Interaction of Curcumin with Phosphatidylcholine: A Spectrofluorometric Study

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Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], the main constituent of the rhizomes of the plant *Curcume longa* L. (turmeric), is a powerful antioxidant in both enzymatic and nonenzymatic systems. The interactions of curcumin with egg and soy phosphatidylcholine were followed by fluorescence spectroscopy. Curcumin had very weak fluorescence in aqueous system, which was enhanced in apolar environments. Curcumin emitted at 490 nm after being excited at 451 nm in phosphatidylcholine micelles. The equilibrium constants for the interaction of curcumin with egg and soy phosphatidylcholine were $(3.26 \pm 0.2) \times 10^5$ and $(2.64 \pm 0.2) \times 10^5$ M^{-1}, respectively. From the Scatchard plot of the fluorometric data, it was inferred that one molecule of curcumin could bind six molecules of phosphatidylcholine. The equilibrium constant for the phosphatidylcholine−curcumin interaction decreased with temperature, indicating the amphiphilic nature of curcumin. The ΔG, ΔH, and ΔS values obtained for the interaction of egg phosphatidylcholine−curcumin were -7.8 ± 0.3 kcal/mol, -9.6 ± 0.4 kcal/mol, and -6.8 ± 0.2 cal/mol/K, respectively. The fluorescence anisotropy measurements of curcumin with phosphatidylcholine suggested that the anisotropy of the curcumin molecule did not change in phosphatidylcholine. The interaction of divalent metal ions with phosphatidylcholine−curcumin in comparison with phosphatidylcholine−1-anilino-8-naphthalenesulfonic acid complex suggested the strong binding of curcumin to metal ions.

Keywords: *Phosphatidylcholine; micelles; fluorescence; curcumin*

INTRODUCTION

Lipid peroxidation of biomembranes has attracted much attention in relation to antioxidative damage leading to a wide variety of pathological conditions. Curcumin, a β-diketone, contains two ferulic acid molecules linked via a methylene bridge at the carbon atoms of the carboxyl groups (Sharma, 1976). Curcumin is the major constituent of the spice turmeric and has received much attention due to its antioxidant and anti-inflammatory activities (Rao et al., 1982; Toda et al., 1985). However, it is not known what part of the curcumin molecule is important for its biological activity, but, it has been suggested that hydroxyl groups of the benzene rings, double bonds in the alkene part of the molecule, and/or the central β-diketone moiety may be responsible for its high biological activity (Osawa et al., 1995). The β-diketone moiety is highly enolized, and it has been suggested that this group can chelate the metal ion (Osawa and Namiki, 1985).

It has been shown that curcumin can access both hydrophobic and hydrophilic environments of a microheterogeneous system; the corresponding radical is stable with respect to oxygen and can be regenerated by both natural antioxidants vitamins C and E in hydrophilic and hydrophobic environments, respectively (Gorman et al., 1997). Under certain conditions, curcumin accelerates oxygen radical formation by reducing ferric ion to give the more active iron(II) (Kunchandy and Rao, 1989). Recently, we have demonstrated that curcumin bound to phosphatidylcholine micelles can inhibit soybean lipoxygenase 1 activity. Its inhibitory activity was due to binding to the active site iron of lipoxygenase (Began et al., 1998). Furthermore, most of the enzymes, which are inhibited by curcumin, are membrane bound and some of them require a metal ion for their activity (Reddy and Aggarwal, 1994; Yamamoto et al., 1997). However, information on the mechanism of the inhibition by curcumin on these enzymes is scanty. In an attempt to understand the mechanism of action of curcumin, we examined its amphilic interaction with phosphatidylcholine micelles and its ability to interact with metal ions after binding to micelles.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine, soy phosphatidylcholine, curcumin, and sodium deoxycholate were purchased from Sigma (St. Louis, MO). Organic solvents and inorganic salts were of analytical reagent grade obtained from Qualigens, India.

Preparation of Mixed Micelles. Mixed micelles were prepared using the mixture of phosphatidylcholine (PC) and deoxycholate (DOC). All solutions had the same applied DOC to PC molar ratio of 2.0. After the PC and DOC had been solubilized in a chloroform/methanol (2:1) mixture, the solvent was evaporated by flash evaporator and dried in the presence of nitrogen gas. The resulting thin film was solubilized in 50 mM Tris-HCl, pH 7.4, and then sonicated for 5 min using a bath-type sonicator.

