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An investigation of the use of Nile Red as a long-wavelength fluorescent probe for the study of α_1-acid glycoprotein–drug interactions

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Abstract

Spectrofluorimetry in the long-wavelength region of the electromagnetic spectrum (600–1000 nm) is a fairly recent development in photoluminescence spectroscopy, which has numerous advantages over measurements in the more conventional ultraviolet and visible spectral region. 9-Diethylamino-5*H*-benzophenoxazine-5-one (Nile Red) is an unchanged, hydrophobic molecule, and long-wavelength fluorescence of which is strongly influenced by the polarity of its environment. When Nile Red was added to solutions of α_1-acid glycoprotein (Orosomucoid. OMD), it showed an enhancement in fluorescence intensity and a shift to blue in emission wavelength, suggesting it was binding hydrophobically to a non-polar site on the protein. The association constant ($12\,261\,000 \pm 900\,000\ M^{-1}$) and number of binding sites (0.746 ± 0.044) were calculated for the probe. Upon addition of both acidic and basic drugs, the Nile Red fluorescence reverted to its unbound form, indicating that OMD probably has one high-affinity, wide and flexible binding area for such drugs. Possible enantiomeric selectivity was shown with ephedrine, and the association constant determined for a racemic mixture of propranolol was found to be comparable to other values obtained with alternative, more conventional techniques.

Keywords: α_1-Acid glycoprotein; Drug displacement; Fluorescence; Long-wavelength; Nile Red

1. Introduction

α_1-Acid glycoprotein or orosomucoid (OMD) was first isolated in 1950 by two separate research groups [1,2]. Since then, a significant amount of research has been carried out on the macromolecule and today it is one of the best characterised serum proteins. Much is known about the genetic regulation of the synthesis of OMD and its polymeric forms. Its primary structure has been identified and a lot of information about its secondary and tertiary structure has been obtained [3].

Although the exact physiological function of OMD is not completely understood, it has demonstrable activity in a number of important physiological systems and interacts with a variety of ligands. For example, OMD is known as an acute-phase protein; increased serum levels have been reported with cancer [4], arthritis [5] and following myocardial infarction [6] and surgery [7], while lower levels have been found during pregnancy [8] and in patients with liver cirrhosis [9] and thyroid disease [10]. In addition, OMD is known to both inhibit and promote platelet aggregation [11], and act as an immunosupressive [12]. Recently, however, a great deal of research has been

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carried out on the binding of drugs to serum OMD. Although albumin is the major binding protein for acidic drugs, increasing evidence suggests that OMD is probably the only high-affinity carrier for basic drugs in the serum [13]. It is believed that all drugs and ligands share one wide and flexible binding area that is perhaps located in a hydrophobic area of the protein [14].

Protein binding to a drug can affect the rates at which it is absorbed, distributed, metabolised and eliminated. This, in turn, may affect the rate and duration of action of the drug, and hence its therapeutic action. Consequently, a number of techniques have been developed to study drug–protein binding. These can be generally divided into two main types: classical and spectrophotometric. Classical methods such as equilibrium dialysis, ultrafiltration and gel separation involve the determination of the free or bound drug using a separation step. Spectroscopic techniques, including nuclear magnetic end electron spin resonance, optical rotatory dispersion, circular dichroism and fluorescence, are homogeneous in nature (i.e. no separation is needed), and entail the measurement of a change in the physical property of the drug and/or protein upon binding.

The use of fluorescence is becoming increasingly common in the study of drug–protein interactions [14–22] because of its many advantages. These include its high selectivity, the great variety of sample handling methods available and most importantly the exceptional limits of detection accessible under favourable circumstances. An important application is the use of fluroescent probes which non-covalently compete for the same protein binding site as the drug molecules, and therefore provide information on the environment, number and affinity of such sites.

This paper describes the use of the dye 9-diethylamino-5*H*-benzophenoxazine-5-one (Nile Red) in OMD binding studies. Nile red is an uncharged, heterocyclic, planar phenoxazine dye that is soluable in organic solvents and lipids, but relatively insoluble in water [23]. It is photochemically stable [24] and is known to be a good hydrophobic probe, because its fluorescence maxima vary depending on the relative hydrophobicity of its environment [25]. For example in cholesterol ester droplets or hydrocarbon solvents, Nile Red fluroesces gold/yellow, whereas in ethanol or phosphatidyl choline vesicles, the dye fluoresces red. However, in aqueous media its fluorescence is red shifted and strongly quenched, probably owing to aggregation of the dye in the polar environment [22]. This solvent dependency facilitates its use as a solvatochromic probe in the measurement of changes in solvent polarity [26], as a hydrophobic stain for intercellular lipids [25,27–29] and in the determination of lipid-related disease in the heart and liver [30]. More recently, Nile Red has been shown to bind to serum lipoproteins [25] and many other proteins, including tubulin [30], lysozyme, ovalbumin, lactoglobulin, bovine serum albumin and β-lactoglobulin [31,32]. Its fluorescence characteristics depend on the hydrophobic character of the drug-binding site.

