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Interaction of alkylpyridinium chlorides with human serum albumin studied by fluorescence techniques

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Abstract

A study has been made on the interaction of cetylpyridium chloride (CPC) and dodecylpyridinium chloride (DPC) with human serum albumin (HSA) in aqueous solution (22 °C, pH = 7.0). The study was performed using techniques based on the effect promoted by the addition of the surfactants on the fluorescence of the protein tryptophan group. From the dependence of the fluorescence quenching with the protein concentration, the binding isotherms of the surfactants with the protein were determined. The results indicate that CPC is considerably more efficient than DPC in quenching the fluorescence of the tryptophan group. However, even for CPC, eight surfactant molecules must be bound (in average) to each HSA molecule to quench half of the protein intrinsic fluorescence, suggesting an association to zones far away from the locus of the fluorescent moiety. Information regarding the locus of CPC and DPC association to HSA was obtained from their effect on the fluorescence of dansyl derivatives bound to the protein. The results show that: (i) CPC binds to site 2 or near it, quenching the fluorescence of the probe bound to this site and increasing the fluorescence of the probe bound to site 1, and (ii) DPC is less efficient in modifying dansyl derivatives fluorescence, being able to interact weakly with compounds located at both sites 1 and 2.

Keywords: Human serum albumin; Alkylpyridinium chlorides; Dansyl derivatives

1. Introduction

A wide range of experimental techniques such as viscosity [1], optical rotatory dispersion [1], calorimetry [2], UV spectroscopy [3], potentiometry [4], EPR [5], fluorescence [6–12], etc. have been used to study the interactions between proteins and surfactants. Among these, fluorescence is the most frequently employed, since the changes in the intensity (and in some cases the spectral distribution) of the emission arising from the tryptophan residues permit to determine the surfactant binding efficiency and to elucidate details of the protein structure. On the other hand, serum albumins are the most abundant of the proteins in blood plasma, accounting for ca. 60% of the total protein; its concentration amounts to ca. 50 g L^{-1} [13,14]. For this reason, and due to its function as a fatty acid carrier [15], studies on protein–surfactant association are mostly performed using albumins. Bovine serum albumin (BSA) and human serum albumin (HSA) present ca. 80% identity in the amino acid sequences, but, from the spectroscopic point of view, the main difference between them is that BSA has two tryptophan residues (W^{131} and W^{214}) which are located in different environments, while HSA has only one (W^{214}) located at site I in the 2A subdomain. It would then be expected that the effect promoted by a given surfactant on the fluorescence of these two proteins be different. This difference will be particularly relevant for those surfactants that are quenchers of the tryptophan fluorescence, such as the alkylpyridium ions employed here [10,16,17]. The presence of only one fluorescent moiety at a well defined site of the protein will simplify the data treatment and could render information regarding the localization of the bound surfactant. Although this point has been stressed in some studies where the binding mode of surfactants with BSA and HSA was compared, very few were performed with surfactants that act as fluorescence quenchers [12] In the present work, we report results of a study of the binding of cetylpyridinum chloride (CPC) and dodecylpyridinium chloride (DPC) with HSA monitored by the fluorescence of the intrinsic tryptophan. The purpose of the study was to investigate the effect of the surfactant chain length on the binding mode.

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Furthermore, the possible locus of binding for each surfactant to HSA was investigated by measuring the effect elicited by surfactant addition on the fluorescence of dansyl derivatives.

2. Experimental

Human serum albumin (HSA, Sigma; fatty acid free, pI = 4.7) was used as received. Cetylpyridinium chloride (CPC), dodecylpiridinium chloride (DPC) and urea were products from Merck. Dansylsarcosine (piperidinium salt) (DS) and dansyl-1-sulfonamide (DNSA), Sigma products, were used as received.

