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Quenching of BSA intrinsic fluorescence by alkylpyridinium cations Its relationship to surfactant-protein association

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Abstract

Alkyl pyridinium ions readily quench the fluorescence arising from Trp groups incorporated to a dipeptide (Ala–Trp) or protein (BSA). The quenching of the dipeptide fluorescence takes place with a Stern–Volmer constant of $730\,M^{-1}$ in aqueous solution, irrespective of the length of the alkyl chain (octyl, dodecyl or hexadecyl). On the other hand, the quenching of BSA fluorescence strongly depends upon the length of the alkyl chain. Octyl pyridinium ion presents a normal behavior, with K_{SV} values of 310 and $280\,M^{-1}$ in aqueous solution and in presence of 8 M urea, respectively. These values are independent of the protein concentration. Dodecyl and hexadecyl pyridinium ions are considerably more efficient as quenchers. Stern–Volmer plots show a strong upward curvature, the quenching efficiency decreases when the protein concentration increases, and is considerably reduced in presence of salt (phosphate) or urea. These results are explained in terms of a static quenching between the adsorbed alkyl pyridinium and the excited Trp groups. From the dependence of the quenching efficiency with the protein concentration it is obtained the alkyl pyridinium/BSA association constant. The values obtained indicate that the binding is dominated by hydrophobic (dependence with the alkyl chain length and urea concentration) effects and, in minor degree, by electrostatic interactions.

Keywords: Bovine serum albumin; Alkylpyridinium salts; Fluorescence quenching; Surfactant-protein association

1. Introduction

The interaction between surfactants and proteins has been extensively studied. A relevant point is which are the factors that determine the association extent and the characteristics of the adsorption isotherms [1–6]. Another relevant aspect is which are the protein conformational changes elicited by the protein-surfactant association [7,8], and how these changes modify the protein function in the case of enzymes, transport proteins or toxins [9–12].

Bovine serum albumin (BSA) is a protein with hydrophobic patches that could be the initial targets of their association to surfactants [1,13]. Several studies have been devoted to study its interaction with surfactants, in particular, anionic surfactants, such as sodium dodecylsulfate [1,4,5]. On the other hand, studies on the effect of cationic surfactants association to proteins are more scarce [1–3]. In general, these surfactants interact less strongly with proteins [4,14], mainly as a consequence of a smaller relevance of electrostatic interactions at the pHs of interest.

Alkyl pyridinium halides constitute a family of cationic surfactants particularly suitable to study surfactants/protein association. The pyridinium moiety constitute an efficient fluorescence quencher [15] that could reduce the intrinsic protein fluorescence. It can be expected that this quenching efficiency would increase if the local surfactant concentration is increased by its association to the protein. In order to assess this possibility, we have performed an analysis of BSA intrinsic fluorescence in water, a phosphate buffer solution and in urea–water solutions, as a function of the concentration of alkyl pyridinium salts. Furthermore, and to evaluate the effect of the alkyl chain length, we compare the results obtained employing octylpyridinium (OP), dodecylpyridinium (DP) and cetylpyridinium (CP) salts. The dipeptide tryptophan–alanine was employed as a low molecular weight model compound.

2. Experimental

Bovine serum albumin (BSA) and the dipeptide tryptophan–alanine (Trp–Ala) were obtained from Sigma and employed as received. Octylpyridinium (OP) bromide was kindly provided by C. Gamboa (University of Chile).

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Dodecylpyridinium chloride (DP), cetylpyridinium chloride (CP), and urea (Merck) were purified by successive recrystallizations from methanol. 9-anthracenemethanol (9-AM) was a Molecular Probe product, employed as received.

Fluorescence measurements were carried out in an Aminco Bowman spectrofluorometer. Excitation was carried out at 285 nm, and emission was measured at 340–348 nm in order to selectively detect Trp group derived fluorescence [16].

Critical micelle concentrations (CMC) of CP under different conditions were derived from fluorescence intensities versus surfactant concentration plots employing 9-anthracenemethanol as probe.

Measurements were carried out at room temperature in aqueous solutions (pH ≈ 7.7 irrespective of BSA concentration) or in phosphate buffer (50 mM, pH 7.0) in presence or in absence of urea.