Spectroscopic Measurements. *Absorption Spectra Measurements.* The interaction of curcumin with PC micelles was followed by recording the absorption spectra of curcumin in

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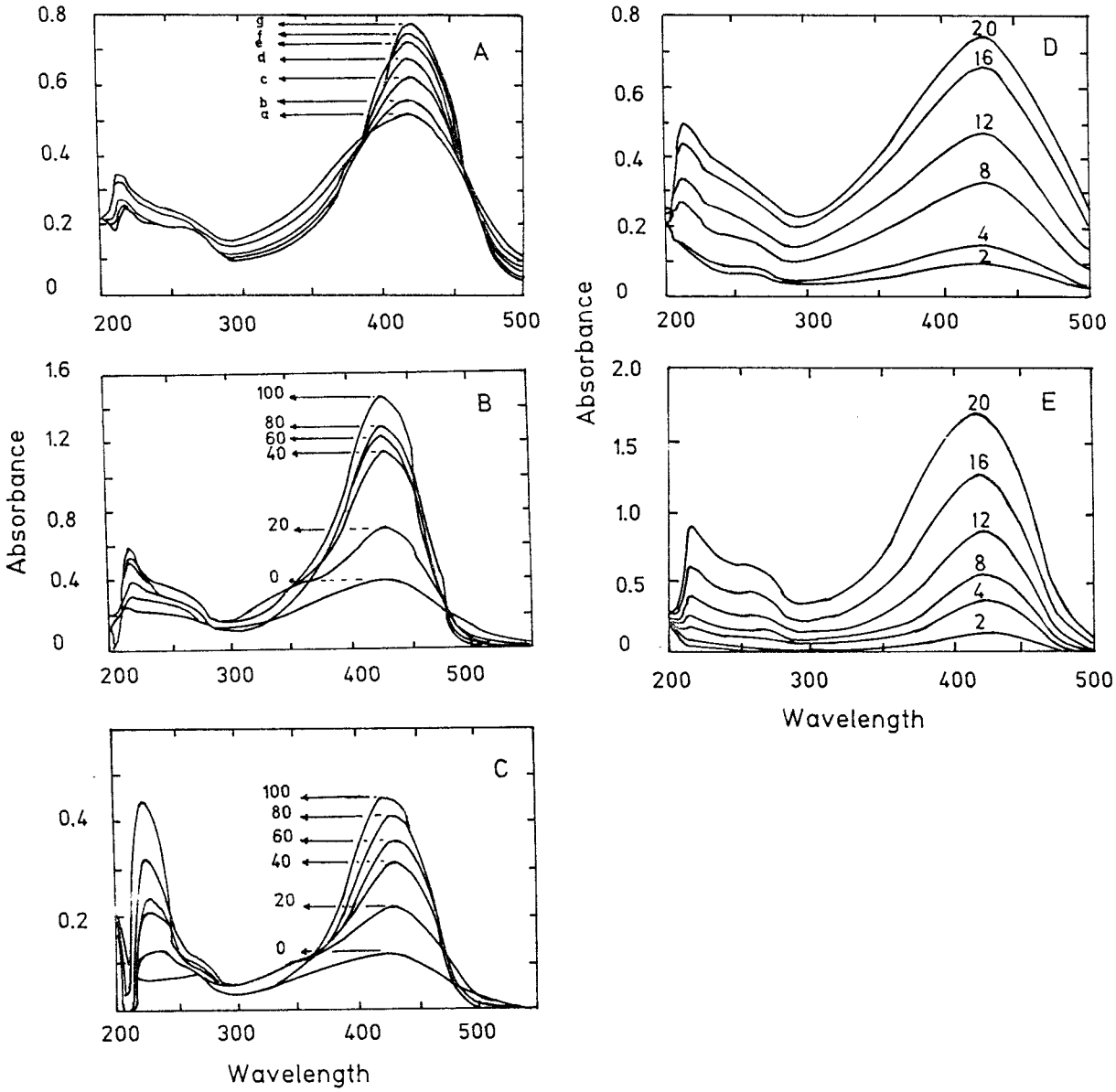