Fluorescence measurements were carried out in an Aminco Bowman spectrofluorometer. Excitation of HSA was carried out at 295 nm and emission was registered at 340–348 nm in order to selectively detect the tryptophan fluorescence [18]. DS and DNSA were excited at 325 nm and 329 nm, respectively. The fluorescences of DS and DNSA were registered at 475 nm and 485 nm, respectively. Experiments were done at a molar ratio between the dansyl derivative and HSA of 0.1.

All measurements were carried out at room temperature in aqueous solution (pH = 7.0) or in phosphate buffer (concentration variable from 10 mM to 100 mM, pH = 7.0).

The association of the surfactants with HSA was determined from the effect of HSA concentration on the I_0/I vs. [surfactant] profiles (being I_0 and I the tryptophan fluorescence intensities in the absence and presence of surfactant). The dependence of the I_0/I vs. [surfactant] profiles with HSA concentration is attributed to the surfactant distribution between the protein and the external medium. According with the pseudophase model [19] a pseudo association constant (K) is defined by Eq. (1).

$$K = \frac{[\text{Surf}]_b}{[\text{Surf}]_{free}[\text{HSA}]} \quad (1)$$

where $[\text{Surf}]_b$ is the analytical concentration of surfactant associated with the protein, $[\text{Surf}]_{free}$ is the concentration of surfactant remaining in the external medium and [HSA] is the protein concentration. Defining $[\text{Surf}]_b/[\text{HSA}]$ as the average number of surfactant molecules bound per HSA molecule, K can be further expressed as in Eq. (2).

$$K = \frac{n}{S_{free}} \quad (2)$$

The method proposed to evaluate K is based on the assumption that the I_0/I value is only determined by the average number of surfactant molecules bound to each protein (n). Since equals n implies equals $[\text{Surf}]_{free}$ (from Eq. (2)), a simple mass balance leads to:

$$[\text{Surf}]_{Analyt} = [\text{Surf}]_{free} + n[\text{HSA}] \quad (3)$$

where $[\text{Surf}]_{Analyt}$ is the total surfactant concentration. For a set of $[\text{Surf}]_{Analyt}$ and [HSA] corresponding to the same I_0/I value, a plot of the left hand side of Eq. (3) vs. [HSA] allows for the evaluation of K from the slope/intercept ratio (Eq. (2)) [20]. The dependence of K with n defines the type of binding isotherm. In particular, cooperativity is evidenced by an increase in the estimated K values with n (or the free surfactant concentration).

3. Results and discussion

Addition of CPC, and to a lesser extent DPC, readily quenches HSA intrinsic fluorescence. Fig. 1 shows representative data obtained in water at pH = 7.0. In this figure are also included for comparison data obtained for BSA under similar experimental conditions. These data show that:

(i) CPC is a quencher considerably more efficient than DPC.
(ii) Quenching by CPC and DPC does not follow a linear Stern-Volmer behaviour. Furthermore, both surfactants present noticeable differences in the quenching profile. In particular, data obtained employing CPC present a noticeable upward curvature when plotted according to a simple Stern-Volmer equation.
(iii) For both surfactants, the quenching is considerably more efficient for BSA than for HSA.

Qualitatively similar conclusions can be drawn from data obtained in presence of phosphate buffer (up to 100 mM). Points (i) and (iii) can be due to differences in quenching efficiencies and/or to differences in the association degree of the surfactants with protein. The noticeable upward curvature shown by the

