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Thermodynamic studies on the interaction of folic acid with bovine serum albumin

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ABSTRACT

Binding of the vitamin folic acid with bovine serum albumin (BSA) has been studied using isothermal titration calorimetry (ITC) in combination with fluorescence and circular dichroism spectroscopies. The thermodynamic parameters of binding have been evaluated as a function of temperature, ionic strength, in the presence of nonionic surfactants triton X-100, tetrabutylammonium bromide, and sucrose. The values of the van't Hoff enthalpy calculated from the temperature dependence of the binding constant agree with the calorimetric enthalpies indicating that the binding of folic acid to the BSA is a two state process without involving intermediates. These observations are supported by the intrinsic fluorescence and circular dichroism spectroscopic measurements. With increase in the ionic strength, reduction in the binding affinity of folic acid to BSA is observed suggesting predominance of electrostatic interactions in the binding. The contribution of hydrophobic interactions in the binding is also demonstrated by decrease in the binding affinity in the presence of tetrabutylammonium bromide (TBAB). The value of binding affinity in the presence of sucrose indicates that hydrogen bonding also plays a significant contribution in the complexation process. The calorimetric and spectroscopic results provide quantitative information on the binding of folic acid to BSA and suggest that the binding is dominated by electrostatic interactions with contribution from hydrogen bonding.

1. Introduction

The interaction between bio-macromolecules and drugs has attracted great interest among researchers for several decades. Among bio-macromolecules, serum albumin is the major soluble protein constituent of the circulatory system and has many physiological functions [1,2]. The most outstanding function of serum albumin is that it serves as a main transport protein for many exogenous compounds. The binding of drugs to various blood proteins and tissues can significantly influence the pharmokinetics of drug, and therefore, the pharmacodynamics and toxicologic action of drugs [3]. The interactions of drugs with protein may result in the formation of a stable drug–protein complex, which can exert important effect on the distribution, free concentration and metabolism of the drug in the blood stream. Thus, the drug–albumin complex may be considered as a model for gaining fundamental insights into the drug–protein interactions. Therefore, studies on the binding of a drug with protein facilitates interpretation of the metabolism and transporting process, and helps to explain the relationship between structure and functions of a protein. While drug binding to plasma was first considered to represent a rather unspecific physiochemical phenomenon, accumulated evidence indicates that drug binding to albumin and α_1-acid glycoprotein (AGP) at low molar drug/protein ratios occurs at only a very few ligand binding sites of both the proteins, respectively [4,5]. Bovine serum albumin (BSA) has been studied extensively, partly because of its structural homology with human serum albumin (HSA). Human serum albumin (HSA) is a major circulatory protein of well-known structure. Bovine serum albumin (BSA), a protein with a molar mass of 66,300 Da is characterized by a low content of tryptophan and methionine, and a high content of cystine and charged amino acids such as aspartic, glutamic, lysine and arginine. The BSA content of glycine and isoleucine is lower than in average proteins. The BSA is composed of 582 amino acids and its sequence contains 17 disulfide bridges and one free cysteine in position 34 [6]. Crystal structure analysis has revealed that the drug binding sites are located in sub-domains IIA and IIIA [7]. A large hydrophobic cavity is present in the IIA sub-domain. The geometry of the pocket in IIA is quite different from that found for IIIA [8–10]. The HSA has one tryptophan (Trp 214) in sub-domain IIA, whereas BSA has two tryptophan moieties (Trp 134 and Trp 214), located in sub-domains IA and IIA, respectively [5].

Folic acid (FA), also known as vitamin B9 or folicin, belongs to group B vitamins. Its chemical name is pteroyl-L-glutamic acid (PGA) [11]. It is composed of 2-amino-4-hydroxy-6-methyl pterin, para-aminobenzoic acid, and L-glutamic acid (figure 1).

It has been reported [12] that folic acid has a high therapeutic effect on the treatment of malignant anemia. Folic acid is necessary to maintain the normal vital movement of an organism. Biochemical and medical studies have confirmed that folic acid as a

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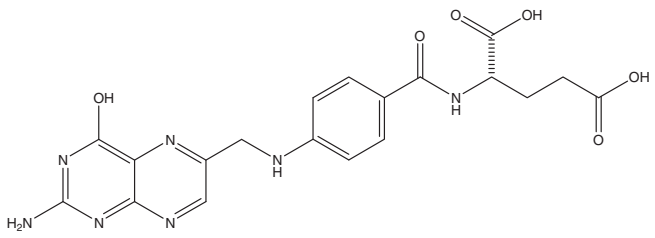

FIGURE 1. The structure of folic acid.

coenzyme is very important to the biosynthesis of purines, pyrimidines, nucleic acid, and protein, and to the division and formation of cells [13]. A shortage of folic acid easily results in the declining of physiological function and some diseases such as megaloblastic anemia, gastrointestinal dysfunction, mental retardation, and neurovascular abnormal newborn.

Folic acid participates in many important metabolic pathways in the human body. The most important function is as a vitamin cofactor for the enzymes that catalyzes the conversion of uracil to thymine *in vivo* [14]. The two main enzymes responsible for this conversion are thymidylate synthase (TS) and dihydrofolate reductase (DHFR) [15]. Rapidly proliferating cells, such as cancer cells need large supplies of thymine for DNA synthesis [16]. For this reason, this pathway is useful for developing the target molecules which are analogous to folic acid that function by inhibiting these enzymes and halting DNA synthesis [17–19]. In this work, we have investigated the interaction of folic acid with BSA by a combination of isothermal titration calorimetry (ITC), fluorescence and circular dichroism spectroscopy.

2. Experimental

2.1. Materials and methods

Fatty acid free bovine serum albumin (mass fraction purity 0.99), folic acid (>0.970), triton X-100 (>0.999) and sodium chloride (>0.99) of extra pure analytical reagent grade were obtained from Sigma–Aldrich Chemical Co Limited, USA. Sucrose (>0.99) was extra pure analytical reagent grade and obtained from Merck, India. Tetrabutyl ammonium bromide (>0.990) was purchased from Spectrochem Private Limited, India. The numbers in parenthesis represent the mass fraction purity of the compounds as listed by vendors. The water used for preparing the solutions was double distilled and then deionised using a Cole-Parmer research mixed bed ion exchange column. The protein stock solutions were prepared by extensive overnight dialysis at $T = 277$ K against $10 \cdot 10^3$ mol $\cdot$ dm^{-3} phosphate buffer at pH 7.0 with three changes. The reported pH is that of the final dialysate determined on a standard control dynamics pH meter at ambient temperature. The concentration of BSA was determined on a Shimadzu double-beam spectrophotometer (UV-265), using an absorbance of $A_1^{1\%} = 6.8$ at 280 nm [20].