Figure 1. Absorption spectra of curcumin under various conditions: (A) absorbance increase of curcumin (20 μM) at different concentrations of PC (a, 0 μM; b, 5 μM; c, 10 μM; d, 15 μM; e, 20 μM; f, 25 μM) in 50 mM Tris-HCl, pH 7.4, and at different concentrations of ethanol (B) and methanol (C) (0, 20, 40, 60, 80, and 100%, v/v); (D) different concentrations of curcumin (2, 4, 8, 12, 16, and 20 μM) in buffer and (E) in fixed concentration of egg PC micelles (100 μM). The spectra presented are an average of three sets of measurements. Measurements were made in the region of 200−500 nm.

the region of 200−600 nm using a Shimadzu UV-160A spectrophotometer. Spectra were recorded in various concentrations of micellar PC and methanol/ethanol solutions. Spectra were recorded for various concentrations of curcumin in a fixed concentration of micellar PC and in buffer solutions. The spectra presented are an average of three sets of measurements.

Fluorescence Studies. Fluorescence measurements were performed with a Shimadzu RF 5000 spectrofluorophotometer. Temperature was maintained at 25 °C by circulating the water through the thermostated cuvette holder. The fluorescence titrations of curcumin and PC micelles were made according to the method of Azzi (1974). The samples were excited at 451 nm, and the emission was recorded at 490 nm. The dissociation constant (K_{cur}) and the number of binding sites on PC micelles were determined using the Scatchard plot.

The interaction of curcumin with PC micelles was characterized by measuring the thermodynamic parameters. The effect of temperature on the K_{eq} constant was measured at 20, 25, 30, 35, and 40 °C. The free energy change (ΔG) was measured using the equation $\Delta G = -RT \ln K_{eq}$. The van't Hoff plot was constructed using the equation $\ln K_{eq} = (-\Delta H/R)/T + (\Delta S/R)$ to determine enthalpy change (ΔH) and entropy change (ΔS), where R is the gas constant and T is the absolute temperature.

The interactions of cations (chloride salts of Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+}) were made by following the fluorescence quenching studies. The PC micelles were saturated with curcumin as reflected in maximum relative fluorescence intensity, F_{max}, at 490 nm. To this PC−curcumin complex were gradually added cations, and the decrease in fluorescence intensity, F, was recorded. The dissociation constant (K_d) for the fluorescence quenching ligand (L) was determined using the equation $F_{max}/F = (1 + K_{cur}/[cur])(1 + [L]/K_d)$ (Aceto et al., 1995), where K_{cur} is the dissociation constant for PC−curcumin determined by Scatchard plot, [cur] is the concentration of curcumin, and [L] is the concentration of cation added during the titration. The reciprocal of dissociation constant was used as equilibrium constant (K_{eq}).

The effect of pH on curcumin fluorescence was made by titrating the curcumin against a fixed concentration of PC micelles at pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The increase in

relative fluorescence intensity was recorded as a function of curcumin concentration.

Polarization experiments were performed using a Shimadzu polarization accessory. Measurements were made at a fixed concentration of egg PC (50 μM) with various concentrations of curcumin (0−10 μM) in 50 mM Tris-HCl, pH 7.4, at 25 °C. The fluorescence intensity components (I_{vv}, I_{vh}, I_{hv}, I_{hh}), in which the subscripts refer to the horizontal (h) or vertical (v) positioning of the excitation and emission polarizers, respectively, were used to calculate the steady-state fluorescence polarization (p):

$$P = [(I_{vv} - GI_{vh}/(I_{vv} + GI_{vh})] \quad (1)$$

where G was the grating factor that corrects for wavelength-dependent distortions of the polarizing system.

$$G = I_{hv}/I_{hh} \quad (2)$$

All results are expressed as mean values of three experiments. The program Microcal Origin (version 4.1, MA) was used for the analysis of data.

RESULTS

The absorption spectra of curcumin with PC micelles shows that by increasing the micellar PC concentration, the absorption at 425 nm increased with a distinct isosbestic point, which suggested the formation of complex of curcumin with PC micelles (Figure 1A). To understand the change in absorbance of curcumin with increasing concentration of PC micelles, the changes in curcumin absorption were followed in ethanol and methanol solutions (Figure 1B,C). The absorption of curcumin at 425 nm increased as the concentration of alcohols increased. The increase in absorption at 425 nm either in PC micelles or in ethanol/methanol suggested the effect of hydrophobic environment on curcumin. At a fixed concentration of PC, the spectrum of curcumin as a function of its concentration was recorded (Figure 1D) and compared with the absorption spectrum in buffer alone (Figure 1E). The increase in absorbance at 425 nm was pronounced in PC micelles, suggesting the change in hydrophobicity (PC versus buffer). The absorption maximum at 425 nm in buffer was blue shifted in PC micelles.