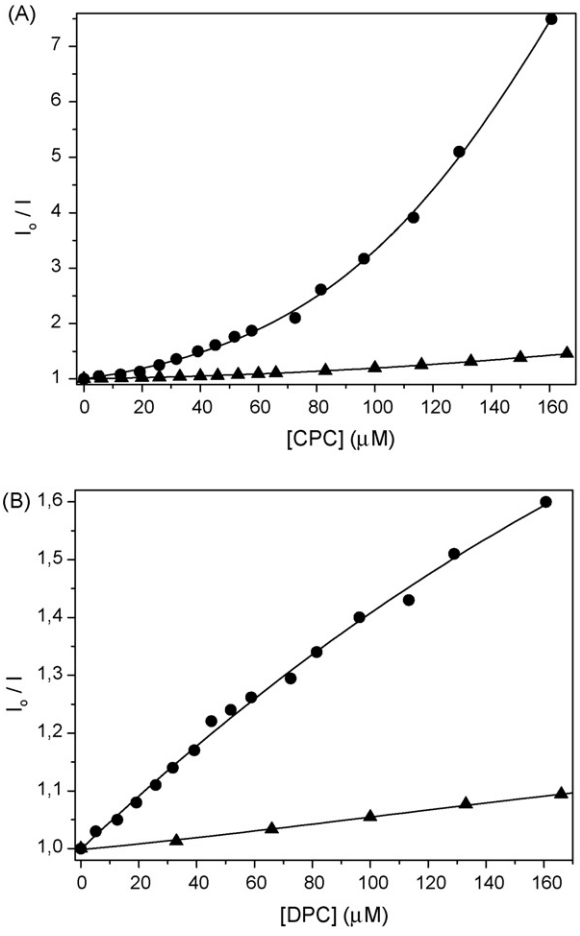

Fig. 1. Quenching of the fluorescence of the protein by alkylpyridinium surfactants: CPC (A) and DPC (B), in an aqueous solution (32 μM; pH = 7.0) of BSA (●) and HSA (▲).

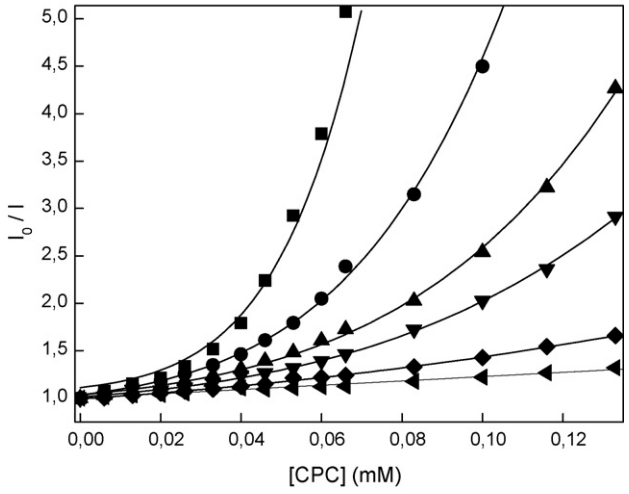

Fig. 2. Effect of CPC addition on the fluorescence of HSA in 10 mM phosphate buffer, pH = 7.0. HSA concentrations: (■) 2 μM; (●) 4 μM; (▲) 6 μM; (▼) 8 μM; (♦) 16 μM; (◄) 32 μM.

CPC data could be ascribed to contribution of static quenching, cooperativity in the binding and/or quenching efficiency of the surfactant. In order to evaluate these possibilities, we have determined the association of the surfactants to the protein by a procedure based on the measurement of the quenching efficiency at different protein concentrations (see Section 2).

Fig. 2 shows the effect of CPC addition on the fluorescence of HSA at different concentrations in 10 mM phosphate buffer at pH 7.0. The dependence of the quenching efficiency with the protein concentration can be used to evaluate the surfactant association extent by plotting the surfactant concentration required to reach a given I_0/I value (I_0 and I are the fluorescence intensities in the absence and in the presence of CPC, respectively) as a function of the protein concentration [10,19]. This type of plot is shown in Fig. 3. The linearity of these plots supports the proposed data treatment. From these plots, the surfactant concentration remaining in the aqueous pseudophase,