2.2. Isothermal titration calorimetry (ITC)

The thermodynamics of the binding of folic acid with BSA was assessed using an isothermal titration calorimeter (VP-ITC Microcal, Northampton, MA). Before loading, the solutions were thoroughly degassed by using a Thermo Vac degassing unit supplied by Microcal, USA. The reference cell was filled with the respective degassed buffer. The sample cell of volume 1.4206 cm^3 was filled with $60 \cdot 10^{-6}$ mol $\cdot$ dm^{-3} protein solution. The syringe of volume $250 \cdot 10^{-6}$ cm^3 was filled with $2.0 \cdot 10^{-3}$ mol $\cdot$ dm^{-3} folic acid solution. The drug solution was added sequentially in $10 \cdot 10^{-6}$ dm^{-3} aliquots (for a total of 25 injections, 20 s duration each) at 4 min intervals. The heat released or absorbed upon each injection was measured, and the data were plotted as integrated quantities. The values of heat of dilution were determined independently and corrections were applied to the main BSA – folic acid heat profiles. The data were analyzed with a single set of binding sites, which is the best representation of the data, using the Origin 7 software provided by Microcal. All the calorimetric titration experiments reported have been performed (at least) in duplicate, and the values represent the average of these independent measurements.

2.3. Fluorescence spectroscopy

Intrinsic fluorescence experiments were performed on a Perkin–Elmer LS-55 spectrofluorimeter with a 3 cm^3 quartz cell that had a path length of 1 cm. The protein concentration in all the experiments was kept at $1.508 \cdot 10^{-6}$ mol $\cdot$ dm^{-3} and the folic acid concentration was varied from $5.0 \cdot 10^{-6}$ mol $\cdot$ dm^{-3} to $16.0 \cdot 10^{-6}$ mol $\cdot$ dm^{-3}. Both the excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at $\lambda = 295$ nm to selectively excite the tryptophan residues, and the emission spectra were recorded in the wavelength range of (300 to 400) nm at a scan rate of 100 nm $\cdot$ min^{-1}. The data thus obtained were analyzed with a modified Stern–Volmer equation [21] to calculate the value of the quenching constant.

2.4. Circular dichroism (CD) spectroscopy

The alterations in the secondary and tertiary structure of the protein in the presence of the drugs were studied on a JASC0-810 CD spectropolarimeter. For the far-UV CD experiments, the protein concentration and path lengths used were $5 \cdot 10^{-6}$ mol $\cdot$ dm^{-3} and 0.2 cm while $20 \cdot 10^{-6}$ mol $\cdot$ dm^{-3} and 1 cm, respectively, were used for the near UV–CD experiments. The spectropolarimeter was sufficiently purged with 99.9% dry nitrogen before starting the instrument. The spectra were collected at a scan speed of 500 nm $\cdot$ min^{-1} and a response time of 1 s. Each spectrum was baseline corrected, and the final plot was taken as an average of three accumulated plots. The molar ellipticity $[\theta]$ was calculated from the observed ellipticity θ as

$$[\theta] = 100 \cdot \left(\frac{\theta}{c \cdot l}\right), \tag{1}$$

where c is the concentration of the protein in mol $\cdot$ dm^{-3} and l is the path length of the cell (in centimeters). These experiments were performed for the folic acid concentration within the range of (5.0 to 16.0) $\cdot 10^{-6}$ mol $\cdot$ dm^{-3}.

2.5. Lifetime measurements

Time-resolved fluorescence measurements were performed at the magic angle using a pulsed Nano-LED based time-correlated single-photon counting fluorescence spectrometer with $\lambda_{ex} = 295$ nm and $\lambda_{em} = 340$ nm. The full width at half-maximum of the instrument response function is $250 \cdot 10^{-12}$ s and the resolution is $56 \cdot 10^{-12}$ s per channel. The data are fitted to a bi-exponential function after de-convolution of the instrument response function by an iterative re-convolution technique by the IBH DAS 6.0 data analysis software using reduced χ^2 and weighted residuals as parameters for goodness of the fit. The BSA concentration in these experiments was kept fixed at $15 \cdot 10^{-6}$ mol $\cdot$ dm^{-3} and the folic acid concentration was varied from (15.0 to 100.0) $\cdot 10^{-6}$ mol $\cdot$ dm^{-3}.

The decay curves were fitted using a nonlinear iterative least square fit method using the following equation,

$$G(t) = \sum_i B_i \exp(-t/\tau_i). \quad (2)$$

The function $G(t)$ is the fitted decay usually assumed to be a sum of exponentials, where B_i is the pre-exponential factor for the ith component, τ_i is the corresponding fluorescence lifetime, and t is time. The data fitted well to a bi-exponential decay where the intensity is assumed to decay as the sum of individual single-exponential decay

$$I(t) = I(0)\left[\alpha_1 \cdot e^{-t/\tau_1} + \alpha_2 \cdot e^{-t/\tau_2}\right]. \quad (3)$$

Here τ_1 and τ_2 are the decay times and α_1 and α_2 are the amplitudes of the components at $t = 0$. The average lifetime $\langle\tau\rangle$ was calculated by following relation

$$\langle\tau\rangle = \alpha_1 \cdot \tau_1 + \alpha_2 \cdot \tau_2. \quad (4)$$

3. Results and discussion

3.1. Isothermal titration calorimetry of the binding of folic acid to BSA at pH 7 and different temperatures

3.1.1. Isothermal titration calorimetry of the binding of folic acid to BSA

A representative calorimetric titration profile accompanying the binding of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $0.060 \cdot 10^{-3}$ mol · dm^{-3} at pH 7 and $T = 298.15$ K is shown in figure 2. Each peak in the binding isotherm represents a single injection of the drug into the protein solution. Panel B of figure 2 shows the plot of the amount of heat liberated per injection as a function of the molar ratio of the drug to the protein. A standard nonlinear least-squares regression binding model, involving a single class of non-interacting sites fitted best to the data. The smooth solid line shown in figure 2 (panel B) is the best fit to the experimental data. The temperature dependence of the thermodynamic parameters accompanying the binding of folic acid with BSA is summarized in table 1. Each value in this table is an average of two to three independent measurements.

The observed enthalpy does not contain a significant contribution from the buffer ionization, because phosphate has a small value for the enthalpy of ionization ($\Delta H_{ioniz} = 3.6 \cdot 10^3$ J · mol^{-1}) [22]. Therefore, the measured enthalpy is taken to be the binding enthalpy of binding of the drug to the protein.