Curcumin in aqueous solution has weak fluorescence. However, with increasing addition of PC micelles for the fixed amount of curcumin, the fluorescence intensity increased (Figure 2A). Similarly, curcumin fluorescence increased with the increasing concentration of methanol and ethanol (Figure 2B,C). A large blue shift was observed when curcumin bound to the PC micelles compared to its corresponding λ_{max} fluorescence intensity in organic solvents, suggesting that curcumin in phosphatidylcholine micelles experiences a nonpolar environment, possibly by binding to the hydrophobic regions of PC micelles. Earlier, it has also been shown that the curcumin fluorescence in organic solvents blue shifts with decreasing solvent dielectric constant (Navas Diza and Ramos Peinado, 1992). It is likely that the phenolic moieties of curcumin occupy a hydrophobic region of the micelles and the β-diketodiene moiety could be located at the interface of the micelles.

To quantitate the binding of curcumin with PC micelles, fluorescence titration measurements with curcumin and egg PC micelles were made (Figure 3). The interaction of curcumin with egg PC micelles was monitored by following the changes in relative fluorescence intensity of curcumin as a function of its concentration (Figure 3A). The concentration of curcumin required for complete saturation with a fixed concentration of PC micelles was 7 μM. Similarly, the concentration of PC micelles required for complete saturation with a fixed concentration of curcumin was 50 μM. The