The dye also has the advantage of high excitation and emission wavelengths, which mean it can be studied in the long-wavelength region (600–1000 nm) of the electromagnetic spectrum. Consequently, problems of absorption and fluorescence from other proteins and cofactors present in the biological matrix, which fluoresce in the 300–400 nm range, are removed. Its large Stokes shift and high wavelength also limit the problems of Raman and Rayleigh scattered light, because of the inverse fourth power relationship and the very long-wavelength water Raman shift. In addition, the significant changes in its fluorescence properties when bound allow the detection of small changes in protein structure and help in the determination of drug–protein interactions.

2. Experimental

2.1. Chemicals

Nile Red (99% pure) was obtained form Eastman Kodak (Rochester, NY, USA). OMD (99% purified from bovine serum) was obtained from Sigma (St. Louis, MO, USA), as were all the drugs utilised.

All buffer salts, i.e. 2-(*N*-morpholino)ethane sulphonic acid (MES), ethylene glycol-bis(b-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA) and magnesium chloride were also obtained from Sigma. De-ionised, triply distilled water from Liqui-Pure Modulab System (Bicester, Oxfordshire, UK) was used throughout. All solvents were of analytical reagent grade and obtained from Fisons scientific equipment (Loughborough, Leics, UK).

2.2. Instrumentation

Fluroescence spectra were obtained using a Perkin-Elmer LS-50 spectrofluorimeter (Beaconsfield, Bucks., UK) which was fitted with a R298 photomultiplier tube (Hamamatsu, Enfield, Middlesex, UK) to enable readings to be taken at high wavelength. The fluorimeter was interfaced to an Epson AX3 personal computer (Hemel Hempstead, Herts, UK).

2.3. Procedures

All measurements were made in 10 mm acrylic cells (Sarstedt, Leics, UK) in MES buffer (pH 6.9; MES (0.1 mol l^{-1})–$MgCl_2$ (1 mmol l^{-1})–EGTA (1 mmol l^{-1}). The Nile Red was stored in dimethyl sulphoxide (DMSO) at −20 °C and brought to room temperature prior to use. All protein and drug preparations were freshly mixed for each experiment. In all cases, excitation was at 550 nm and the excitation and emission slits were set at 5 nm.

A standard regimen for all binding and displacement titrations was performed in which was adapted from previous studies involving Nile Red [22,25,27–32]. The required concentrations of OMD, Nile Red and drug were aliquotted into 5 ml volumetric flasks and made up to the mark with buffer. Nile Red was added from the relevant stock solutions such that the additions were less than 0.5% of the total volume, to negate any possible effects of the organic solvent. Using this regimen, the fluorescence of Nile Red has been found to be linearly proportional to the dye concentration in aquous media up to 15 μM [25]. Therefore, the solubility of the dye was not a problem at the concentrations used.

For the binding and displacement calculations, measurements were always taken at a fixed wavelength, normally the emission maximum of the Nile Red–OMD complex. All experiments were repeated five times. All the spectra were corrected and obtained at room temperature.

2.4. Data analysis

Analysis of the binding data was achieved by using the Scatchard plot [33], which was adapted for fluorescence [34]:

$$\frac{1}{1-R} \times \frac{1}{K_a} = \frac{C_L^\circ}{R} - nC_P^\circ$$

where K_a is the association constant, n the number of binding sites per protein molecule, C_L° the total concentration of probe added and C_P° the total concentration of protein added. R is the fraction of the protein sites occupied. It can be obtained from fluorescence measurements and is equal to F/F°, where the unbound probe is assumed not to be fluorescent; F° is the fluorescence intensity due to fully bound protein–probe complex and F is the fluorescence intensity at any point in the titration. A plot of $1/1-R$ versus C_L°/R gives a straight line if K_a is constant. The intercept of the C_L°/R axis is equal to nC_P°. C_P° is known and therefore n can be evalutaed.