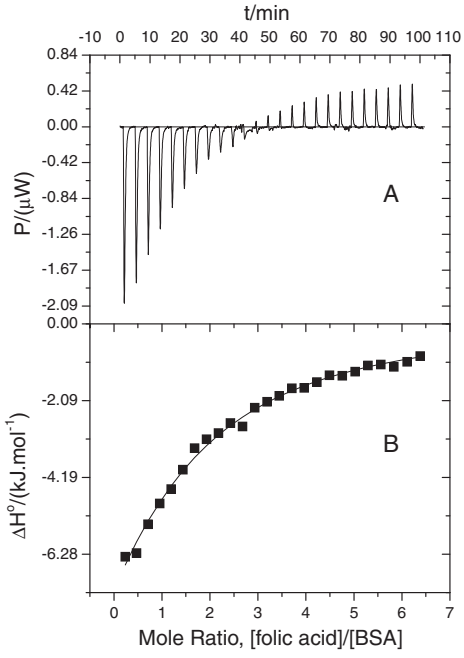

FIGURE 2. (A) Raw data for the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA at pH 7.0 and $T = 298.15$ K, showing the calorimetric response in power (P) units as successive injections of the ligand are added to the sample cell. (B) Integrated heat profile of the calorimetric titration shown in panel A. The solid line represents the best nonlinear least squares fit to the measured heats (■) according to single binding-site model.

TABLE 1
Binding constant (K), standard enthalpy change ($\Delta H°$) and standard entropy change ($\Delta S°$) of binding as determined by the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} bovine serum albumin (BSA) at pH 7.0 at different temperatures (T).

T/K	K/M^{-1}	$\Delta H°$/ (kJ · mol^{-1})	$\Delta S°$/ (J · K^{-1} · mol^{-1})	$\Delta G°$/ (kJ · mol^{-1})
288.15	$(7.19 \pm 0.25) \cdot 10^3$	−27.9 ± 1.0	−23.3 ± 0.9	−21.27 ± 0.08
293.15	$(5.77 \pm 0.28) \cdot 10^3$	−26.9 ± 1.1	−20.0 ± 3.6	−21.10 ± 0.12
298.15	$(5.31 \pm 0.77) \cdot 10^3$	−27.9 ± 2.7	−22.4 ± 5.6	−21.26 ± 0.36
303.15	$(3.17 \pm 0.73) \cdot 10^3$	−27.1 ± 1.4	−22.4 ± 5.0	−20.31 ± 0.58
308.15	$(3.07 \pm 0.62) \cdot 10^3$	−29.2 ± 6.9	−27.9 ± 9.6	−20.57 ± 0.52

As seen in figure 2, the titrations of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA yielded negative heat deflection after correction for the dilution effects, which indicated that the binding is an exothermic process. The value of K varies from $(7.19 \pm 0.25) \cdot 10^3$ M^{-1} at $T = 288.15$ K to $(3.07 \pm 0.62) \cdot 10^3$ at $T = 308.15$ K. The value of the stoichiometry of the binding is fixed to one based upon fitting of the ITC profile to a single binding site model. The binding is entropically opposed but enthalpically favoured over the temperature range studied. The enthalpy of binding is in the range of $-(26.9 \pm 1.6) \cdot 10^3$ J · mol^{-1} at $T = 293.15$ K to $-(29.2 \pm 6.9) \cdot 10^3$ J · mol^{-1} at $T = 308$ K. Thus, the main contribution to change in the standard Gibbs's energy change, $\Delta G°$ is from enthalpy.

The enthalpy of binding does not vary much with the change in temperature which shows that the value of ΔC_P is $-(0.06 \pm 0.05)$ kJ · K^{-1} · mol^{-1}. This shows that there is insignificant burial or exposure of hydrophobic groups due to interaction of folic acid with serum albumin. The strong exothermicity observed in the binding suggests the involvement of electrostatic interactions in the binding process. Folic acid contains 2-amino-4-hydroxy-6-methyl pterin, para-aminobenzoic acid, and L-glutamic acid groups. Thus, it has both hydrophilic hydroxyl and amino groups, and hydrophobic side chains. Since folic acid consists of amino, carboxyl and carbonyl groups, it can interact via hydrogen bonding [23]. The value of the stoichiometry of the binding is 1 to 1 based upon best fitting of the ITC profile to a single binding site model. At pH 7.0, folic acid is comprised of both positively

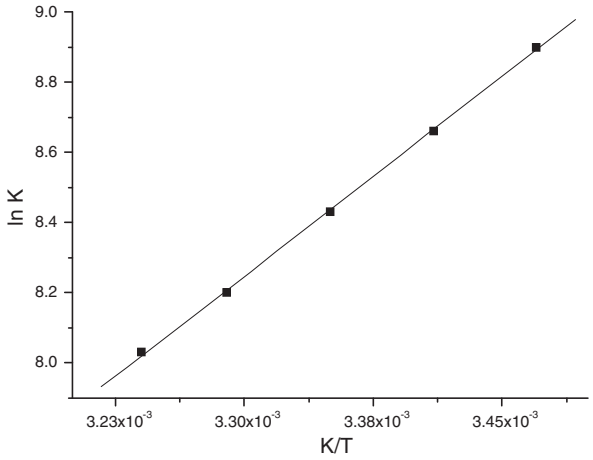

FIGURE 3. Plot of ln K *versus* $1/T$ for the binding of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA at pH 7.0 and various temperatures.

TABLE 2
Binding constant (K), standard enthalpy change ($\Delta H°$) and standard entropy change ($\Delta S°$) of binding as determined by the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} bovine serum albumin (BSA) at pH 7.0 in presence of NaCl and sucrose at $T = 298.15$ K.

	K/M^{-1}	$\Delta H°$/ (kJ · mol^{-1})	$\Delta S°$/ (J · K^{-1} · mol^{-1})	$\Delta G°$/ (kJ · mol^{-1})
		c(NaCl)/(mol · dm^{-3})		
0.01	$(8.28 \pm 0.01) \cdot 10^3$	− 22.7 ± 1.9	−1.0 ± 6.7	−22.36 ± 0.03
0.02	$(2.62 \pm 0.01) \cdot 10^3$	−27.6 ± 0.04	−27.2 ± 3.4	−19.51 ± 0.09
0.05	No binding			
		c(Sucrose)/(mol · dm^{-3})		
1.0	$(1.46 \pm 0.58) \cdot 10^3$	−11.7 ± 0.06	21.4 ± 3.3	−18.06 ± 0.99

charged amine groups and negatively charged carboxyl groups and is expected to bind at a site that are comprised of both positive and negative charged residues.

