Fluorescence Studies of Binding of Dansylglycine and Dicoumarol to Bovine Serum Albumin

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Abstract

Dansylglycine was used as a fluorescent probe to study the binding of dicoumarol to bovine serum albumin (BSA) at pH 7.4. The fluorescence of dansylglycine was greatly enhanced on binding to BSA with a blue shift in wave- length of the emission maximum. Fluorescence data indicated that dansylglycine was bound tightly to one high – affinity binding site with an association constant of (2.6 ± 0.2) x 10^6 M^{-1} in addition to several low – affinity secondary sites. The fluorescence of dansylglycine: BSA complex was quenched by the binding of dicoumarol. This indicates a competition for the binding sites between dicoumarol and dansylglycine in favour of dicoumarol. The fluorescence quenching data indicated that BSA had one high – affinity binding site for dicoumarol with an association constant of (1.46 ± 0.03) x 10^6 M^{-1}. It was concluded that the binding sites for these drugs are located in the hydrophobic region of BSA.

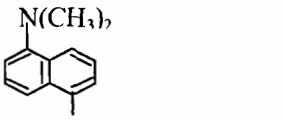
Key Words: Bovine Serum albumin, dansylglycine, dicoumarol, hydrophobic sites

Introduction

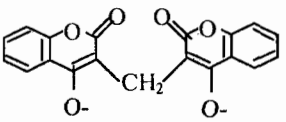
The binding of a drug to plasma proteins affects drug efficacy since the unbound plasma concentration of a drug corresponds to the pharmacologically active fraction (Herve *et al.*, 1984; Brodie and Hogben, 1957). The binding phenomenon is therefore, an important factor in drug distribution, drug transport and drug availability at target sites (Goldstein, 1949; Herve *et al.*, 1982). The serum albumin molecule is considered the major carrier – protein in blood and binds a wide variety of drugs and other biological substances (Attallah and Lata, 1968). One high affinity binding site has been reported on serum albumin for several drugs with some of them binding to a second site of lower affinity (Hsu *et al.*, 1974; Sudlow *et al.*, 1975).The high affinity site of bovine serum albumin (BSA) has been analyzed by NMR spectroscopy (Jardetzky and Wade-Jardetzky, 1965; Tanaka *et al.*, 1991a,b) and fluorescent probe techniques (Flanagan and Ainsworth, 1968; Jun *et al.*, 1971, 1972).

Dansylglycine[I] has been used as a fluorescent probe to detect binding at the hydrophobic sites of human serum albumin (Chignell, 1970a) and chicken serum albumin (Anunuso, 1988). In the present investigation, the binding of dansylglycine and dicoumarol [II] to

BSA was studied and the competition between the two drugs for the binding sites was examined.



[I]



[II]

Materials and Methods

Materials: Dansylglycine, dicoumarol and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic (Aldrich, England). The pH values were checked at room temperature using a suitably standardized digital pH meter (Knick-647, Germany). Standard buffer tablets were obtained from BDH Chemicals Ltd. England. All other chemicals were of reagent grade. Water used in this study was double distilled in an all glass apparatus.

Methods: The binding of dansylglycine to bovine serum albumin (BSA) was studied by measuring the increase in fluorescence following the titration of the protein solution with dansylglycine as described by Essassi *et al.* (1990) and modified as described below. Dicoumarol binding was determined by titrating BSA solution with the drug in the presence of dansylglycine. The fluorescence measurements were made at 480 nm with the excitation wavelength at 350 nm. All solutions were made up in 0.05 M sodium phosphate buffer at pH 7.4. The concentration of BSA was determined by measuring the absorbance at 280 nm using a =0.66 ($E^{1\%}_{1cm}$ = 6.6); the molecular weight of 66,000 was used to determine molar concentrations (Jun *et al.*, 1971).

Binding of Dansylglycine to BSA: The use of fluorescence to measure the binding of dansylglycine to BSA required the following two steps.

