Thermochimica Acta 482 (2009) 21–29

Contents lists available at ScienceDirect

Thermochimica Acta

journal homepage: www.elsevier.com/locate/tca

Binding of streptomycin with bovine serum albumin: Energetics and conformational aspects

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ARTICLE INFO

Article history:
Received 9 July 2008
Received in revised form 1 October 2008
Accepted 3 October 2008
Available online 1 November 2008

Keywords:
Bovine serum albumin
Streptomycin
Isothermal titration calorimetry
Spectroscopy
Binding constant

ABSTRACT

Thermodynamics of the binding of antibiotic streptomycin to bovine serum albumin have been studied using isothermal titration calorimetry in combination with fluorescence, UV–vis and circular dichroism spectroscopies. The values of van't Hoff enthalpy calculated from the temperature dependence of the binding constant do not agree with the calorimetric enthalpies indicating temperature dependent conformational changes in the protein upon binding. With increase in the ionic strength, reduction in the binding affinity of streptomycin to BSA is observed suggesting the predominance of electrostatic interactions in the binding. The contribution of hydrophobic interactions in the binding is also demonstrated by decrease in binding affinity in the presence of tetrabutylammonium bromide (TBAB). The value of binding affinity in the presence of sucrose indicates that hydrogen bonding is not a significant contribution in complexation. The results have permitted quantitative evaluation of the interaction of streptomycin with bovine serum albumin.

1. Introduction

The interaction of a drug with blood components influences its bioavailability and can affect the functions of several biomolecules [1,2]. Ligand–protein interactions play a key role in the distribution and transport of small molecules in biological systems. Understanding the molecular basis of these interactions is important in the rational design of new and more efficient therapeutic agents than can recognize and bind to specific biological targets for improved drug activity. Serum albumins are important blood proteins which have ability to transport multitude ligands such as fatty acids, amino acids, steroids, metal ions and drugs. Animal experiments are indispensable in providing basic information on the pharmacological actions, bio-transformation, bio-distribution, etc. of drugs. Bovine serum albumin (BSA) is well suited to these studies, since it has been extensively characterized. Studying binding phenomenon with BSA further helps in explaining the relationship between structures and functions of proteins. BSA, one of the major components in the plasma protein, is a single-chain 582 amino acid globular nonglycoprotein cross-linked with 17 cystine residues (eight disulfide bonds and one free thiol). It is divided into three linearly arranged, structurally distinct, and evolutionarily related domains (I–III), each of which is composed of two subdomains (A and B) [3–5]. The capability of serum albumins to bind aromatic and heterocyclic compounds is largely dependent on the existence of two major binding regions, namely Sudlow site I and site II [6,7], which are located within specialized cavities in subdomains IIA and IIIA, respectively [8,9]. Both these domains are characterized by the presence of a central cavity formed from six amphipathic helices arranged in a myoglobin like fold.

Antibiotics are an important class of ribosomal ligands [10]. Among them, streptomycin belongs to amino glycosides category of drugs which promotes misreading of messenger RNA [11]. Streptomycin, 5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)-4-[4,5-dihydroxy-6-(hydroxymethyl)-3-methylamino-tetrahydropyran-2-yl] oxy-3-hydroxy-2-methyl-tetrahydrofuran-3-carbaldehyde, is a bactericidal antibiotic (Fig. 1) which is used in the treatment of tuberculosis [12]. It is known to interfere with bacterial protein synthesis through interaction with 30S subunits and 70S ribosome [13–15]. It also inhibits polypeptide chain initiation and increases the errors in polypeptide chain elongation [16].

The studies on the interaction of antibiotics with serum albumin are important in understanding the drug delivery process. Though the interactions of some antibiotics with the serum albumin are reported in literature [11,17–19], quantitative studies on the interaction of streptomycin with serum albumin addressing the energetics and type of interactions is not available in literature. A combination of isothermal titration calorimetry and fluorescence spectroscopy has yielded valuable information on binding interactions in biologically important systems both quantitatively and qualitatively [20–25]. In this work, we have used isothermal titration calorimetry in determining binding affinity, enthalpy, entropy and stoichometry

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doi:10.1016/j.tca.2008.10.012

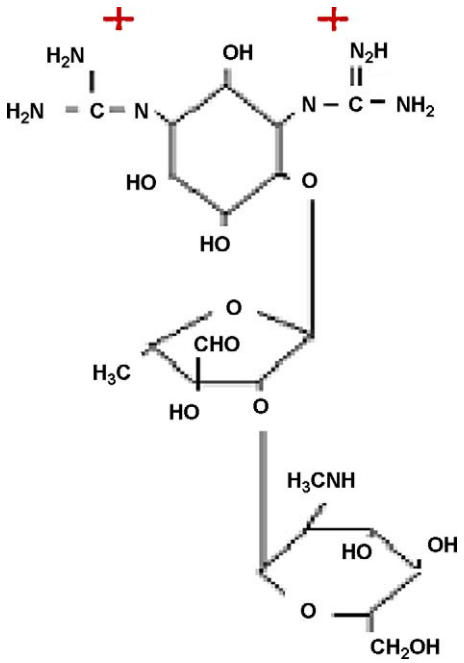

Fig. 1. Structure of streptomycin.

of binding of streptomycin with bovine serum albumin at 283.15 K, 293.15 K, 298.15 K, 303.15 K and 308.15 K. We have used fatty acid free BSA to avoid interference due to the binding capacity of fatty acids to the protein. The corresponding conformational changes in the protein as the result of binding have been monitored by UV–vis, fluorescence and circular dichroism spectroscopies.

2. Experimental

2.1. Material and methods

Fatty acid free bovine serum albumin and streptomycin sulphate were purchased from Sigma–Aldrich Chemical Private Limited, USA. Tetrabutylammonium bromide (TBAB) was purchased from Spectrochem Private Limited, India, and sodium chloride and sucrose were extra pure analytical reagent grade obtained from Merck, India. The water used for preparing the solutions was double distilled and then deionized using a Cole Parmer research mixed bed ion exchange column. The protein stock solutions were prepared by extensive overnight dialysis at 4 °C against 10 mM 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.