The two state behaviour of the binding can be assessed by comparing the values of van't Hoff enthalpy ($\Delta_{vH}H$) with the calorimetric enthalpy. The latter can be analyzed by using the vant' Hoff relation

$$\left(\frac{\partial \ln K}{\partial T}\right)_p = \frac{\Delta H^0}{RT^2}. \quad (5)$$

Generally for an exothermic process, an increase in temperature leads to a reduction in the value of the binding constant in accordance with the LeChatelier principle. In this case, a plot of ln *K versus* l/*T* via least squares gave a correlation coefficient of 0.999 as shown in figure 3.

Using the data presented in table 1 and equation (4), the value of the van't Hoff enthalpy calculated from the temperature dependence of the binding constant *K*, is $-(33.9 \pm 5.7) \cdot 10^3\ J \cdot mol^{-1}$ which is close to the observed calorimetric enthalpy within the standard deviation at each temperature. This comparison shows that the binding of folic acid to BSA follows two state binding processes without involving intermediates and that the binding of folic acid to the BSA does not alter its conformation appreciably.

3.1.2. Ionic strength dependence of the binding of folic acid to BSA

The salt dependence of the biomolecular association is often used to assess the contribution of charge–charge interactions to the free energy of the binding. To understand the role of electrostatic interactions in the binding process, the ionic strength dependence of the binding of folic acid with BSA was studied. The experiments were performed in the presence of 0.01, 0.02, and 0.05 mol · dm^{-3} NaCl at pH 7.0 and *T* = 298.15 K. The binding parameters thus obtained are listed in table 2.

For folic acid, an increase in the ionic strength from (0.01 to 0.02) mol · dm^{-3} leads to a decrease in the binding affinity of the drug to the protein, as reflected by the decrease in the value of *K* by a factor of 4. The stoichiometry of binding remains the same. When the concentration of NaCl is increased to 0.05 mol · dm^{-3}, no binding was observed (see figure 4(C)).

These results are consistent with the dominant involvement of electrostatic interactions in the binding process which are disrupted in the presence of NaCl. Even though there are only two points in the NaCl concentration used, the value of d(lg *K*)/d(lg[Na^+]) = −1.72, which is equivalent to the number of counter-ions released upon drug binding [24]. The possibilities of the ionic strength dependence include the binding of the ions to the charged amino acid residues and also forming an ion-pair with the drug. Folic acid has two carboxyl groups attached to L-glutamic acid side chain which can be responsible for the stronger electrostatic interactions.

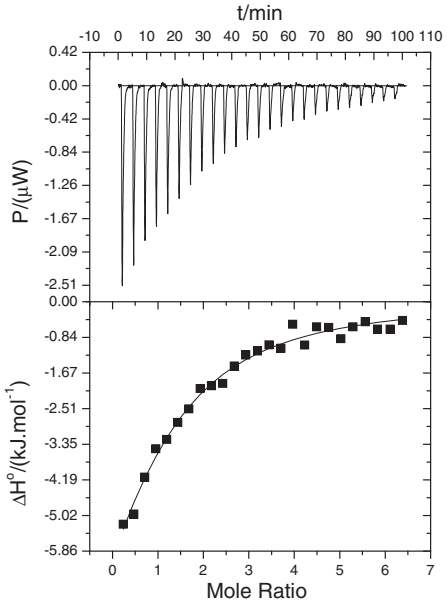

FIGURE 5. Isothermal titration calorimetric (ITC) profile showing the calorimetric response in power (*P*) units for the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA in the presence of 1 mol · dm^{-3} sucrose at pH 7.0 and *T* = 298.15 K. The symbol (■) represents the heat change at each injection.

3.1.3. Binding of folic acid to BSA in the presence of sucrose

The involvement of hydrogen bonding in the binding process can be assessed by using sucrose as a co-solute as it has a number of hydroxyl groups, which can interfere in the binding process. The ITC profile and the corresponding data for the binding of folic acid to BSA in the presence of sucrose are given in figure 5 and table 2.

The value of K at *T* = 298.15 K in the absence and presence of sucrose are $(5.31 \pm 0.70) \cdot 10^3\ M^{-1}$ and $(1.46 \pm 0.58) \cdot 10^3\ M^{-1}$. Hence in the presence of 1.0 mol · dm^{-3} sucrose, the binding affinity decreases. The binding become less exothermic with an increase in entropy compared to that in the absence of sucrose. Since the value of *K* decreases in the presence of sucrose, it indicates involvement of hydrogen bonding in the binding process. However the positive change in entropy observed in presence of 1.0 mol · dm^{-3} sucrose shows that the solvent structure is altered significantly in the presence of sucrose.

