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A Unique Photoreaction of Hypericinate Bound to Human Serum Albumin, Lipids, or Vesicles

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Summary. Hypericinate bound to human serum albumin is effectively photo-bleached. This reaction occurs to a lesser extent for hypericinate incorporated into dipalmitoyl-phosphatidyl-cholin vesicles or lecithin. Surprisingly, the photo-bleaching was strongly retarded for the complex of hypericinate with bovine serum albumin. In homogeneous solutions in a variety of solvents, micellar systems, and in ethanolic solutions containing additives mimicking possibly active parts of the mentioned hosts, almost no photoreaction was discernible. No products besides hypericin could be recovered from a bleached system. It was concluded that the main photoreaction involves photo-reduction or photo-addition in which the product becomes specifically stabilized by the host, but reverts partly to hypericinate upon destruction of the complex.

Keywords. Hypericin; Photochemistry; Bleaching; Fluorescence; Absorption.

Introduction

Photochemical reactions of hypericin (**1**) and its derivatives have been described in a few instances only. Thus, 3-benzylhypericin in presence of proton sponge has been shown to undergo a photo-rearrangement leading to a condensed pleiadene chromophore [1] and 3,4-dibenzylhypericin under the same conditions yielded a stable radical ion pair [2]. So far, hypericin (**1**) itself has not been thought to undergo photochemical reactions besides the sensibilization of singlet oxygen and the formation of short-living activated species [3]. However, very recently it has been observed that **1** adsorbed to human serum albumin or certain whole cells was bleached upon excessive irradiation [4]. Bleaching of standard solutions of **1** by ambient light with a half life of about 380 days has also been recorded [5]. In addition, we observed that certain fossils containing the hypericin-related fringelites bleached over the years when exposed to daylight. This phenomenon is of

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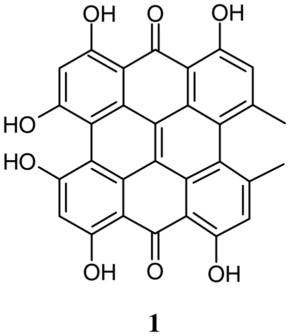
high interest with respect to the use of **1** as a phototherapeutic agent [3], but of course, also regarding the general photochemistry of the phenanthroperylene quinones [3]. Therefore, we investigated this phenomenon in more detail.



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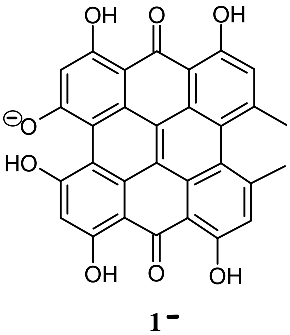

1$^-$

Results and Discussions

The first question we addressed was if the bleaching of **1** (or more precisely of **1**$^-$ [3]) adsorbed to human serum albumin is specific. Thus, homogeneous solutions of **1**$^-$ in several solvents (methanol, ethanol, pyridine, acetonitrile, acetone, *DMSO*, *DMF*) or an ethanolic solution of **1**$^-$ containing an at least tenfold molar excess of aminoethanol, mercaptoethanol, phenol, imidazol, cystein methyl ester, tyrosine, tryptophane, lysine, and in addition, micellar solutions of **1**$^-$ (Tween-60, Triton X-100, and *N,N*-didodecyl-*N,N*-dimethylammonium bromide) were irradiated ($\lambda > 580$ nm) under conditions and for times (about 6 hours), which led to a stationary photo-conversion (bleaching) of **1**$^-$ adsorbed to human serum albumin (Fig. 1). Only a very slight bleaching (<3%/h as compared to 40%/h for **1**$^-$) could be observed in all cases regardless if the reaction was carried out on degassed samples or under admittance of air. When **1**$^-$ was complexed to polyvinylpyrrolidone in absence or presence of mercaptoethanol or to polylysine, again