Table 1
Binding constant (K_a), enthalpy (ΔH°) and entropy(ΔS°) binding accompanying the titration of 5×10^{-3} mol dm^{-3} streptomycin with 0.045×10^{-3} mol dm^{-3} BSA at pH 7.0 at different temperatures according to Eq. (5).

T (K)	K_a (M^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J K^{-1} mol^{-1})	ΔG° (kJ mol^{-1})
283.15	$(2.39 \pm 0.12) \times 10^3$	-24.06 ± 0.42	-20.25	-18.33
293.15	$(2.30 \pm 0.41) \times 10^3$	-24.27 ± 2.34	-19.00	-18.87
298.15	$(2.23 \pm 0.07) \times 10^3$	-26.36 ± 1.59	-24.81	-19.12
303.15	$(2.12 \pm 0.05) \times 10^3$	-35.27 ± 2.09	-52.72	-19.29
308.15	$(1.10 \pm 0.07) \times 10^3$	-67.66 ± 0.04	-161.92	-17.95

2.2. UV–vis spectroscopic measurements

The concentration of BSA was determined on a Shimadzu double-beam spectrophotometer (UV-265), using extinction coefficient of $E^{1\%}_{280} = 6.8$ [26]. If the extinction coefficient of the protein were to be in error by 5%, the calculations suggest that the maximum error in the thermodynamic quantities reported in Tables 1 and 2 will be within 10%. The thermal denaturation of bovine serum albumin in the presence of streptomycin was also carried out on this spectrophotometer with a Cole Parmer constant – temperature circulator attached to it. With this arrangement, the temperature of the solutions in the cuvettes was stable to ±0.05 K. Before loading into the cuvettes, all the solutions were degassed and the water loss due to vaporization was made up by adding appropriate amount of deionized and degassed water. The reference solution was buffer when the measurements were done made in buffer, or buffer containing drug when the experiments were carried out in the presence of the drug. To check the reversibility of thermal denaturations, the sample in the first scan was heated to a temperature slightly above the complete denaturation temperature, cooled immediately, and then reheated. The data on absorbance as a function of temperature was analyzed with the EXAM program of Kirchoff [27] and thermodynamic parameters were evaluated.

2.3. Isothermal titration calorimetry

The energetics of the binding of streptomycin to BSA was assessed using an isothermal titration calorimeter (VP-ITC Microcal, Northampton, MA). Before loading, the solutions were thoroughly degassed by using Thermo Vac degassing unit supplied by Microcal, USA. The reference cell was filled with the respective degassed buffer. The protein solution was kept in the sample cell, and aqueous streptomycin was filled in the syringe of volume 250 μL. The drug solution was added sequentially in 10 μL aliquots (for a total of 25 injections, 20 s duration each) at 4 min intervals. The values of heat of dilution were determined independently and corrections were applied to the main BSA–streptomycin heat profiles. The data were analyzed for obtaining the values of binding constant K_a, enthalpy ΔH, entropy ΔS, free energy ΔG° and

Table 2
Binding constant (K_a), enthalpy (ΔH°) and entropy(ΔS°) binding accompanying the titration of 5×10^{-3} mol dm^{-3} streptomycin with 0.045×10^{-3} mol dm^{-3} BSA at pH 7 and 25.0 °C in presence of NaCl, TBAB and Sucrose according to Eq. (5).

Concentration (mol dm^{-3})	K_a (M^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J K^{-1} mol^{-1})	ΔG° (kJ mol^{-1})
[NaCl]				
0.1	$(1.74 \pm 0.33) \times 10^3$	-5.44 ± 0.67	-44.43	-18.49
0.35	$(0.36 \pm 0.16) \times 10^3$	-35.56 ± 0.33	-122.17	-14.60
0.5		No binding observed		
[TBAB]				
0.02	$(0.84 \pm 0.31) \times 10^3$	-69.87 ± 4.14	-180.12	-16.69
0.05	$(0.50 \pm 0.12) \times 10^3$	-38.41 ± 1.97	-76.99	-15.40
0.1		No binding observed		
[Sucrose]				
1.0	$(2.28 \pm 0.02) \times 10^3$	-37.91 ± 2.72	-62.97	-19.16

stoichiometry of binding with a single set of binding sites using the Origin 7 software provided by Microcal [28]. According to the single site binding model, the total heat content Q of the solution contained in the active cell volume V_0 (determined relative to zero for the unligated species) at fractional saturation Θ is given by,

$$Q = n\Theta M_t \Delta H V_0 \quad (1)$$

where, ΔH is the molar heat of ligand binding, M_t is the total concentration of the macromolecule and n is the number of binding sites in the macromolecule. The binding constant K_a is related to the fractional saturation Θ and free concentration of the ligand $[X]$ by,

$$K = \frac{\Theta}{(1-\Theta)[X]} \quad (2)$$

The heat released $\Delta Q(i)$ from the ith injection for an injection volume dV_i is then given by the following equation:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i)+Q(i-1)}{2}\right] - Q(i-1) \quad (3)$$

2.4. Fluorescence spectroscopy

Intrinsic fluorescence experiments were performed on a PerkinElmer LS-55 spectrofluorimeter with a 3 mL quartz cell that had a path length of 1 cm. The protein concentration in all the experiments was kept at 1.508×10^{-6} mol dm^{-3} and the [Streptomycin]/[BSA] molar ratio was varied from 0.07 to 53.33. Both the excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at 295 nm to selectively excite the tryptophan residues, and the emission spectra were recorded in the wavelength range of 300–400 nm at a scan rate of 100 nm min^{-1}. The data thus obtained were analyzed by modified Stern–Volmer equation to calculate the value of binding constant.

2.5. 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 5×10^{-6} mol dm^{-3} and 0.2 cm while 20×10^{-6} mol 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 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

$$[\theta] = 100 \times \left(\frac{\theta}{c \times l}\right) \quad (4)$$

where c is the concentration of the protein in mol dm^{-3} and l is the path length of the cell (in centimeters). These experiments were performed for the [Streptomycin]/[BSA] molar ratios in the range of 1.0–25.0.

