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Spectrofluorometric Study of the Interaction of Coumarin Derivatives with Bovine Serum Albumin

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The mechanism of interaction of four coumarin derivatives (CDS) with bovine serum albumin (BSA) was studied using spectrofluorometric technique. It was found that the coumarin ring common to all CDS makes major contribution to interaction. Binding affinities could be related to parachor values of CDS. Stern-Volmer plots indicated the presence of static component in the quenching mechanism. Results also showed that both tryptophan residues of protein are accessible to CDS. The high magnitude of rate constant of quenching indicated that the process of energy transfer occurs by intermolecular interaction forces and thus CDS binding site is in close proximity to tryptophan residues of BSA. Binding studies in the presence of the hydrophobic probe, 8-anilino-1-naphthalein-sulfonic acid showed that there is hydrophobic interaction between CDS and the probe and they do not share common sites in BSA. Thermodynamic parameters obtained from data at different temperatures showed that the binding of CDS to BSA involve hydrophobic bonds predominantly. The effects of various metal ions on the binding of CDS with BSA were also investigated.

key words: Coumarin derivatives, Bovine serum albumin, interaction studies

INTRODUCTION

Serum albumin, the most abundant protein in the circulatory system, has been one of the most extensively studied of all proteins [1]. It is the major transport protein for unesterified fatty acids, but is also capable of binding an extraordinarily diverse range of metabolites, drugs and organic compounds. The remarkable binding properties of albumin accounts for the central role, it can play in the efficacy and rate of delivery of drugs. Many drugs, including anti-coagulants, tranquilisers and general anesthetics, are transported in the blood while bound to albumin [2]. This has simulated a great deal of research on the nature of the drug binding sites and investigations of whether fatty acids, natural metabolites and drugs compete with one another for binding to the protein [1]. The proteins contain tryptophan residues and have the intrinsic fluorescence. Information about the protein can be obtained by the measurement of intrinsic fluorescence intensity of protein tryptophan residues before and after addition of drug. Some reports are devoted to the study of the reaction between drug and protein [3-7].

Coumarins constitute an important group of natural products and many of their analogues are found to be biologically active. 4-Methylcoumarins have been found to possess a wide range of biological activities *viz.* chloretic, analgesic, antispermatogenic, antifungal, anticoagulant and diuretic properties [8]. 4-Methylcoumarins bearing different functionalities inhibit effectively the rat liver microsome mediated aflatoxin B_1-DNA binding in vitro. The coumarin anticoagulants have been used for many years for prophylactic purposes in thromboembolism. Many of these agents are bound to serum proteins, especially serum albumin. This binding affects their pharmacologic and pharmacokinetic properties. The agents, which have been studied, generally, have been bound to a high extent, in part, contributes to their long duration of action [8].

The interactions of some 7-aminocoumarins with human serum have been carried out by Mishra et al [8]. Based on the experimental studies they have reported that 7-aminocoumarins bound to domain II of HSA. However, no literature is available for the interactions between the four coumarin derivatives employed in the present study *viz.*, 3-nitroso-4-hydroxy coumarin (3N-4H-C), 4-hydroxy coumarin (4H-C), 4-methyl-6-hydroxy coumarin (4M-6H-C) and, 4-methyl-7-hydroxy coumarin (4M-7H-C) and BSA. In view of the biological importance of CDS, we have planned to carry out the detailed investigations on the interactions of these CDS with BSA using spectrofluorometric technique.

MATERIALS AND METHODS

Fluorescence measurements were performed on a Hitachi spectrofluorometer Model F-2000 equipped with a 150W Xenon

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lamp and slit width of 10 nm. A 1.00 cm quartz cell was used throughout.

Bovine serum albumin (BSA, fraction V, approximately 99%; protease free and essentially γ-globulin free) and 8-anilino-1-naphthalene sulfonic acid sodium salt, $C_{16}NSO_3H_{13}Na$ (ANS) were obtained from Sigma Chemical Company, St Louis, USA. Coumarin derivatives are obtained from s.d fine chemicals Ltd., Mumbai, India. All other materials were of analytical reagent grade. The solutions of CDS and BSA were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solution was prepared based on molecular weight of 65,000.

RESULTS AND DISCUSSION

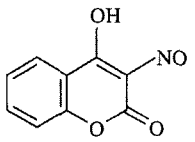
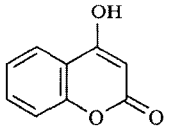
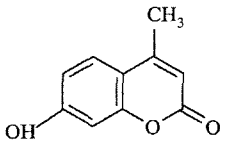
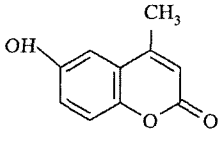
The structures of CDS employed in the present study are shown in Table 1.

