Biochemical Pharmacology, Vol. 44, No. 4, pp. 824–826, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Fluorescence quenching of human serum albumin by xanthines

(*Received* 6 *September* 1991; *accepted* 18 *May* 1992)

Abstract—A study of the fluorescence quenching of human serum albumin (HSA) by caffeine, theophylline and theobromine, based on temperature dependence, has shown that it is predominately static. This quenching mechanism is due to the formation of a xanthine–HSA non-fluorescent complex. The Stern–Volmer equation let us determine the association constants. It seems that the quenching of the protein fluorescence depends on the number and position of the methyl groups. The temperature dependence of the association constant is used to estimate the values of the thermodynamic parameters involved in the interaction of the drugs with HSA. All three binding processes are exothermic and probably hydrophobic, and hydrogen bonds play a significant role in the stabilization of such complexes. The enthalpy and entropy changes observed appear to compensate each other to produce a relatively small Gibbs free energy.

Caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) are of particular interest for their clinical, pharmacological and biological properties [1, 2].

The binding of several of these compounds to both bovine serum albumin (BSA*) and human serum albumin (HSA) has been studied in detail, proving that interaction with HSA is dependent on the nature of substituents at the 1 and 7 position and is affected by the pH of the medium [3]. By measuring fluorescence quenching, Nishijo *et al.* [4] found that theophylline binds to the tryptophan residue on BSA by van der Waals forces, but details of the quenching mechanism were not known.

This paper reports a physicochemical study of the quenching mechanism of the tryptophan fluorescence of HSA by xanthines, taking as a model the review presented by Eftink and Ghiron [5], and studies by other workers with this technique [6, 7]. Furthermore, a thermodynamic analysis based on the temperature dependence of the apparent binding constant was performed in order to elucidate molecular details of the binding process.

Materials and Methods

The following high purity compounds were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.): fatty acid-free human albumin and theobromine; and from Aldrich-Chemie: caffeine and theophylline. Solutions were prepared in 0.05 M phosphate buffer, pH 7.4 ± 0.1. A typical binding experiment was performed at a constant protein concentration of about 2.5×10^{-6} M, generally following the method described earlier [8] and with the same instruments. Three temperatures were tested: 15, 25 and 37 (°C).

Data on fluorescence intensities were analysed according to the Stern–Volmer equation:

$$F_0/F = 1 + KM \quad (1)$$

by plotting F_0/F versus M, where F_0 and F are the fluorescence intensities in the absence and in the presence of quencher M respectively, and K is the Stern–Volmer quenching constant.

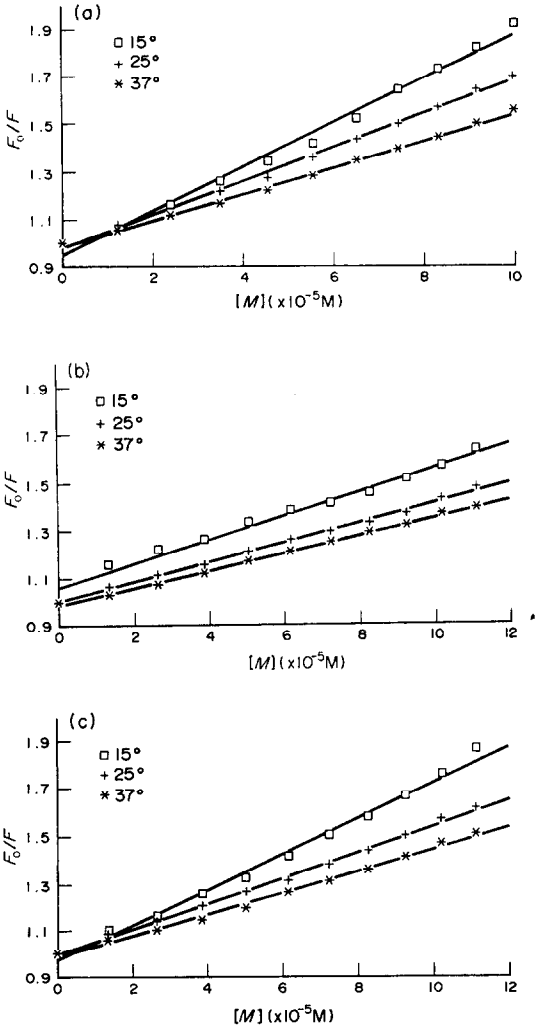

Fig. 1. Stern–Volmer plots for the quenching of the fluorescence of HSA by: (a) caffeine, (b) theophylline and (c) theobromine, at various temperatures (°C). The slopes of these representations show the temperature dependence of the Stern–Volmer constant to differentiate between static and dynamic quenching mechanisms.

* Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; F_0, fluorescence intensity in the absence of quencher; F, fluorescence intensity in the presence of quencher; M, quencher; K, Stern–Volmer constant or binding constant; ΔG, the Gibbs free energy change; ΔH, the binding enthalpy change; ΔS, the entropy change; T, temperature (°K).

Table 1. Binding constants and thermodynamic parameters for the interaction of caffine, theophylline and theobromine with HSA

	T (°K)	K (M^{-1})	ΔG (kJ/mol)	ΔS (J/mol)
Caffeine*	288	9320	−21.86	14.82
	298	7168	−21.99	14.69
	310	5577	−22.23	14.89
Theophylline†	288	5006	−20.40	20.42
	298	4077	−20.60	20.40
	310	3291	−20.87	20.51
Theobromine‡	288	7580	−21.39	15.96
	298	5464	−21.32	15.19
	310	4612	−21.74	15.96

ΔG was calculated from the relation $\Delta G = -RT \ln K$. The enthalpy was obtained from the slope of van't Hoff plots and the entropy evaluated from the relation $\Delta S = -(\Delta G - \Delta H)/T$.

