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Thermodynamic aspects of the molecular recognition of drugs by human serum albumin ☆

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Abstract

Binding sites on human serum albumin (HSA) for some typical anionic drugs (Site I- and Site II-bound drugs) have been thermodynamically characterized by flow microcalorimetry. The binding and the thermodynamic parameters were computed directly from the calorimetric titration data at 37°C in a phosphate buffer (pH 7.4) using one- and two-class binding models. From compensation analysis by plotting the molar enthalpy change ($\Delta H_{m,i}$) versus the molar free energy change ($\Delta G_{m,i}$), and the molar entropy change ($\Delta S_{m,i}$) for every class of HSA binding sites, drug binding was classified into groups S1, S2 and S3, which agreed with those of fatty acid binding. Groups S1 and S2 included high-affinity binding sites (the first class of binding sites) and low-affinity binding sites (the second class of binding sites) for Site II-bound drugs, respectively, and group S3 contained the high-affinity binding sites for Site I-bound drugs. In each group, the $\Delta H_{m,i}$–$\Delta S_{m,i}$ plot gave an excellent linear relationship, where the value of T/β calculated from the intercept interpreted the molecular recognition by HSA as a quantitative measure of the hydrophobic interaction upon complex formation. Groups S1 and S2 were characterized by large negative values of $\Delta H_{m,i}$ and $\Delta S_{m,i}$, reflecting van der Waals interaction and hydrogen bonding formation in low dielectric media. The main force stabilizing the binding complex in group S3 was hydrophobic interaction characterized by small negative $\Delta H_{m,i}$ values, minor or positive $\Delta S_{m,i}$ values, and a large positive value of $T\Delta S_0$ (32.4 kJ mol^{-1}).

Keywords: Albumin; Drug; Enthalpy; Entropy; Human serum albumin

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1. Introduction

The drug binding and displacement reactions on human serum albumin (HSA) strongly influence the distribution, elimination and pharmacological effect of drugs [1–4]. Most drugs bind reversibly to a limited number of binding regions of albumin and some workers have adopted the view that there are at least two major binding sites: Site I (azapropazone site) and Site II (benzodiazepine site) [2,5–7]. Recent descriptions of the three-dimensional structure of crystalline albumin have revealed that HSA comprises three contiguous domains (denoted I, II and III), each containing two subdomains (A and B) that possess common structural motifs [8–10]. It is suggested that the principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA. The crystal structure map of the binding region on HSA is a major step forward in understanding this transport protein, but has not yet yielded detailed information about the molecular basis for its unique drug-binding capabilities and factors forming and stabilizing drug–HSA complexes in the blood.

The binding of ligands to proteins such as drug–HSA complexation occurs mostly through weak non-covalent interactions, namely hydrophobic interaction, van der Waals forces, and hydrogen bonding in low dielectric medium [11–13]. Thermodynamic parameters can potentially provide valuable insight as to the nature of the interactions and the forces that stabilize the complexes. Recently, microcalorimetry has become a versatile technique in studying the reaction between various compounds and biopolymers, and several methods for determining binding constants, stoichiometries and enthalpy changes have been developed using graphical techniques and calorimeters equipped with some software programs [14–17]. The application of the methods, however, has been limited to the study of site-specific ligand–protein associations with 1 : 1 and/or 1 : 2 stoichiometric ratios in a single-class binding model. They are not suitable for systems that involve a multiple stepwise equilibrium such as blood proteins and cells which contain classes of binding sites with different affinities and capacities for a drug.

Our recent studies on the interaction thermodynamics of ionic drugs with human blood components have shown that the thermodynamic parameters obtained in a multiple-class binding model are sensitive functions of binding modes and molecular recognitions, and demonstrated that a good-to-excellent linear relationship exists in the enthalpy and entropy compensation effect for drug binding to the common binding sites of the components [18–20]. Furthermore, using compensation analysis for the multiple class of binding sites for consecutive mono- and di-aliphatic carboxylic fatty acids on HSA, we reported that HSA binding sites are characterized into three groups, resulting in correlationships between thermodynamic functions and alkyl-chain length/hydrophobicity [21,22]. In this work, some typical anionic drugs, phenylbutazone, oxyphenbutazone, sulfinpyrazone and warfarin (Site I-bound drugs), clofibric acid, ibuprofen, ethacrynic acid and flufenamic acid (Site II-bound drugs) and acetylsalicylic acid and salicylic acid, were chosen to study the binding characteristics to HSA. We first reported the thermodynamic parameters for the individual drug binding sites with high- and low-affinities of

drug binding to HSA, and discuss the factors controlling the complex stability from the thermodynamic point of view. We also wish to demonstrate that, as in the case of fatty acid binding to HSA [22], compensation analysis is a convenient and versatile tool for molecular recognition by HSA.

2. Experimental

2.1. Materials

Defatted human serum albumin (Fraction V, "essentially fatty acid free", less than 0.005% fatty acids, HSA) obtained from Sigma Chemical (St. Louis, MO, USA) was used without further purification. HSA was dissolved in a 1/30 M phosphate buffer with analytical grade regents, pH 7.4, and its concentration was calculated using a molecular weight of 69 000. Clofibric acid (CA), ethacrynic acid (EA), flufenamic acid (FA), ibuprofen (IP), oxyphenbutazone (OB), phenylbutazone (PB), sulfinpyrazone (SP), warfarin (WA), salicylic acid (SA), and acetylsalicylic acid (AS) were purchased from Sigma Chemical.

2.2. Microcalorimetry

Titration calorimetry was performed in a thermostated water bath at 37 ± 0.001°C, using isothermal microcalorimeters, a TAM 2277-204 flow-mix cylinder (ThermoMetric, Sweden) [23] and a differential flow microcalorimeter with twin-cell flow systems [24]. The instruments were calibrated both electrically and chemically with a sensitivity on the 3–10 μW scale. All solutions were prepared in a 1/30 M phosphate buffer, pH 7.4. The concentrations of HSA (mol. wt. 69 000) in the initial and final calorimetric solutions were determined by the UV absorption at 278 nm using an extinction coefficient E (1% 1 cm) of 5.30. The total concentrations of drugs in the final calorimetric solutions were measured by UV absorption. Consequently, the final concentrations of HSA and drugs were equal to half of the initial concentrations.

The heat of binding (Q_r) is proportional to the amount of drug–HSA complex formed with the total HSA concentration fixed at P_t. Assuming that a drug interacts with HSA by stepwise equilibrium, Q_r is derived from the law of mass action as follows

$$Q_r = \Delta H(Fr)P_t \sum_{i=1}^{m} \frac{n_i K_i D_f}{1 + K_i D_f} \quad (1)$$

where ΔH is the apparent enthalpy per mole of a drug, D_f is the free drug concentration at constant flow rate (Fr), m is the number of classes of independent binding sites, and n_i the number of binding sites in the ith class with the binding constant K_i. The binding and thermodynamic parameters, K_i, n_i and ΔH, for single-class ($m = 1$) and two-class ($m = 2$) binding models were computed from actual measurements by a non-linear least-squares regression program for minimizing the value of $\Sigma (Q_{r,exp} - Q_{r,calc})^2$ [18,19,22].

2.3. Compensation analysis

In order to characterize each class of binding sites on HSA which has a multiple class of binding sites, compensation analysis can be examined by plotting $\Delta S_{m,i}$ and $\Delta G_{m,i}$ against $\Delta H_{m,i}$, representing the molar entropy, the molar free energy and the molar enthalpy changes for the ith class of HSA, respectively. The values were calculated by the following expressions

$$\begin{aligned} \Delta H_{m,i} &= n_i \Delta H \\ \Delta G_{m,i} &= -RT \ln(n_i K_i) \\ \Delta S_{m,i} &= (\Delta H_{m,i} - \Delta G_{m,i})/T \end{aligned} \tag{2}$$

where R is the gas constant and T is the temperature in K.

The expression of the enthalpy–entropy compensation relationship in its simplest form (Eq. (3)) allows the derivation of two integrated equations (Eqs. (4) and (5))

$$\delta(\Delta H_{m,i}) = \beta \delta(\Delta S_{m,i}) \tag{3}$$

where the parameter β is a constant with the dimensions of absolute temperature. Integration of Eq. (3) gives Eq. (4), where $1/\beta$ refers to the slope in $\Delta H_{m,i}$–$\Delta S_{m,i}$ plots

$$\Delta S_{m,i} = \frac{1}{\beta} \Delta H_{m,i} + \Delta S_0 \tag{4}$$

The sequential substitution of the $\Delta S_{m,i}$ term into the differential form of the classical Gibbs expression, $\delta(\Delta G_{m,i}) = \delta(\Delta H_{m,i}) - T\delta(\Delta S_{m,i})$, where δ denotes a small change in the system undergoing a process, followed by integration yields Eq. (5)

$$\Delta G_{m,i} = \left(1 - \frac{T}{\beta}\right) \Delta H_{m,i} + \Delta G_0 \tag{5}$$

Both ΔS_0 and ΔG_0 are integration constants and represent the value of the left side of the respective equation when the independent variable of the equation is zero. Eq. (4) indicates that the entropy change consists of two terms, one of which is proportional to the enthalpy change and the other independent of it. From Eq. (5), when the value of β is equivalent to the temperature of measurement or when T/β is equal to unity, compensation will be total and ΔG will have a constant value independent of the treatment.

3. Results

3.1. Thermodynamics of anionic drug binding to HSA

The heat of binding reaction of anionic drugs to HSA was measured in the same scale of the calorimetric range at pH 7.4 and 37°C. The initial concentrations of

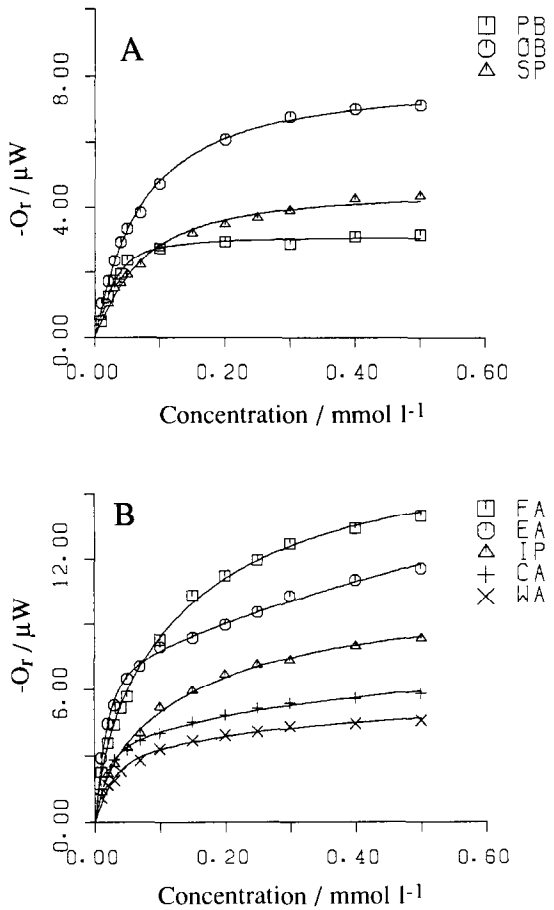

Fig. 1. Calorimetric titration curves of anionic drugs with HSA at 37°C and pH 7.4 in 1/30 M phosphate buffer. Points show experimental values from a single titration and solid lines in the figures A and B represent computer-fitting curves assuming one- and two-class binding model, respectively. The initial concentrations of HSA were about 0.5% (w/v) for PB, OB and SP, 0.2% (w/v) for WA, CA, IP, EA and 0.1% (w/v) for FA. Abbreviations in the figures refer to Table 1.

HSA were 0.5% for PB, OB and SP, 0.20–0.25% for CA, IP, EA, WA, AS and SA, and 0.1% for FA. The calorimetric data for the binding of all drugs were examined to fit both one- and two-class binding models ($m = 1$ and 2 in Eq. (1), respectively). The best-fit values of ΔH and the binding parameters are listed in Table 1. The saturated titration curves as shown in Fig. 1A fitted better to the one-class than the two-class binding model, because the values of n_1 obtained from the two-class binding model were below 0.3, indicating that in the two-class binding model the first class was unavailable for binding. Thus, PB, OB and SP are bound to HSA at only one class of binding site ($i = 1$) with binding affinity of the order of $K = 10^4$ to 10^5 M^{-1} at a molar ratio of 1:1 ($n = 1$). The other titration curves in Fig. 1B and

Table 1
Binding and thermodynamic parameters calculated from the calorimetric titration curves of anionic drugs with HSA at pH 7.4 and 37°C [a]

Drug	K_1 in 10^5 M^{-1}	n_1	K_2 in 10^3 M^{-1}	n_2	$-\Delta H$ in kJ mol^{-1}
Phenylbutazone (PB)	4.22 ± 0.23	0.9 ± 0.2	–	–	24.8 ± 1.3
Sulfinpyrazone (SP)	0.436 ± 0.08	1.1 ± 0.1	–	–	23.1 ± 1.1
Oxyphenbutazone (OB)	0.753 ± 0.09	1.4 ± 0.1	–	–	23.2 ± 1.6
Warfarin (WA)	1.40 ± 0.31	0.9 ± 0.2	2.30 ± 0.22	1.7 ± 0.2	48.8 ± 2.0
Clofibric acid (CA)	0.742 ± 0.03	1.4 ± 0.2	1.86 ± 0.10	1.1 ± 0.1	57.6 ± 2.3
Ibuprofen (IP)	1.10 ± 0.13	1.0 ± 0.2	5.17 ± 0.59	1.6 ± 0.4	66.5 ± 3.1
Ethacrynic acid (EA)	1.65 ± 0.14	1.1 ± 0.2	3.89 ± 0.76	3.2 ± 0.6	71.0 ± 3.4
Flufenamic acid (FA)	1.56 ± 0.08	1.5 ± 0.4	8.26 ± 1.67	2.4 ± 0.4	75.9 ± 4.7
Acetylsalicylic acid (AS)	1.30 ± 0.21	1.4 ± 0.2	1.38 ± 0.13	4.9 ± 0.4	20.3 ± 2.0
Salicylic acid (SA)	1.28 ± 0.18	1.1 ± 0.1	1.80 ± 0.09	7.4 ± 0.6	19.1 ± 1.3

[a] Each value is the mean of the best fit values computed from three calorimetric titration curves.

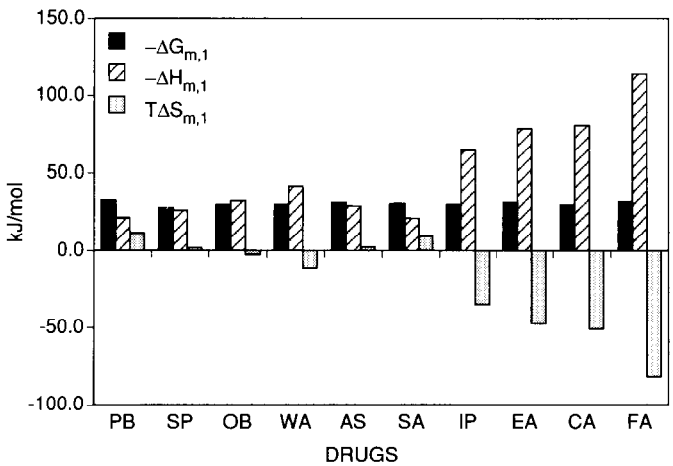

Fig. 2. Molar free energy ($-\Delta G_{m,1}$), molar enthalpy ($-\Delta H_{m,1}$), and molar entropy ($T\Delta S_{m,1}$) changes of HSA for the first class of binding sites of anionic drugs in 1/30 M phosphate buffer (pH 7.4) at 37°C.

those of AS and SA were analyzed only by assuming the two-class binding model. The anionic drugs, except for PB, OB and SP, are bound to HSA on at least two classes of independent binding sites containing one high-affinity site with a binding constant of $K_1 = 10^5$ M^{-1} (first class of binding sites) and some low-affinity binding sites with the order of $K_2 = 10^3$ M^{-1} (second class of binding sites).

Fig. 2 shows the enthalpic ($-\Delta H_{m,1}$) and entropic ($T\Delta S_{m,1}$) contributions to the free energy change ($-\Delta G_{m,1}$) calculated from Eq. (2) for the first class of binding sites. Although the values of $\Delta G_{m,1}$ obtained for all drugs were almost constant

(-30.5 ± 1.3 kJ mol^{-1}), Site II-bound drugs (CA, IP, EA and FA) form typical enthalpy-driven complexes with large negative entropic gains, reflecting van der Waals and/or hydrogen bonding in low dielectric media, whereas Site I-bound drugs (PB, SP, OB and WA), and also both AS and SA give entropy-driven complexes with minimal negative or positive entropic contributions, reflecting hydrophobic interaction [11].

3.2. Classification of drug binding sites on HSA

To characterize the binding sites, compensation analysis was undertaken for each class of drug binding site of HSA. Fig. 3A shows the $\Delta H_{m,i}-\Delta G_{m,i}$ relationships in the ith class of binding sites for anionic drugs, together with those for fatty acids quoted from previous data [21,22]. The solid lines represent 95% confidence ellipses of the classification into groups S1, S2, and S3 for fatty acid binding sites on HSA molecule: group S1 suggests the binding sites of short- or medium-chain fatty acids; group S2, the second class of binding sites for long-chain fatty acids; and group S3, the first class of binding sites for long-chain fatty acids [22]. All the binding sites of anionic drugs are clearly located within the same regions of groups S1, S2 and S3: the first classes of CA, EA, FA and IP in group S1; the second classes of CA, EA, FA, IP and WA in group S2; and the first classes of OB, PB, SP and WA in group S3. The first and second classes of binding sites for AS and SA were located in the regions of groups S3 and S2, respectively.

The $\Delta H_{m,i}-\Delta S_{m,i}$ plots for drug binding sites of the groups S1, S2 and S3 display excellent linear relationships with correlation coefficients of 0.999, 1.000, and 0.963, respectively, although the available data points are fairly limited. These linear compensation plots as shown in Fig. 3B can be generated with the slopes just above or below the experimental temperature (310 K), and thus T/β in Eq. (5) can be estimated to be very close to unity. The values of T/β and $T\Delta S_0$ calculated from the slope and intercept for each group of drug binding to HSA are compared with those of fatty acid [22] in Table 2. The agreement of linear $\Delta H_{m,i}-\Delta S_{m,i}$ plots for every group seems to be stronly indicative of the common binding sites on HSA and/or the same mechanisms between drug– and fatty acid–HSA complexation.

Table 2
The slope (T/β) and intercept ($T\Delta S_0$) of $\Delta H_{m,i}-\Delta S_{m,i}$ plots for drugs and fatty acid binding to HSA and pH 7.4 and 37°C

	Group	T/β	$T\Delta S_0$ in kJ mol^{-1}	r [a]
Anionic drug–HSA	S1	0.964	27.7	0.999
	S2	0.973	19.41.000	
	S3	1.068	32.40.963	
Fatty acid–HSA [22]	S1	0.976	26.7	0.988
	S2	0.958	18.90.999	
	S3	0.989	32.60.780	

[a] Correlation coefficient.

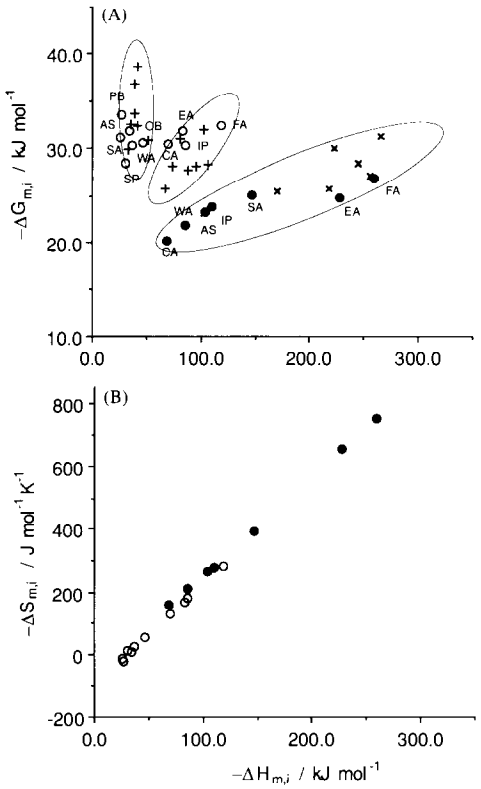

Fig. 3. Compensation analysis using (A) molar enthalpy change ($-\Delta H_{m,i}$) and molar free energy change ($-\Delta G_{m,i}$) coordinates and (B) molar enthalpy/molar entropy change ($-\Delta S_{m,i}$) for the ith class of drug binding sites on HSA. Symbols (○) and (●) are the first and the second class, respectively, of anionic drug binding sites on HSA; (+) and (×) are first and the second class of fatty acid binding sites, respectively [22]. The solid lines represent the 95% confidence ellipses for groups S1 to S3 of fatty acids binding sites.

4. Discussion

The compensatory $\Delta H-\Delta S$ relationship has already been observed in individual thermodynamic studies using some of the ligand–macromolecule (receptor) interactions [12,25–28]. In most cases, however, only the linear enthalpy–entropy relationship was reported and sometimes the isoequilibrium temperature was calculated, but the possible meanings of the slope and intercept obtained have not been discussed in further detail. The $\Delta H-\Delta G$ correlations, which would imply that there is a common mechanism controlling the binding of all ligands to all receptors [25], have been neglected except for use to evaluate the experimental errors on ΔH [29]. The compensation effect is quite useful in the explanation of individual complexation behavior, but is not always applicable to different ligand–receptor interactions and does not appear to lead to a global understanding of the complex-

ation behavior. In this context, the general validity of the compensation effects (ΔS and ΔG vs. ΔH), originally proposed for the fatty acid binding to HSA, is discussed for drug binding sites on HSA and the factors forming and stabilizing drug–HSA complex.

The linear $\Delta H_{m,i}-\Delta S_{m,i}$ compensation as shown in Fig. 3B indicates that the enthalpic gain/loss from any changes in the ligand, protein, or solvent (water) is almost cancelled out by the entropic loss/gain arising from the structural fitting/loosening on the binding sites of the ligand–protein complexation. The slope $1/\beta$ in Eq. (4) is a quantitative measure of the entropic cancelling of the enthalpic gain from the ligand–protein complexation. In other words, only a proportion $(1 - T/\beta)$ of the increment in $\Delta H_{m,i}$ contributes toward raising the complex stability ($-\Delta G_{m,i}$) on the binding sites (Eq. (5)). Eq. (4) further indicates that, provided the intercept (ΔS_0) is positive as is indeed the case with all the drugs and fatty acids binding to HSA as shown in Table 2, the complex formation can take place even in the absence of enthalpic gain. This situation is quite likely to occur when the entropic contribution from the releasing process of water molecule (hydrophobic interaction) is the major factor governing the complexation. From these considerations and the actual data in Table 2, it is concluded that the value of $T\Delta S_0$ can be used as a quantitative measure of the hydrophobic interaction upon complex formation.

Site I-bound drugs are structurally bulky, heterocyclic compounds with a negative charge localized in the middle of the molecule. The polar charged groups must be solvated in polar protic solvents such as water and buffer. According to Carter and He [9,10], these drugs must be bound in a hydrophobic crevice within the cavity where the distribution of hydrophobic and hydrophilic residues is distinctly asymmetric through extensive desolvation (releasing water). Group S3 including the first classes of Site I-bound drugs and fatty acids with long alkyl-chains is characterized by a relatively constant $\Delta H_{m,i}$ (-28.6 and -35.5 kJ mol^{-1}, respectively) and the largest values of $T\Delta S_0$ as listed in Table 2. By contrast, Site II-bound drugs are aromatic carboxylic acids with a negative charge on the carboxyl group at one end of the molecule away from the hydrophobic center. Possessing flexible methylens, a carboxylic group and/or ether oxygens connecting rigid aromatic moieties, these drugs can easily alter their conformation upon complexation and those with polar/negatively charged groups must be heavily bound through ion (dipole)–dipole and/or van der Waals interactions particularly in the polar cationic group of HSA. Group S1, the first class of binding sites for Site II-bound drug, is characterized by larger negative $\Delta H_{m,i}$ values ($-64.8 \geq \Delta H_{m,1} \geq -113.8$ kJ mol^{-1}) and constant values of $\Delta G_{m,i}$ which are nearly equal to those of Site I-bound drugs located in group S3 (Fig. 2), and the $\Delta H_{m,i}-\Delta S_{m,i}$ plot gives distinctly smaller values of T/β and $T\Delta S_0$ than those of group S3. In group S2 including the second class of binding sites with lower affinity and higher capacity, the values of $\Delta H_{m,i}$ and $\Delta S_{m,i}$ may be distributed over relatively wide intervals (approximately $-60 \geq \Delta H_{m,2} \geq -250$ kJ mol^{-1} and $-0.1 \geq \Delta S_{m,2} \geq -0.7$ kJ mol^{-1} K^{-1}), resulting in small values of $\Delta G_{m,2}$ (-22.9 ± 2.4 kJ mol^{-1}) because of an enthalpy–entropy compensation effect for which any decrease in binding enthalpy is compensated by a parallel decrease in binding entropy. This suggests the interesting possibility that

both $\Delta H_{m,2}$ and $\Delta S_{m,2}$ are controlled by the hydrogen bond rearrangements occurring during the complexation. The formation of hydrogen bonding may restrict the rotational and transformational freedom of the alkyl side chains and fixes it rigidly in the HSA molecule. Indeed, hydrogen bonding formation has recently been shown to play a significant role in ligand–protein complexation [30,31].

Finally, it is interesting to note that the values of T/β calculated from the slopes of $\Delta H_{m,i}-\Delta S_{m,i}$ relationships are nearly equal to unity, with the result that the drug binding to HSA is dependent on the compensation balance. In view of the interaction and mechanism involved in drug–HSA complexation, the validity of the compensation analysis by microcalorimetry may be confirmed as a general tool for classification of the binding sites on HSA.

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