For displacement titrations, the data was anlaysed using a simple equation based on the mass action law [34]:

$$r/R = K\left(\frac{(C_D^\circ/C_B^\circ)-r}{(C_L^\circ/C_B^\circ - R}\right)$$

where C_D° is the total concentration of drug added and C_B° is the total concentration of binding sites ($C_B^\circ = nC_P^\circ$). R and r are the fractions of binding sites occupied by Nile Red and the drug respectively, i.e. $r = F^\circ - F/F$. A plot of the equation has a slope of K, which is the ratio of the association constant of the drug K_b to that of the probe K_a. K_a has been determined, and hence K_b can be calculated.

All the data was converted to binding and displacement plots using the spread-sheet software package Quatro Pro (Borland, Twyford, Berks., UK).

3. Results and discussion

3.1. Nile Red fluorescence

As previously reported [22,31], the fluorescence intensity of Nile Red decreases with time in aqueous media (Fig. 1). A possible explanation for this is that because of its poor solubility, Nile Red forms aggregates in water and consequently these polymers precipitate out. The phenomenon takes place over a period of time because initially, as the hydrophobic Nile Red is dissolved in DMSO, which acts as a solubuliser, aggregation will not be favoured in this less polar, more hydrophobic environment. However, as the water molecules begin to replace the DMSO shell around the Nile Red, the dye molecules begin to aggregate and precipitate. Therefore, in all experiments an incuba-

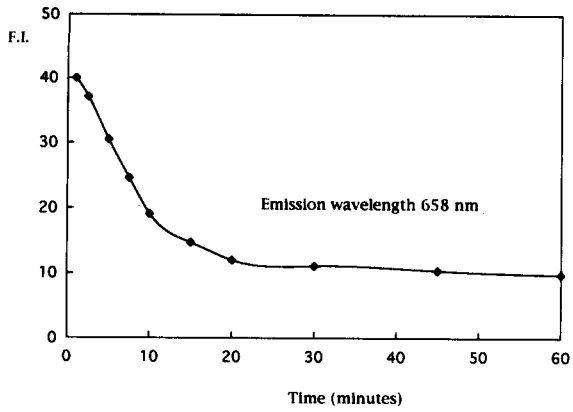

Fig. 1. Fluroescence decay of Nile Red (1 μM) in MES buffer (pH 6.9).

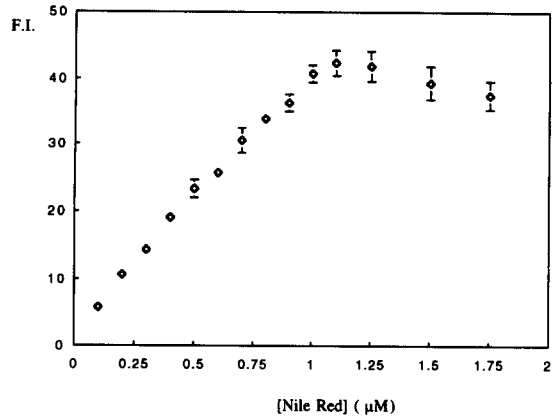

Fig. 3. Binding of Nile Red to OMD (1 μM) in MES buffer (pH 6.9).

tion period of 20 min was allowed to ensure that any background from the unbound dye was at a minimum [30,31].

3.2. Nile Red binding studies

Fig. 2 shows the effect of the addition of 1 mg ml^{-1} (25 μM) OMD to the fluorescence of 10 μM Nile Red. In the presence of OMD, Nile Red undergoes an approximate ten-fold enhancement in fluorescence intensity (F.I.) and approximate 25 nm hyposchromic or blue shift in emission wavelength, suggesting that the Nile Red is situated in a hydrophobic binding site on OMD.

The effect of DMSO on Nile Red binding was investigated, as it has been reported that several different organic modifiers such as methanol, ethanol, 1-propanol and acetonitrile, at concentrations between 1 and 15%, can influence the binding of several ligands to immobilised OMD [35]. It was found that the fluorescence intensity and λ_{em} of Nile Red when bound to OMD were unaffected when the DMSO concentration was changed from 0.05% (F.I. = 58.4 ± 4.32 at λ_{max}) to 0.75% (F.I. = 56.9 ± 3.14 at λ_{max}). Significant differences in fluorescence were only found at DMSO concentrations above 1% and indicate that DMSO has a negligible effect on the binding of Nile Red to OMD at the concentrations used.