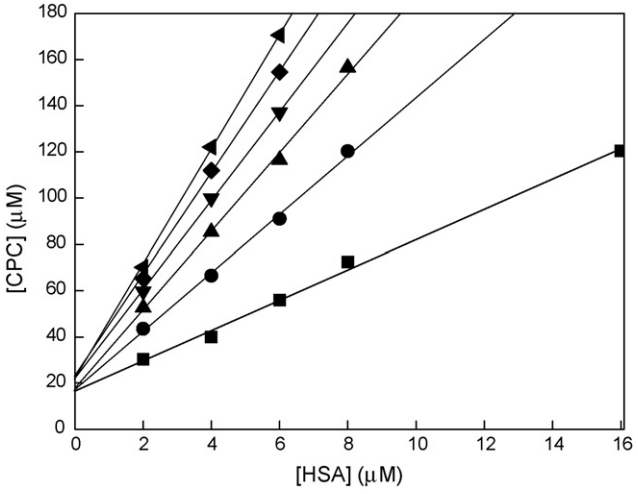

Fig. 3. Plot of the CPC concentration needed to reach a given I_0/I value as a function of HSA concentration. I_0/I values: (■) 1.5; (●) 2.0; (▲) 2.5; (▼) 3.0; (♦) 3.5; (◄) 4.0.

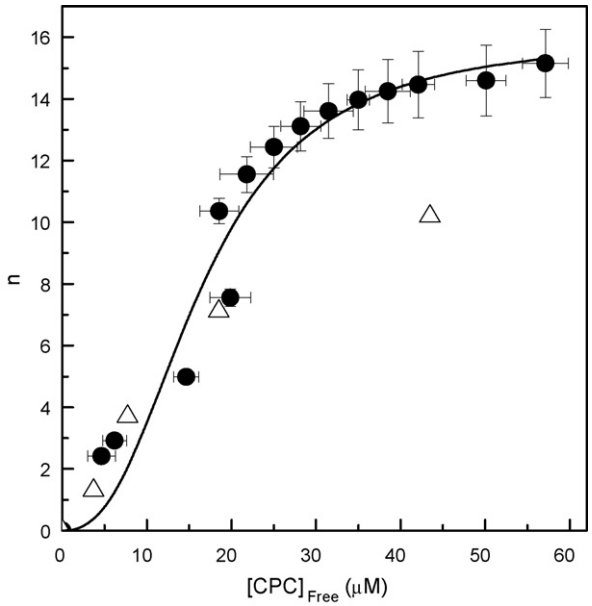

Fig. 4. Number of CPC molecules bound per protein molecule versus the concentration of free CPC. HSA (●); BSA (△) (Ref. [10]). The line represents the fitting of HSA data to a Hill equation with a cooperativity parameter $n = 2.5$.

$[CPC]_{free}$ (from the intercept) and the average number of surfactant molecules bound to each protein (n) from the slope are obtained. Values of n as a function of the free surfactant are shown in Fig. 4 (for CPC) and Fig. 5 (for DPC).

The data obtained employing DPC shows a linear relationship between the bound and free surfactant, allowing an estimation of the binding constant ($K = 1 \times 10^5\ M^{-1}$) over all the concentration range considered (up to $n = 14$). This value is even larger than that previously reported for BSA ($K = 0.16 \times 10^5\ M^{-1}$ Ref. [10]). This implies that the more efficient quenching elicited in this protein (Fig. 1) is not due to a more efficient binding, and

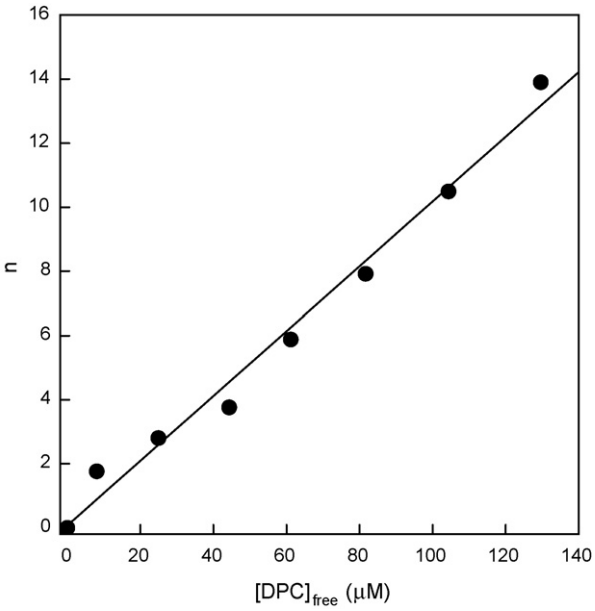

Fig. 5. Number of DPC molecules bound per HSA molecule versus the concentration of free DPC.

