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Biothermodynamic characterization of monocarboxylic and dicarboxylic aliphatic acids binding to human serum albumin: A flow microcalorimetric study

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Abstract

Thermodynamic parameters have been evaluated for the binding of unbranched monocarboyxlic aliphatic acids (MCAs) of 4 to 16 carbons (MC4 to MC16) and dicarboxylic aliphatic acids (DCAs) of 4 to 16 carbons (DC4 to DC16) to human serum albumin (HSA) on the basis of microcalorimetric measurement at pH 7.4 and 37°C by computer-fitting to single- and two-class binding models. Long-chain MCAs (MC10 to MC16) and DCAs (DC14 and DC16) had the first class of binding sites with high affinity (large binding constant) of 10^5 to 10^6 M^{-1} and the second class with lower affinity and high capacity (large numbers of binding sites). Short- or medium-chain MCAs and DCAs bound to HSA at some low affinity binding sites. The binding constants of MCAs were ten times larger than those of DCAs. All the relationships between the thermodynamic parameters and alkyl-chain length of the acids showed clear-cut inflections in their plots around eight or nine methylene units. The free energy change of the first class of binding sites ($-\Delta G_1$) became more negative with an increment of -1.0 kJ mol^{-1} CH_2^{-1} as the alkyl-chain length increased, but there were steep rises between MC9 and MC11 with -2.90 kJ mol^{-1} CH_2^{-1} and between DC9 and DC12 with -2.02 kJ mol^{-1} CH_2^{-1}. The enthalpy change ($-\Delta H$) increased at the rate of -7.4 kJ mol^{-1} CH_2^{-1} to the maximum at MC9 and DC10, then decreased due to hydrophobicity of the alkyl-chains. From compensation analyses (ΔH vs. ΔS and ΔG), HSA binding sites were characterized into three groups.

Keywords: Carboxylic aliphatic acids–HSA binding; Alkyl-chain-length hydrophobicity; Biothermodynamics; Microcalorimetry

1. Introduction

The investigation of drug–protein interaction, especially those with albumin, is necessary for a better understanding of their possible influence on the biological activity or pharmacokinetic behavior of drugs [1–3]. Physicochemical parameters such as the binding constant (K) and the number of binding sites (n) have been determined for various drugs [4–7]. The next but more fundamental issue concerns the nature of the binding interaction. Hydrogen bonding, van der Waals' and hydrophobic forces are generally considered to be important for drug–protein interactions [8–11].

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Unbranched carboxylic aliphatic acids have been frequently used to study the characteristics of drug–protein binding, as they provide a convenient homologous series of ligand molecules with the carboxy anion group but with varying the alkyl-chain length/ hydrophobicity. Monocarboxylic aliphatic acids (MCAs) interact primarily with various binding sites on human serum albumin (HSA) *in vivo* [6,12]. Dicarboxylic aliphatic acids (DCAs) binding sites on the albumin molecule, depending on their chain length, are characterized in order to understand the potential effects of drugs and other ligands on DCAs binding and their toxity *in vivo* [13,14]. Their binding isotherms [12–25] and locations are associated with enhanced or reduced binding of the drug ligands to HSA [26–29], where allosteric effects [30–32] and competitive displacement [33–36] have been extensively studied. However, no evidence as yet has been presented of any systematic relation among thermodynamic parameters. In only a few instances, the enthalpy and entropy changes of MCA binding have been determined using the van't Hoff relationship between K and temperature [17–19,28].

The experimental binding data described by fitting K and n are often highly variable owing to methodological difference and/or mathematical modeling in the interaction. For example, in the literature little agreement exists concerning the affinity of capric acid (MC10) binding to HSA. Ashbrook et al. determined the first stoichiometric binding constant in terms of multiple stepwise equilibria to be $10^5\ M^{-1}$ by equilibrium dialysis at 37°C in calcium-free Krebs–Ringer phosphate buffer, pH 7.4 [16]. Honoré and Brodersen reported the value $10^7\ M^{-1}$ at 37°C in 66 mM sodium chloride phosphate buffer, pH 7.4, by using a dialysis exchange rate method [24]. The high-affinity binding constant from Scatchard plots was $10^6\ M^{-1}$ according to Lee and McMenamy, determined by ultrafiltration technique at 25°C in phosphate buffer with 100 mM potassium chloride, pH 6.0 [28]. Koh and Means used an acetylation method and determined that K was about $10^7\ M^{-1}$ at 25°C in triethanolamine/HCl, with an ionic strength 0.02 M and pH 7.5 [27].

