenvironment. We also note that the intensity of this band relative to 1’s main absorption at ~360 nm is rather low as compared with free molecules isomerizing in solution (compare with Fig. S4 in the Supporting Information). Based on the relative intensities of the two bands, we estimate that the yield of the ring-opening reaction (1SP→1oc) is on the order of ~20%. This result suggests that binding within HSA stabilizes the closed-ring isomer of 1 (i.e., 1sp), in agreement with the fluorescent properties of the switch (vide infra).

Notably, the photoisomerization process was reversible: exposure to visible light (a fluorescent bulb with an intensity of ~1.0 mW·cm^-2) induced the UV-Vis spectrum to return to its original shape (Fig. 1d; dotted purple trace), and the process could be repeated.

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Next, we studied the effect of HSA binding on the emission properties of 1. A steady-state fluorescence spectrum of HSA-1 in the PBS buffer is shown as pink traces in Fig. 2. The pronounced band in the red/near infrared region is due to the S1 → S0 transition typical for merocyanines, and it is an indication that the switch is partially present in the MC form, which can be attributed to 1SP → 1MC isomerization inevitably induced by exciting the molecules with the fluorometer beam (λex = 390 nm). The most surprising feature of the emission spectrum is the presence of an intense band centered at ~450 nm, which is probably due to the emission of the T1 state of the closed-ring form of 1. This band indicates that 1SP triplet emission efficiently competes with the 1SP → 1MC ring-opening reaction (typically in the sub-ps time regime), and it suggests that binding within HSA provides a conformational stabilization to the 1SP isomer (unfortunately, attempts to grow single crystals of HSA from solutions containing 1 with the goal of conducting X-ray crystallography studies proved unsuccessful). Triplet emission (phosphorescence) of the closed-ring isomer was previously observed at low temperatures and in media of high viscosity, including glasses, ionic liquids, and electrospun fibers – however, the emergence of the ~450 nm band as a consequence of interactions with biomacromolecules is, to the best of our knowledge, unprecedented.

To determine whether HSA was unique in stabilizing the closed-ring form of 1, we also studied the emission properties of 1 interacting with insulin fibrils (see the Experimental Section). Similar to HSA-bound 1, emission spectra (red traces in Fig. 2) revealed an intense emission in the long-wavelength region, which can be attributed to the extended planar structure of 1MC binding to the hydrophobic domains of fibrillated insulin (analogously to the fibrils interacting with the fluorescent dye thioflavin T). This intense band at 620-660 nm was not accompanied by any appreciable emission in the higher-energy part of the spectrum, suggesting a lack of strong interactions between 1SP and the insulin fibrils. Control experiments with insulin monomers revealed that although they did provide 1 with good water solubility, only weak fluorescence in the red region was observed under the same conditions. We also tested two other common proteins – namely, lysozyme and glucose oxidase – as possible stabilizers of 1 in an aqueous environment. Similar to insulin monomers, these proteins facilitated the solubilization of 1. These results are presented in Section S4 in the Supporting Information.

To complement the steady-state emission measurements, we studied the time-resolved emission of 1 (Fig. 3) in the two emission regimes: at 650 nm (Fig. 3a) and 450 nm (Fig. 3b). It follows from the results in Fig. 3a that when bound to either protein, 1MC at 650 nm displays fluorescence decay with two lifetime components. By using exponential fitting, we estimate that
the lifetimes for the short-lived components are \( \tau_{MC} = 76\pm6 \) ps and 
\( 72\pm4 \) ps, and for the long-lived component \( \tau_{MC} = 2.09\pm0.05 \) ns and 
\( 2.16\pm0.02 \) ns for HSA and insulin fibrils, respectively. The 
short-lived component – which is the predominant one – can be 
ascribed to the lifetime of the merocyanine \( S_1 \) state, in agreement 
with previous literature reports (e.g. Ref. 30, which found \( \tau_{MC} = 
67\pm5 \) ps, and Ref. 40, where \( \tau_{MC} = 57\pm6 \) ps), whereas the long-
lifetime one could be attributed to the MC \( T_1 \) state. The rapid 
(nanoseconds) decay in the 450 nm regime due to phosphorescence 
of 1 was reported before.\(^{40}\)

Finally, we considered the possibility that the photosomerization of 
1 affects the properties of the protein “host”. In this context, 
we studied the electronic conduction via HSA monolayers and, 
following up on the recently introduced\(^{41-43}\) concept of doping 
proteins to alter their conductivity properties, we measured how 
modifies the conduction via the monolayer. We found (Fig. 4) 
that the conduction via a monolayer of HSA-1\(^{SP}\) increased fivefold 
(at \( \pm1 \) V) as compared with native HSA. Notably, a similar increase 
in conduction was observed upon the binding of retinoic acid to 
HSA (1-2 molecules of retinoic acid per each HSA molecule).\(^{44}\)

Following UV irradiation (which was performed in solution during 
the deposition process; we found that exposing our devices to UV 
light had deleterious effects on protein monolayers), we observed 
a slight increase in the conduction value (6.5-fold increase at \( \pm1 \) V 
as compared with the currents across native HSA), which could 
be rationalized by a higher degree of electronic conjugation in the \( 1_{MC} \) 
isomer (as opposed a structural transition of the protein, as in the 
previously reported photoinduced increase in conduction via 
bacteriorhodopsin\(^{44}\)). Notably, taking into account that the 
photostability of UV light caused isomerization of only a fraction of 
the photoswitch molecules (see above) suggests that the intrinsic 
conductivity via HSA \( 1_{MC} \) could be significantly higher than through 
HSA \( 1_{SP} \).