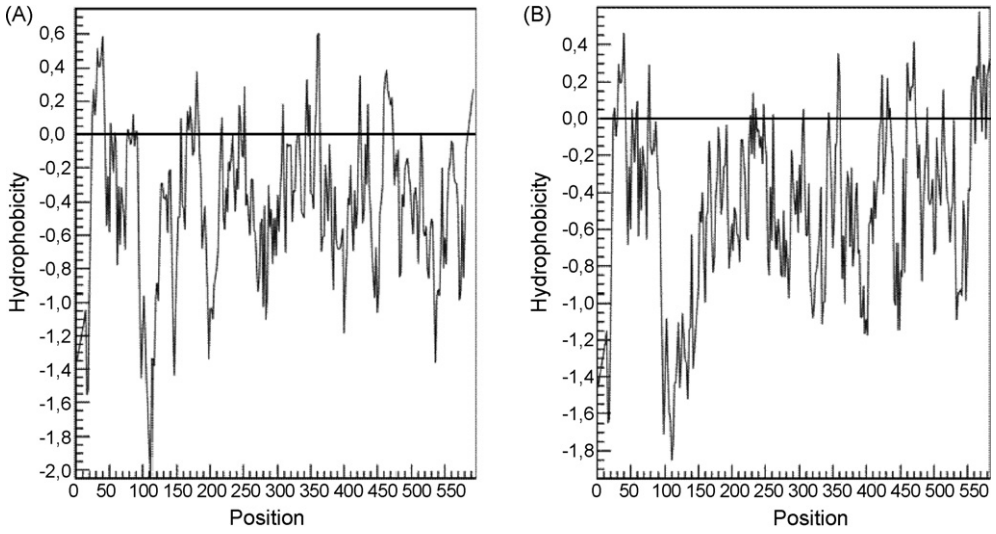

Fig. 6. Kyte-Doolittle hydrophobicity plots for HSA (A) and BSA (B); size window = 21.

can be ascribed to the presence of a much more exposed Trp group in BSA.

The data obtained employing CPC (Fig. 4) are somehow different than that reported for DPC (Fig. 5). In fact, the binding isotherm shows some cooperativity, is similar to that previously reported for BSA, and presents a clear saturation at high CPC concentrations. This saturation of the protein takes place at $[CPC]_{free}$ well below the surfactant CMC and would indicate that approximately 14 CPC molecules is the largest amount that a HSA molecule can accommodate.

The association of CPC to HSA is nearly four times larger than that estimated for DPC. For example, four surfactant molecules are bound to each protein when the free concentrations are 10 and 40 μM for CPC and DPC, respectively. An even larger difference has been reported for the association to BSA and explained in terms of a hydrophobic contribution [10]. The predominance of hydrophobic interactions, particularly for CPC, can explain the similarities between HAS and BSA isotherms (Fig. 4) given the correspondence between the hydrophobicity plots of both proteins (Fig. 6).

The presence of CPC also modifies the wavelength of maximum emission. In particular, a small shift towards shorter wavelengths is observed. In fact, the position of the centre of the emission band changes from 342 nm to 339 nm when the CPC concentration increases. This change was also observed in the interaction of CPC with BSA [10]. In this case, the effect was interpreted in terms of two possibilities: (a) selective quenching of the more exposed tryptophan groups, or (b) a change in the protein conformation. Since HSA shows the same behaviour in spite of having only one tryptophan, it could be assigned to the fact that the emitting Trp groups are more protected than in the unperturbed protein. This could be due to shielding by the tails of the bound CPC and/or to overfolding of the protein resulting from the surfactant association [7]. This explanation is in line with the maximum of the emission observed at 348 nm in the presence of urea (corresponding to the open structure).