Two mathematical models have been mainly used to analyze MCA binding to HSA. Brodersen's group has studied the binding of caproic (MC6) up to myristic acids (MC14) with HSA, using the dialysis exchange rate method. They determined the binding constants using multiple equilibrium analysis [23]. The first stoichiometric binding constants were $7.1 \times 10^4\ M^{-1}$ for MC6; $1.6 \times 10^6\ M^{-1}$ for MC8; $1.1 \times 10^7\ M^{-1}$ for MC10; $8.31 \times 10^6\ M^{-1}$ for MC12; and $3.24 \times 10^7\ M^{-1}$ for MC14. The K value of MC12 is smaller than that of MC10. This could be because the binding constant of the high-affinity site cannot be calculated exactly from the given values of the stoichiometric constant [37]. Taking a closer view at the Scatchard plots for MC8 and MC10, they often show that points below an r-value (r represents the molar ratio of bound MCA ligand to HSA) of 0.6 describe a straight line intersecting the abscissa below 1. Thus, the variation of the r/L_f ordinate (L_f is the free ligand concentration) increases toward an infinite limiting value as r and L_f approach zero. Furthermore, the stochastic errors in the experimental data will necessarily induce severe scattering of points in the low-r region of the plot, which renders determination of the K value by the extrapolation and intercept procedure unreliable [38].

Recently we have reported the usefulness of differential flow microcalorimetry for the study of ligands binding to blood components [39–43]. Isothermal titration calorimetry is a versatile technique which: (1) is universally applicable to all reactions regardless of the chemical nature or size of the macromolecule (e.g., HSA) and ligand, (2) has short equilibration and analysis times, (3) allows simultaneous determination of the enthalpy and entropy changes as well as the binding constant and stoichiometry in a single experiment, and (4) thereby avoids the necessity of partitioning between HSA and the ligands [39,44]. It is especially advantageous for carboxylic aliphatic acids to bind to albumin, where the spectroscopic approach has been limited to the aromatic residues owing to the weak absorption of carboxylic acids in the UV and visible region.

We report here a comparative study of the binding of MCAs and DCAs to HSA, using flow

microcalorimetry at 37°C and pH 7.4, in order to provide detailed thermodynamic descriptions based up on the complex-forming reaction.

2. Materials and methods

2.1 Materials

HSA (Fraction V, "Essentially fatty acid free", less than 0.005% fatty acids) obtained from Sigma (St. Louis, MO, USA) was used without further purification. HSA was dissolved in 1/30 *M* phosphate buffer, pH 7.4 and its molecular weight was assumed to be 69,000. All MCAs and DCAs were supplied from Nippon Chromato Kogyo Co., Ltd. (Tokyo, Japan). A relatively concentrated solution of each acid dissolved in 0.01 *N* NaOH was prepared and diluted with 1/30 *M* phosphate buffer, pH 7.4 to provide the desired initial concentration (10^{-6} to 10^{-3} *M*). The initial pH of all solutions was adjusted to within the range of 7.40 to 7.46.

2.2 Microcalorimetry and data analysis

Isothermal calorimetric titrations were carried out at 37.0 ± 0.005°C using an LKB microcalorimeter (2277 BioActivity Monitor, Boromma, Sweden) [45] and a differential flow microcalorimeter with a twin-cell structure. Details of this calorimeter and its operation mode have been described previously [39]. The reaction solutions were introduced at a constant flow rate (0.12 ml min^{-1}) into the calorimeter through Tygon tubing, using a four-channel peristaltic pump (Gilson, Villers-Le-Bel, France). A base line was established by flowing phosphate buffer and HSA in the buffer solution, corresponding to the heat of dilution of HSA. The initial and final concentrations of HSA were determined by the absorption at 278 nm ($E_{278}^{0.1\%} = 0.531$). The final HSA concentrations were equal to half of the initial concentrations. The solutions of carboxylic aliphatic acid at different concentrations were then introduced sequentially into the buffer flow line in the reaction cell. The heat effect was proportional to the recorded steady-state value. The calorimetric calibration was carried out by introducing a known quantity of electric power into the electric calibration heater.

The experiment recorded during the mixing of the two reagents is equal to the sum of the heats of dilution of the components and the heat of the carboxylic acid ligand binding to HSA. As noted before, the heat of dilution of HSA can be instrumentally eliminated. After subtraction of the heat of dilution of the ligand measured, the heat of reaction (W_r) is proportional to the quantity of the ligand–HSA complex formed with the fixed concentration of HSA (P_t), as follows:

$$W_r = \Delta H L_b F_r \tag{1}$$

where ΔH is the binding enthalpy per mole of ligand and L_b is the bound concentration of the ligand at a constant flow rate of F_r. The equilibrium aspect of such interactions was correlated through the mass action law, yielding the familiar expression:

$$L_b/P_t = \sum_{i=1}^{m} (n_i K_i L_f)/(1 + K_i L_f) \tag{2}$$

where m denotes the number of classes of independent binding sites such that each class (i) has n_i sites with the binding affinity K_i, and L_f is the free ligand concentration related to the equation,

$$L_t = L_b + L_f \tag{3}$$

This procedure for the simple case of one and two classes of binding sites ($m = 1$ and 2) can be calculated by the use of the following equations, respectively [46].

$$L_b^2 + AL_b + B = 0 \tag{4}$$

$$L_f^3 + CL_f^2 + DL_f + E = 0 \tag{5}$$

where $A = -(1/K_1 + n_1P + L_t)$, $B = P_t n_1 L_t$, $C = P_t(n_1 + n_2) + (1/K_1 + 1/K_2) - L_t$, $D = P_t(n_1/K_2 + n_2/K_1) - L_t(1/K_1 + 1K_2) + (1/K_1)(1/K_2)$, and $E = -L_t\,(1/K_1)(1/K_2)$.

The binding and thermodynamic parameters, K_i, n_i and ΔH, can be computed from the actual calorimetric data with an iterative non-linear least-squares using a FACOM M-380R computer program [47] for minimizing the value of

$\sum(W_{r,exp} - W_{r,calc})^2$. The initial value of ΔH was estimated experimentally from the slope of the initial linear part of an enthalpic titration curve.

3. Results and discussion

3.1 Heat of binding of carboxylic aliphatic acids to human serum albumin

The enthalpic titrations of HSA with consecutive MCAs and DCAs at 37°C and pH 7.4 are shown in Fig. 1. The titration data were directly fitted to one- and two-class binding models. The solid lines represent the computer-generated best fit curves according to a two-class binding model. In the cases of MCA from MC4 to MC9 and DCA from DC4 to DC13, the data were fitted to both models. The values of n_1 computed from the two-class binding model were below 0.5, indicating that the first class in the two-class binding model was either weakly or unavailable for binding. The values of K_2 and n_2 for the second class agreed with those of K and n obtained from the one-class binding model. By contrast, the data for MCAs with 10 or more carbons (MC10 to MC16) and DCAs with 14 and 16 carbons (DC16 and DC14) did not fit the one-class binding model. Using the two-class binding model with independent binding sites, a better fit was observed between the enthalpic titration data and the curves generated with most probable values of K_1, K_2, n_1 and n_2 in Table 1.

The short- or medium-chain MCA bound to only one class of binding sites of HSA at a ratio of 2:1 with K in the order of 10^4 M^{-1}. The long-chain MCA had at least two classes of independent binding sites containing one strong binding site with K_1 of 10^5 to 10^6 M^{-1} and some weak sites with K_2 of 10^3 to 10^4 M^{-1}. DCAs from DC8 to DC13 bound to the single class of binding sites ($n = 2$ to 3) with lower binding affinity than MCA. Neither of DC4 and DC6 bound to the specific binding sites of HSA because the very large numbers of the binding site ($n \geqq 9$) indicated the extended binding area. Only DC14 and DC16 had two classes of independent binding sites on HSA. These results indicate that the nonpolar alkyl-chain is much more important for carboxylic acid binding to HSA than the carboxyl anion groups, which probably play only a minor role in forming carboxylic acid–HSA complexes.

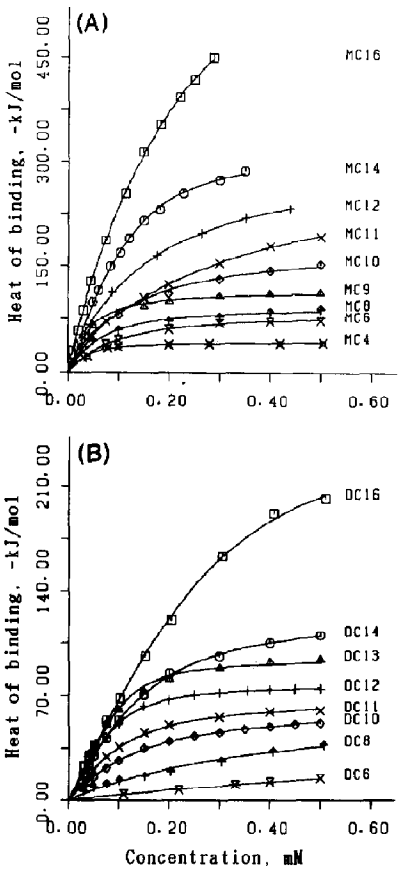

Fig. 1. Calorimetric titrations of monocarboxylic acids (A) and dicarboxylic acids (B) with HSA at 37°C and pH 7.4 in 1/30 M phosphate buffer. The initial concentrations of HSA used were about 0.2% (w/v) and the final concentrations in the calorimetric solutions were 1.41 to 1.50×10^{-5} M. Each point is an average of three measurements and solid lines represent computer-fitting curves assuming a two-class binding model. Abbreviations in the figures refer to Table 1.

3.2 Comparison of the present results with the previous data

Since the binding constants are temperature dependent, the free energy changes (ΔG_1) estimated from the highest affinities for MCA binding to HSA were compared with those from earlier studies (Fig. 2). For comparison, we recalcu-

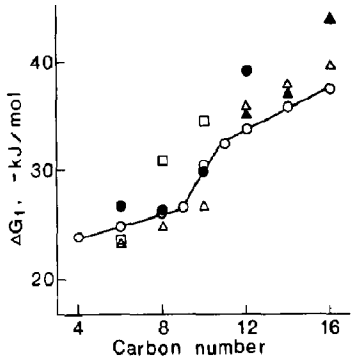

Fig. 2. Comparison of ΔG_1 values obtained from the present results with those of previous data. (○) Present results at 37°C; (▲) data of Goodman [15] at 23°C; (△) Ashbrook et al. [16,17] at 37°C; (●) Honoré and Brodersen [24] at 37°C; and (□) Lee and McMenamy [28] at 25°C. The solution conditions were different in these studies. The data reported by Ashbrook et al. [16,17] and by Honoré and Andersen [24] were recalculated by Scatchard analysis using a two-class binding model.

lated the binding data reported from Ashbrook et al. [16,17] and from the Pedersen group [22–25], by computer using the Scatchard model. The values of $-\Delta G_1$ increased with increasing the alkyl-chain length but with a disproportionately increase between MC9 and MC11. According to the report by Spector [12], the deviation from linearity between MC10 and MC12 reflects the configurational adaptability of the albumin binding sites. Differences in the absolute values of ΔG_1 in the long-chain MCA could be explained by differences in the solutions used, since Goodman [15] and Ashbrook et al. [16], using a phase distribution with n-heptane, have determined the concentration of free MCA anion in the aqueous phase by measuring the concentration of MCA in the organic phase. But it is an advantage to use a method in which organic phases are absent, because organic solvents such as n-heptane binding to HSA, might complicate the analysis.

3.3 Effect of alkyl-chain length on the thermodynamic parameters for carboxylic aliphatic acid binding to human serum albumin

Figure 3 shows the relationship between the thermodynamic parameters and the carbon number of the carboxylic acid. The values of $-\Delta G_1$

Table 1

Binding and thermodynamic parameters of unbranched aliphatic carboxylic acid binding to HSA at pH 7.4 and 37°C

Carboxylic acids	$-\Delta H$ (kJ mol^{-1})	K_1 (M^{-1}) 10^{-4}	n_1	K_2 (M^{-1}) 10^{-3}	n_2
Butyric acid (MC4)	23.74±2.27	1.110±0.161	2.3±0.2	–	–
Caproic acid (MC6)	36.22±2.55	1.652±0.096	2.3±0.1	–	–
Caprylic acid (MC8)	53.45±1.05	2.579±0.476	1.9±0.2	–	–
Pelargonic acid (MC9)	60.36±1.18	3.106±0.927	1.5±0.1	–	–
Capric acid (MC10)	51.71±2.02	14.404±1.368	0.9±0.0	4.829±0.187	3.2±0.1
Undecylic acid (MC11)	46.35±0.13	29.410±0.264	0.8±0.0	5.922±0.122	5.4±0.2
Lauric acid (MC12)	42.73±2.66	49.825±4.034	0.8±0.2	9.008±0.007	5.6±0.3
Myristic acid (MC14)	39.61±2.59	113.22 ±0.564	1.1±0.3	17.090±1.003	5.5±0.6
Palmitic acid (MC16)	38.90±2.60	216.80 ±2.680	1.2±0.3	22.655±1.055	6.7±0.4
Succinic acid (DC4)	5.09±0.15	0.114±0.012	10.3±0.2	–	–
Adipic acid (DC6)	6.04±0.58	0.120±0.008	9.3±0.1	–	–
Suberic acid (DC8)	17.57±1.10	0.320±0.063	3.5±0.3	–	–
Azelaic acid (DC9)	26.23±0.63	0.358±0.003	2.0±0.0	–	–
Sebacic acid (DC10)	36.90±0.48	1.019±0.017	1.7±0.0	–	–
Undecanedioic acid (DC11)	29.71±0.16	2.006±0.144	2.3±0.0	–	–
Dodecanedioic acid (DC12)	29.37±0.47	5.001±0.254	2.6±0.1	–	–
Tridecanedioic acid (DC13)	29.65±0.51	5.968±0.320	3.3±0.0	–	–
Tetradecanedioic acid (DC14)	28.12±1.57	9.110±0.321	1.0±0.2	1.784±0.006	3.5±0.4
Hexadecanedioic acid (DC16)	28.33±1.96	22.812±0.835	1.1±0.3	2.357±0.014	7.5±0.1

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

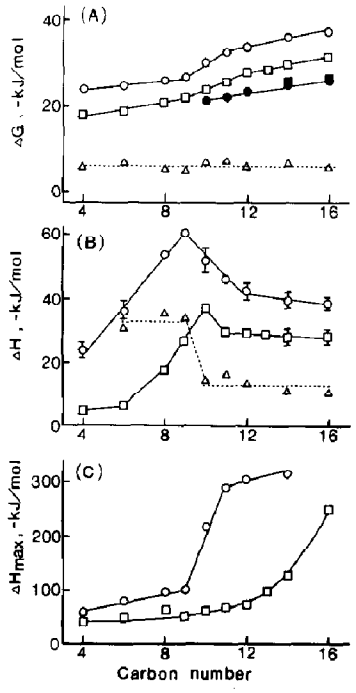

Fig. 3. Influence of unbranched carboxylic aliphatic acid chain length on thermodynamic parameters of fatty acid binding to HSA. The value of the free energy change (ΔG) was calculated from the binding constant listed in Table 1 using the equation, $\Delta G = -RT \ln K$. The enthalpy change per mol of carboxylic acid (ΔH) and per mol of HSA (ΔH_{max}), were determined directly from individual calorimetric titrations. (○) Monocarboxylic acids; (□) dicarboxylic acids; (△) difference between monocarboxylic acid and dicarboxylic acid with same carbon number; (●) and (■) ΔG_2 values of monocarboxylic and dicarboxylic acids, respectively.

increased relatively with the increasing alkyl-chain length of the carboxylic acid. There was a significant increase in $-\Delta G_1$ with 2.90 kJ mol^{-1} CH_2^{-1} between MC9 and MC11 and with 2.02 kJ mol^{-1} CH_2^{-1} between DC9 and DC12. The other steady increments per methylene group in $-\Delta G_1$ were 0.54 kJ mol^{-1} (MC4 to MC9), 1.02 kJ mol^{-1} (MC11 to MC16), 0.77 kJ mol^{-1} (DC4 to DC9), and 1.01 kJ mol^{-1} (DC12 to DC16). In contrast, $-\Delta G_2$ in MCAs binding to HSA increased proportionately with the increment of 0.70 kJ mol^{-1} CH_2^{-1} from MC10 to MC16. Comparing the values of $-\Delta G_1$ in carboxylic acid involving the same carbon number, the $-\Delta G_1$ for DCA was lower by 6.10 ± 0.66 kJ mol^{-1} (the average of the differences for nine groups).

In the relationship between the enthalpy change per mole of carboxylic acid (ΔH) and the carbon number, the absolute values of $-\Delta H$ for MCAs increased at the rate of 7.47 kJ mol^{-1} CH_2^{-1} to a maximum at MC9, then decreased as the chain length increased (Fig. 3B). A similar tendency was shown in the $-\Delta H$ for DCA binding to HSA, where there was a maximum at DC10. The increment of 7.31 kJ mol^{-1} CH_2^{-1} from DC6 to DC10 agreed with that of MCA. The difference of ΔH between MCA and DCA with the same carbon number was 33.4 ± 2.4 kJ mol^{-1} between 4 and 9 carbons, and 13.4 ± 2.2 kJ mol^{-1} between 10 and 16 carbons.

The parameter ΔH_{max}, referes to the molar enthalpy change of HSA for the complete binding of all sites [39], and was obtained from a plateau value ($W_{r,max}$) of the enthalpic titration curve using the equation:

$$W_{r,max} = \Delta H_{max} F_r P_t \quad (6)$$

The ΔH_{max} values were almost equal to those of $\Delta H \sum n_i$, indicating the extent of the binding area or capacity of HSA molecule, and changed largely both from MC9 to MC11 and from DC13 to DC16.

These clear-cut inflections in ΔG, ΔH and ΔH_{max} around nine or ten carbons in the alkyl-chain reflected the different interactions taking part in the complex formation of carboxylic acid with HSA.

3.4 Characteristics of the binding sites of human serum albumin

To characterize each class of binding sites on HSA, compensation was examined by plotting ΔS_{mi} and ΔG_{mi} against ΔH_{mi}, representing the molar entropy, the molar free energy and the molar enthalpy changes of HSA of the ith class, respectively. The values were calculated by the following expressions [37].

$$\Delta H_{mi} = n_i \Delta H$$

$$\Delta G_{mi} = -RT \ln(n_i K_i)$$

$$\Delta S_{mi} = (\Delta H_{mi} - \Delta G_{mi})/T \quad (7)$$

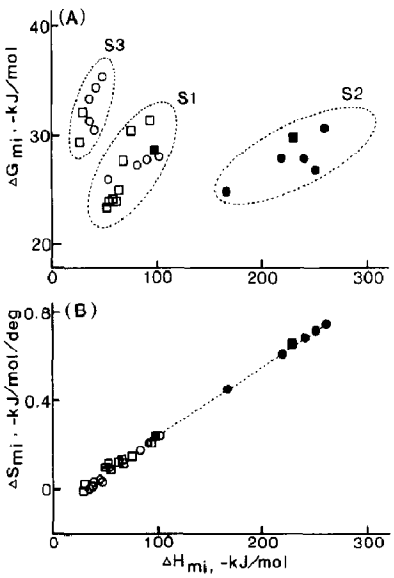

Fig. 4. Compensation analysis using (A) molar enthalpy (ΔH_{mi}) and molar free energy (ΔG_{mi}) coordinates, and (B) molar enthalpy/molar entropy (ΔS_{mi}) coordinates for ith class of HSA binding sites of unbranched carboxylic aliphatic acids. Symbols (○) and (●) are the first and the second class of binding sites of monocarboxylic acids to HSA; (□) and (■) the first and second classes of binding sites of dicarboxylic acids to HSA, respectively; and (···) represents the 95% confidence ellipses.

where R is the gas constant and T is the temperature in Kelvin. As shown in Fig. 4, the plots of ΔG_{mi} against ΔH_{mi} were classified into three binding sites groups (S1, S2 and S3), and ΔS_{mi} vs. ΔH_{mi} plots were represented by two independent linear relations. The groups S1 and S2 lay on the same straight line with a coefficient of correlation $r = 0.9996$ ($\Delta H_{mi} = 0.313\ \Delta S_{mi} - 25.8$ kJ mol^{-1} of HSA). The short- and medium-chain carboxylic acids (MC4 to MC9 and DC4 to DC13) having only one class of binding sites belonged to group S1. In group S2, the second class of the long-chain carboxylic acids (MC10 to MC16 and DC16) having the lower affinity K_2, reflected large negative values of ΔH_{m2} (-261.4 to -167.0 kJ mol^{-1} of HSA) and ΔS_{m2} (-744 to -458 J K^{-1} mol^{-1} of HSA). The first class of binding sites with high affinity K_1 belonged to group S3, and was characterized by a relatively constant enthalpy change ($\Delta H_{m1} = -38.4 \pm 6.9$ kJ mol^{-1} of HSA) and small entropy change ($-47.4 \leqslant \Delta S_{m1} \leqslant +4.2$ J mol^{-1} K^{-1} of HSA. The values of $-\Delta G_{m1}$ were increased with increasing the alkyl-chain length.