In summary, we studied non-covalent interactions between 
the photoconvertible spiropyran 1 and HSA – hence, our report 
complements earlier studies, which focused on interfacing other 
photochromic compounds with serum albumins.\(^{45-47}\) We found 
that spiropyran can bind to HSA and a variety of other proteins, 
including insulin monomers and fibrils, lysozyme, as well as 
glucose oxidase. In all cases, binding stabilizes spiropyran in 
aqueous media, in which aggregation otherwise occurs. Binding 
to HSA and insulin fibrils resulted in a strong emission of 1 in the 
red region of the spectrum; in addition, HSA provided 1 with an 
additional intense emission peak in the blue region, which could 
be attributed to spiropyran phosphorescence. The unique emission 
pattern of 1 within HSA exemplifies how the binding of photochromic 
molecules can alter their photophysical properties, and it suggests 
that the protein binding sites may provide environments suitable 
for constraining synthetic molecules. Conversely, the binding of 
1 affected the properties of HSA: we showed that in the presence 
of 1, the electrical conduction via the protein increases by fivefold, 
and that an additional increase could be achieved by photosomerizing 
1. We foresee that our results pave the way towards developing 
new materials featuring an intimate coupling of biomacromolecules 
and molecular switches.

Figure 3. a) Time-resolved emission of 1 bound to HSA (green) and insulin 
fibrils (blue) at the detection wavelength of 650 nm (±2 nm). Time-resolved 
emission of 1 bound to HSA at the detection wavelength of 450 nm (±2 nm). In 
all cases, the excitation wavelength was 390 nm. Decay of the ~450 nm 
emission due to SP phosphorescence in the nanosecond regime was reported 
previously; see, e.g., Ref. 40. c) A series of time-resolved emission spectra of 
HSA 1 showing a relatively fast decay of the red emission as compared to the 
blue emission.

Figure 4. Electrical conductance through HSA, HSA 1, and HSA 1 exposed to 
UV light. The measurements were performed at room temperature.
Experimental Section

Preparation of HSA: HSA (Sigma-Aldrich, Fatty acid-free; Globulin free, ≥99%) was dissolved in PBS buffer (5 mM phosphate buffer, 70 mM NaCl, pH = 8) to a final concentration of 30 μM. A stock solution of 1 was prepared by dissolving 1 in DMSO (Sigma-Aldrich, ≥99%) to a final concentration of 3.5 mM. Aliquots from the stock solution of 1 were added to 1 mL of the HSA solution to afford ligand/protein molar ratios in the range 0.05-1, which corresponds to the addition of 0.4-8.6 μL from the 1 stock solution. Following the addition of 1 to HSA, the samples were incubated with mild shaking in the dark and at room temperature for at least 4 h and up to overnight. Controls of only HSA and only 1 in the same buffer (at a concentration of 30 μM) were also incubated with the other samples.

Preparation of insulin fibrils: Freeze-dried powder of recombinant human insulin (Sigma-Aldrich) was dissolved in a 100 mM aqueous solution of NaCl at pH = 1.6 to a final concentration of 2 mg/mL. The insulin solution was heated to 65 °C for 4-5 h in order to form the fibrous structures. Following the formation of the fibrils, the acidic buffer was replaced by spinning down the fibrils and replacing the supernatant with the same buffer, as were used for the HSA samples. For the preparation of insulin fibrils-1, an aliquot of 15 μL of the stock solution of 1 was added to 1 mL of insulin fibrils in the PBS buffer at pH = 8.

Photoisomerization of 1: Photoisomerization of protein-bound 1 to 1c was induced by placing the samples under a UV lamp (365 nm; UVP, LLC; model number UVGL-25) for ~1 min. We verified that longer irradiation times (up to 5 min) did not induce further changes in the absorbance spectra of the samples. The reverse reaction was induced by placing the samples under a strong fluorescent lamp for 4-5 min.

UV-Vis absorption measurements were performed on a Cary 5000 UV-Vis-NIR spectrophotometer. Pure PBS buffer was used as a blank.

Steady-state fluorescence measurements were performed on a FluoroLog-Modular Spectrofluorometer (Horiba Jobin Yvon).

Time-resolved fluorescence measurements were acquired with a cavity-dumped Ti:sapphire femtosecond laser system (Mira, Coherent), which provides short (120 fs) pulses of variable repetition rates. For the excitation of 1, the system was operated at its second-harmonic-generation (SHG) frequency (spectral range of 380-420 nm), with a repetition rate of 800 kHz. Time-resolved fluorescence of 1, HSA, and insulin fibrils-1 was obtained by the time-correlated single-photon-counting (TCSPC) technique, which is based on a Hamamatsu 3809J photomultiplier, which collects resolved fluorescence of 1, which is based on a Hamamatsu 3809U photomultiplier, which collects the samples. The reverse reaction was induced by placing the samples under a UV lamp with a capillary on top of the protein monolayer. The current-voltage dependence (at room temperature) was acquired by a Keithley source-meter SMU system (model 6430).

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