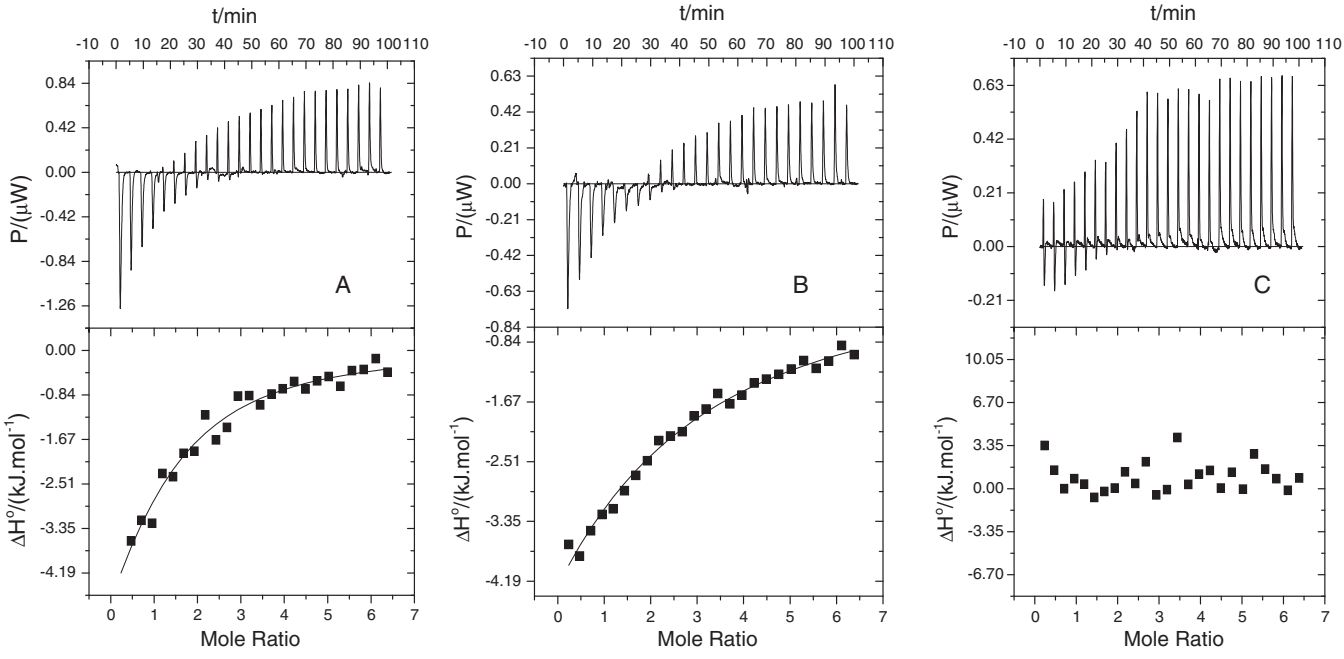

FIGURE 4. Isothermal titration calorimetric (ITC) profiles showing the calorimetric response in power (*P*) units for the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA in the presence of (A) 0.01 (B) 0.02, and (C) 0.05 mol · dm^{-3} NaCl at pH 7.0 and *T* = 298.15 K. The symbol (■) represents the heat change at each injection.

TABLE 3
Binding constant (K), standard enthalpy change ($\Delta H°$) and standard entropy change ($\Delta S°$) of binding as determined by the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} bovine serum albumin (BSA) at pH 7.0 in the presence of tetrabutylammonium bromide (TBAB) and triton X-100 (TX-100) at T = 298.15 K.

	K/M^{-1}	$\Delta H°$/ (kJ · mol^{-1})	$\Delta S°$/ (J · K^{-1} · mol^{-1})	$\Delta G°$/ (kJ · mol^{-1})
		c(TBAB)/(mol · dm^{-3})		
0.02	$(2.81 \pm 0.89) \cdot 10^3$	−20.5 ± 0.2	−2.75 ± 3.02	−19.68 ± 0.88
0.10	No binding			
		c(TX-100)/(mol · dm^{-3})		
0.15	$(6.95 \pm 0.58) \cdot 10^3$	−21.5 ± 0.3	1.5 ± 1.2	−21.92 ± 0.21
0.35	$(4.95 \pm 0.21) \cdot 10^3$	−30.8 ± 0.2	−32.7 ± 0.8	−21.08 ± 0.11

3.1.4. Effect of tetrabutyl ammonium bromide on the binding of folic acid to BSA

The use of TBAB as co-solute can give better insights into role of hydrophobic and electrostatic interactions in the binding process since TBAB can affect both the charge–charge and hydrophobic interactions [16]. The binding experiments were done in the presence of TBAB and the results obtained are listed in table 3 and the ITC profiles are shown in figure 6.

In the presence of 0.02 mol · dm^{-3} TBAB, the binding affinity of folic acid with BSA decreases from $K = (5.31 \pm 0.77) \cdot 10^3$ M^{-1} to $(2.81 \pm 0.89) \cdot 10^3$ M^{-1}. On further increasing the concentration of TBAB to 0.1 mol · dm^{-3}, no binding profile is observed. On comparing the results of the titration of folic acid with BSA in the presence of 0.05 mol · dm^{-3} NaCl and 0.1 mol · dm^{-3} TBAB, it is seen that the binding is almost lost in the former at 0.05 mol · dm^{-3} NaCl. This indicates that electrostatic interactions play significant role in the binding process. The reduction of the affinity in the presence of the TBAB ion will also have contributions from competitive binding of folic acid and tetrabutyl ammonium cations in the same negatively charged hydrophobic pocket.

3.1.5. Effect of nonionic surfactant triton X-100 on the binding of folic acid to BSA

To understand the effect of non-columbic interactions on the binding of drug to BSA and the extent of overlap of binding sites on BSA for the drugs and surfactants, experiments were performed on the binding of folic acid to BSA at pH 7.0 and T = 298.15 K in presence of TX-100.

Figure 7 shows the binding of folic acid to BSA in the presence of nonionic surfactant TX-100 in the pre- and post-micellar concentration range of the surfactant (CMC = $0.261 \cdot 10^{-3}$ mol · dm^{-3}) [25]. As seen in this figure 7, the measured enthalpies cross from exothermic to endothermic effects. This can happen if the heat of dilution of the protein is different from the heat of dilution of the drug–protein complex. The value of the binding constant in the presence of TX-100 is the same at 0.15 mol · dm^{-3} as compared to that in absence of TX-100 at T = 298.15 K. Because TX-100 is a neutral molecule and it can bind via polar and non-polar interactions with equal preference, it seems, from data, that at the higher concentration the polar groups of TX-100 is blocking the binding sites for folic acid on BSA where the drug can bind to the protein via hydrogen bonding.

3.2. Fluorescence spectroscopy

The binding of folic acid to BSA was also investigated by monitoring the intrinsic fluorescence of the BSA. Figure 8 shows the fluorescence emission spectra of BSA in the presence of varying concentrations of folic acid. It is seen that the addition of folic acid to BSA leads to a reduction in the fluorescence intensity indicating that the binding of folic acid to BSA quenches the intrinsic fluorescence of tryptophan. The native BSA shows a maximum of fluorescence emission intensity (λ_{max}) at 346.5 nm, which is concordant with the literature [26]. In the presence of folic acid, the value of λ_{max} was observed to decrease with a maximum blue shift of 3 nm at $16 \cdot 10^{-6}$ mol · dm^{-3} concentration of the drug. This indicates that the binding of folic acid is associated with change in the local dielectric environment in the BSA, which suggests that after the addition of folic acid, the chromophore was placed in a more hydrophobic environment. The quenching of fluorescence is known to occur mainly by a collision process (dynamic quenching) or by formation of a complex between the quencher and fluorophore (static quenching). The fluorescence quenching data were analyzed according to the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{sv} \cdot [Q], \tag{6}$$

where F_0 is the fluorescence intensity in the absence of folic acid, F is the fluorescence intensity in the presence of the molar concentration of the quencher [Q] and K_{sv} is the Stern–Volmer quenching constant. Linear Stern–Volmer plots show an upward curvature as seen in figure 9 implying the simultaneous operation of more than one mechanism.

The modified Stern–Volmer equation [21] was therefore applied to this heterogenous system.