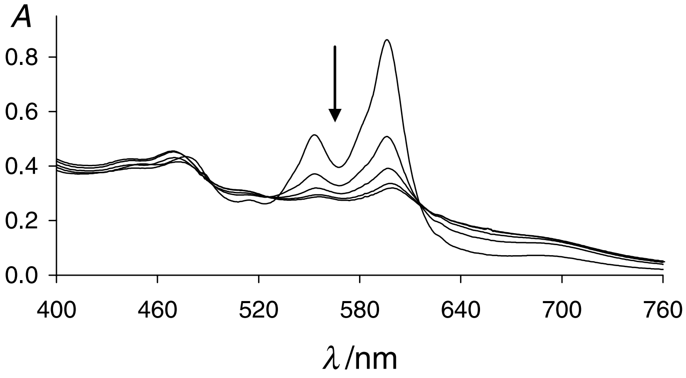

Fig. 1. UV-Vis spectra of **1**$^-$ adsorbed to human serum albumin dissolved in H_2O ($c = 5 \cdot 10^{-5}$ mol dm^{-3}) in the dark and after irradiation ($\lambda > 580$ nm) for 1.5, 3, 4.5, and 6 h (arrow); additional irradiation caused no further changes in absorption

no bleaching was observed upon irradiation. Very surprisingly in contrast to the human serum albumin case, the complex between bovine serum albumin and $\mathbf{1}^-$ proved to be also rather photo-stable (only about 15% bleaching) under these conditions. However, the complex of $\mathbf{1}^-$ and lecithine and $\mathbf{1}^-$ incorporated into vesicles of *D*,*L*-α-dipalmitoyl-phosphatidyl-cholin [6], which displayed an absorption spectrum similar to the albumin complex, showed significant bleaching (30%) under the irradiation conditions of Fig. 1. This result is in accord with the observation [4] that $\mathbf{1}^-$ is also bleached when adsorbed to certain whole cells.

Judged from these findings, $\mathbf{1}^-$ is specifically and efficiently bleached only when adsorbed to human serum albumin (Fig. 1), although besides that some bleaching was also observed with the vesicular and lipid systems. Interestingly enough, the intense fluorescence of $\mathbf{1}^-$ at 616/650 nm in the case of human serum albumin was replaced by a weak fluorescence at 603/649 nm, which has been attributed to the photoproduct [4]. However, the excitation spectrum of this photo-bleached material was found to be quite similar to the absorption spectrum of **1**, and the absorption correlated fluorescence intensity was similar to that of **1** thus making a change in dissociation of a presumptive residual hypericinate ($\mathbf{1}^-$) a more plausible interpretation.

Regarding the adsorption of $\mathbf{1}^-$ to human serum albumin it has been shown that a specific 1:1 complex ($K_b = 7.5 \cdot 10^4\,\mathrm{M}^{-1}$ [6]) is formed, which can be isolated [7]. From a study of the fluorescence polarization of this complex it has been inferred that $\mathbf{1}^-$ is bound very rigidly to its protein environment although only partial chiral discrimination of the two molecular helices is encountered [7]. Probing the association with competitors, like diazepam, warfarin, or bilirubin, a selective association of $\mathbf{1}^-$ to the IIIA domain has been deduced [7]. In addition, by means of surface enhanced resonance spectroscopy and resonance *Raman* experiments a contact of $\mathbf{1}^-$ with a tryptophane residue of the IIA domain of albumin has been inferred [8]. The latter result might not necessarily be interpreted by an association of $\mathbf{1}^-$ to the IIA domain [8], but to our opinion, the observed correlation might be derived from the close spacial vicinity [9] of this residue to $\mathbf{1}^-$ bound to domain IIIA.

From the results described above it became obvious that the functionalities provided by the adsorptive site of human serum albumin that may be needed in the course of the photochemical reaction or which stabilize the resulting photoproduct have to reside under more or less defined sterical requirements. Providing $\mathbf{1}^-$ with the functional groups of the active site of albumin in homogeneous solution did not result in photo-bleaching.