3. Results

3.1. CMC of CP under the employed experimental conditions

The fluorescence of 9-anthracenemethanol is poorly quenched by submilimolar concentrations of the pyridinium salts. A noticeable increase in quenching efficiency is observed after the CMC, due to a favorable partitioning of the probe towards the micellar pseudophase. A typical plot of the ratio between the fluorescence intensities in absence and in presence of CP (I_o/I) (being I_o and I the fluorescence intensities in the absence and presence of the surfactant, respectively) versus the surfactant concentration is shown in Fig. 1. From this type of plot it can be obtained the surfactant CMC. The values obtained under several conditions are summarized in Table 1. The value obtained in water is close to that (0.9 mM) previously reported [17]. The data given in Table 1 show, as expected, a decreased CMC in buffer, and increased values when the urea concentration increases.

3.2. Quenching of the model compound Trp–Ala

Trp–Ala was selected as a model compound in order to evaluate the quenching efficiency of the surfactants under conditions of minimal fluorophore/surfactant interaction. Although Stern–Volmer like plots show a rather big scatter, due to the small quenching (<20%) at the accessible surfactant concentrations, the data obtained can be summarized as:

(i) linear plots were obtained;
(ii) the quenching extent was, in a given medium, similar for OP, DP and CP;
(iii) the quenching was rather similar in water and buffer, but it was significantly faster in a 4 M urea solution. The values obtained are collected in Table 2;
(iv) the quenching extent does not depend on the Trp–Ala concentration (data not shown).

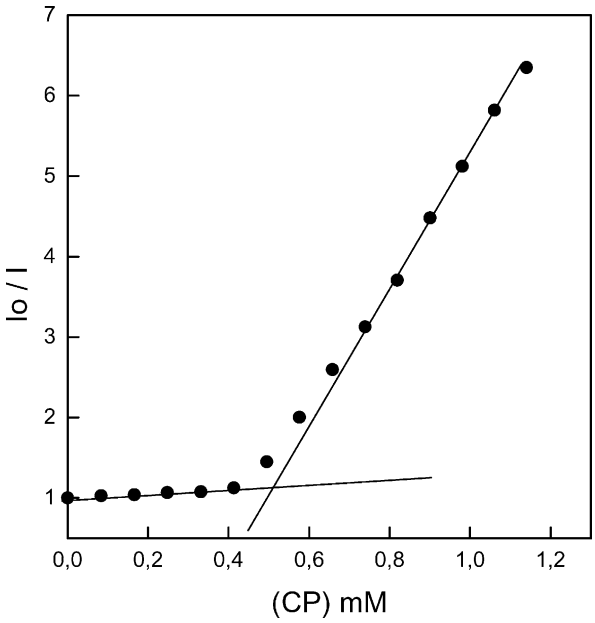

Fig. 1. Plot of 9-antrhacenemethanol fluorescence intensity as a function of CP concentration. Data obtained in water.

Table 1
Cetyl pyridinium CMC values

Solvent	CMC (mM)
Water	0.48
Buffer[a]	0.075
Urea 2 M in water	1.22
Urea 4 M in water	1.9
Urea 8 M in water	>2.0[b]
Urea 4 M in buffer	0.35

[a] Buffer phosphate 50 mM, pH 7.0.
[b] Turbidity observed at higher concentrations.

3.3. Quenching of BSA fluorescence by OP

Quenching of BSA fluorescence by OP are smaller, but of the same order of magnitude, than that of the model compound Trp–Ala. The Stern–Volmer constants (K_{SV}) obtained in the different media are collected in Table 3. The low K_{SV} values, and the fact that they are very little dependent on the media, are compatible with a negligible association between this short alkyl chain surfactant and the protein.