3.5 *Thermodynamic aspect of carboxylic aliphatic acid–human serum albumin binding interactions*

The thermodynamic parameters can be interpreted in terms of the forces received to stabilize carboxylic acid–HSA complex. From a purely pragmatic point of view, ΔG determines the direction of any spontaneous change in the complex equilibrium under specific conditions and can be brought about by either a decrease in ΔH, an increase in ΔS, or by a combination of both changes. The contribution to positive ΔH and ΔS arises from the occurrence of a large degree of hydrophobic interaction, while the sources of negative ΔH and ΔS arise from van der Waals' interaction and hydrogen bond formation. Although the electrostatic forces contribute to the positive ΔS, the value of ΔH is expected to be very small or almost zero for purely electrostatic interaction [10]. The existence of an enthalpy–entropy compensation effect is tempting to rationalize the linearities on the bases of the nature of the common binding sites.

As shown in Fig. 4, the compensation plots for each class of binding sites on HSA molecule provide evidence that two different binding sites and three mechanisms exist on HSA. In group S3, the hydrophobic interaction plays a significant role to stabilize carboxylic acid–HSA complexation, reflecting the positive or small negative values of ΔS_{m1} and the constant ΔH_{m1}. The longer alkyl-chain has greater freedom of rotation around its single bonds, and thus is more flexible. The increased freedom of movement allows the hydrocarbon side chain to be present on the surface of HSA molecule (the more positive the ΔS), causing ΔG_1 to be become more negative with an increment of about -1.0 kJ mol^{-1} of CH_2 group (Fig. 3). Thus, carboxylic acids (MC10 to MC16 and DC14 to MC16) bind one strong binding site with high affinity, in the order of 10^5 to 10^6 M^{-1}.

For groups S1 and S2, a linear compensation plot with a slope approximately equal to the

experimental temperature (310 K) was observed between ΔH_{mi} and ΔS_{mi}. Binding with large negative values of ΔH_{mi} and ΔS_{mi} may be affected by van der Waals' interaction and hydrogen bonding formation in low dielectric media. The shorter alkyl-chain has comparatively little freedom of movement, thus the effect of each additional methylene group to van der Waals' interaction increases ΔH negatively with increasing chain-length at the rate of about 7.4 kJ mol^{-1} CH_2^{-1} up to an alkyl-chain was of eight units (MC9 and DC10). The formation of hydrogen bonding may restrict the rotational and transformational freedom of the alkyl-chain and fixes it rigidly in the HSA molecule (the more negative the ΔS). Such interaction might be expected from the pK_a of carboxylic acids (4.0 to 4.9) [48]: it should exsist predominantly in an anionic form bound to HSA at pH 7.4, thus permitting formation of hydrogen bonding with the positively charged amino acid chains of HSA. Ge et al. proposed a double-hydrogen bond mechanism for binding of long-chain MCAs to bovine serum albumin, as a result of ESR studies [49]. The small increment of $-\Delta G_2$ (0.61 kJ mol^{-1} CH_2^{-1}) from MC11 to MC16 and the large negative values of ΔH_{m2} and ΔS_{m2} reflect hydrogen bonding formation due to long-chain carboxylic acids (MC10 to MC16 and DC16) in the second class of binding sites of HSA.

4. Conclusions

The binding of carboxylic acid to HSA is dependent upon the balance of enthalpy and entropy changes (compensation), which may result in apparently linear relationships between thermodynamic functions and chain length. The clear disparity in the thermodynamic parameters at eight-alkyl chain length (MC9 and DC10) of carboxylic acid is interpreted as indicating the difference in the primary binding sites on HSA, supporting the proposal of separate high-affinity binding sites of long-chain and short- or medium-chain carboxylic acids [6,25–30], and/or different interactions taking part in the complex formation of carboxylic acid with HSA. The primary binding for short- or medium-chain MCA is located at the same region as the binding sites of DCA, where one of the carboxy anion groups at both ends of DCA probably plays a minor role based on van der Waals' interaction. The binding of long-chain MCA to HSA is characterized by hydrophobic interaction due to the long-alkyl chain and by hydrogen bonding formation, reflecting the large values of the molar enthalpy and entropy changes of HSA.

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