Several arguments can be put forward to support the proposal that quenching must be due to bound surfactant molecules. In particular, if it is assumed that quenching arises from free surfactant diffusing towards the protein, data such as that shown in Fig. 1A would require k_q values larger than $10^{13}\ M^{-1}\ s^{-1}$, i.e., larger than the diffusion controlled limit.

The data derived from plots like those shown in Fig. 3 allow an evaluation of I_0/I as a function of n. This type of plot makes possible a comparison of the quenching of different surfactants towards a given protein, as well as a comparison of data obtained with a given surfactant and different proteins. The first type of comparison is shown in Fig. 7. From this figure it is possible to conclude that:

(i) CPC is considerably more efficient than DPC.
(ii) CPC does not follow a simple Ster-Volmer behaviour, with a very strong upward curvature in the I_0/I vs. n plot.
(iii) On the average, nearly eight CPC molecules are bound to each HSA molecule when the fluorescence intensity is reduced to one half.
(iv) Bound CPC molecules are less efficient quenchers for HSA than for BSA. For this last protein nearly four bound CPC molecules are needed to reduce to one half the unperturbed fluorescence. Easy access to the exposed Trp moiety in BSA could explain, at least partially, this difference.

HSA has two well defined binding sites (site 1 and site 2) and less defined binding places where strong hydrophobic interactions could be operative [1,21–23]. The Trp moiety is located in site 1 and strong quenching by an additive can be considered as indicative of a preferential association to it. Point (i) would indicate a dissimilar distribution of the two probes, with CPC residing nearer (or more distributed towards) the Trp moiety. The fact that, even for CPC, so many bound molecules are needed to quench half the Trp fluorescence would indicate that even this surfactant is not interacting predominantly with site 1. This could be a site of low affinity for CPC due to unfavourable

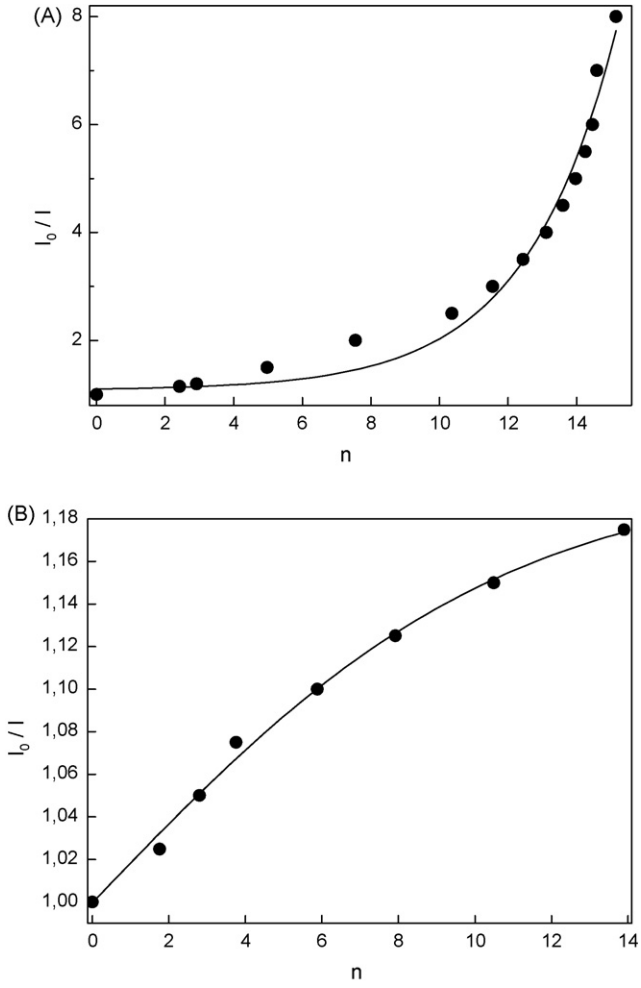

Fig. 7. Quenching of the tryptophan fluorescence in HSA by alkylpyridinium chlorides versus the number of surfactant molecules bound per HSA. (A) CPC; (B) DPC.