This led us to study the bleaching reaction of $\mathbf{1}^-$ in the human serum albumin adsorbed state in further detail. As a first step we investigated a possible recovery of $\mathbf{1}^-$ from its complex before and after irradiation. It turned out that the most effective way to recover $\mathbf{1}^-$ from the albumin complex with $\mathbf{1}^-$ was to precipitate the albumin complex by addition of ammonium sulfate and then to extract $\mathbf{1}^-$ with acetone. This operation led to a recovery of $\mathbf{1}^-$ of about 20% in the case of a non-irradiated sample and of about one tenth of this value for the bleached sample (lowest trace in Fig. 1). In both cases the $m/z = 504$ signal of **1** could be recorded from the ammonium sulfate precipitate by methane CI mass spectrometry. Unfortunately, neither human serum albumin nor its complex with $\mathbf{1}^-$ gave reliable mass spectra in ES mass experiments [10] prohibiting direct identification of the irradiation induced chemical modification of albumin or **1**. Experiments to detect or isolate any other product than $\mathbf{1}/\mathbf{1}^-$ from the bleached sample (extraction with organic solvents, extraction after precipitation, extraction after denaturation of the protein under alkaline or acid conditions) failed. With respect to the radical or neutral/charged nature an ESR spectrum of the bleached system was recorded. However, no signal pointing to a radical species comparable to the one found for the photoproduct of dibenzyl-hypericin [2] could be found. This means that there are no stabilized radical species involved in the photo-bleaching of $\mathbf{1}^-$ adsorbed to human serum albumin.

The following step aimed at the question if a specific complex between $\mathbf{1}^-$ and human serum albumin is the prerequisite of the bleaching reaction. Accordingly, solutions of albumin and $\mathbf{1}^-$ complexed in 5:1, 1:1, and 1:5 molar ratios (it should be noted that $\mathbf{1}^-$ is complexed to human serum albumin also in the case of a 5:1 molar ratio without any sign of homo-association [7] of $\mathbf{1}^-$, and thus there is also non-specific association of $\mathbf{1}^-$ to human serum albumin outside the IIIA domain) with the same absolute concentration of $\mathbf{1}^-$ in every case were irradiated. No difference in the bleaching kinetics and the final absorption spectra could be observed. This result could mean that either the adsorption has not to be specific for the reaction to occur, or that the bleached $\mathbf{1}^-$ at the specific site of domain IIIA is displaced by unreacted $\mathbf{1}^-$ residing at an unspecific site up to this moment.

To decide between these two possibilities we studied the time dependence of the specific complex formation between $\mathbf{1}^-$ and human serum albumin. As shown in Fig. 2 the time course of the complexation is rather slow ($\tau_{1/2} = 10$ min). However, this complexation reaction is fast compared to the irradiation times necessary under the given experimental conditions to bleach $\mathbf{1}^-$ (*i.e.* more than 1 h is needed to bleach half of the complex; *cf.* Fig. 1). Accordingly, we may conclude that it is the specific complexation site with its functional groups and the steric environment it provides, which might be necessary to lead to the observed photo-bleaching of $\mathbf{1}^-$.