Table 2
Stern–Volmer constants, K_{SV}, for the quenching of Trp–Ala fluorescence by pyridinium salts in different media

Solvent	K_{SV} (M^{-1})
Water	730 ± 80
Buffer	1000 ± 300
4 M urea in water	2200 ± 150

Table 3
Stern–Volmer constants, K_{SV}, for the quenching of BSA by OP

Solvent	K_{SV} (M^{-1})
Water	250 ± 30
Buffer	430 ± 30
Urea 4 M in water	500 ± 50
Urea 8 M in water	280 ± 50

3.4. Quenching of BSA fluorescence by DP and CP salts

The quenching of the fluorescence by long chain surfactants, particularly CP, are widely different. In the first place, the quenching is considerably faster (Fig. 2). In the second place, the quenching efficiency decreases when the urea concentration increases and decreases in presence of buffer (Fig. 3). In the third place, the quenching efficiency decreases when the protein concentration increases (Fig. 4). All these facts are indicative of a strong surfactant-protein association, particularly for CP. In fact, the dependence with the protein concentration can be utilized to evaluate the association extent by plotting the surfactant concentration required to reach a given I_o/I value as a function of the protein concentration [5]. This type of plot is shown in Fig. 5. From these plots are obtained the surfactant concentration remaining in the aqueous pseudophase (from the intercept) and the average number of surfactant molecules bound to each protein (**n**) from the slope. If a pseudo association constant (K) is defined by

$$K \equiv \boldsymbol{n}/[S]_f \quad (1)$$

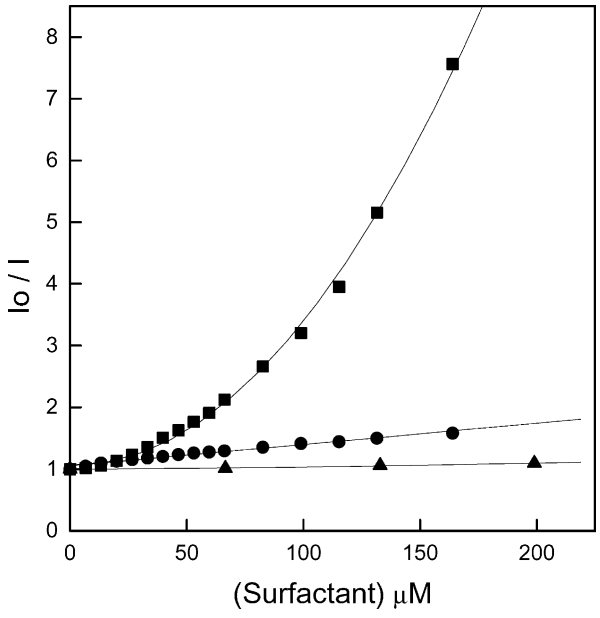

Fig. 2. Quenching of the fluorescence from an aqueous BSA (32 μM) solution by alkylpyridinium surfactants: (■) CP; (●) DP; (▲) OP.

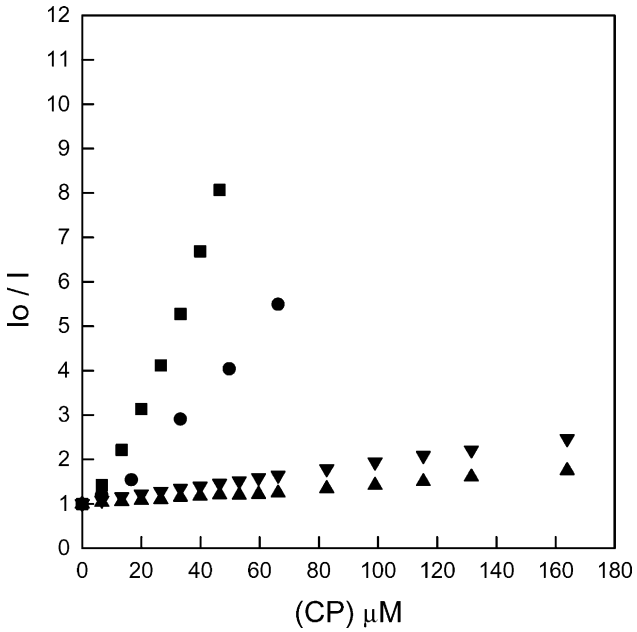

Fig. 3. Quenching of BSA (32 μM) florescence by CP. Data obtained in: (■) water; (●) buffer; (▼) urea 4 M; (▲) urea 8 M.

its value can be obtained from the quotient between the slope and the intercept. The values of K obtained by this procedure are collected in Table 4.