Fluorescence studies

Some preliminary studies were carried out to select optimum protein and CDS concentrations for CDS-protein interaction. On the basis of preliminary experiments, BSA concentration was kept fixed at 10 μM and CDS concentration was varied from 10-140 μM. Fluorescence spectra were recorded at room temperature (29°C) in the range 300-500 nm upon excitation at 296 nm in each case.

Fluorescence spectra of BSA were recorded in the presence of increasing amounts of CDS. The spectra of one of the representative CDS, the 4H-C are shown in Fig. 1. It was observed that the interaction of CDS with serum albumin resulted in noticeable change in λ_{max} of tryptophan fluorescence in albumin. However, all the CDS were observed to quench

Table 1. Structures of coumarin derivatives.

Coumarin Derivative	Structure
3-Nitroso-4-hydroxy coumarin	
4-Hydroxy coumarin	
4-Methyl-7-hydroxy coumarin	
4-Methyl-6-hydroxy coumarin	

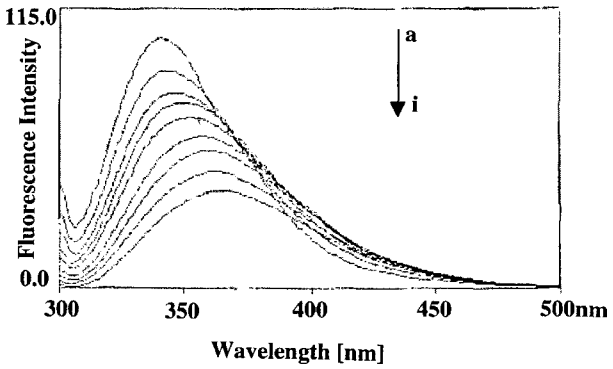


Figure 1. Fluorescence spectra of BSA in the presence of 4H-C. BSA concentration was kept fixed (10 μM). 4H-C concentration was a-0, b-10, c-20, d-30, e-40, f-60, g-80, h-100 and i-140 μM.

the fluorescence of albumin with bathochromic shift in the λ_{max} values i.e., from 342 to 352 nm and from 342 to 354 nm for 3N-4H-C and 4M-6H-C, respectively, while it is from 342 to 364 nm for 4H-C and, from 342 to 358 nm and a new peak at 446 nm for 4M-7H-C. The fraction of CDS bound, è, was determined according to Weber and Young [9] and Maruyama et al. [10] using the equation, $\theta = (F_o\text{-}F)/F_o$ --------- (1), where F and F_o denote the fluorescence intensities of protein in a solution with a given concentration of CDS and without CDS, respectively. Fluorescence data was analyzed using the method described by Ward [11]. It has been shown that for equivalent and independent binding sites,

$$\frac{1}{(1-\theta)K} = \frac{[D_t]}{\theta} - n[P_T] \qquad (2)$$

where, K is the association constant for CDS-protein interaction, n is the number of binding sites, $[D_t]$ is the total drug concentration and $[P_T]$ is the total protein concentration. From the plot of $1/(1\text{-}\theta)$ *versus* $[D_t]/\theta$ the values of K and n were evaluated from the slope and intercept (Table 2). A representative graph of CDS, the 3N-4H-C is shown in Fig. 2. The K values were of the order of 10^3 to 10^4 M^{-1} and the number of binding sites varied from 1.97 to 4.3. Since the data fit equation 2 in all cases, it may be concluded that under the conditions of the experiment, all the binding sites are equivalent and independent. The order of K value is in consistent with non-covalent interactions [12]. These values are supported by standard free energy change (ΔG^0) values obtained from the relationship, $\Delta G^0 = -2.303RT \log K$ and are seen to be close to -24 kJ mol^{-1} (Table 2). This shows that the coumarin ring common to all CDS perhaps makes major contribution to interaction. The coumarin is the primary hydrophobic portion of the molecule but substituents on the ring may have an effect on surface activity of the molecule. The binding is not directly related to hydrophobicity or hydrophilicity of substituents on the ring. However, it plays an indirect role by affecting the intramolecular interaction in the molecule. For different CDS samples, the K values vary in the

Table 2. Binding parameters for the interactions of various coumarin derivatives with bovine serum albumin.