2.6. 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 λ_{ex} = 295 nm and λ_{em} = 340 nm. The full width at half-maximum of the instrument response function is 250 ps and the resolution is 56 ps per channel. The data are fitted to biexponential function after deconvolution of the instrument response function by an iterative reconvolution 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 15×10^{-6} mol dm^{-3} and the [streptomycin]/[BSA] ratio was varied from 1:1 to 1:10.

3. Results and discussion

3.1. Isothermal titration calorimetry of the binding of streptomycin to BSA

A representative isothermal titration calorimetric heat profile for the binding of streptomycin with BSA at pH 7.0 and 298.15 K is shown in Fig. 2A. Each peak in the binding isotherm represents a single injection of the drug into the protein solution. The thermodynamic parameters accompanying the binding of streptomycin to BSA at different temperatures are summarized in Table 1.

As seen in Fig. 2A, the titration of streptomycin with BSA yielded positive heat deflection. The dilution of 5×10^{-6} mol dm^{-3} streptomycin is also significantly endothermic process (Fig. 2B). The integrated heat profile shown in the bottom panel of Fig. 2A is corrected for all the dilution effects. The overall binding as reflected by the overall titration profile after correction for the dilution effects shows that the binding of streptomycin to BSA is an exothermic process accompanied by an unfavorable change in the entropy of binding. The value of K varies from $(2.39 \pm 0.12) \times 10^3$ M^{-1} at 283.15 K to $(1.10 \pm 0.07) \times 10^3$ M^{-1} at 308.15 K. It is recommended [29] that for the most reliable ITC measurements, the values of c parameter (protein concentration K) should be between 1 and 1000. Experiments were also performed with 0.33 mM BSA (20 mg ml^{-1}) to raise the value of c parameter, and the results obtained were essentially same (see supplementary information). The disadvantage of using such a high concentration of BSA in the binding experiments is that the results will be affected by the aggregation of the protein. Further, the agreement of the ITC results with those obtained from fluorescence measurements supports the accuracy of the results obtained. The value of the stoichiometry of the binding is fixed to one based upon the goodness of the fit to ITC data and the fluorescence observations as discussed further in the text. Therefore we represent the binding reaction in the form of following equation.

$$\text{Streptomycin} + \text{BSA} = \text{Streptomycin} \cdot \text{BSA} \quad (5)$$

The enthalpy of binding is observed to vary non-linearly with temperature suggesting that heat capacity of binding is temperature dependent. The data presented in Table 1 indicates that though the binding of streptomycin to the BSA is exothermic, the value of the binding constant decreases with rise in temperature. Hence the van't Hoff dictates are not followed. The inconsistency between the molar enthalpy of reaction calculated from the van't Hoff equation and calorimetrically determined molar enthalpy of reaction could be due to conformational changes in the protein as a function of temperature, as well as upon ligand binding.

Complete enthalpy–entropy compensation resulting in linear relationship is generally obtained when a group of similar species interact [30,31]. Enthalpy–entropy compensation is typically assigned to solvation effects, solvent structure, and more specifically, this is usually ascribed to be a property of water. The values of entropy of binding varies from -4.84 J K^{-1} mol^{-1} at 283.15 K to -38.70 J K^{-1} mol^{-1} at 308.15 K. The large unfavorable entropy changes for streptomycin binding are atypical for water loss on binding to a hydrated pocket and for desolvation of the ligand. The thermodynamic parameters presented in Table 1 associated with the binding of streptomycin with BSA show a linear relationship between ΔH and $T\Delta S$ (Fig. 3) with the slope of the plot as 0.99 and a correlation coefficient of 0.999.

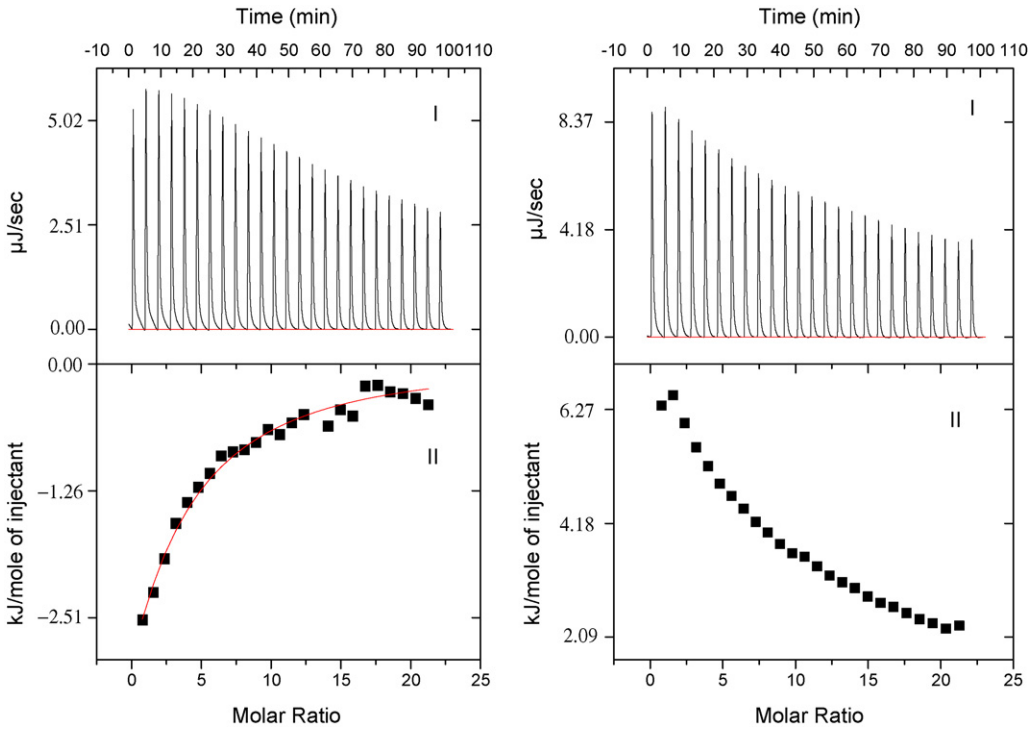

Fig. 2. (A) (I) Raw data for the titration of 5×10^{-3} mol dm^{-3} streptomycin with 0.045×10^{-3} mol dm^{-3} BSA at pH 7 and 298.15 K. (II) Integrated heat profile of the calorimetric titration shown in panel I. The solid line represents the best nonlinear least- squares fit to a single binding site model. (B). Raw data for the titration of 5×10^{-3} mol dm^{-3} streptomycin with buffer (II) Integrated heat profile of the calorimetric titration shown in panel I.