(i) ***Titrations to Measure the Limiting Fluorescence Enhancement:*** A titration of dansylglycine with BSA was used to establish the limiting fluorescence enhancement when all the dansylglycine was bound. A 2.0 ml solution of 1.43×10^{-6}M BSA was successively titrated with a solution of 1×10^{-4}M dansylglycine so that the protein concentration remained constant while the dansylglycine concentration increased. After each addition the dansylglycine fluorescence was measured at 480 nm with the excitation wavelength at 350 nm. At high dansylglycine concentrations, the dansylglycine fluorescence reached a plateau which was taken as the limiting fluorescence enhancement when that concentration of dansylglycine was completely bound. A parallel dansylglycine titration in the absence of BSA was run with 2.0 ml of phosphate buffer. At the emission and excitation wavelengths, the fluorescence of dansylglycine was neglible.

(ii) ***Titration of BSA with Dansylglycine:*** The limiting fluorescence enhancement (i.e. when dansylglycine is completely bound) was then used to determine the amount of bound and free dansylglycine at each point during the titration. Aliquots (10 µl) of a solution containing 1×10^{-4}M dansylglycine in phosphate buffer at pH 7.4 were added to 2.0 ml BSA solution (1.43×10^{-6}M) in a 1cm light – path silica cuvet.

The fluorescence was measured at 480 nm with excitation at 350 nm and corrected for dilution from a blank titration with buffer solution. To minimize loss of fluorescence through photodecomposition of BSA that may occur under the conditions of continuous illumination, albumin solutions were exposed to radiation for only the short measurement period (Essassi *et al.*, 1990).

Treatment of data: The concentration of bound dansylglycine at each point along the titration curve was calculated using the following equation:
Dansylglycine $_{bound}$ = (F_o/F_m) $1x10^{-4}$M
where F_o is the observed fluorescence and F_m is the limiting fluorescence enhancement when the concentration ($1x10^{-4}$M) of dansylglycine is completely bound. The concentration of free dansylglycine was calculated by subtraction of the bound from the total concentration. Results were plotted according to the method of Scatchard (1949) using the following expression:
$r/A = nk_a - rk_a$ where ***r*** and ***A*** are the bound and free dansylglycine concentrations, respectively, ***n*** is the number of binding sites and K_a is the association constant.

BSA Fluorescence quenching titration: Successive aliquots of 10 µl of dansylglycine solution ($1x10^{-4}$M) were added directly to the cell which contained 2.0 ml of BSA solution ($1.43x10^{-6}$M) in phosphate buffer (pH 7.4). After each titration, fluorescence intensity was measured at 348 nm with excitation at 285 nm.

Binding of dicoumarol to BSA: The quenching of the dansylglycine:BSA – induced fluorescence in the presence of dicoumarol was employed to investigate dicoumarol binding to BSA. Initially fluorescence cells contained dansylglycine ($1x10^{-4}$M) together with 2.0 ml of $1.43x10^{-6}$M BSA solution. Fluorimetric titrations were performed with successive additions of 2 µl of $1x10^{-2}$M solution of dicoumarol. After each addition, the fluorescence intensity was measured at 480 nm with the excitation wavelength at 350 nm.

Results and Discussion

Binding of dansylglycine to bovine serum albumin: Figure 1 shows the fluorescence emission spectra of dansylglycine in the presence and absence of BSA. The fluorescence intensity of dansylglycine in aqueous solution was minimal; but in the presence of BSA ($1.43x10^{-6}$M) the intensity was greatly enhanced with a marked hypsochromic shift in wavelength of the emission maximum. The area under an emission curve is proportional to the fluorescence quantum yield (Jun *et al.*, 1971). The quantum yield of dansylglycine increased considerably in the presence of BSA. These changes were due to alterations in the environment of dansylglycine and they present a strong evidence of the binding between dansylglycine and BSA. The high quantum yield of bound dansylglycine and the resultant blue shift in the emission maximum are indicative of a hydrophobic interaction

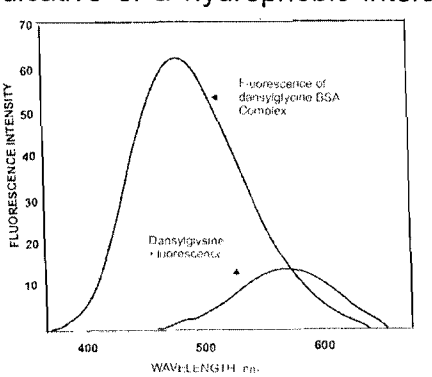


Fig.1 Fluorescence emission spectra of free (lower curve) and bound (upper curve) dansylglycine. The cuvet contained $1x10^{-4}$ M dansylglycine ($1.43x10^{-6}$ M BSA in the case of bound) in a total volume of 3 ml of 0.05 M phosphate buffer at pH 7.4. The spectra were obtained with a Shimadzu RF-500 spectrophotometer. Excitation was at 350 nm for emission. Bandwidths of excitation emission were 3 nm and 10 nm, respectively