Coumarin derivative	Association constant (M^{-1})	Number of binding sites (n)	Standard free energy change (ΔG°, kJ $mole^{-1}$)	Parachor $(Nm^{-1})^{1/4}$ m^3
3N-4H-C	$1.97X10^4$	1.92	-24.64	381.5
4H-C	$8.12X10^3$	4.30	-22.40	322.3
4M-6H-C	$8.74X10^3$	3.41	-22.63	350.3
4M-7H-C	$1.74X10^4$	2.64	-24.35	354.3

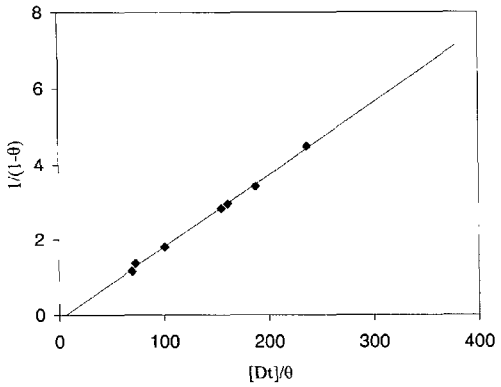


Figure 2. 1/(1- θ) versus $[D_t]/\theta$ plot for the binding of 3N-4H-C to BSA.

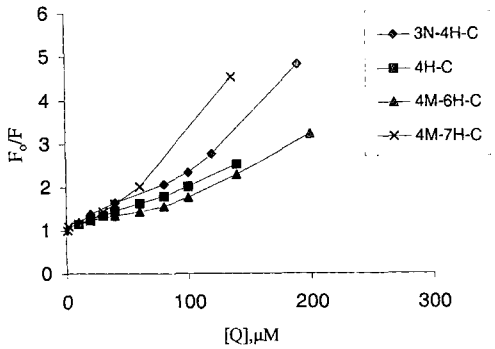


Figure 3. Stern-Volmer plot of F_o/F *versus* [Q] for the binding of coumarin.

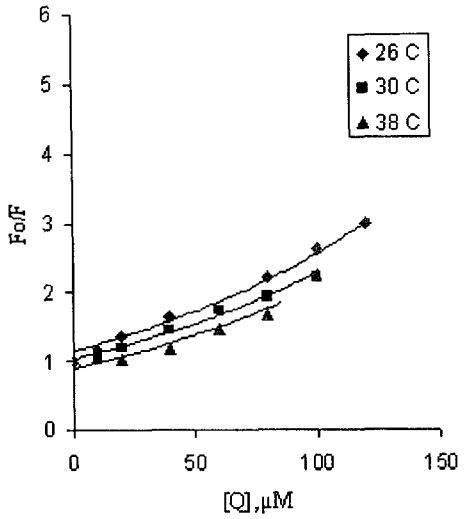


Figure 4. Stern-Volmer curves for 3N-4H-C at different temperatures.

order 3N-4H-C>4M-7H-C >4M-6H-C>4H-C. The 3N-4H-C has nitroso group and has the highest association constant followed by 4M-7H-C. This shows that coumarin with nitroso group as substituent will bind stronger than hydroxyl and methyl substituents.

Parachor, which is a measure of molar volume of drug, was calculated for each CDS from the atomic parachors and other structural features [13]. The values for different samples varied in the order 3N-4H-C> 4M-7H-C> 4M-6H-C> 4H-C. The order of parachor values is in close agreement with the order of K values.

Fluorescence intensity data was also analyzed according to Stern-Volmer law, $F_o/F = 1 + K_q [Q]$ --------- (3) by plotting F_o/F *versus* [Q], where F_o and F are the steady state fluorescence intensities at 344 nm in the absence and presence of quencher (CDS), respectively, and [Q] is the total CDS concentration. The Stern-Volmer plot (Fig. 3) showed positive deviation from straight line, suggesting the presence of a static component in the quenching mechanism [14]. According to Eftink and Ghiron [15] upward curvature in the Stern-Volmer plot indicates that both tryptophan residues of BSA are exposed to quencher and the quenching constant of each tryptophan residue is nearly identical, while downward curvature indicates buried tryptophan residues. The Stern-Volmer graph for a CDS, 3N-4H-C is shown in Fig. 4 at various temperatures which shows that the slopes decrease with increasing temperature indicating the presence of static quenching interaction [16]. This was further examined by analyzing the data by modified Stern-Volmer plot, $F_o/(F_o-F) = 1/f_a + 1/[Q]\, f_a K_{SV}$ --------- (4) where F_o and F are fluorescence intensities at 344 nm in the presence and absence of quencher, respectively, [Q] is equal to the total concentration of CDS, K_{SV} is the Stern-Volmer quenching constant and f_a is the fraction of fluorophore (protein) accessible to the quencher (CDS). From the plot of $F_o/(F_o-F)$ *versus* 1/[Q], the values of f_a and K_{SV} were determined. A representative graph is shown in Fig. 5. The value of f_a was found to be close to unity indicating thereby that both tryptophan residues of BSA are involved in the drug-protein interaction. The maximum quenching was obtained by extrapolating a plot of $F_o/(F_o-F)$ *versus* 1/[Q] to 1/[Q] = 0 corresponding to infinite concentration of CDS. We have observed that at infinite concentration of CDS, fluorescence quenching was more than 89% in each case. This again shows that both the tryptophan residues of BSA are accessible to CDS molecules.