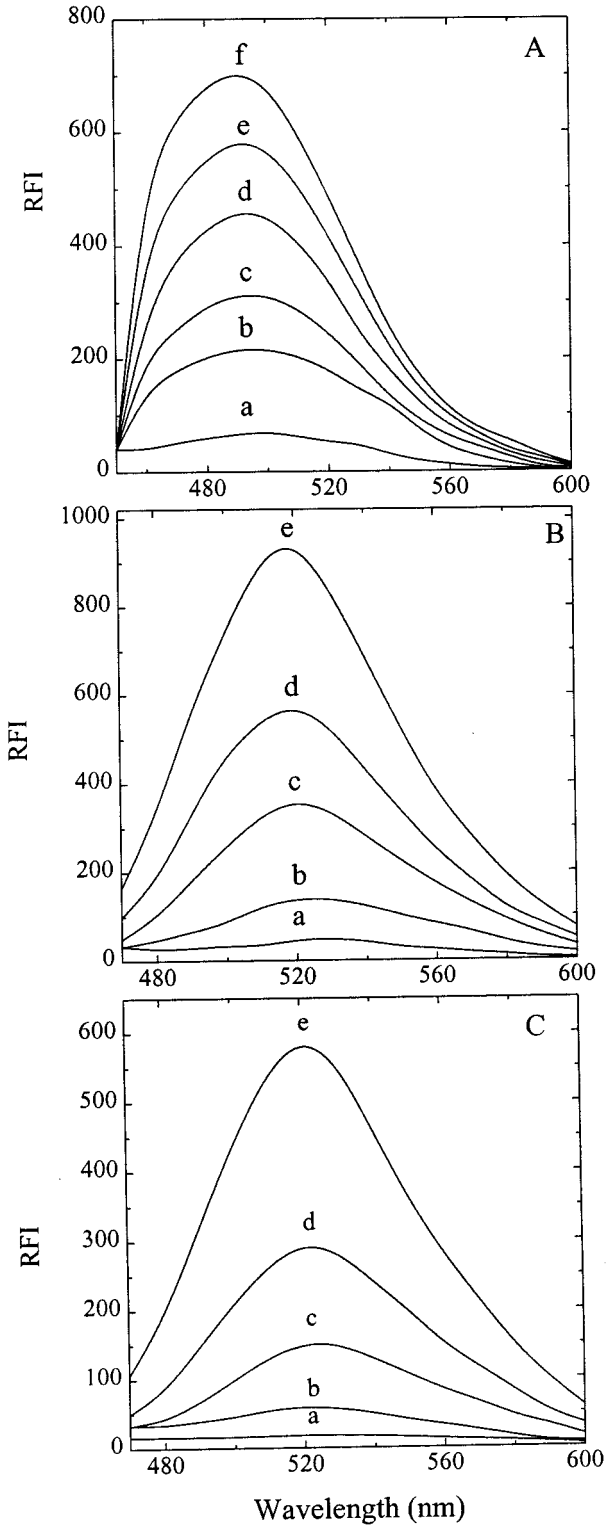

Figure 2. Fluorescence spectra of curcumin (5 μM): (A) measurements were made in various concentrations of egg PC micelles (a, 0 μM; b, 10 μM; c, 20 μM; d, 30 μM; e, 40 μM; and f, 50μM) and also in ethanol (B) and methanol (C) (a, 0%; b, 20%; c,40%; d, 60%; e, 80%, v/v). The excitation wavelength was set at 451 nm, and spectra were recorded at 25 °C. The spectra presented are an average of three sets of measurements.

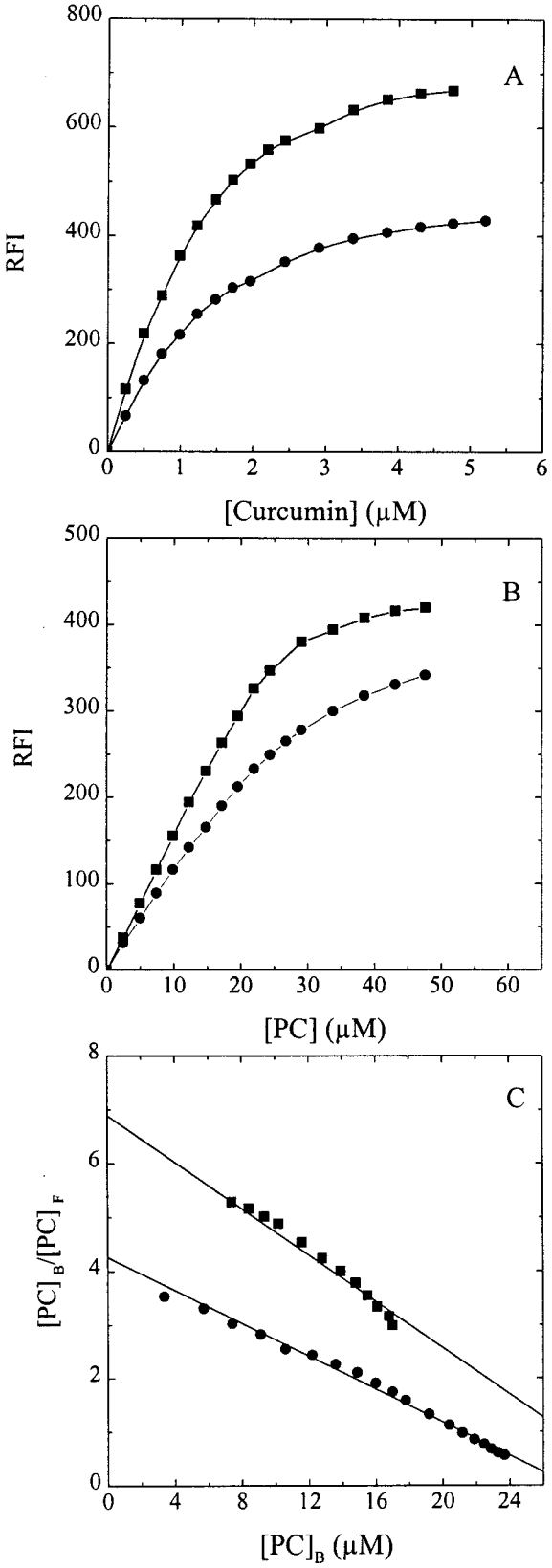

Figure 3. Fluorescence titration of PC micelles with curcumin in 50 mM Tris-HCl, pH 7.4: (A) 50 μM PC was titrated with increasing concentration of curcumin until the saturation point; (B) similarly, various concentrations of PC were added to the fixed concentration of curcumin (5 μM). The RFI_{max} was calculated from the titration curve of the first titration (A) using a double-reciprocal plot. A Scatchard plot was constructed using the second titration curve (B) and RFI_{max} value (C) (■, egg PC micelles; ●, soy PC micelles). Results represent the mean ± standard deviation of three titrations.

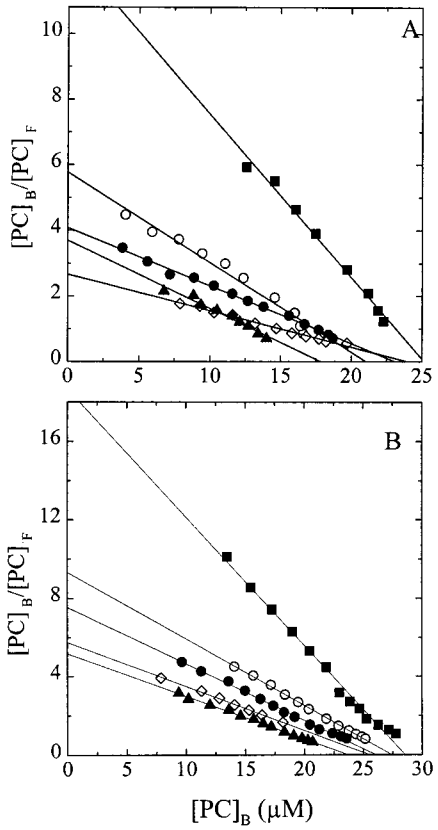

Figure 4. Scatchard plot for the interaction of curcumin with egg PC micelles (A) and soy PC micelles (B) at different temperatures (■, 20 °C; ○, 25 °C; ●, 30 °C; ◇, 35 °C; ▲, 40 °C). Curcumin concentration was 5 μM, and titration was carried out in 50 mM Tris-HCl, pH 7.4, at 25 °C. Results represent the mean ± standard deviation of three titrations.

Table 1. Changes in Free Energy (ΔG) at Different Temperatures

T (K)	EPC[a] $K_{eq} \times 10^5$ (M^{-1})	SPC[b] $K_{eq} \times 10^5$ (M^{-1})	EPC ΔG (kcal/mol)	SPC ΔG (kcal/mol)
293	6.0 ± 0.3[c]	5.3 ± 0.3	−7.7 ± 0.3	−7.6 ± 0.3
298	3.3 ± 0.2	2.6 ± 0.2	−7.8 ± 0.3	−7.4 ± 0.3
303	2.9 ± 0.1	2.0 ± 0.1	−7.5 ± 0.3	−7.4 ± 0.3
308	2.2 ± 0.1	1.7 ± 0.1	−7.5 ± 0.3	−7.4 ± 0.3

[a] EPC, egg PC micelles. [b] SPC, soy PC micelles. [c] Mean equilibrium constants ± standard deviations; $n = 3$.

maximum relative fluorescence intensity (RFI_{max}) obtained by the reciprocal plot was 1000. From the Scatchard plot of PC−curcumin interactions, it was inferred that one molecule of curcumin was bound to six molecules of PC with an equilibrium constant of $(3.26 \pm 0.2) \times 10^5\ M^{-1}$ (Figure 3C). However, the affinity of curcumin with soy PC was slightly decreased [$K_{eq} = (2.64 \pm 0.2) \times 10^5\ M^{-1}$] without altering the stoichiometry of the interaction, suggesting a high degree of unsaturation in soy PC could reduce the affinity of curcumin due to decreased micelle hydrophobicity.

The interaction of curcumin with PC micelles was characterized by measuring the equilibrium constant (K_{eq}) as a function of temperature (Figure 4). K_{eq} values were decreased for both egg and soy PC micelles with the increase in temperature. The change in free energy (ΔG) at various temperatures was determined (Table 1). The van't Hoff plot was used to determine ΔH and ΔS values for the interaction of curcumin with egg PC and soy PC micelles. The calculated binding enthalpy,