3.2. Ionic strength dependence of the binding of streptomycin to BSA

The salt dependence of a bimolecular association is often used to assess the contribution of charge–charge interactions to the free energy of binding. To understand the role of electrostatic interactions in the binding process, the ionic strength dependence of the binding of streptomycin to BSA was studied. The experiments were performed in the presence of 0.1–0.5 mol dm^{-3} NaCl and the corresponding binding parameters thus obtained are listed in Table 2.

For streptomycin, an increase in the ionic strength from 0.1 mol dm^{-3} to 0.35 mol dm^{-3} leads to a decrease in the value of binding affinity of the drug as reflected by fall in the value of K by a factor of nearly 5. On further increasing the ionic strength to 0.5 mol dm^{-3}, no detectable binding is observed (Fig. 4). The fall in the value of binding constant with an increase in ionic strength indicates that the association of streptomycin with BSA involves ionic interactions since the ions of NaCl can interfere in the binding process.

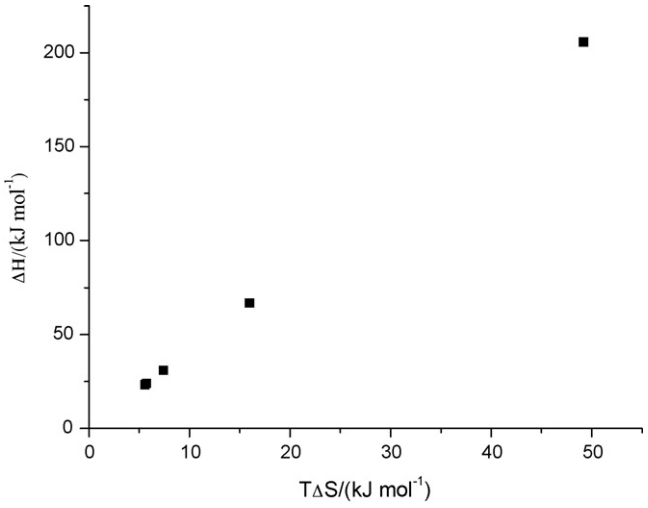

Fig. 3. Plot of enthalpy–entropy compensation for the binding of streptomycin to BSA at pH 7.0 and various temperatures.

3.3. Effect of tetrabutylammonium bromide on the binding of streptomycin to BSA

TBAB as cosolute can give better insights into role of hydrophobic and electrostatic interactions in the binding process since TBAB can affect its both charge–charge and hydrophobic interactions [32]. The binding experiments were done in the presence of 0.02–0.1 mol dm^{-3} TBAB and the results obtained are listed in Table 2.

Fig. 5 shows the ITC profiles obtained for the binding of streptomycin to BSA in the presence of TBAB. In the presence of 0.02 mol dm^{-3} and 0.05 mol dm^{-3} TBAB, the binding affinity of streptomycin with BSA decreases. On further increasing the concentration of TBAB to 0.1 mol dm^{-3}, no binding profile is observed. On comparing the results of titration of streptomycin with BSA in the presence of 0.1 mol dm^{-3} NaCl and 0.1 mol dm^{-3} TBAB, it is seen that the binding is almost lost in the latter where as the value of K in the former is $(1.74 \pm 0.33) \times 10^3$ M^{-1}. This indicates that hydrophobic interactions also play significant role in the binding process since the value of K decreases due to interference of the bulky tetrabutyl groups of TBAB.

3.4. Effect of sucrose on the binding of streptomycin to BSA

Studies on streptomycin–BSA interactions in the presence of sucrose will provide information on the involvement hydrogen