For a bimolecular quenching process, $K_q = k_q \tau_o$, where τ_o is

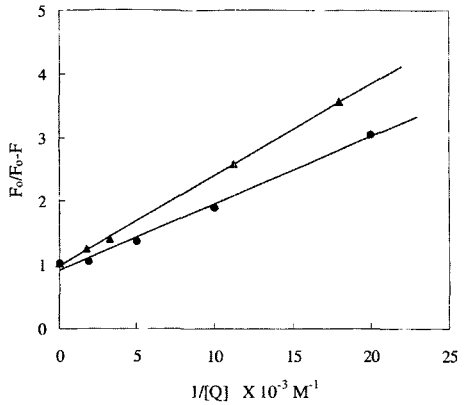


Figure 5. Plot of $F_o/(F_o-F)$ versus 1/[Q] for 4H-C(–▲ –) and 4M-6H-C (–●–).

the lifetime in the absence of quencher and k_q is the rate constant for quenching. As τ_o value for tryptophan fluorescence in proteins [3] is known to be equal to 10^{-9} s, the k_q would be of the order of 10^{13} M^{-1} s^{-1}. The value of k_q depends on the probability of a collision between fluorophore and quencher. This probability depends on their rate of diffusion (D), their size and concentration. It can be shown that $k_q = 4\pi aDN_aX10^{-3}$ --------- (5), where D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii and N_a is the Avogadro's number. The upper limit of k_q expected for a diffusion-controlled bimolecular process [4] is 10^{10} M^{-1} s^{-1}. The high magnitude of k_q in the present study (10^{13} M^{-1} s^{-1}) can probably be attributed to increase in the encounter radii of tryptophan-coumarin derivatives. This can happen only by the process of energy transfer and hence the quenching of tryptophan fluorescence occurs by intermolecular interaction forces [3].

Thermodynamics of CDS -protein interactions

Thermodynamic parameters for the binding of CDS to BSA were determined by carrying out the binding studies at four different temperatures, 13°, 26°, 30° and 38°C by spectrofluorometric method. The association constant, K was found to decrease with increase in temperature since,

$$\text{Log } K = -\Delta H^\circ / 2.303RT + \Delta S^\circ / 2.303R \quad (6)$$

Log K *versus* 1/T plot enabled the determination of standard enthalpy change, ΔH° and standard entropy change, ÄS° for the binding process. The ΔH°, ΔS° and ΔG° values found to be in the range of +4.78 to +38.29 kJ mol^{-1}, +91.14 to +204.1 J $K^{-1}mol^{-1}$, and -22.15 to -61.16 kJ mol^{-1}, respectively. The positive values of standard enthalpy and entropy changes indicate that the hydrophobic contribution is the predominant intermolecular force stabilizing the BSA-coumarin complexes [17]. These results together with spectral changes in the fluorescence emission spectra of BSA induced by CDS suggest that the interaction may take place in subdomain I and II since these subdomains have been proposed to bind drugs and other hydrophobic materials [18].

Binding studies in the presence of ANS

Fluorescence spectra of 10 µM BSA in the presence of increasing amounts of CDS/ANS (4 to 25 µM) were determined upon excitation at 296 nm. Both CDS and ANS quench the fluorescence of BSA, but the magnitude of decrease in fluorescence intensity was much larger for ANS as compared to that for CDS. The ANS bound to BSA calculated from the fraction of occupied sites (θ) was 76% where as the CDS bound to BSA was 18.2 to 27.3%. It is known that excitation at 296 nm involves fluorescence due only to tryptophan residues of BSA. Further, under conditions of the experiment, tryptophan residues of BSA are partially exposed and their accessibility depend upon the nature of molecules of the interacting species [18]. It thus appears that whereas, tryptophan residues are fully accessible to the hydrophobic probe, ANS they are only partially accessible to the CDS which have partially hydrophilic character.