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a \cdot K_{sv} \cdot [Q]}. \tag{7}$$

In the above equation, f_a stands for the fraction of accessible tryptophan and K_{sv} is the Stern–Volmer quenching constant, other terms having the same significance as before. A plot of $[F_0/(F_0 - F)]$ *versus* $[Q]^{-1}$ in accordance with the above equation is found to be linear with the intercept on the ordinate as shown in figure 10.

The value of f_a is found to be 0.28 and that of K_{sv} is $1.55 \cdot 10^4$ M^{-1} · s^{-1}. The value of τ_0 for the fluorescence of the tryptophan is equal to 10^{-9} s, the bimolecular quenching constant K_q for BSA in presence of folic acid is of the order of 10^{13} M^{-1} · s^{-1} which is calculated from $K_q = K_{sv}/\tau_0$, where τ_0 is the average life time of BSA in the absence of the drug. The probability of collision between the fluorophore and quencher depends on the diffusion coefficient (D), their size and concentration [27].

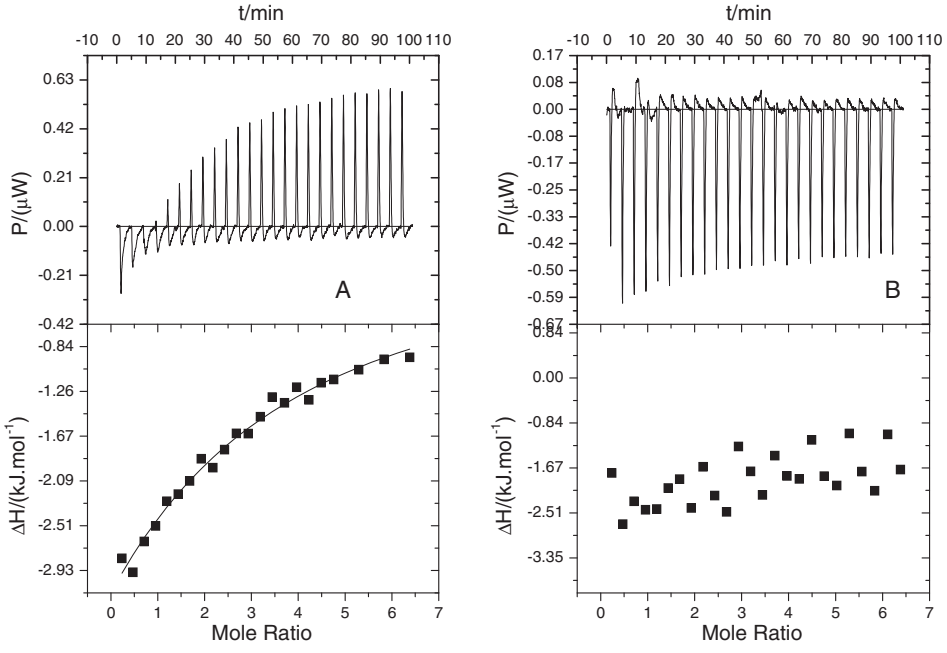

FIGURE 6. Isothermal titration calorimetric (ITC) profile showing the calorimetric response in power (P) units for the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA in the presence of (A) 0.02 and (B) 0.1 mol · dm^{-3} tetra butyl ammonium bromide at pH 7.0 and T = 298.15 K. The symbol (■) represents the heat change at each injection.

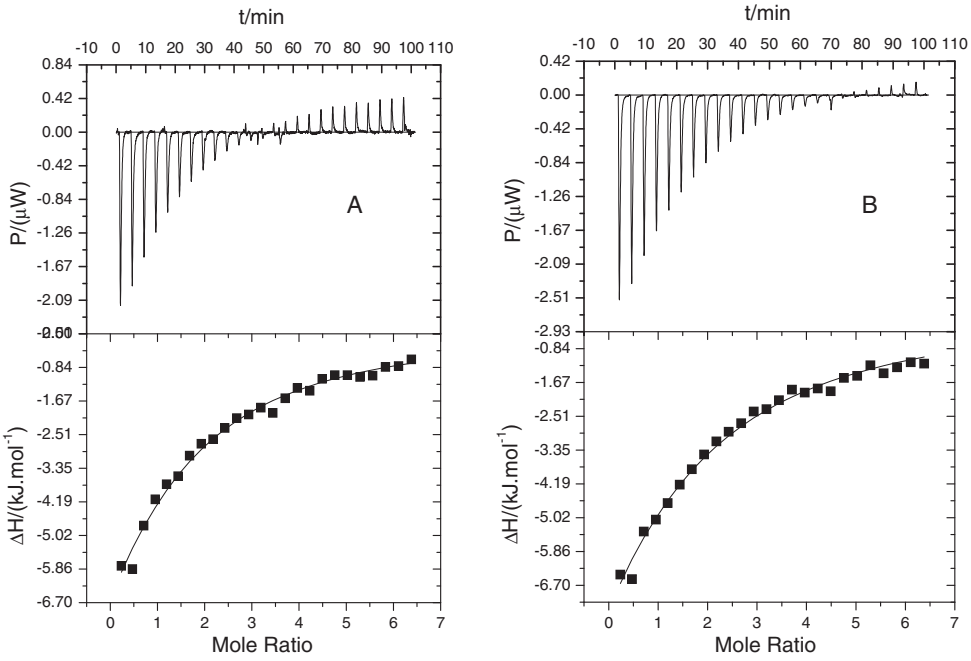

FIGURE 7. Isothermal titration calorimetric (ITC) profile showing the calorimetric response in power (P) units for the titration of $2 \cdot 10^{-3}$ mol · dm^{-3} folic acid with $60 \cdot 10^{-6}$ mol · dm^{-3} BSA at pH 7.0 and T = 298.15 K, in the presence of (A) 0.15, (B) 0.35 mol · dm^{-3} Triton X-100. The symbol (■) represents the heat change at each injection.

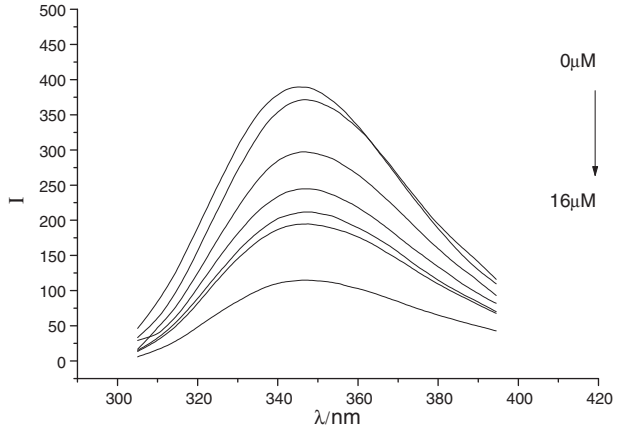

FIGURE 8. Intrinsic fluorescence emission spectra of $1.5 \cdot 10^{-6}$ mol · dm^{-3} BSA in the presence of increasing concentrations of folic acid.