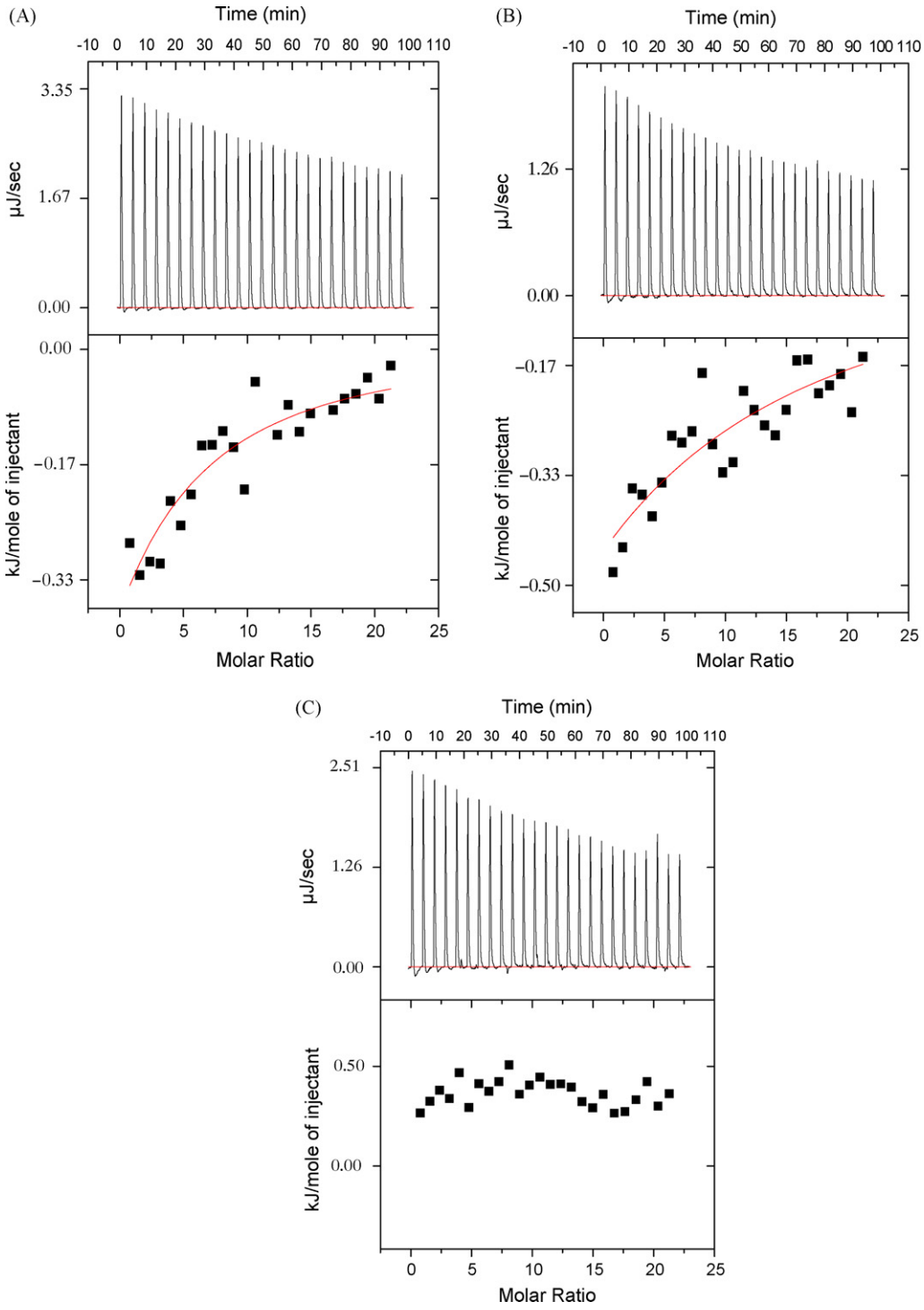

Fig. 4. ITC profiles for the titration of 5×10^{-3} mol dm^{-3} streptomycin to 0.045×10^{-3} mol dm^{-3} BSA in the presence of (A) 0.1 (B) 0.35 and (C) 0.5 mol dm^{-3} NaCl at pH 7 and 298.15 K.

bonding in the binding process since sucrose has hydroxyl groups which can interfere in the hydrogen bond formation between the drug and protein. In the presence of 1 mol dm^{-3} sucrose, the binding affinity of the streptomycin to the protein remains the same as in the absence of sucrose (Fig. 6, Table 2) though binding is more exothermic. Since the binding affinity does not change, it indicates that sucrose does not interfere in the binding of streptomycin with BSA. However the increase in exothermicity of binding may have contributions from the structural changes of water in the presence of sucrose.

3.5. Fluorescence measurements

The binding of streptomycin with BSA was also assessed by intrinsic fluorescence measurements (Fig. 7). The maximum fluorescence intensity for BSA in the absence of the streptomycin is observed at 345.3 nm. A steady increase in the intensity of the emission maxima is observed upon addition of streptomycin with a maximum shift in the value of $\lambda_{max} = 2$ nm. These fluorescence results indicate that the binding of streptomycin does not cause a significant alteration in the local environment of the tryptophan