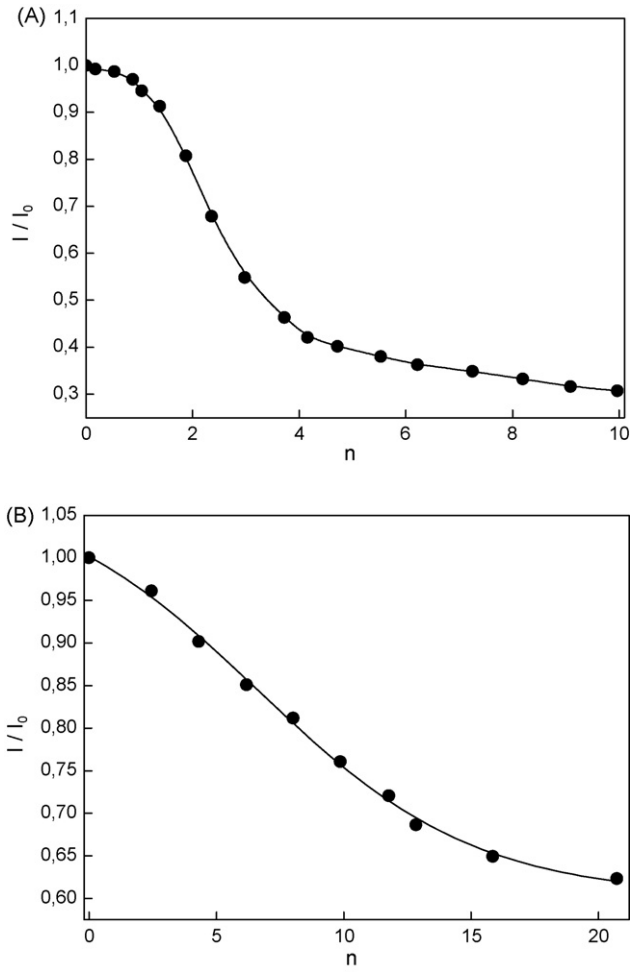

Fig. 8. Changes in the fluorescence of DSA bound to HSA as a function of the number of surfactant molecules bound per HSA. Surfactants: (A) CPC; (B) DPC.

electrostatic interactions [1]. Only when other sites are occupied does the protein allow adsorption of surfactant molecules with the pyridinium moiety near the fluorescent group, thereby quenching the protein intrinsic fluorescence. This could explain the strong upward curvature evidenced in Fig. 7.

In order to gain further information regarding the locus of CPC and DPC association to HSA, we evaluated the effect of their addition upon the fluorescence of dansyl derivatives bound to the protein. Data obtained employing dansylsarcosine (DS) and dansyl-1-sulfonamide (DNSA) are shown in Figs. 8 and 9, respectively. In these figures, I/I_0 values are given as a function of the surfactant concentration, expressed as number of surfactant molecules bound per protein. These values could be slightly overestimated since they were obtained assuming that the presence of the dansyl derivative does not modify the binding efficiency of the surfactant. However, if it is considered that the ratio dansyl derivative/protein is 0.1, the effect of the probe upon the surfactant binding must be relatively small.

Data given in Fig. 8 show similar profiles for CPC and DPC. The decrease in DSA fluorescence can be ascribed to direct quenching and/or displacement of the probe by the surfactants. In any case, these data would indicate that both surfactants can associate to (or near to) site 2, where DS molecules are specifically adsorbed [24,25]. Remarkable aspects of these data are the sigmoidal profiles and the high values of n required to quench the fluorescence. This would indicate that surfactants are very poor at displacing DS molecules, and that most bound surfactant molecules are not able to directly quench the probe fluorescence. Furthermore, the data indicate that CPC is considerably more efficient than DPC. In fact, when $n = 2$, I/I_0 values of 0.96 and 0.8 can be estimated for DPC and CPC, respectively. A predominant binding of both surfactants to site 2 would explain their low efficiency to quench excited tryptophan moieties.

The results presented in Fig. 9 for DNSA fluorescence present striking differences among surfactants and with those given in Fig. 8. This would confirm that these two surfactants bind differently to HSA. In particular, the results of CPC could be interpreted by assuming that its initial association to the protein increases the lipophilic character of site 1, where DNSA molecules are preferentially adsorbed. At higher n values, significant adsorption of CPC into site 1 would expel the probe (decreasing its emission) and/or quench its fluorescence. Furthermore, this late association to site 1 can explain the upward

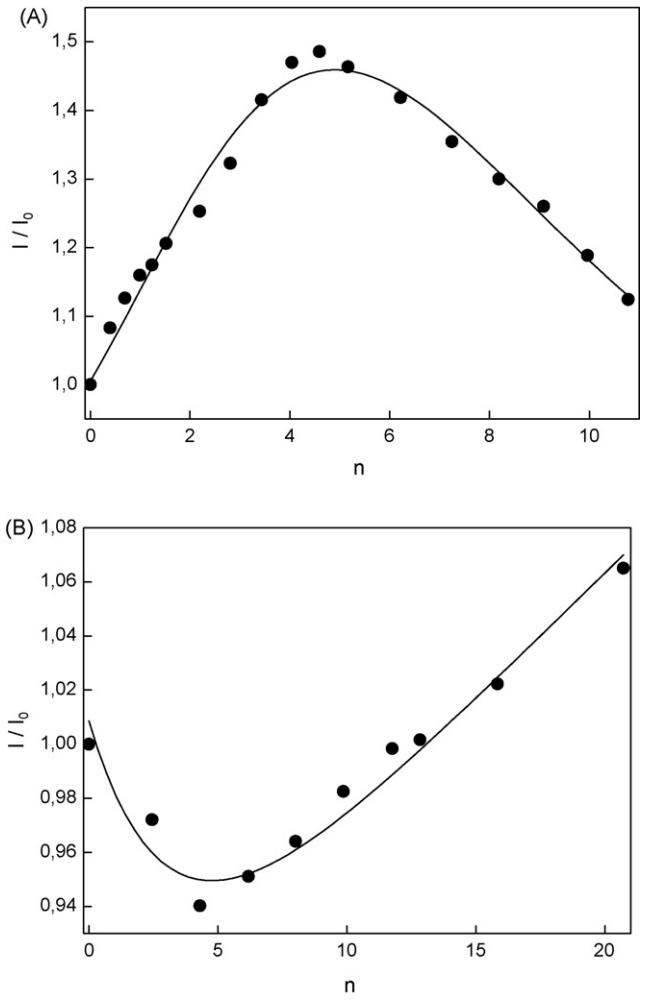

Fig. 9. Changes in the fluorescence of DNSA bound to HSA as a function of the number of surfactant molecules bound per HSA. Surfactants: (A) CPC; (B) DPC.

curvature in Fig. 7A. Data obtained employing DPC show a small initial decrease, attributable to association into site 1, followed by a noticeable increase in DNSA fluorescence at high surfactant concentrations. The variety of factors that could lead to this increase in fluorescence (HSA conformational changes, formation of hydrophobic microdomains, shielding of the probes by the surfactant tails) does not allow further interpretation of this behaviour. However, the change in the slope of the plots is compatible with the proposal of Bordbar and Taheri-Kafrani [12] that the degree of binding depends upon the substrate concentration. In conclusion, at low occupation numbers both surfactants bind to different locations. In particular, the displacement experiments suggest that:

- CPC and DPC bind to site 2 or near it, quenching and/or displacing the probe bound to this site (DS) and increasing the fluorescence of the probe bound to site 1 (DNSA).
- DPC is less efficient in modifying dansyl derivatives fluorescence, weakly interacting with both sites 1 and 2.

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