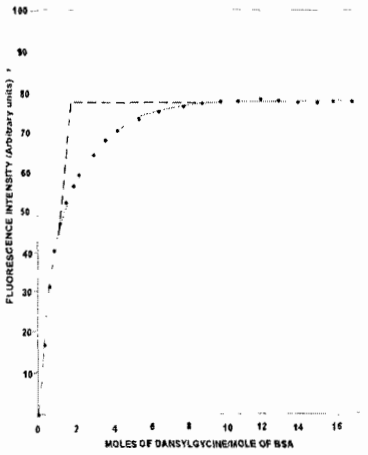


Fig. 2. Fluorescence titration curve of bovine serum albumin with dansylglycine. The increase in intrinsic dansylglycine fluorescence was measured at 480 nm with excitation at 350 nm. The BSA concentration was 1.43×10^{-6} M and the dansylglycine concentration was 1×10^{-4} M in 0.05 M phosphate buffer at pH 7.4. Bandwidths of excitation and emission were 3 nm and 10 nm respectively. Each point is the mean of three measurements.

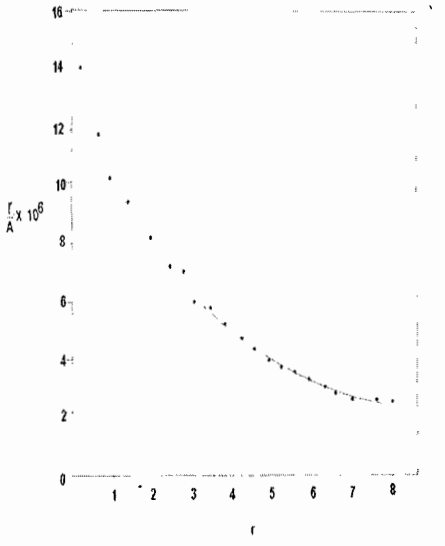


Fig. 3. Scatchard plot for the binding of dansylglycine to bovine serum albumin. The points for the curve were calculated from the data presented in Fig. 2. The BSA concentration was 1.43×10^{-6} M and the dansylglycine concentration was 1×10^{-4} M in 0.05 M phosphate buffer at pH 7.4. *A* is molar concentration of free dansylglycine, *r* is number of moles dansylglycine bound per mole of BSA. Ka x $(2.6 \pm 0.2) \times 10^6$ M^{-1}

(Jun *et al.*, 1971, 1972). Figure 2 shows the fluorescence titration curve for the binding of dansylglycine to BSA. Extrapolation of the linear portions of the titration curve indicates that two moles of dansylglycine were bound per mole of BSA. The Scatchard plot for the binding of dansylglycine to BSA (Figure3) showed that dansylglycine was bound to one high affinity binding site (K_a = $2.6 \pm 0.2 \times 10^6 M^{-1}$) in addition to an indeterminate number of secondary binding sites of low affinity. The low affinity binding sites probably resulted from configurational changes that occurred in the BSA molecule at higher dansylglycine concentrations, in which unfolding of the peptide chains led to exposure of more binding sites (O'Reilly, 1967).

The binding of dansylglycine to BSA also quenched the native fluorescence of the protein measured at 348 nm with excitation at 285 nm (Figure 4). The fluorescence titration curve showed that quenching resulted from the binding of dansylglycine to a single site on BSA. However, the downward slope of the titration curve indicated that binding of dansylglycine to other sites on BSA also caused some fluorescence quenching. The quenching of BSA fluorescence by dansylglycine was probably due to energy transfer from the protein tryptophan residues to the bound dansylglycine. According to Chignell (1970b) this resonance energy transfer was feasible because the emission band of BSA overlapped the absorption band of dansylglycine (Figure 5).