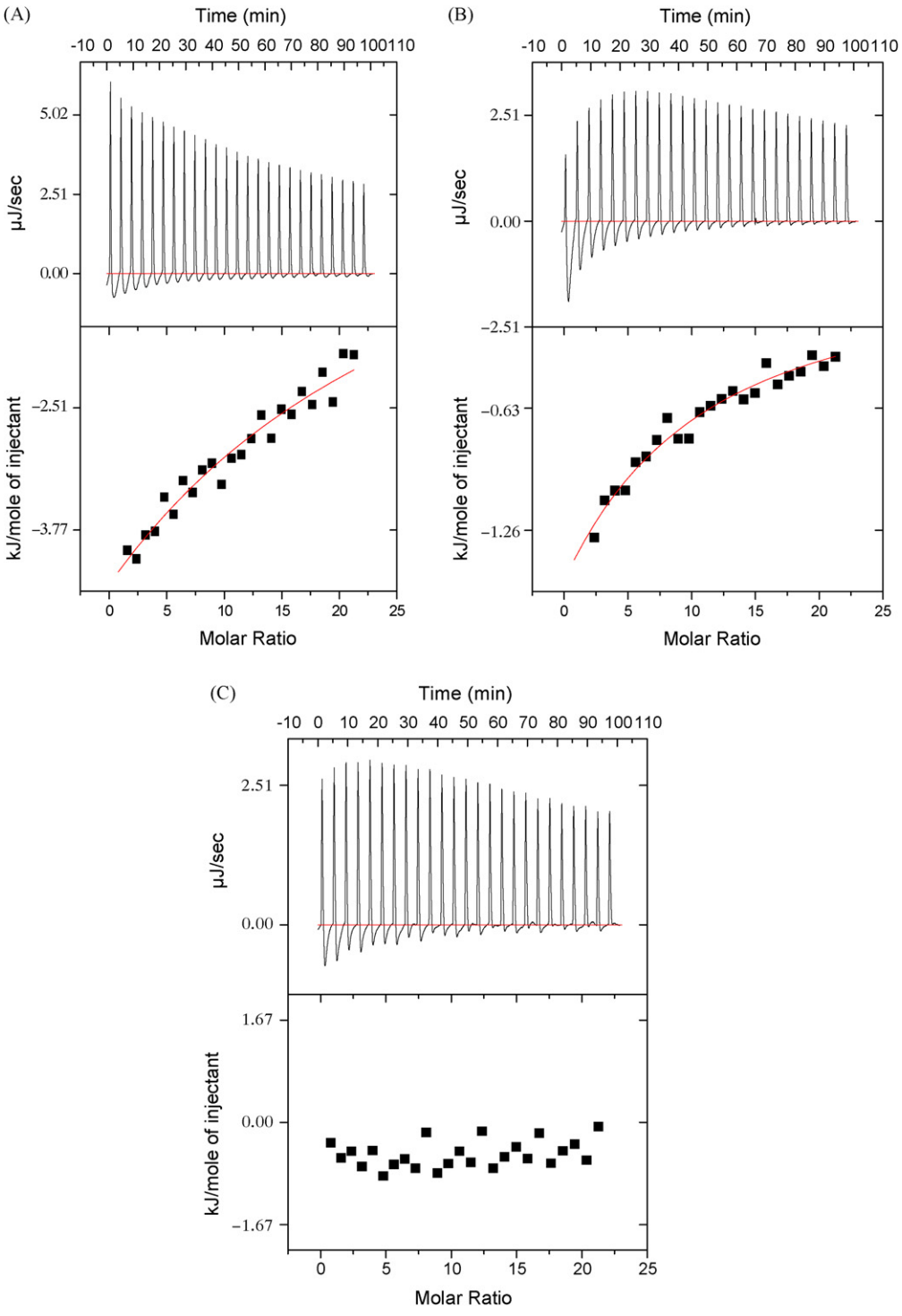

Fig. 5. Isothermal titration calorimetric profile for the titration of 5×10^{-3} mol dm^{-3} streptomycin to 0.045×10^{-3} mol dm^{-3} BSA in the presence of (A) 0.02 (B) 0.05 and (C) 0.1 mol dm^{-3} TBAB at 298.15 K.

moieties, which are located at positions 134 and 212 in the sub domain IA and IIA, respectively [8].

A steady increase in BSA fluorescence intensity with increase in streptomycin concentration was used in the determination of binding constant and number of binding sites on BSA as described by Tedesco et al. [33–35]. According to this method the BSA fluorescence intensity (F) scales up with increase in streptomycin concentration according to the following relation [36]:

$$\frac{F_0 - F}{F - F_\infty} = \left(\frac{[\text{Streptomycin}]}{K_b}\right)^n \quad (6)$$

The binding constant K_b is obtained by plotting $\log(F_0 - F)/(F - F_\infty)$ versus log [Streptomycin], where F_0 and F_∞ are the relative fluorescence intensities of BSA alone and BSA saturated with streptomycin, respectively. The slope of the double-logarithm plot (Fig. 8) gives the number of the binding site (n), whereas the value of

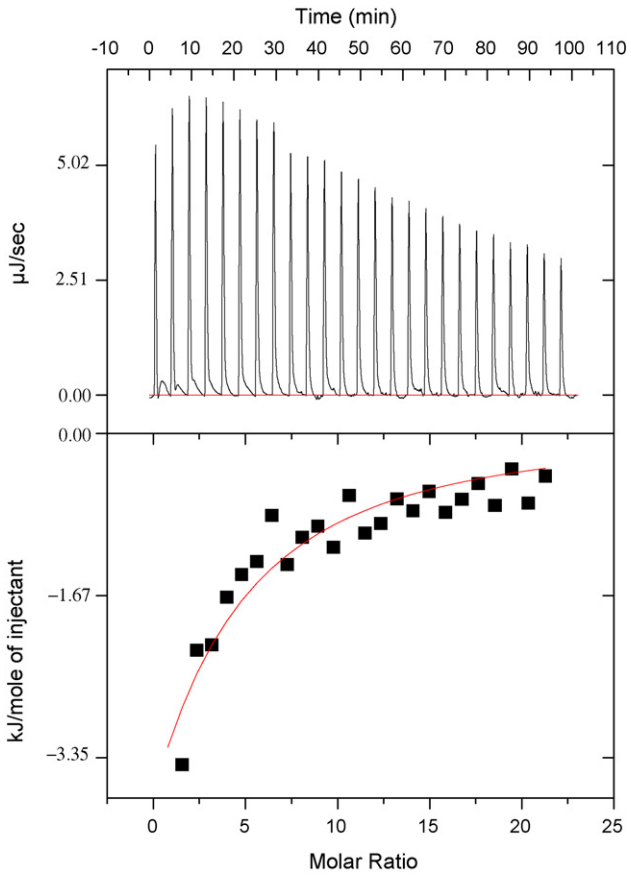

Fig. 6. ITC profile for the titration of 5×10^{-3} mol dm^{-3} streptomycin to 0.045×10^{-3} mol dm^{-3} BSA in the presence of 1 mol dm^{-3} Sucrose at 298.15 K.

log [streptomycin] at log $[(F_0 - F)/(F - F_\infty)] = 0$ equals the logarithm of the binding constant (K_b). The value of K_b and n thus obtained are 6.23×10^3 M^{-1} and 0.92, respectively. Since the fluorescence results suggest nearly 1:1 stoichometry for the streptomycin complex, the value of N in the ITC data analysis was fixed to 1.0.

3.6. Lifetime measurements

Fluorescence lifetime measurements were carried out for BSA in the absence and presence of streptomycin. The sample containing

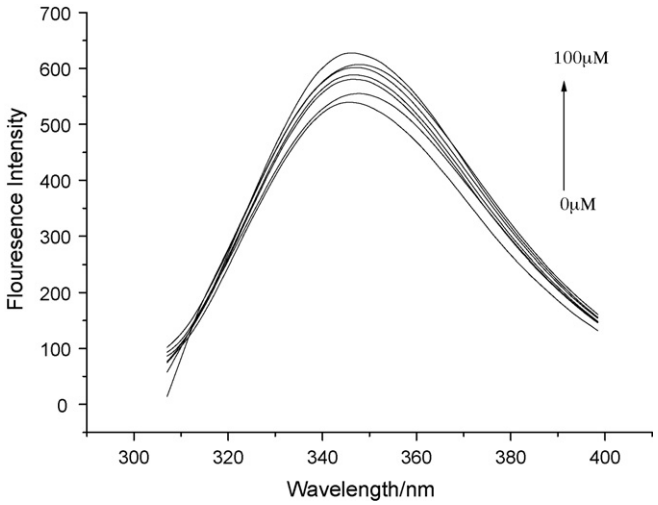

Fig. 7. Intrinsic fluorescence emission spectra of 1.5×10^{-6} mol dm^{-3} BSA in presence of various concentrations of streptomycin.