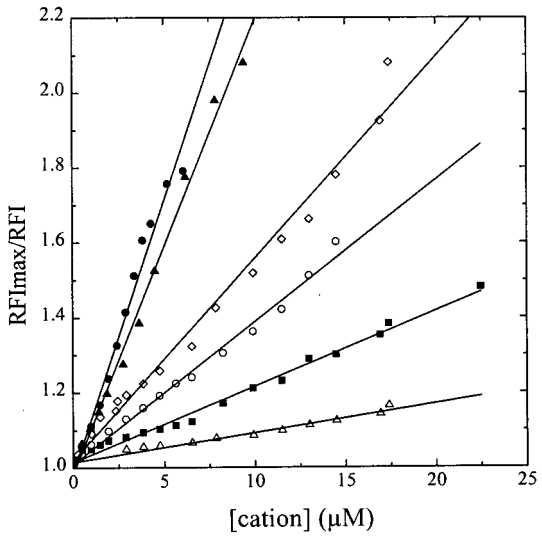

Figure 5. Curcumin fluorescence quenching by different cations. Chloride salts of Na^+ (△), K^+ (■), Mg^{2+} (○), Ca^{2+} (◇), Fe^{3+} (▲), and Al^{3+} (●) were titrated against curcumin−PC complex. Curcumin concentration is 5 μM, and the egg PC concentration is 50 μM. Measurements were made in 50 mM Tris-HCl, pH 7.4, at 25 °C. Results represent the mean ± standard deviation of three titrations.

ΔH, values were −9.6 ± 0.5 kcal/mol and −13.1 ± 0.8 kcal/mol for egg and soy PC micelles and the corresponding entropy changes, ΔS, were −6.8 ± 0.5 and −18.7 ± 1.2 cal/mol/K, respectively. The ΔH values for the transfer of curcumin from water to the micelles were distinctly negative, and these values were larger than the total free energy change, ΔG, indicating that the transfer of these molecules from water to the micelles was "enthalpy driven". The K_{eq} constant for the egg PC−curcumin interaction was higher than that for the soy PC, indicating that the hydrophobic interactions favor the strong binding of curcumin with PC micelles. Such interactions have also been studied in several other systems (Seelig, 1997; Seelig and Ganz, 1991). Thus, the dependency of the K_{eq} constant on the nature of the PC and the negative enthalpy values pointed to the contribution of hydrogen bonding apart from hydrophobic interactions to curcumin−PC interactions. It is conceivable that the −OH groups of phenolic rings are involved in hydrogen bonding, whereas the aromatic rings could be involved in the hydrophobic interactions.

Fluorescence polarization measurements were made with the curcumin−egg PC micelle system. The anisotropy of curcumin was not significantly changed by PC micelles over the concentration range studied. However, the anisotropy values were 0.3 ± 0.05, suggesting the rotation of the phenolic groups of curcumin was restricted with respect to bound PC, indicating the strong interaction of phenolic groups with the PC moiety.

The fluorescence of curcumin bound to the PC micelles can be quenched by cationic metal ions. The relationship of the magnitude of the fluorescence quenching to the ionic radius of the free concentrations of metal ions such as K^+, Na^+, Mg^{2+}, Ca^{2+}, Fe^{3+}, and Al^{3+} is represented in Figure 5. The fluorescence quenching was minimum for Na^+ and maximum for Al^{3+}. However, these results are in contrast to the report that binding of cationic metal ions to 8-anilino-1-naphthalenesulfonate (ANS) (bound to PC micelles) enhances the ANS fluorescence (Vander Kooi and Martonoxi, 1969; Flanagan and Hesketh, 1973). The K_{eq} constant for the binding of cations to the curcumin−PC micelles is shown in the Table 2.

Table 2. K_{eq} Values for the Binding of Cations to the Curcumin−PC Micelles

cation	$K_{eq} \times 10^5$ (M^{-1})	cation	$K_{eq} \times 10^5$ (M^{-1})
NaCl	0.16 ± 0.02[a]	$CaCl_2$	1.00 ± 0.04
KCl	0.40 ± 0.03	$FeCl_3$	2.22 ± 0.05
$MgCl_2$	0.69 ± 0.03	$AlCl_3$	2.70 ± 0.05

[a] Mean equilibrium constants ± standard deviations; $n = 3$.

