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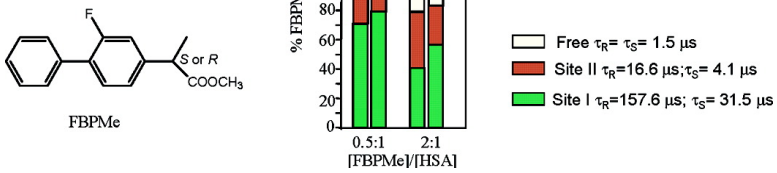

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Triplet Excited States as Chiral Reporters for the Binding of Drugs to Transport Proteins

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Human serum albumin (HSA) is the most important transport protein. One of its main functions is to carry different endogenous and exogenous agents (including drugs) around the blood stream to achieve their efficient and selective delivery to the specific targets.[1]

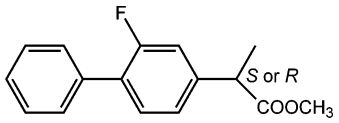
The pioneering work performed by Sudlow and co-workers three decades ago revealed that there are two major and structurally selective binding sites in HSA, namely, site I and site II.[2] Although more recent studies have found evidence supporting the existence of several subdomains, consensus still exists that there are basically two high affinity binding sites for small heterocyclic or aromatic compounds in this protein.[3]

As the knowledge of drug−HSA interactions is essential for understanding the biodistribution, metabolism, elimination, and pharmacological effect of drugs in the body, a number of techniques have been used to address this problem. They include equilibrium dialysis, ultrafiltration, ultracentrifugation, calorimetry, fluorescence quenching, circular dichroism, liquid chromatography, capillary electrophoresis, NMR diffusion measurements, etc.[4,5] Each method presents advantages and limitations; the latter are mainly related to sensitivity, interferences, diffusion problems, or lack of reproducibility arising from a complicated workup.

Arylpropionic acid derivatives are the main group of nonsteroidal anti-inflammatory drugs (NSAIDs). They are currently marketed as racemic mixtures (with few exceptions such as naproxen); however, their anti-inflammatory activity is mainly attributed to the *S*-isomer.[6] Those drugs bind preferentially (and often stereoselectively) to site II of HSA by means of hydrogen bonding and electrostatic interactions.[7] Upon methylation of the carboxy group, binding occurs preferentially to site I, where hydrophobic interactions predominate.[8]

The aim of the present work has been to explore the suitability of triplet excited states as chiral reporters for the important issue of drug binding to transport proteins. As the properties of these states are very sensitive to the experienced microenvironment,[9] they should allow one to study the drug distribution among the bulk solution and the different protein binding sites. More interestingly, triplet lifetime measurements would provide valuable information on the nature of drug−HSA complexes, such as strength of the interaction, conformational restrictions, protection from attack by oxygen or other reagents, stereochemical requirements, etc. Previously, laser flash photolysis (LFP) has been employed for the study of carprofen−HSA complexes.[10] However, in this case, fast reaction of the drug triplet state with the Trp unit of site I limits its possibilities to be used as a probe. To circumvent this problem, an "inert" triplet would be more adequate as reporter, while residing as observer in the HSA binding sites. Flurbiprofen (FBP) is an NSAID of the 2-arylpropionic acid class used for several medical purposes, such as for the treatment of osteoarthritis, rheumatoid arthritis, post-operative dental pain, and ocular disorders.[11] The lowest triplet excited state of FBP has recently been characterized,[12] and its lifetime has been found to be highly sensitive to the medium. In the present work, the triplet state of FBP methyl ester (FBPMe, Chart 1) has been used as probe for the binding sites of HSA.

Chart 1



The choice of FBPMe has been done for experimental convenience since its hydrophobic character should favor inclusion within HSA (preferentially in site I, as stated above). Both (*R*)- and (*S*)-FBPMe have been investigated in order to obtain valuable information about the possible stereodifferentiation in the interaction of drugs with proteins.

Aqueous solutions containing (*S*)-FBPMe and HSA (molar ratio from 10:1 to 0.5:1) were prepared in neutral buffer (0.01 M PBS) and submitted to laser flash photolysis. The transient absorption spectrum obtained after laser excitation (λ_{exc} = 266 nm) of an aerated 2.5×10^{-5} M solution of (*S*)-FBPMe displayed a maximum at 360 nm, assigned to the first triplet excited state.[12] Its decay (Figure 1) followed a first-order exponential law with a lifetime of 1.5 μs.

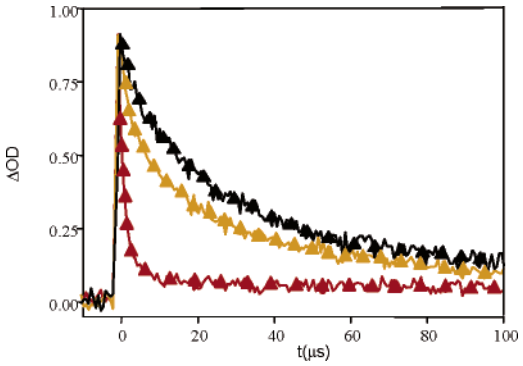


Figure 1. Laser flash photolysis (λ_{exc} = 266 nm) of (*S*)-FBPMe (burgundy triangles), (*S*)-FBPMe/HSA 2:1 (gold triangles), and (*S*)-FBPMe/HSA 0.5:1 (black triangles) in 0.01 M PBS. Normalized decays monitored at 360 nm. The concentration of (*S*)-FBPMe was 2.5×10^{-5} M in all cases.

Remarkably, in the presence of HSA, this species lived much longer (see Figure 1) and did not exhibit a first-order exponential decay. Thus, when the [(*S*)-FBPMe]/[HSA] ratio was between 1:1 and 0.5:1, the signal followed a second-order exponential decay with lifetimes of 4.1 and 31.5 μs. The negligible contribution of the 1.5 μs lifetime component indicated the absence of free (*S*)-FBPMe in solution under these conditions.

It is noteworthy that protein-bound (*S*)-FBPMe has dramatically longer lifetimes than the noncomplexed form (τ_T values up to 21-

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fold higher). This can be attributed to a slower deactivation via nonradiative processes, due to important restrictions in the degrees of freedom inside the HSA binding sites, where a microenvironment is provided that protects the triplet excited state from attack by oxygen or other reagents. In this context, the fact that two different τ_T values were measured in the presence of HSA can be correlated with the existence of two types of binding sites. At higher [(*S*)-FBPMe]/[HSA] ratios (from 1.25:1 to 10:1), three lifetime values (1.5, 4.1, and 31.5 μs) were necessary to obtain a good fitting of the decay signal (also shown in Figure 1). This can be clearly associated with the presence of free (*S*)-FBPMe in solution, in addition to the two HSA-bound species.

Parallel experiments were carried out for (*R*)-FBPMe. In PBS solution, the transient absorption spectra and the triplet lifetimes did not differ from those of the *S*-enantiomer. By contrast, when included within HSA, two components with τ_T values of 157.6 and 16.6 μs were found for (*R*)-FBPMe. These values are much higher than those obtained for (*S*)-FBPMe, indicating a remarkable stereodifferentiation between the two enantiomers in the protein binding process. A comparison between the decays monitored at 360 nm for (*S*)-FBPMe, (*S*)-FBPMe/HSA, and (*R*)-FBPMe/HSA is shown in Figure 2.

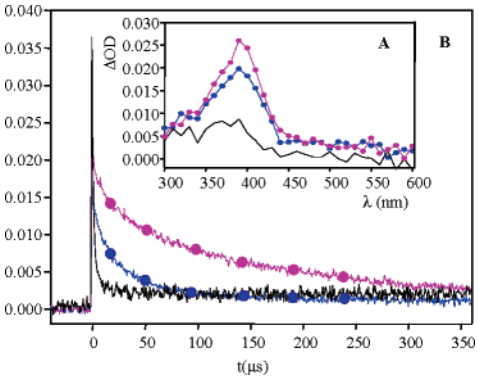

Figure 2. Laser flash photolysis (λ_{exc}= 266 nm) of 2.5 × 10^{-5} M (*S*)- or (*R*)-FBPMe (—), as well as 1:1 solutions of (*S*)-FBPMe/HSA (blue lines) and (*R*)-FBPMe/HSA (magenta lines) in 0.01 M PBS, under air. (A) Spectra obtained 3.7 μs after the laser pulse. (B) Decays monitored at 360 nm.

Regression analysis of the decay curves for several [FBPMe]/[HSA] ratios was performed to obtain the values of the A_1, A_2, and A_3 coefficients (components with different lifetimes). On the basis of the known fact that the high affinity site of 2-arylpropionic acid methyl esters is site I,[8] the major (longer-lived) component under nonsaturating conditions was assigned to FBPMe within this site, while the minor (shorter-lived) component was assigned to site II-bound FBPMe. To obtain independent evidence supporting this assignment, additional LFP experiments were performed in the presence of warfarin, a well-established probe for site I.[13] In agreement with our initial assumption, addition of this probe resulted in a reduced contribution of the longer lifetime components (attributed to site I). Thus, at 1:1.1 [FBPMe]/[HSA]/[warfarin] molar ratio this value dropped to ca. 50% (as compared with ca. 70% in the absence of warfarin).

The trends clearly show (Figure 3A) that, for the same [FBPMe]/[HSA] ratio, inclusion within HSA is slightly favored for the *S*-isomer. Besides, a significant stereodifferentiation is observed when comparing the occupation level of the two sites, with higher preference for site I in the case of (*S*)-FBPMe. In general, when the [FBPMe]/[HSA] ratio is low (far from saturation), all of the drug is protein-bound and the high affinity site I is selectively populated. Progressive increase of the [FBPMe]/[HSA] ratio results in saturation of site I and (subsequently) site II. This leads to a decrease of site selectivity (Figure 3B) and the appearance of increasing amounts of free FBPMe.

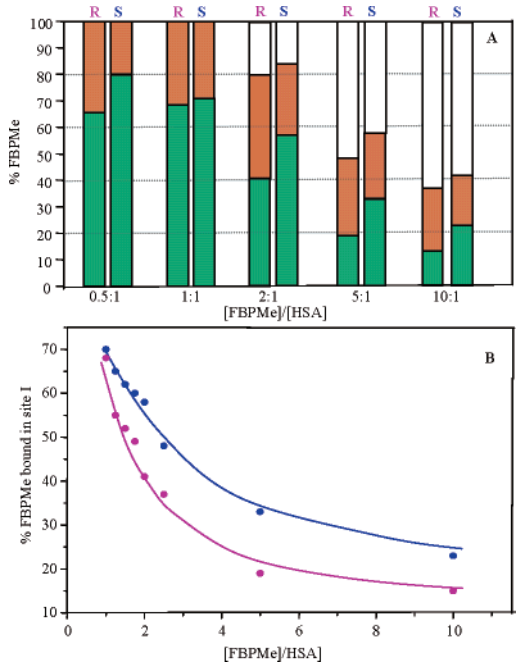

Figure 3. (A) Percent of (*S*)-FBPMe and (*R*)-FBPMe: free (white), in site I (green), and in site II (brown). (B) Percent of (*R*)-FBPMe (magenta circles) and (*S*)-FBPMe (blue circles) in site I of HSA at different [FBPMe]/[HSA] ratios.

In summary, the triplet excited state of FBPMe has been used as a chiral reporter for the two binding sites of HSA. The occupation level of the binding sites in HSA has been estimated from regression analysis of the triplet decays at several [FBPMe]/[HSA] ratios. A remarkable stereodifferentiation has been found in the triplet lifetimes within the protein microenvironment.

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