Binding of dicoumarol to bovine serum albumin: The binding of dicoumarol to BSA was studied by measuring the decrease in fluorescence of bound dansylglycine following displacement by dicoumarol. A decrease in fluorescence of the probe – protein complex in the presence of a drug is an indication of the competition between the probe and the drug for the same binding site (Jun *et al.*, 1971; Brand *et al.*, 1967). Competition between dansylglycine and dicoumarol could not have occurred if the binding sites were not

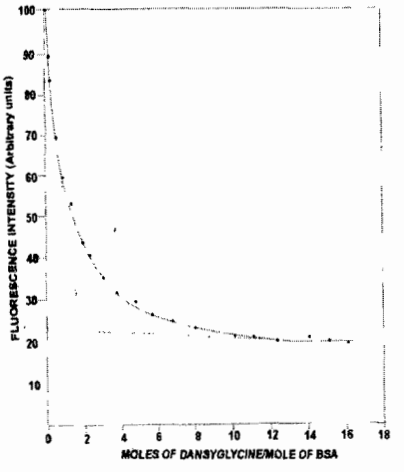


Fig 4. Fluorescence quenching titration of BSA with dansylglycine. BSA concentration in 0.05 M phosphate buffer, pH 7.4, was 1.43×10^{-6} M and dansylglycine concentration was 1×10^{-4} M. Fluorescence was measured at 348 nm with excitation at 285 nm. Bandwidths of excitation and emission were 3 nm 10 nm, respectively.

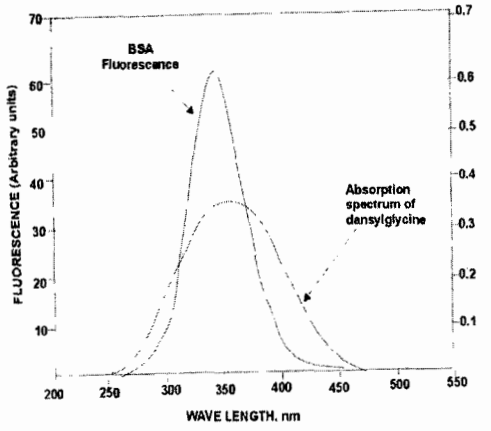


Fig 5 Spectra of dansylglycine and bovine serum albumin. The absorption spectrum was measured as the excitation spectrum by scanning the excitation while emission wavelength was set at 580 nm for dansylglycine (1×10^{-4} M). The fluorescence emission spectrum was measured by exciting BSA (1.43×10^{-6} M) at 285 nm. Bandwidths of excitation and emission were 3 nm and 10 nm, respectively

common. Since dansylglycine has been shown to bind at hydrophobic sites of proteins (Anunuso, 1988), its displacement by dicoumarol therefore indicates that the BSA binding site for dicoumarol is located in a hydrophobic region of the protein. Chignell (1970a), using circular dichroism techniques, found that both coumarin rings of dicoumarol took part in the binding of the drug to albumin. In the light of this, it follows that dicoumarol may bind to the hydrophobic region of BSA by the phenyl groups of the coumarin rings.

The binding of dicoumarol to BSA quenched the native fluorescence of the protein. The quenching may be due to non-radiative energy transfer from the excited state of tryptophan residues to the bound drug followed by emission of some of the photons as fluorescence. The fluorescence titration curve (Figure 6) suggests that quenching resulted from the binding of dicoumarol to a single site on BSA. However, the shape of the titration curve indicates that binding of dicoumarol to secondary sites on BSA also caused some fluorescence quenching. This implied that some energy transfer may have occurred at these sites leading to quenching of fluorescence. The quenching could also be due to changes in the conformation of the protein (Attallah and Lata, 1968), which usually occur at high drug to protein ratios (O'Reilly, 1967).

The data presented in Figure 6 showed that BSA had high binding capacity for dicoumarol. Extrapolation of the linear portions of the fluorescence titration curve (Figure 6) indicated that 10 moles of dicoumarol were bound per mole of BSA. The observed high mole ratios of bound dicoumarol to BSA cannot, however, be explained as due simply to interaction with tryptophan residues since there are only two tryptophan and about 20 tyrosine residues per molecule of BSA (Attallah and Lata, 1968). Tyrosine 128 has been identified as a dicoumarol–binding site in rat liver NAD(P)H:quinones oxidoreductase (Ma *et al.*, 1992). It is therefore suggested that tyrosine may have taken part in the binding of dicoumarol to BSA. This suggestion is in accordance with the results of Attallah and Lata (1968) who also noted the involvement of tyrosine in the binding of certain steroids to BSA. The high mole ratio of bound

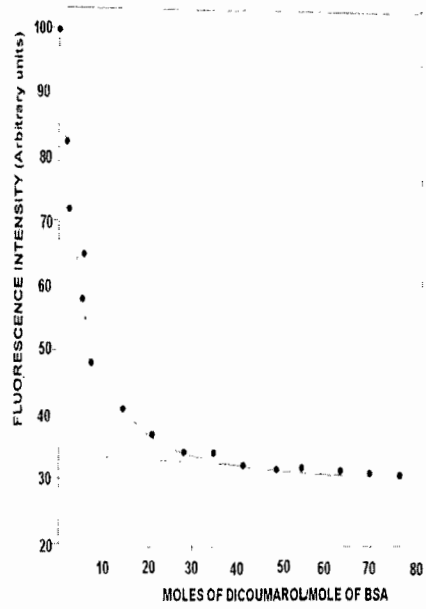


Fig. 6. Fluorimetric titration of bovine serum albumin by dicoumarol. BSA (1.43×10^{-6} M) was titrated with dicoumarol (1×10^{-2} M) in the presence of 1×10^{-4} M dansylglycine in 0.05 M phosphate buffer at pH 7.4. Fluorescence was measured 480 nm with excitation at 350 nm at 28°C. Bandwidths of excitation and emission were 3 nm and 10 nm respectively. Each point is the mean three measurements

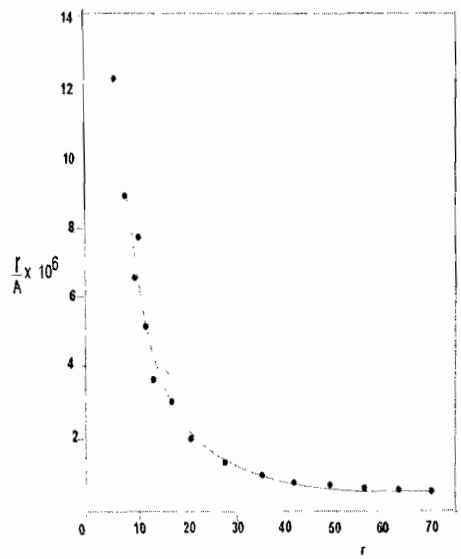


Fig. 7. Scatchard plot for the binding of dicoumarol to bovine serum albumin. The points for the curve were calculated from the data presented in Fig.6. Fluorescence was measured at 480 nm with excitation at 350 nm. A is the molar concentration of free dicoumarol, r is the number of moles of bound dicoumarol per mole of BSA. Ka = (1.46 ± 0.03) $\times 10^6$ M^{-1}

dicoumarol to BSA can be further explained on the basis of protein configurational changes at high drug concentrations which unfold more binding sites (O'Reilly, 1967). Quenching of the native fluorescence of BSA's tryptophan and tyrosine residues by dicoumarol provides evidence for molecular interaction between dicoumarol and the amino acid residues.

The Scatchard plot for the binding of dicoumarol to BSA (Figure 7) indicates that the protein has one high affinity site for dicoumarol with an association constant of (1.46 ± 0.03) $x10^6M^{-1}$ in addition to secondary binding sites of weaker affinity. The plot followed a curvilinear course that bent sharply near the abscissa. This is suggestive of rapid binding of dicoumarol to primary sites followed by protein configurational changes, which exposed secondary binding sites. These secondary sites were excluded by the first order configurational disposition of the protein. A binding free energy of –35.5 KJ mol^{-1} was calculated for the binding of dicoumarol to BSA, indicating a spontaneous displacement of dansylglycine from binding sites on BSA.

Conclusion

The present study shows that dansylglycine can be used as a fluorescent probe to measure the binding of dicoumarol to BSA. It further demonstrates the usefulness of the probe technique in the evaluation of competitive drug interactions for the binding sites. Our results show that the interactions were of a hydrophobic nature and that both dansylglycine and dicoumarol were bound tightly to a single site on BSA in addition to weaker binding to a number of secondary sites.

Acknowledgement

This paper forms part of a doctoral thesis by SAO at the Federal University of Technology, Owerri, Nigeria. We acknowledge the gift of bovine serum albumin by Dr. Sam Achilefu.

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