The binding of increasing concentrations of Nile Red to OMD is shown in Fig. 3. The lower concentrations used were necessary to avoid any problems with the inner filter effect, which results in fluorescence quenching at concentrations which an absorbance greater than 0.05 abs. units. When maximum binding was reached ($F°$), the fluorescence began to fall away rather than reach a plateau. This is probably explained by the polymerisation of the excess, unbound Nile Red and possible excimer quenching. The adapted Scatchard plot is

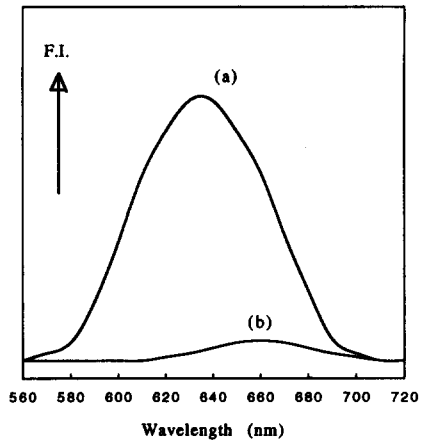

Fig. 2. Fluroescence spectra of Nile Red (10 μM) with (a) and without (b) OMD (1 mg ml^{-1}) in MES buffer (pH 6.9).

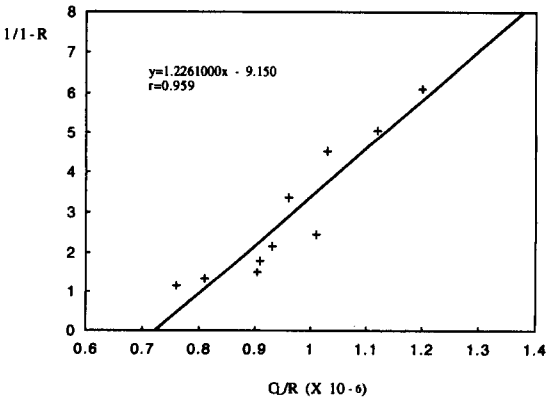

Fig. 4. Scatchard plot of Nile Red binding to OMD (1 μM).

Table 1
The association constant (K_a) and number of binding sites for Nile Red bound to OMD (1 μM) in MES buffer (pH 6.9)

$K_a M^{-1}$	n
12 261 000 ± 90 0000	0.746 ± 0.044

shown in Fig. 4, which the association constant (K_a) and number of binding sites (n) in Table 1.

The results indicate that Nile Red has one high-affinity binding site on OMD. The association constant is much higher than that found with other fluorescent probes, such as the positively charged Auramine O and acridine orange dodecyl bromide [14], which have been previously used for the fluorescence determination of human OMD in serum.

3.3. Drug displacement studies

The effects of both basic and acidic drugs on the fluorescence of Nile Red bound to OMD are shown in Table 2.

The results show that upon the addition of most of the drugs tested, there was a resultant decrease in F.I. and a bathochromic or red shift in λ_{em} of Nile Red back to that of its unbound form. This indicates that the drugs displaced Nile Red from its binding site. The fact that this competitive displacement was achieved with both basic and acidic drugs would support previous suggestions that OMD possesses one high-affinity, wide and flexible binding site that can bind both acidic and basic drugs [14,36,37]. The high concentrations of some of the drugs required to displace Nile Red support the previous findings of the strong hydrophobic binding of the dye to OMD.

Table 2
Effect of various drugs (250 μM) on the fluroescence of a Nile Red–OMD complex (2:4 μM) in MES buffer (pH 6.9)

Drug	% decrease in F.I.	Shift to red in λ_{em}
Basic		
Amitriptyline	85.1	9
Ephedrine	62.6	7
Propranolol	86.8	11
Acidic		
Ethacrynic acid	64.5	6
Flufenamic acid	84.6	10
Phenylbutazone	70.2	7
Salicylic acid	10.6	0
Sulphadiazine	19.9	2
Sulphamethoxazole	23.2	2
Warfarin	55.3	6

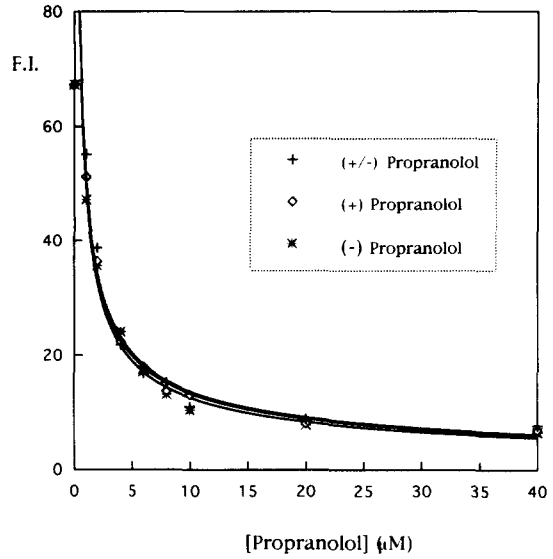

Fig. 5. Displacement of Nile Red (2 μM) from OMD (4 μM) by various chiral forms of propranolol in MES buffer (pH 6.9).

3.4. Stereoselective drug displacement of Nile Red

Although originally it was believed that the high-affinity binding site of OMD did not show stereoselectivity, it has recently suggested that stereoselective binding to OMD occurs for a number of drugs including isoporterenol, verapamil, tilidine and warfarin [37]. In fact, OMD has been frequently used as an immobilised protein for the resolution of enantiomers of racemic drug mixtures from many different classes of compounds [35].

This was investigated by studying the effects of the (+) and (−) chiral forms of both propranolol and ephedrine on Nile Red binding. Figs. 5 and 6 show the results obtained for the propranolol and ephedrine displacement of Nile Red from OMD respectively, while Table 3 shows the calculated association constants for the different chiral forms of the drugs.

It would appear that (+)-ephedrine has a much smaller affinity than (+/−) and (−)-ephedrine for the Nile Red binding site on OMD, indicating the possibility that OMD shows enantiomeric selectivity for ephedrine. However, no such evidence was found for the binding of propranolol to OMD.

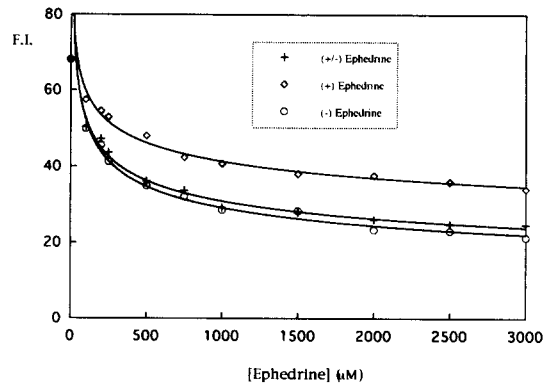

Fig. 6. Displacement of Nile Red (2 μM) from OMD (4 μM) by various chiral forms of ephedrine in MES buffer (pH 6.9).

The stereospecific binding of propranolol and its racemers is probably one of the most studied attachments to OMD owing to the fact that the (−) form is approximately 100 times more therapeutically active as a β-blocker than the (+) racemer. It has been reported that both human [38–40] and rat [41–43] OMD show a stronger binding of the (+) form than the (−) form, which may explain the difference in efficacy. However, although this technique may not be sensitive to determine such a difference, the results in Table 3 are comparable with the values obtained using other techniques. Association constant values for racemic mixtures of propranolol binding to human and rat OMD using equilibrium dialysis and chiral column separation [43–47] range between 1×10^6 and $3.5 \times 10^6\ M^{-1}$, although values above and below this range for different racemic forms are also quoted [38,39,41]. Any slight difference between these literature values and those obtained by the fluorescent probe displacement technique could be explained by the difference in the sources of OMD or by the recent findings that the determination of binding constants is dependent on receptor concentration [48]. For example, with this fluorescence technique it is necessary to use much lower concentrations of OMD (4 μM) than with some of the classical techniques (10–20 μM) to avoid problems with the inner filter effect, and this may help to explain any discrepencies in the K_a values obtained.

Table 3
Association constants determined for the different enantiomeric forms of propranolol and ephedrine bound to OMD (4 μM) in MES buffer (pH 6.9)

	$K_a(M^{-1})$
Propranolol	
(+/−)	355 6000 ± 440 000
(+)	386 2000 ± 390 000
(−)	399 6000 ± 510 000
Ephedrine	
(+/−)	78 850 ± 3600
(+)	22 680 ± 1500
(−)	82 510 ± 4200

4. Conclusions

In conclusion, the results presented in this paper show that Nile Red is an extremely useful hydrophobic, long-wavelength probe for the study of drug interactions with OMD. It is shown that Nile Red binds to one high-affinity binding site on OMD. Displacement studies involving acidic and basic drugs showed that both types share this binding site. The results indicate the presence of a wide and flexible hydrophobic binding area on the protein. Although evidence was found for the stereoselective binding of ephedrine at this site, the technique was not sensitive enought to obtain any evidence for the racemic forms of propranolol. However, the value obtained for the association constant of the racemic mixture of propranolol was comparable to those found in previous studies. Obviously, this technique warrants further investigation, as the application a long-wavelength fluorescent dye such as Nile Red could be an extremely useful alternative to the more established techniques for the determination of OMD and other proteins, and their binding to drugs in the body.

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