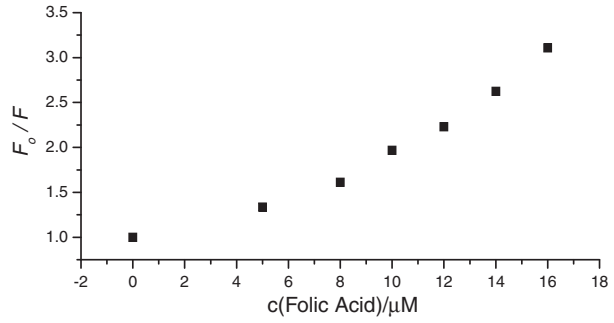

FIGURE 9. The plot of F_0/F represented as (■) against concentration of folic acid in $1.5 \cdot 10^{-6}$ mol · dm^{-3} BSA. Here, F_0 and F are the fluorescence intensities in the absence and presence of folic acid.

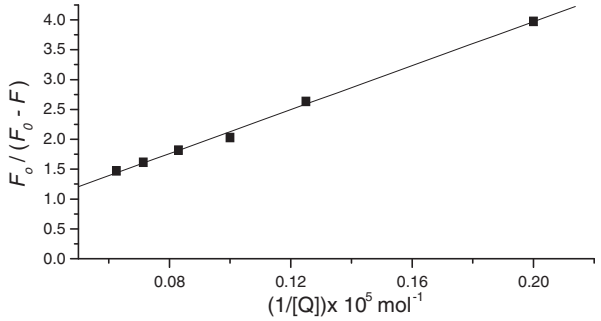

FIGURE 10. The plot of $\frac{F_0}{F_0-F}$ represented as (■) against concentration of folic acid in $1.5 \cdot 10^{-6}$ mol · dm^{-3} BSA Here, F_0 and F are the fluorescence intensities in the absence and presence of folic acid.

$$K_q = 4 \cdot \pi \cdot a \cdot D \cdot N_A \cdot 10^{-3}. \quad (8)$$

In this equation, D is the sum of the diffusion coefficients of fluorophore and quencher, 'a' is the molecular size and N_A is the Avogadro number. The maximum limit of K_q for a diffusion controlled bimolecular reaction is 10^{10} M^{-1} · s^{-1}. The higher values of K_q for BSA and folic acid (10^{13} M^{-1} · s^{-1}) in this study show that quenching is not initiated by a collision process, but by complex formation.

3.3. Time resolved fluorescence spectroscopy

Time resolved fluorescence lifetime measurements were carried out in order to study further the role of static or dynamic quenching in the presence of the drug. Figure 11 shows the time resolved fluorescence decays of BSA in $10 \cdot 10^3$ mol · dm^{-3} phosphate buffer at pH 7.0. The fluorescence decay of BSA at λ = 346 nm has been measured at different mole ratios of folic acid to get an idea about the change in micro environment of the fluorophore upon binding to BSA. The values of the fluorescence lifetime and amplitude are listed in table 4.

The average lifetime decreases from (5.72 to 3.72) · 10^{-9} s at the maximum concentration ($100 \cdot 10^{-6}$ mol · dm^{-3}) of folic acid, thereby indicating fluorescence quenching has a dynamic component.The average lifetime is defined as

$$\langle \tau \rangle = \alpha_1 \cdot \tau_1 + \alpha_2 \cdot \tau_2. \quad (9)$$

Here τ_1 and τ_2 are the decay times and α_1 and α_2 are the amplitudes of the component at t = 0. In the bi-exponential model, the intensity is assumed to decay as the sum of individual single exponential decays in equation (3). The average lifetime was calculated by using equation (9). It can be seen from figure 11 and table 4 that

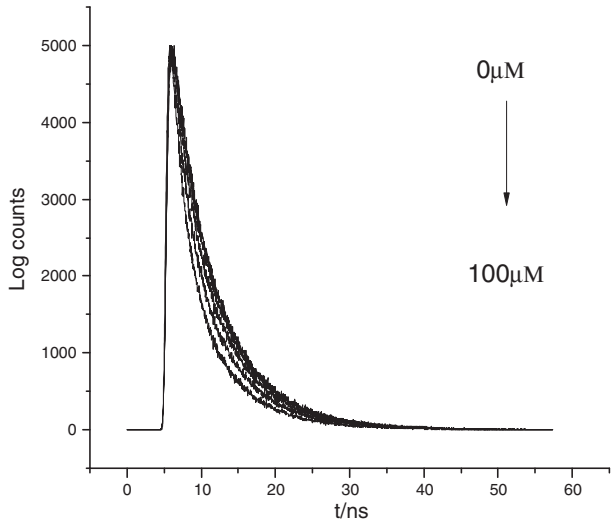

FIGURE 11. Fluorescence decay profiles of $0.15 \cdot 10^{-7}$ mol · dm^{-3} BSA in the presence of different molarities of folic acid, (A) 0, (B) 15, (C) 45, and (D) $100 \cdot 10^{-6}$ mol · dm^{-3}.

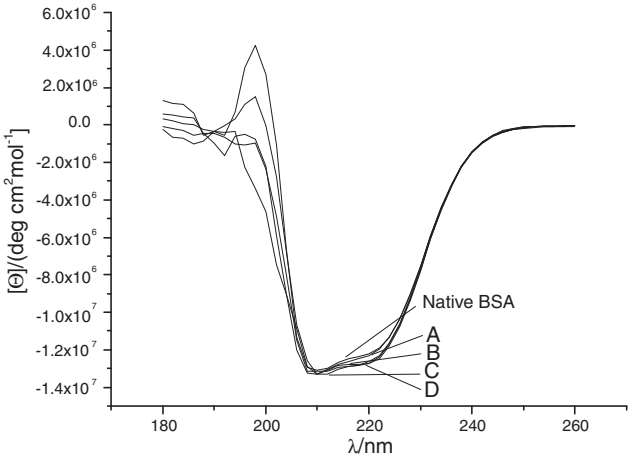

FIGURE 12. Far UV CD spectra of BSA at different molarities of folic acid at pH 7.0: 5(A), 10(B), 12(C), and $16(D) \cdot 10^{-6}$ mol · dm^{-3}.