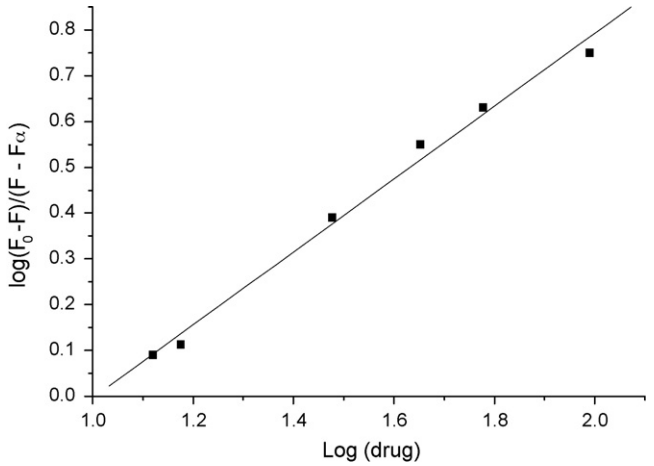

Fig. 8. Plot of log $[(F_0 - F)/(F - F_\infty)]$ against log [streptomycin] to determine the value of binding constant.

15×10^{-6} mol dm^{-3} BSA was excited at 295 nm and the emission intensity was monitored at 340 nm. The decay curves in Fig. 9 fitted well to biexponential function yielding relative fluorescence lifetimes of 2.92 ns and 6.77 ns, and an average lifetime (τ_0) of 5.57 ns. It can be seen from Fig. 9 and Table 3 that the decay remains biexponential [37]. The fluorescence lifetime of BSA did not change significantly with the rise in the concentration of streptomycin suggesting formation of static complex between the drug and the protein.

3.7. Thermal denaturation of BSA with the streptomycin

Since the ITC results show weak binding pattern for the streptomycin–BSA interactions, the thermal denaturation of BSA in the presence of the drug was also performed. These experiments were done to understand if the drug provides any thermal stability to the protein as a result of binding. Fig. 10 shows the thermal denaturation profiles of BSA in the absence and presence of different concentrations of streptomycin at pH 7.0 and the corresponding thermodynamic parameters are given in Table 4. The mid point

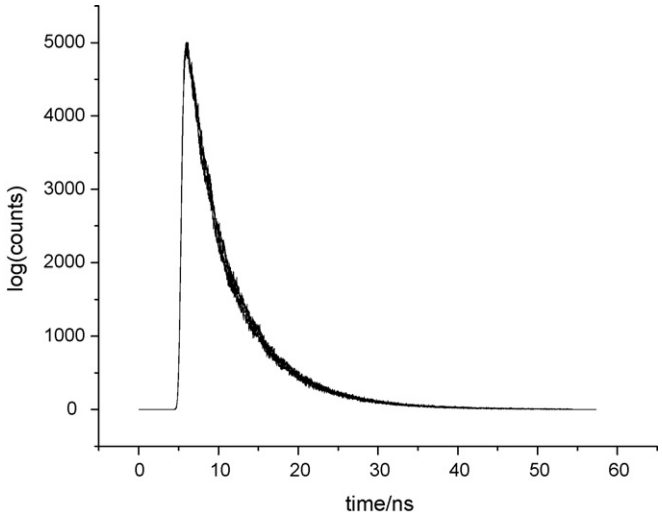

Fig. 9. Fluorescence decay profiles of 15×10^{-6} mol dm^{-3} BSA in the absence and presence of streptomycin in 10×10^{-3} mol dm^{-3} phosphate buffer of pH 7.0. $\lambda_{ex} = 295$ nm and $\lambda_{em} = 344$ nm. BSA concentration was fixed at 15×10^{-6} mol dm^{-3} and BSA–streptomycin in ratio 1:1, 1:5 and 1:10.

Table 3
Lifetime of fluorescence decay of 15×10^{-6} mol dm^{-3} BSA in phosphate buffer of pH 7.0 at different concentration of streptomycin.

Streptomycin	Lifetime (ns)		Amplitude		Average lifetime (ns)	χ^2
(μM)	τ_1	τ_2	A_1	A_2		<τ>
0	2.92	6.77	31.7	68.3	5.57	1.12
15	4.38	7.34	47.7	52.3	5.98	1.18
75	4.42	7.45	51.6	48.40	5.93	1.09
150	4.53	7.56	49.30	50.07	6.03	1.06

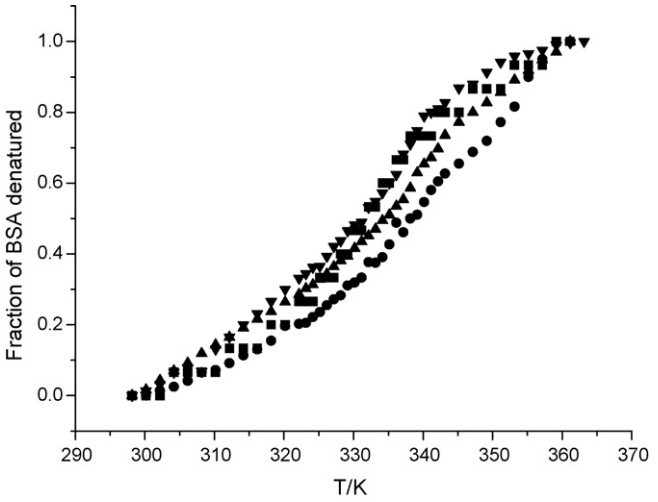

Fig. 10. Thermal denaturation of 7.25×10^{-6} mol dm^{-3} BSA in the absence and presence of varying concentration of streptomycin/10^{-3} mol dm^{-3} at pH 7.0: (●) 0, (▲) 3, (■) 4 and (▼) 6.

of thermal transition for BSA was observed at (334.8 ± 0.3) K. The maximum increase in the value of transition temperature of BSA observed in the presence of 0.8×10^{-3} mol dm^{-3} streptomycin is (339.5 ± 0.2) K.