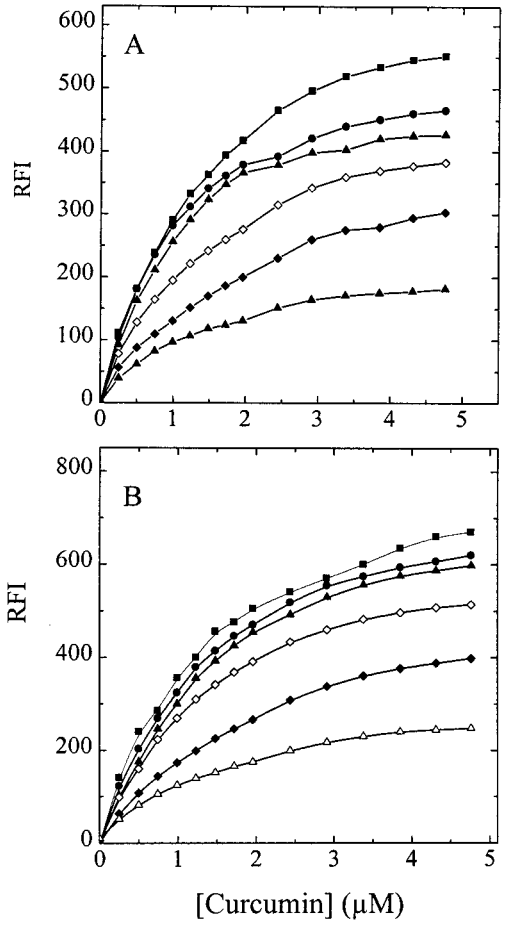

Figure 6. Fluorescence titration of curcumin (0−50 μM) with soy (A) and egg (B) PC micelles (50 μM) at different pH values (■, 5.5; ●, 6.0; ▲, 6.5; ◇, 7.0; ◆, 7.5; △, 8.0) having the same ionic strength of 0.05. Buffers used were 50 mM sodium acetate (pH 5.5 and 6.0) and 50 mM Tris-HCl (pH 6.5, 7.0, and 8.0). Results represent the mean ± standard deviation of three titrations.

The stability of curcumin in aqueous solution is highly pH dependent. Furthermore, the fluorescence of membrane-bound ANS has been shown to be highly pH dependent (Vander Kooi and Martonoxi, 1969; Flanagan and Hesketh, 1973). Therefore, the effect of pH on the affinity of curcumin with egg and soy PC micelles was studied. The results showed that curcumin's maximum fluorescence increased ~3-fold as the pH was lowered from 8 to 6 (Figure 6). The decrease in curcumin incorporation in PC micelles with the increase in pH could be mainly due to ionization of curcumin, as it has been shown that curcumin in aqueous solution is highly unstable above pH 7.0 (Navas Diaz and Ramos Peinado, 1992).

DISCUSSION

The results of our experiments clearly show the strong interaction of curcumin with PC micelles. Both absorp-

tion and fluorescence measurements showed that curcumin, on average, occupies a hydrophobic region of the PC micelle. Earlier, the interaction of curcumin with detergent micelles was shown, and it was suggested that the amphiphilic character and stability/repair behaviors of curcumin are important for its anticarcinogenic effect. The thermodynamic analysis of the interaction of curcumin with PC micelles indicated that the binding process was characterized by large exothermic enthalpies. Therefore, the reaction is driven by enthalpy and not by entropy alone, suggesting that curcumin forms both hydrophobic and hydrogen bonds with PC micelles.

The results of the present study suggest that curcumin upon binding with PC micelles can efficiently bind metal ions. It has been shown that most β-diketones are stong chelating agents. However, the hydrophobic side chains on both sides of the β-diketones are essential for its antioxidative properties (Osawa et al., 1985). It has been suggested that the high antioxidant activity of curcumin may be due to metal chelation on a β-diketone moiety. The strong binding of metal ions to PC micelle bound curcumin suggested that curcumin could inhibit many of the membrane-bound enzymes such as phospholipase D, protein kinase C, and $pp60^{c-src}$ tyrosine kinase, and these enzymes require Mg^{2+} and Ca^{2+} for their activity (Reddy and Aggarwal, 1994; Yamamoto et al., 1997). Our recent observations on the inhibition of LOX1 by curcumin suggested the reversible binding of curcumin with the active site iron(III) of LOX1 (Began et al., 1998). The inhibition of lipid peroxidation could be due to chelation of Fe(III)/Fe(II) ions by curcumin. The high K_{eq} constant (Table 2) of curcumin toward the Al^{3+} suggests that curcumin, being in a hydrophobic environment, can chelate Al^{3+} ions very effectively. It has recently been reported that aluminum ion stimulates lipid peroxidation in phospholipid liposomes (Ohyashiki et al., 1996) and membranes such as rat liver microsomes (Quinlan et al., 1988), human erythrocytes (Gutteridge et al., 1985) and mouse brain membranes (Oteiza et al., 1993).

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