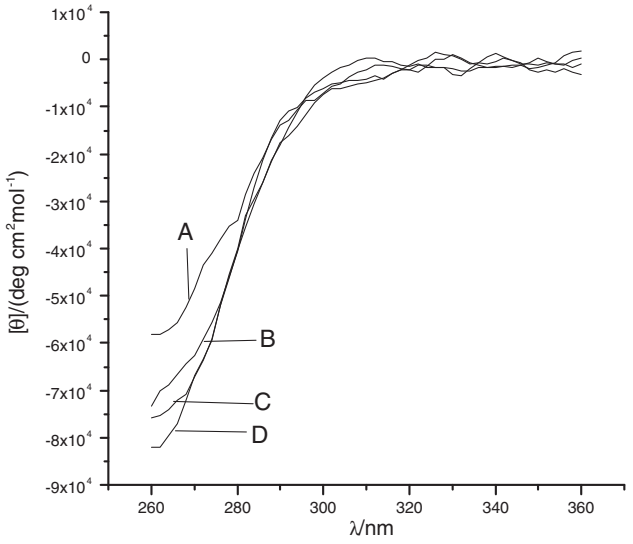

FIGURE 13. Near UV CD spectra of BSA at different molarities of folic acid at pH 7: 0(A), 5(B), 10(C), and $16(D) \cdot 10^{-6}$ mol · dm^{-3}.

the decay remains bi-exponential. The fluorescence life time of BSA changes significantly with the rise in the concentration of folic acid suggesting the formation of a dynamic complex between the drug and the protein. On comparing the value of K_{sv} with the value of K obtained from ITC, it is seen that the values do not match with the ITC values. This difference in result can be due to involvement of dynamic quenching in the binding of folic acid to BSA.

3.4. Circular dichroism (CD) spectroscopy

The changes in the secondary and tertiary structure of the protein in the presence of the drug were studied in the far UV CD and near UV CD regions at the increasing [folic acid]/[BSA] molar ratio. The CD spectrum of the native BSA showed minima at λ = 209 nm and 222 nm [28] which are indicative of α helical structure of the protein. This is in accord with the literature [28]. Figure 12 shows the far UV–CD spectra of BSA in the presence of increasing concentration folic acid the protein. The near UV–CD spectra are used to observe the tertiary structural features of protein. Figure 13 shows the near UV–CD spectra of BSA in the presence of increasing concentration of folic acid. In the presence of drug, no appreciable perturbation of the tertiary structure of the protein is observed. However, the secondary structure shows a slight strengthening with the rise in the concentration of folic acid. This result is consistent with a blue shift observed in the fluorescence experiments.

4. Mode of interactions and probable binding sites of folic acid with BSA

In addition to the quantitative measurements, the results on binding thermodynamics allow us to draw important conclusions on the nature of the molecular forces taking part in the complexation of folic acid with BSA. With increase in ionic strength, reduction in the binding affinity of folic acid to BSA indicates involvement of electrostatic interactions in the complexation. The contribution of hydrophobic interactions in the binding process is also demonstrated by a decrease in the binding affinity of the folic acid to BSA in the presence of TX-100. The reduction in the binding affinity in the presence of higher concentrations of TX-100 can be due to blockage of the hydrophobic binding sites of BSA by the neutral molecule, TX-100. However, the fact that the loss of binding in the presence of NaCl occurs at 0.05 mol · dm^{-3} level indicates that NaCl strongly interferes in the binding and the involvement of electrostatic interactions is more predominant. Since folic acid contains 2-amino-4-hydroxy-6-methyl pterin, para-aminobenzoic acid, and L-glutamic acid groups and it is comprised of hydrophilic hydroxyl, amino groups, and hydrophobic side chains, it can interact via hydrogen bonding through several hydroxyl groups. The presence of sucrose interferes in the binding thereby suggesting that hydrogen bonding contributes to the binding process. This is also reflected in the relatively low enthalpy of binding since hydrogen bond formation is always accompanied by heat evolution in the range of $(6 \text{ to } 84) \cdot 10^3$ J · mol^{-1}. The standard enthalpy change of binding in the presence of 1 mol · dm^{-3} sucrose is $11.7 \cdot 10^3$ J · mol^{-1}. This is consistent with very weak hydrogen bonding. Electrostatic interactions lead to a decrease in the entropy of the system where as hydrophobic interactions of organic anions with serum albumin have been reported to lead to an increase in entropy [23]. In the

TABLE 4
Lifetimes (τ_1, τ_2), average lifetime ($\langle\tau\rangle$) and amplitudes (A_1, A_2) of $0.15 \cdot 10^{-7}$ mol · dm^{-3} BSA in the presence of folic acid.

Folic acid/ ($10^6 \cdot$ mol · dm^{-3})	Lifetime/ns		Amplitude		Avg. lifetime/ (10^{-9} s) $\langle\tau\rangle$	χ^2
	τ_1	τ_2	A_1	A_2		
0	3.13	6.69	26.9	73.1	5.72	1.03
10	2.41	6.50	29.0	71.0	5.31	1.05
25	1.91	6.25	32.7	67.2	4.82	1.07
50	2.48	6.60	46.1	53.9	4.69	1.10
100	1.91	6.03	54.3	45.6	3.78	1.08

present work, the entropy of binding in all the experiments is observed to be negative except in case of sucrose thereby indicating the predominance of electrostatic interactions in the binding.

Crystallographic analysis of HSA revealed that protein contains three homologous α-helical domains (I, II and III), each of which contains sub-domain (A and B). The principal regions of binding sites of albumin are located in hydrophobic cavities in sub-domains IIA and IIIA. The tryptophans occupy the 135 and 214 positions in the BSA polypeptide chain. In the tertiary structure of BSA, they are located in the sub-domain IB and IIA [29]. The quenching of BSA fluorescence is accompanied by a blue shift of fluorescence. This indicates the decrease in the polarity of tryptophan environment. Folic acid can form a complex with BSA via $\pi-\pi$ interactions between aromatic rings of folic acid and aromatic residues Phe 309, Phe 330 and Tyr 353 present in sub-domain II B. Similar interactions have been described for the complex of methotrexate and BSA which has same chemical structure as folic acid [30]. The ITC results show that binding constant decreases in the presence of NaCl indicating ionic/polar interaction. This suggests possible electrostatic interaction between negatively charged groups of folic acid and Arg 199 or Lys 222 [9,31]. The carbonyl group of folic acid can form hydrogen bond with Arg 237 and Ser 287 residues of sub-domain II A.

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