It is well known that protein thermal stability is modified by ligand binding due to coupling between two mutual processes under equilibrium. Relatively small enhanced thermal stability of BSA in the presence of the streptomycin supports the ITC results that streptomycin binds weakly to the native BSA. The enthalpy of unfolding also does not change significantly which suggests further weak binding of the drug to the protein.

Table 4
The transition temperature ($T_{1/2}$) and enthalpy (ΔH) change parameters accompanying the thermal unfolding of 14×10^{-6} mol dm^{-3} BSA at the different concentration of streptomycin.

Streptomycin (10^{-3} mol dm^{-3})	$T_{1/2}$ (K)	ΔH (kJ mol^{-1})
0	334.8 ± 0.3	225.5 ± 4.6
0.2	334.5 ± 0.2	220.3 ± 16.5
0.3	336.7 ± 0.3	233.0 ± 13.2
0.4	337.3 ± 0.2	239.0 ± 8.6
0.5	338.6 ± 0.1	248.6 ± 3.9
0.6	339.1 ± 0.0	268.9 ± 4.4
0.8	339.5 ± 0.2	269.0 ± 5.8

Experiments were conducted on the effect of TBAB on the thermal stability of BSA. The value of $T_{1/2}$ in the presence of 0.1 M TBAB is 329.2 K which is 5.6 K lower than that of the protein in the absence of TBAB. However, NaCl is reported [38] to stabilize BSA at pH 7.0 by about 14 K at 0.5 M concentration level where no binding of streptomycin to BSA is observed by us. These results indicate that the effect of decreased binding in the presence of these additives is not due to denaturation of the enzyme in the presence of TBAB and NaCl in the studied concentration range.

3.8. Circular dichroism spectroscopy

Circular dichorism is a sensitive technique to monitor the conformational changes in the protein upon interaction with the ligand. The CD spectra of BSA in the absence and presence of streptomycin are shown in the Fig. 11. The CD spectra of BSA exhibited two negative bands in the far UV CD region at 208 nm and 220 nm, which are characteristics of an α-helical structure of the protein [39]. Upon increasing concentration of streptomycin in BSA, the secondary structure of BSA is observed to increase. However, there is no appreciable change in the tertiary structure of BSA. An increase in the secondary structural content is consistent with the rise in the transition temperature and calorimetric enthalpy of denaturation.

4. Mode of interaction

In addition to the quantitative measurements, the results on binding energetics obtained have allowed us to draw important conclusions on the nature of molecular forces taking part in the complexation of streptomycin with BSA. With increase in ionic strength, reduction in the binding affinity of streptomycin to BSA indicates involvement of electrostatic interactions is in the

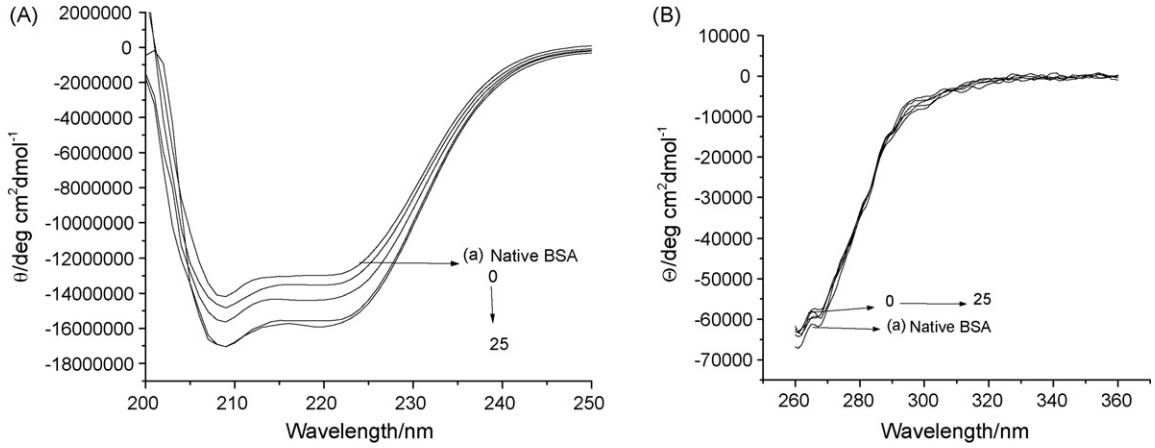

Fig. 11. Circular dichorism (CD) spectra of BSA in the presence of streptomycin: (A) Far UV CD spectroscopy and (B) near UV CD spectroscopy. The concentration used are represented as [streptomycin]/[BSA] molar ratio was 1 (b) 10 (c) 20 (d) and 25 (e).

complexation. The contribution of hydrophobic interactions in the binding process is also demonstrated by decrease in the binding affinity of the streptomycin to BSA in the presence of TBAB which has four bulkier hydrophobic tetrabutyl groups. The reduction in the binding affinity in the presence of higher concentrations of TBAB can also have contributions from its partial denaturating ability on the protein. However, the fact that the loss of binding in the presence of NaCl occurs at 0.5 mol dm^{-3} level, it indicates that the involvement of electrostatic interactions are significant and more predominant. Though streptomycin has several hydroxyl groups, the presence of sucrose does not interfere in the binding thereby suggesting that hydrogen bonding is probably not significant contribution in the complexation. This is also reflected in the relatively low heat of binding since hydrogen bond formation is always accompanied by heat evolution in the range of 6–84 kJ mol^{-1}. 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 increase in entropy [40]. In the present work, the entropy of binding in all the experiments is observed to be negative thereby indicating predominance of electrostatic interactions in the complexation. Upon binding to streptomycin, the thermal stability of BSA is enhanced as a result of strengthening of its secondary structure.

Acknowledgement

Financial support from Council of Scientific and Industrial Research, and Department of Science and Technology New Delhi is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tca.2008.10.012.

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