The presence of the surfactant also modifies the wavelength of maximum emission. In particular, it is observed a small shift towards shorter wavelengths in the presence of CP. In fact, the position of the center of the fluorescence band changes from 342 to 339 nm when the surfactant

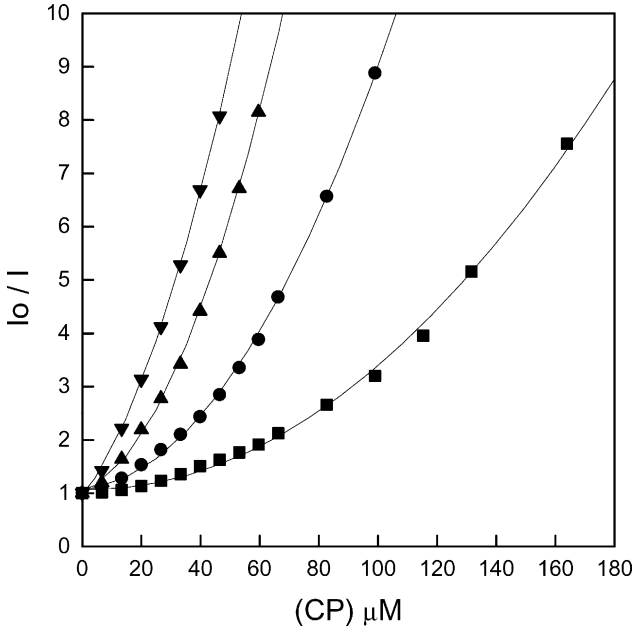

Fig. 4. Plot of I_o/I as a function of CP concentration. Data obtained in water at different BSA concentrations: (■) 32 μM; (●) 16 μM; (▲) 8 μM; (▼) 4 μM.

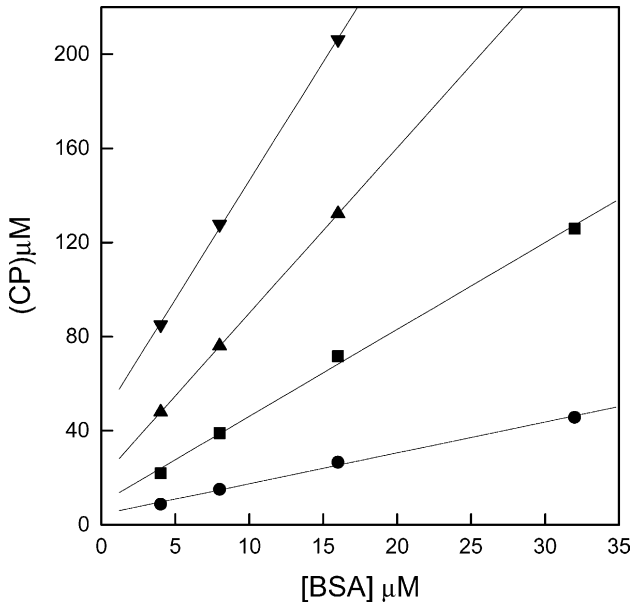

Fig. 5. Plot of the surfactant (CP) concentrations needed to reach a given I_o/I value as a function of the protein concentration. I_o/I values: (●)1.25; (■) 2.06; (▲) 4.0; (▼) 8.06.

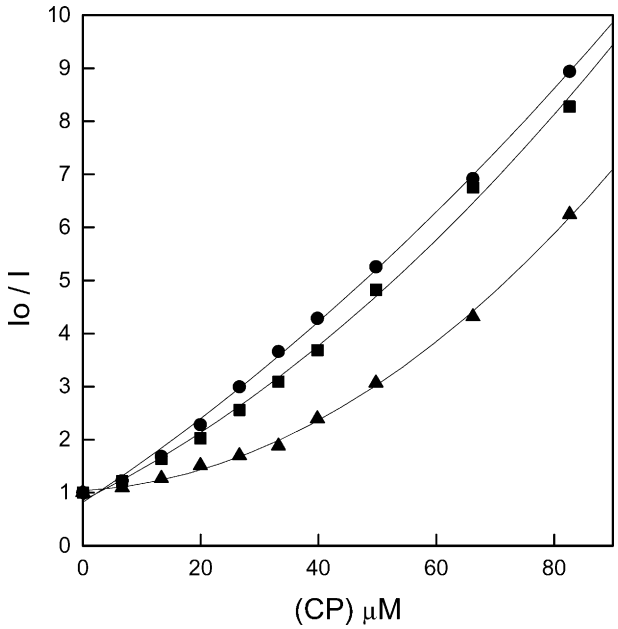

Fig. 6. Dependence of BSA fluorescence quenching by CP on the solution pH. Data obtained at pH 8 (●); pH 7 (■); pH 6 (▲).

