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Dedicated to Professor Ionel Haiduc
on the occasion of his 75th anniversary

BINDING AFFINITY OF THE 3-CARBOXY-5,6-BENZOCOUMARINIC ACID TO HUMAN SERUM ALBUMIN: AN ISOTHERMAL TITRATION CALORIMETRY STUDY

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The binding affinity of the 3-carboxy-5,6-benzocoumarinic acid (BzCum) to human serum albumin (HSA) was investigated at 7.4 pH, in phosphate buffer by isothermal titration calorimetry (ITC) at several temperatures, using both the stepwise and continuous titration method. From fitting the ITC data, it resulted that the BzCum binding occurs at two classes of independent binding sites. The first class contains a single high-affinity site, characterized by a binding constant of about 1.5×10^6 M^{-1} at 25ºC. The second class is characterized by at least two sites with a binding constant of about 5.9×10^4 M^{-1}. The binding was an exothermic process characterised by an enthalpy variation of about -7 Kcal mol^{-1} for the high-affinity site and about -4 Kcal mol^{-1} for the low-affinity sites. The heat capacity change (ΔCp) of binding was lower for the high-affinity sites and negative for both sets of sites. The enthalpy and entropy dependence on temperature variation show that the interaction occurs by enthalpy- and entropy-driven mechanisms at 15°C and 25°C and became entirely enthalpy-driven at 35°C.

INTRODUCTION

The interaction of several ligands or drugs with serum albumins was widely studied using several spectral methods like absorption, fluorescence and circular dichroism spectroscopy, with the main purpose to determine the number of binding sites (n) and the binding constant (K).[1-7] Performing spectral experiments at several temperatures, the thermodynamic parameters, the enthalpy of binding (ΔH) and the entropy of binding (ΔS) can be obtained, allowing for the characterization of the main forces implied in the binding process.[8]

During the last years we were interested in the binding process of some heteroaromatic carboxylic acids with human and bovine serum albumins. All the compounds used were characterized by pKa values of the acid base equilibrium in the range 3.0-4.7, implying that in buffer solutions suitable for protein studies, pH =7.4, the dissociated form, the carboxylate ion, is the main species present in the system. Our previous experiments were performed by the fluorescence and circular dichroism methods monitoring either the intrinsic properties of the protein (the tryptophan emission band at 340 nm and the dichroic signal in the range of 205-225 nm) or of the ligand.[9-13]

In the followings we were interested in the interaction of a coumarin carboxylic derivative, the 3-carboxy-5,6-benzo-coumarinic acid (Fig. 1) with HSA. Based on the fact that another derivative of coumarin, the warfarin, a common anticoagulant medicine, with the hydrophobic molecular surface 355.36 $Å^2$ and polar surface area 66.43 $Å^2$, binds

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with high affinity at a specific site on HSA, we can assume that BzCum (hydrophobic surface 248.6 Å^2), can also be accommodated, based on hydrophobic forces, in the warfarin binding site situated within subdomain IIA (Sudlow's site-I). Another possible site for BzCum binding is Sudlow's site II located in subdomain IIIA, were typical bind aromatic carboxylates with an extended conformation such as ibuprofen (hydrophobic surface 319.4 Å^2). Other possible ways of binding of BzCum are the secondary binding site of ibuprofen located on domain II and the digoxin-binding site (site-III) on HSA (digoxin`s hydrophobic surface 972.4 Å^2).[14-22]

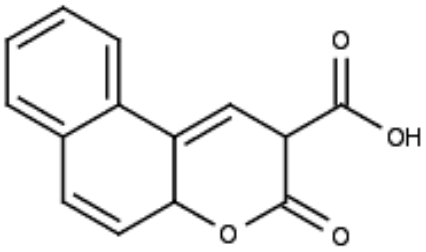

Fig. 1 – Structure of 3-carboxy-5,6-benzocoumarinic acid.

The binding process will be studied by the isothermal titration calorimetry. The ITC represents a method that permits the determination of all the binding parameters (n, K and ΔH) in a single experiment, by measuring the heat generated or absorbed during the association of a ligand with its binding partner. The Gibbs free energy of binding process (ΔG), and ΔS are determined from K, thus solving, the enthalpic and entropic components of binding affinity. The temperature dependence of the ΔH parameter, measured by performing the titration at varying temperatures, describes the binding ΔCp term.[23-29]

MATERIALS AND METHODS

Materials

Fatty acid and globulin-free HSA was obtained from Sigma Chemical (St. Louis, MO, USA), and 3-carboxy-5,6-benzocoumarinic acid was obtained from ABCR (Germany) and were used without further purification. The stock solution of phosphate buffer pH 7.4, was prepared using distilled and deionized water. The HSA (0.0237 mM) and BzCum (1.072 mM) solutions in buffer, were prepared by weight.

ITC

Titration experiments were performed using an iTC200 high-sensitivity microcalorimeter (MicroCal Inc., Northampton, MA, USA). This calorimeter is of power compensation type and is designed to work with small volumes of solutions. The calorimeter was verified by titration EDTA with $CaCl_2$. The test results were consistent with the acceptance criterion of standard sample tests on the Manual iTC200. The sample cell was filled with 280 μL of HSA and the titrant syringe was filled with 40 μL of BzCum. The samples were degassed under vacuum, prior to using for ITC runs. The reference cell was filled with deionized and degassed water. In incremental ITC, nineteen injections of 2 μL each (except 0.5 μL for the first injection) were performed at a rate of 0.5 μL per second, spaced 150 s apart to allow for a complete return to the baseline. The first injection was excluded from the analysis due to the backlash effect and dilution across the injection needle tip in the time of final baseline equilibration and delay period. The following settings were used: stir speed 750 rpm, which ensured rapid mixing and did not caused foaming on the protein solution, reference power 6 μCal per second, feedback mode/gain set to high, initial delay 600 seconds and filter period 2 seconds. In single injection method (continuous ITC) the following settings were used: stir speed 750, reference power 6 μCal per second, feedback mode/gain set to high, initial delay 60 seconds, injection volume were 39.2 μl, performed in 1003.6 seconds and filter period 5 seconds. The measurements were performed at three temperatures, 15, 25 and 35ºC.

The same setting parameters, were also used for the control runs for dilution. The heats of dilution of the ligand and the HSA were small compared with the heat of binding and were subtracted from the experimental titration results. The experimental data were then processed and integrated using the Origin for ITC v.7.0 SR4 software (Microcal) distributed with the instrument. The volume change in the mixing cell due to the injection of the ligand solution was corrected automaticaly by software. Data were then fitted using a nonlinear least squares routine using a two sets of sites binding model with the same Origin package, allowing for the determination of n, K, ΔH and ΔS.

RESULTS AND DISCUSSION

The experimental results obtained using the two previously discussed calorimetric titration methods,

the incremental and the single injection method, are presented in Figs. 2 and 3. In both figures, panel A presents representative ITC profiles for titration of HSA with BzCum at 25ºC in phosphate buffer pH 7.4. It can be seen that HSA is saturate in Bzcum at the ratio molar over 6:1 ligand:protein. Panel B depicts the binding isotherm of the calorimetric titration shown in panel A. The best fit of the experimental ITC data was obtained for the two independent set of sites binding model, implemented in the Origin for ITC software.[30] The results are listed in Tables 1 and 2.

The values in Tables 1 and 2 shows that using both calorimetric titration methods, in the limit of experimental errors, the results are quite similar. BzCum binds to two classes of independent binding sites, class 1 being characterized by a single site with a binding constant at 25ºC of $1.5x10^6$ M^{-1} and class 2 being characterized by at least two sites with a lower binding constant, of about $5.9x10^4$ M^{-1}.

The values of $\Delta H < 0$ and $\Delta S > 0$ at 15°C and 25°C, show that both enthalpy and entropy contribute favorably to the binding process such that, the interaction was enthalpy- and entropy-driven. According to the thermodynamic parameters ($\Delta H < 0$, $\Delta S > 0$ and $\Delta G < 0$) the main contributions on the binding forces in the BzCum-HSA system are provided by both hydrogen bonding and hydrophobic forces. Usually, the increase of entropy in drug-HSA interactions is related to the release of water of solvation from both the drug and albumin molecules. Increasing the temperature at 35ºC, the entropy contribution to ΔG becomes negative but it is overcome by a large binding enthalpy and the process became entirely enthalpy-driven.

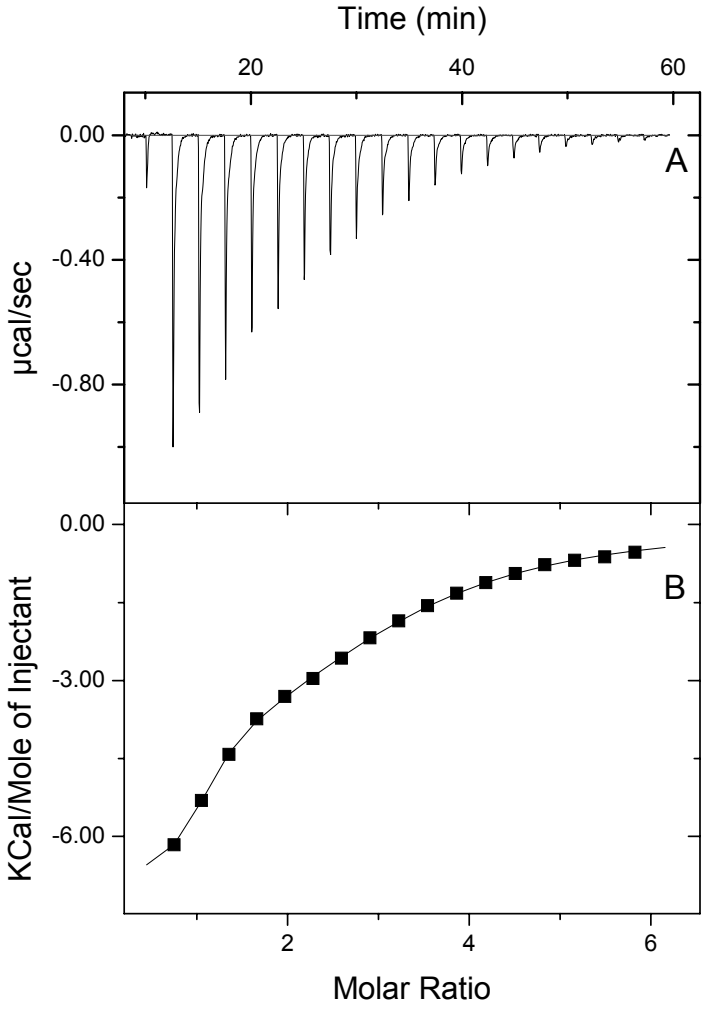

Fig. 2 – (A) Differential power (µcal/sec) versus time for titration of HSA with BzCum at 25ºC in phosphate buffer, pH 7.4, showing the calorimetric response as successive injections of ligand are added to the reaction cell. (B) Heat per injection from integration of the peak areas in part (A) per mole of injectant versus molar ratio. The continuous line represents the least-squares fit of the data to a two sets of sites binding model.

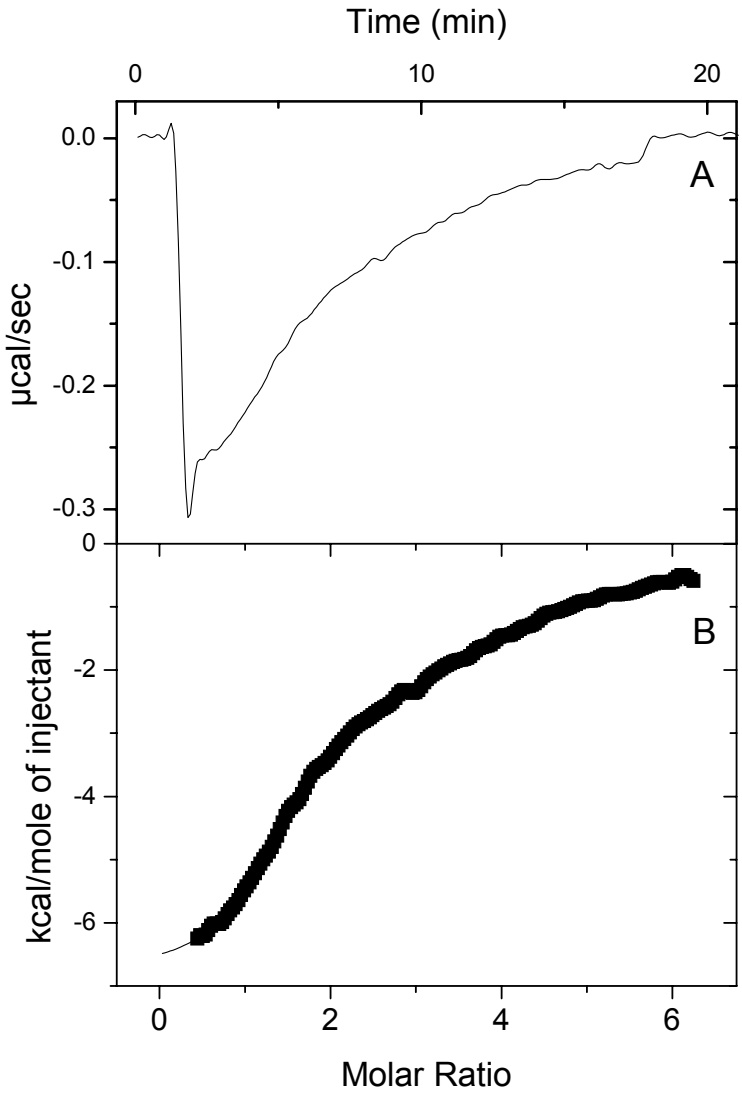

Fig. 3 – (A) Differential power (µcal/sec) versus time for titration of HSA with BzCum at 25ºC in phosphate buffer, pH 7.4, showing the calorimetric response as single injection of ligand is added to the reaction cell. (B) Heat per injection from integration of the peak areas in part (A) per mole of injectant versus molar ratio. The continuous line represents the least-squares fit of the data to a two sets of sites binding model.

Table 1

Thermodynamic parameters for the binding of BzCum to HSA from secvential ITC experiments

Temp ºC	n_1 Sites	K_1 (M^{-1})	ΔH_1 (cal·mol^{-1})	ΔS_1 (cal·mol^{-1}·grad^{-1})	ΔG_1 (cal·mol^{-1})	ΔC_{p1} (cal·mol^{-1}·grad^{-1})
15	0.82±0.02	2.54E6±5.15E5	-6890±231	5.39±0.89	-8438.74±116.01	
25	0.84±0.02	1.55E6±3.44E5	-7383±299	3.56±1.09	-8439.17±131.4	-89.7±23.3
35	0.85±0.04	5.25E5±9.63E4	-8684±337	-2.01±1.15	-8059.74±112.24	
Temp ºC	n_2 Sites	K_2 (M^{-1})	ΔH_2 (cal·mol^{-1})	ΔS_2 (cal·mol^{-1}·grad^{-1})	ΔG_2 (cal·mol^{-1})	ΔC_{p2} (cal·mol^{-1}·grad^{-1})
15	2.44±0.07	9.97E4±8.56E3	-3609±231	10.3±0.81	-6586.07±49.12	
25	2.67±0.13	5.87E4±5.88E3	-4125±376	7.98±1.27	-6501±59.3	-153.4±58.7
35	2.28±0.2	2.80E4±2.35E3	-6676±966	-1.32±3.13	-6266.07±51.35	

Table 2

Thermodynamic parameters for the binding of BzCum to HSA from continuous ITC experiments

Temp ºC	n_1 Sites	K_1 (M^{-1})	ΔH_1 (cal·mol^{-1})	ΔS_1 (cal·mol^{-1}·grad^{-1})	ΔG_1 (cal·mol^{-1})	ΔC_{p1} (cal·mol^{-1}·grad^{-1})
15	0.90±0.01	2.38E6±2.12E5	-6456±114	6.78±0.43	-8401.51±50.96	
25	0.76±0.02	1.59E6±4.14E4	-7028±306	4.80±1.03	-8454.25±15.41	-104.8±27.5
35	1.08±0.01	7.19E5±4.00E4	-8552±96.4	-0.95±0.33	-8252.17±34.04	
Temp ºC	n_2 Sites	K_2 (M^{-1})	ΔH_2 (cal·mol^{-1})	ΔS_2 (cal·mol^{-1}·grad^{-1})	ΔG_2 (cal·mol^{-1})	ΔC_{p2} (cal·mol^{-1}·grad^{-1})
15	2.15±0.04	8.03E4±3.38E3	-3414±130	10.6±0.46	-6462.24±24.08	
25	2.47±0.11	6.04E4±5.21E3	-4410±367	7.08±1.24	-6517.9±51.07	-161.15±35.5
35	2.34±0.09	2.80E4±1.16E3	-6637±413	-1.19±1.34	-6266.07±25.35	

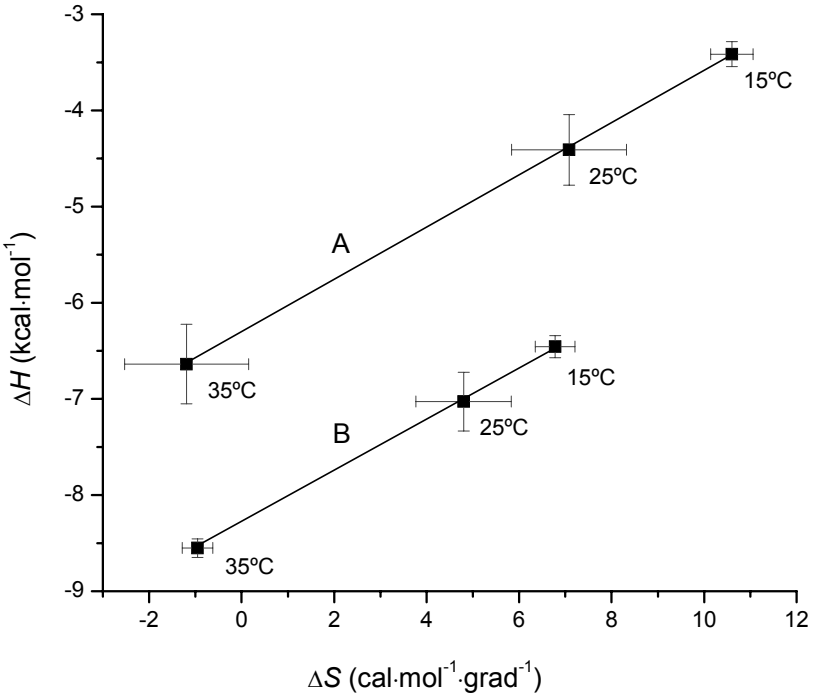

Fig. 4 – Thermodynamic enthalpy-entropy compensation (values from Table 2) on temperature variation for: A. low-affinity sites and B. high-afinity site.

The dominant negative enthalpy suggests that there are a large number of favourable hydrogen bond contacts between the water of solvation released from hydrophobic surfaces and water in bulk. The binding free energy (ΔG), calculated as $\Delta G = -RT\ln K$, shows the compensation phenomena between the contributions of enthalpy and entropy changes at the temperature increases. Entropy decreases with temperature but the effect is compensated by the binding enthalpy (Fig. 4), the linear enthalpy–entropy relationship indicating the participation of water in ligand-protein binding processes.[31] The binding heat capacity change (ΔCp) for each site was obtained by plotting the binding enthalpy vs temperature (T). Considering a linear dependence, the slope gives the value of ΔCp. The ΔCp was lower for the high-affinity sites and negative for both sets of sites. $\Delta Cp < 0$ usually correlated with the burial of hydrophobic surfaces. A negative ΔCp means that the net thermodynamic driving force for association will shift from being entropic to enthalpic with increasing temperature.[32]

CONCLUSIONS

The calorimetric investigation by both sequential and continuous titration showed that the interaction of BzCum with HSA can be described by two stoichiometric binding constants corresponding to at least three binding sites, one high-affinity site and at least two relatively low-affinity sites. BzCum binds at different binding

sites on HSA, being so able to influence the binding of common drugs and natural ligands.

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REFERENCES

1. A. H. Hegde, B. Sandhya, S. S. Kalanur and J. Seetharamappa, *J. Solution. Chem.,* **2011**, *40*, 182-197.
2. F. Mohammadi, A.-K. Bordbar, A. Divsalar, K. Mohammadi and A. A. Saboury, *Protein J.*, **2009**, *28*, 189-196.
3. Y. Liu, P. Mei, Y.-Z. Zhang, X.-H. Sun and Y. Liu, *Biol. Trace Elem. Res.,* **2010**, *138*, 125-138.
4. Y.-Z. Zhang, J. Zhang, F.-F. Li, X. Xiang, A.-Q. Ren and Y. Liu, *Mol. Biol. Rep.*, **2011**, *38*, 2445-2453.
5. Y. He, Y. Wang, L. Tang, H. Liu, W. Chen, Z. Zheng and G. Zou, *J. Fluoresc.*, **2008**, *18*, 433-442.
6. X. Fang, C. Liu, R. Liu and Y. Teng, *J. Fluoresc.*, **2011**, *21*, 1069-1074.
7. R. Punith, A. H. Hegde and S. Jaldappagari, *J. Fluoresc.*, **2011**, *21*, 487-495.
8. P.D. Ross and S. Subramanian*, Biochemistry*, **1981**, *20*, 3096-3102.
9. l. Birla, A. M. Cristian, D. Gavriliu, O. Maior and M. Hillebrand, *Rev. Roum.Chim*., **2002**, *47*, 769-775.
10. A. Varlan and M. Hillebrand, *Molecules*, **2010**, *15*, 3905-3919.
11. A. Varlan and M. Hillebrand, *Cent. Eur. J. Chem.*, **2011**, *9*, 624-634.
12. A. Varlan and M. Hillebrand, *Rev. Roum. Chim.*, **2010**, *55*, 69-77.
13. C. Stoica, A. Jelea and M. Hillebrand, *J. Photochem. Photobiol. A: Chemistry,* **2006**, *183*, 89-97.
14. http://www.chemicalize.org/structure/#!mol=warfarin& source = calculate
15. http://www.chemicalize.org/structure/#! mol=structureId% 3A1161728302131&source=fp
16. http://www.chemicalize.org/structure/#!mol=ibuprofen& source =calculate
17. http://www.chemicalize.org/structure/#!mol=digoxin& source =calculate
18. G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.,* **1975**, *11*, 824-832.
19. H. Watanabe, U. Kragh-Hansen, S. Tanase, K. Nakajou, M. Mitarai, Y. Iwao, T. Maruyama and M. Otagiri, *Biochem. J*., **2001**, *357*, 269-274.
20. I. