Journal of Fluorescence, Vol. 3, No. 3, 1993

Use of Nile Red as a Long-Wavelength Fluorophore in Dual-Probe Studies of Ligand–Protein Interactions

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Received October 18, 1993

The long-wavelength fluorescence probe, Nile Red, has polarity-dependent fluorescence intensity and wavelength properties that can be used to monitor the binding of drugs and other ligands to plasma proteins such as albumin and α-1 acid glycoprotein. This paper shows that it can be used in tandem with another fluorescence probe, 1-anilinonaphthalene-8-sulfonic acid, to study two or more types of ligand binding sites simultaneously. Some ligands displace one or the other probe from the protein/dual-probe complex, other ligands displace both probes. In each case the resulting decrease in fluorescence can be used to estimate the numbers of binding sites and their association constants.

KEY WORDS: Nile Red; 1-anilinonaphthalene-8-sulfonic acid; fluorescence probes; ligand–protein binding.

INTRODUCTION

Fluorescence techniques provide a simple and sensitive approach to the study of ligand binding to macromolecules. Probe molecules, whose fluorescence is normally much enhanced and blue-shifted in emission wavelength on binding to sites on the surfaces of proteins and other macromolecules, are displaced from such sites by competing ligands, the resulting decrease in fluorescence being used to measure the ligand binding characteristics. The best known such probe is 1-anilinonaphthalene-8-sulfonic acid (ANS) (1), which fluoresces strongly at about 480 nm when bound to nonpolar sites. Recent work has shown that the phenoxazone dye Nile Red shows analogous probe properties, but its emission wavelength is in the 620- to 660nm region (2). Here it is shown that the disparity in emission wavelength of the two probes allows their use simultaneously in studies of proteins such as albumin and α-1 acid glycoprotein, which have two or more classes of binding site. Drugs and other ligands competing for the binding sites occupied by Nile Red and/or ANS displace them to different degrees. Such *simultaneous* dual-probe studies have apparently not been reported previously.

EXPERIMENTAL

Human and bovine serum albumins (defatted), bovine α-1 acid glycoprotein, ANS, and all the drugs except sulfadiazine (Merck, Poole, Dorset, UK) were obtained from Sigma (Poole, Dorset, UK) and Nile Red (99.9% pure) was a product of Eastman Kodak. Protein–ligand interactions were studied in 0.1 *M* Mes buffer, pH 6.9. Fluorescence measurements were made using a Perkin–Elmer MPF44B or LS-50B spectrometer. All reagents were analytical grade and were dissolved in triply distilled deionized water.

RESULTS AND DISCUSSION

Nile Red showed fluorescence enhanced by about 10- and 15-fold, respectively, when bound to bovine and

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1053-0509/93/0900-0129$07.00/0

Table I. Displacement of ANS and Nile Red from Bovine Serum Albumin by Various Ligands (% fluorescence decrease)

Ligand	Nile Red	ANS
Flufenamic acid	50	92
Ethacrynic acid	1	64
Sulfadiazine	2	4
Digitoxin	1	16
Stearic acid	63	7
L-Tryptophan	3	4

human albumins, with emission wavelengths of 630 and 635 nm, respectively (λ_{ex} = 554 nm). Scatchard analysis showed the presence of two classes of binding site. When bound to the albumins, ANS showed intense fluorescence at 470 and 475 nm, respectively. The long-wavelength tails of these emission bands overlapped to some degree the excitation spectrum of Nile Red, but the use of submicromolar concentrations of each probe eliminated spurious inner filter effects, i.e., reabsorption by Nile Red groups of photons emitted by ANS ligands. Under these conditions no evidence was found for energy transfer between ANS and Nile Red ligands: The binding of each fluorophore to the albumin surfaces was effectively independent of the other fluorophore, indicating that they occupied separate binding sites.

The addition of an excess of competing ligands to 1:2:1 Nile Red:ANS:albumin mixtures resulted in the fluorescence decreases shown in Table I. Several ligands displaced both probe molecules, some displaced neither probe, several preferentially displaced ANS, and one—stearic acid—preferentially displaced Nile Red. These results confirmed that ANS and Nile Red occupy separate sites on the surface of the albumins and suggest that the Nile Red site is a relatively nonpolar one. Affinity constants were determined for several of these ligands by Scatchard analysis: such detailed studies (3) show differences between human and bovine albumins and can be interpreted in detail in the light of present knowledge on albumin ligand-binding sites (to be published).

Analogous results were obtained with bovine α-1 acid glycoprotein. In this case the enhancement of ANS fluorescence was not so marked, but again, the two dyes bound to the protein independently. In competitive binding studies, most ligands displaced both probes to some degree, tending to confirm the belief that this protein has a large binding area containing several subsites.

These results show that the increasing interest in long-wavelength and near-infrared fluorophores may have benefits over and above those of reduced background and scattered light interference and simpler instrumentation: The combination of such long-wavelength fluorophores with conventional uv–visible ones may provide unexpected applications. The widespread availability of very fast scanning spectrometers will allow studies of such dual fluorophore systems with no loss of convenience.

ACKNOWLEDGMENT

We thank the Science and Engineering Research Council for the award of a Research Studentship to M.B.B.

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