Table 4
Values of the free surfactant $[S]_f$ and association constants (K) measured a the given I_o/I values

Surfactant	Solvent	I_o/I	$[S]_f$ (μM)	K (10^5 M^{-1})
CP	Water	1.2	4.4	3.0 ± 0.7
	Buffer	1.5	10.3	1.5 ± 0.7
	Urea 4 M, water	1.2	5.1	1.0 ± 0.8
	Urea 8 M, water	1.2	49	0.07 ± 0.01
DP	Water	1.2	26	0.16 ± 0.02
	Buffer	1.05	16	0.5 ± 0.4

concentration increases. The change in band position can be related to a selective quenching of the more exposed Trp groups, and/or to a change in the protein conformation. If it is considered that the position of the band of the open structure (in urea 8 M) is 348 nm, this could indicate an overfolding of the protein resulting from the surfactant association [4].

The quenching efficiency of CP is also pH dependent. Data obtained at three pHs are shown in Fig. 6. These data show a noticeable reduction in the quenching efficiency at low pH. This pH dependence is not observed when Trp–Ala is employed as fluorophore (data not shown).

4. Discussion

4.1. Hydrophobic contribution to the surfactant binding to BSA

All the data obtained in the present work support a surfactant-protein interaction in which hydrophobic forces play a dominant role. In particular, the effect of the surfactant chain length (quantified by the association constants) and the effect of urea upon CP binding constants point to a relevant role of hydrophobic forces. In fact, chain length and urea effects upon the binding constant are similar to the effect of these factors upon the surfactant CMCs. So, changing the alkyl chain from dodecyl to hexadecyl increases the binding constant by a factor of near 20, while it decreases the CMC from 14.7 [17] to 0.48 mM (a factor near 30). Similarly, the presence of 4 M urea increases the CMC value of CP by a factor 4.0, while it decreases the binding constant between the surfactant and BSA by a factor 3.0. All these data suggest that, when bound to BSA, the alkyl chain of the surfactants is strongly protected from the external solvent and anchored in hydrophobic protein pockets.

4.2. Relevance of surfactant-BSA electrostatic interactions

The presence of buffer (phosphate, 50 mM, pH 7.0) has opposite effects on the CMC and the BSA-surfactant binding. In fact, while the high ionic strength favors the formation of the micelles, lowering in a factor 6.4 the CMC of CP, it reduces the value of CP binding constant to BSA (the error in DP binding constant in buffer is too large to allow a comparison with the data in water). The decrease in CP binding constant with the solution ionic strength can be explained in terms of the low cooperativity of the surfactant-BSA association (see following) and favorable interactions between negatively charged groups in the protein and the cationic surfactant. Under these conditions, the ionic strength effect would be to reduce the binding. On the other hand, and due

Table 5
Free surfactant concentrations (mM) required to give an I_o/I value of 1.2

Solvent	OP	DP	CP
Water	0.64	0.026	0.0044
Buffer	0.46	0.047	0.005
Urea 4 M	0.4	–	0.005
Urea 8 M	0.7	–	0.05

to the high cooperativity of micelle formation, the presence of salt would reduce the electrostatic interaction among the surfactant heads, leading to the expected decrease in CMC values. The lowering in CP quenching efficiency observed at lower pHs (Fig. 6) is fully compatible with this interpretation.

4.3. Quenching by the adsorbed CP molecules

The quenching of BSA fluorescence by the surfactants can be due to the free surfactant and/or to the bound molecules. An estimate of the contribution of the free surfactant can be obtained by a comparison of the data obtained with compounds of different hydrophobicity. These data are collected in Table 5, where are given the free surfactant concentrations when I_o/I reaches a value of 1.2. The data given in this Table were derived by assuming that OP is negligible bound to the protein. The very large difference between the quenching efficiency of BSA for the different surfactants (not observed in the quenching of Trp–Ala, Table 2), as well as the decreased quenching efficiency of CP in presence of 8 M urea (effect not observed when OP is employed as quencher, Table 3) indicates that quenching by DP and CP is mostly due to the adsorbed surfactant. This is further supported by the fact that the data given in Table 4 allow to conclude that similar **n** values are required to produce the same I_o/I value employing DP or CP as BSA fluorescence quenchers.

The noticeable upward curvature in the Stern–Volmer plot of the data obtained in the CP/BSA system (Fig. 2) can be due to a strong cooperativity in the adsorption and/or to static quenching by the bound surfactant. From a series of plots like those shown in Fig. 5 it can be obtained both the pseudo association constant as a function of the free surfactant, and I_o/I values as a function of the bound surfactant (Fig. 7). The values of K are very little dependent with the free surfactant concentration This would indicate a low cooperativity in the adsorption. Actually, K_p values decrease from 0.30 to 0.22 when the free surfactant increases from 4 to 50 μM. This small decrease of K_p with free surfactant could be related to changes in BSA conformation and/or to an increase in the electrostatic repulsion when the number of bound surfactants per protein increases from ca. 1.3 to 10.1.

In order to test the relevance of static quenching, $\ln(I_o/I)$ values were plotted against n in Fig. 7. The linearity of the plot would indicate an almost totally static quenching by the bound surfactant. However, it is interesting to note that the slope of the plot is far from one, as expected if quenching

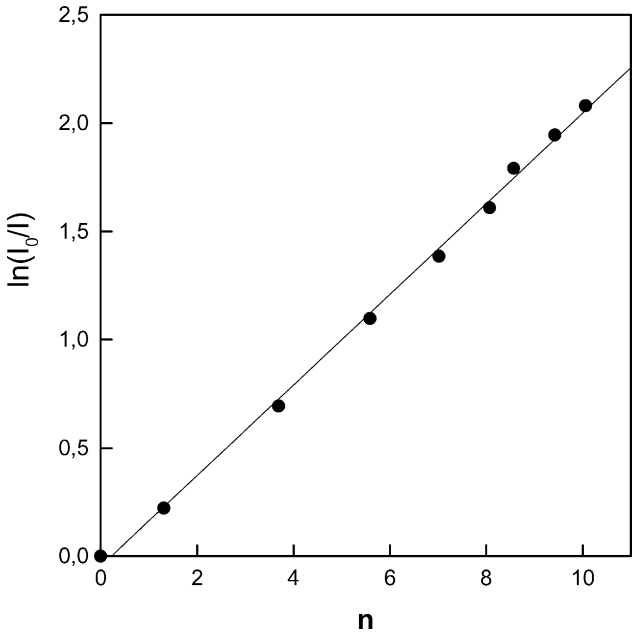

Fig. 7. Plot of $\ln(I_o/I)$ against the average number (***n***) of surfactant molecules bound per BSA molecule. Data obtained in water employing CP as quencher.

takes place if at least one CP molecule is bound to the protein bearing the excited Trp moiety. In fact, the slope of Fig. 7 is nearly 0.2. This can be explained if each protein has (on the average) five distinct cages or, in other words, that each protein must have at least five bound CP molecules (calculated assuming Poisson distribution) when static quenching takes place. This static quenching can hardly be explained in terms of resonance energy transfer (Förster type) due to the lack of significant overlap of donor (emission) and acceptor (absorption) bands, and to their low oscillator strengths. It can be better understood by considering (five) distinct "binding sites" on each protein, and that quenching efficiently occurs if at least one quencher molecule is incorporated to the critical one. Interestingly, the data given in Table 4 indicate that, in urea 8 M, the value of ***n*** required to reach a given I_o/I value (1.2) is nearly four times smaller than in pure water. This would indicate that in the expanded protein there are less binding sites than in the native macromolecule. This, together with the decreased free energy of the surfactant in the presence of urea, could contribute to the decreased binding constant measured in presence of the denaturant.

5. Conclusions

Alkylpyridinium ions are efficient quenchers of the fluorescence of Trp residues in BSA and small peptides. The quenching is particularly effective for long alkyl chain ions and BSA. This is due to association of the alkylpyridinium ions to the protein. This association is mainly driven by hydrophobic forces.

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