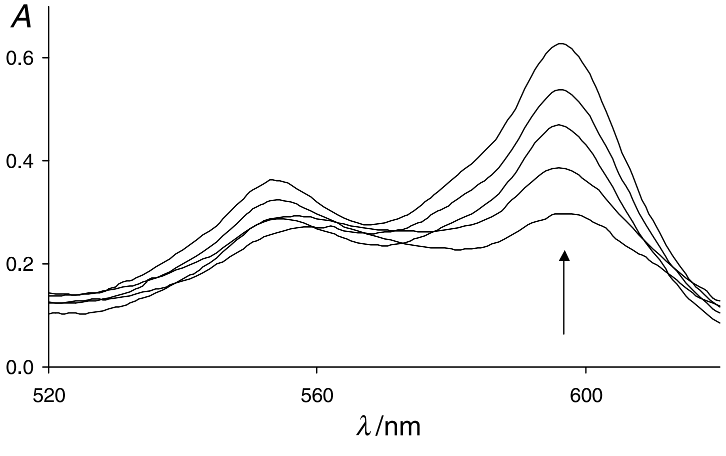

Fig. 2. Time dependence (UV-Vis spectra taken after mixing: 0, 0.5, 5, 10, and 60 min – following the direction of the arrow) of complexation of $\mathbf{1}^-$ with human serum albumin ($c = 1.5 \cdot 10^{-5}$ mol dm^{-3}); upon irradiation after 60 min no further absorption changes were observed

In conclusion we might speculate about which mechanistic paths in the photobleaching of $\mathbf{1}^-$ adsorbed to human serum albumin are involved. We tend to infer that in this process either photoreduction comparable to the one observed for anthraquinones [11] or photoaddition of a donor to the vinylogous oxo system of the hypericinate ion might occur. In homogeneous systems the product of this photoreaction immediately would revert back to hypericin, whereas in the adsorbed states with a suitable steric and functional environment these photoproduct(s) may become stabilized to a certain degree and revert back partly only upon destruction of the complex (*cf.* the small recovery of $\mathbf{1}^-$ from an irradiated sample described above). This process appears to be specific for human serum albumin and to a lesser extent for bovine serum albumin and for lipid or vesicular complexed $\mathbf{1}^-$. The photoreaction of $\mathbf{1}^-$ in a long term bleaching experiment [5] or when incorporated in vesicular or lipid systems could follow the same course as for human serum albumin. However, in these cases the stabilization of the photoproducts is lower and the reversion is partly prohibited by their secondary long time reactions.

Experimental

The UV-Vis and fluorescence spectra were recorded on the Hewlett Packard P 8453, Varian Cary 100Bio, and Hitachi F-4010 spectrometers using SiO_2 cells, $d = 1$ cm. The fluorescence excitation wavelength was set at the long wavelength absorption maximum and the monitor wavelength to record the excitation spectrum was held at the fluorescence maximum. Mass spectra were recorded by means of a Fisons MD 800 instrument using negative chemical ionization (methane) and a source temperature of 150°C. Otherwise a Mariner TOF/ES mass spectrometer (Perkin Elmer) in the positive and negative ion mode using a nano electrospray sample interface instrument was used for the ES experiments (*cf.* Ref. [10]).

Hypericin (**1**) was prepared and purified according to Ref. [12]. Lecithin (from soja; SERVA), *N,N*-didodecyl-*N,N*-dimethylammonium bromide (KODAK), Triton X-100 (Rohm & Haas), Tween 60 (Merck), and *D,L*-α-dipalmitoyl-phosphatidyl-cholin (SIGMA) were obtained from commercial sources. Solvents and other chemicals were of analytical or synthesis grade. Human serum albumin ($M = 66439$ [13]) and bovine serum albumin (M 66267 [13]) were obtained from Sigma. The complexes were prepared either by adding $\mathbf{1}^-K^+$ to the serum albumin dissolved in phosphate buffer $pH = 7.0$ according to Ref. [7], or by adding a *DMSO* solution of **1** to the albumin solution in *PBS* buffer $pH = 7.2$ according to Ref. [5]. Degassing of solutions was achieved by means of three freeze-pump-thaw cycles and gassing with Ar. All the differences in handling described above were without influence on the results. Irradiation of the water/ice cooled stirred samples contained in quartz cells in a simple merry go round apparatus was executed using an air blow cooled 700 W high-pressure mercury lamp with a cutoff filter ($\lambda < 580$ nm).

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