In another set of experiments, BSA-ANS interaction was studied in the presence and absence of 5, 10, 15, and 20 µM each of CDS by monitoring ANS fluorescence upon excitation at 370 nm. It was found that for a given concentration of ANS, fluorescence intensity increases when CDS is added to BSA-ANS system. It is known [14] that the ANS shows greatly increased fluorescence as a result of hydrophobic interactions with proteins and other macromolecules due to transfer of probe from an aqueous to non-polar environment. Increase in fluorescence intensity of BSA-ANS system on the addition of CDS can be explained as follows: when CDS is added to BSA-ANS system, it can compete with ANS for the hydrophobic sites on the surface. In that case it would inhibit the binding of ANS, i.e. displaces ANS from its binding sites and the fluorescence intensity should decrease. But, the fluorescence intensity actually increases. This shows that ANS and CDS do not share common sites in BSA. Increase in fluorescence intensity shows that the CDS has highly hydrophobic character, and thus, further shifts the fluorescence intensity of ANS to higher values.

Surface activity

Surface tension of coumarin solutions (each of 0.8% separately prepared in phosphate buffer of pH 7.4 containing 0.15 M NaCl solution) at 27°C was determined by drop weight and drop number methods using a stalagmometer. Surface tension data was expressed as surface activity, which in turn expressed as surface pressure, π, which is the difference between surface tension of the solvent and that of the solution. Thus, surface activity values, expressed as surface pressure, $\pi = \gamma_{solvent} - \gamma_{solution}$, were found to be 6.95 $X10^{-3}$, 8.21$X10^{-3}$, 3.93 $X10^{-3}$, and 8.27 $X10^{-3}$ for 3N-4H-C, 4H-C, 4M-6H-C and 4M-7H-C, respectively. Reduction in surface tension of solvent or increase in surface pressure is attributed to hydrophobicity of the CDS molecule. However, the order of π value suggests that the CDS have hydrophobic character.

Table 3. Association constants of coumarin derivative-BSA systems in the presence of metal ions.

Sample	Association Constant, M^{-1}			
	3N-4H-C	4H-C	4M-6H-C	4M-7H-C
BSA+ CDS	$1.97X10^4$	$8.12X10^3$	$8.74X10^3$	$1.74X10^4$
BSA+ CDS +Zn^{2+}	$1.65X10^4$	$7.25X10^3$	$8.56X10^3$	$1.66X10^4$
BSA+ CDS+Mg^{2+}	$1.54X10^4$	$7.87X10^3$	$7.24X10^3$	$1.68X10^4$
BSA+ CDS+V^{2+}	$1.64X10^4$	$7.74X10^3$	$7.01X10^3$	$7.56X10^3$
BSA+ CDS +Fe^{3+}	$2.97X10^3$	$6.65X10^3$	$6.98X10^3$	$7.89X10^3$
BSA+ CDS+Co^{3+}	$1.69X10^4$	$8.05X10^3$	$6.65X10^3$	$9.58X10^3$
BSA+CDS+K^+	$7.78X10^3$	$6.69X10^3$	$7.99X10^3$	$1.41X10^4$

Effects of metal ions

The fluorescence spectra of albumin-coumarin were recorded in presence of various metal ions *viz.*, K^+, Mg^{2+}, Co^{2+}, V^{5+}, Zn^{2+} and Fe^{3+} at 344 nm upon excitation at 296 nm. The concentration of BSA and metal ion was fixed at 10 μM while that of CDS was varied in the range 10-80 μM. The fluorescence emission spectrum of CDS in the presence of metal ions shows that there is no interaction between metal ions and CDS. But, there is a binding reaction between metal ion and protein and the presence of metal ion directly affects the binding between CDS and protein. Further, in order to study the effect of metal ions on the binding between CDS and BSA, binding constants were determined in presence of various metal ions. The results are shown in Table 3. The competition between metal ions and CDS decreased the binding constant between protein and CDS, implying that the binding force between protein and CDS also decreased. Thus, shortening the storage time of the CDS in blood plasma and enhancing the maximum effectiveness of the drug [16].

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