* $\Delta H = -17.62$ kJ/mol.

† $\Delta H = -14.52$ kJ/mol.

‡ $\Delta H = -16.79$ kJ/mol.

Results and Discussion

As can be seen in Fig. 1, the slopes decrease with temperature for all three xanthines studied here, a characteristic that is consistent with the static type of quenching mechanism. Static quenching arises from the formation of a dark complex between fluorophore and the quenching agent, and the quenching constant can be interpreted as the association constant or binding constant of the complexation reaction in all three systems [9]. The association constant may be described in its general form as:

$$K = \frac{[\text{xanthine–HSA}]}{[\text{xanthine}]\,[\text{HSA}]} \qquad (2)$$

It has been pointed out previously that for caffeine–HSA and theophylline–HSA interactions [3, 4], according to the Scatchard plot, there is a single binding site in the protein and the values given for the binding constants agree very closely with those obtained by us in Table 1. In addition, Nishijo *et al.* [4] observed a similar temperature dependence of the theophylline–BSA binding constant. Hence, the above considerations allow us to assume that the ligands bind to only one site on the protein.

Despite the very high molar ratios between the ligands and albumin, the relatively small values of K found in this work may be due to the low affinity of the xanthine compounds to HSA, since this agrees with findings showing that caffeine binds poorly to plasma albumin, at about 10–30% (see Ref. 1, p. 48).

The linearity of the Stern–Volmer plots indicates that a single mechanism probably dominates the quenching process. In these representations the intercept values are in the range 0.971–1.059. An analysis of variance (ANOVA) was also performed in all cases showing that the differences between the slopes of the lines are statistically significant.

Fig. 2 is an example of the fluorescence quenching on HSA by 0.1 mM caffeine, and 0.11 mM theophylline and theobromine, at 25°. This graph allows us to analyse two effects. First, the addition of quencher changed neither the shape nor the maximum wavelength of the protein emission spectra, suggesting that no conformational changes are induced in HSA by xanthines under the conditions studied here. Second, the fluorescence intensity was quenched by caffeine > theobromine > theophylline, implying that the quenching of protein fluorescence depends on the number and position of the methyl groups. Caffeine has three methyl groups at positions 1, 3 and 7 and fluorescence quenched was about 50% while theophylline and theobromine have only two methyl groups at positions 1,3 and 3,7 respectively, and fluorescence quenched was 30 and 40%, respectively. A greater increase in quenching appears to depend on the methyl group at the 7 position because theobromine is a better quencher than theophylline. As a comparison, Lehrer [10] had observed that a greater increase in quenching appears to depend on the protonation of certain groups in the quencher.

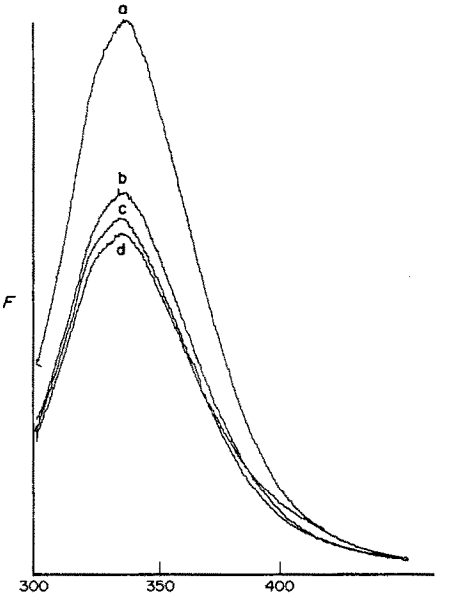

Fig. 2. Effect of xanthines on the fluorescence emission of HSA at 25°. (a) Fluorescence emission spectra in the absence of xanthines; (b, c, d) in the presence of 0.11 mM theophylline, 0.11 mM theobromine and 0.1 mM caffeine, respectively. The excitation and emission wave lengths were 290 and 340 nm, respectively, and the slit-width was 4 nm.

The static quenching constant is a measure of the ability of a quencher molecule to remain in contact with the tryptophan residue on HSA, suggesting the possibility that 3,7 xanthine derivatives could be accommodated inside this protein better than 1,3 derivatives.

The temperature dependence of the binding constant also allows us to estimate the thermodynamic parameters by a van't Hoff plot, in analogy with other drug interactions [11, 12]. On the basis of the results of the thermodynamic parameters presented in Table 1, it can be seen that xanthine–HSA interactions are exothermic with values of ΔH in the range of -14.52 to -17.62 kJ/mol. These values have the same sign and a similar magnitude as those values obtained for other drug–HSA interactions [13, 14]. Thus, it is obvious that the major contributing factor in the stabilization of the complexes is enthalpic rather than entropic in origin. It is possible that hydrogen bonding may occur as a result of the complexation reactions.

As in our case, a positive entropy has been reported for many interactions between organic molecules and albumin [15, 16] suggesting that hydrophobic bonds can also be involved in the association process. In fact, the tryptophan residue in HSA lies in a microenvironment which is essentially hydrophobic and in this region fats, drugs and other hydrophobic material are bound [17]. Hence, hydrophobic interactions and hydrogen bonds could be the dominant physical forces behind the formation and stabilization of caffeine–HSA, theophylline–HSA and theobromine–HSA complexes.

Acknowledgements—The authors wish to thank Prof. Dr Manuel Cortijo for his skillful technical assistance.

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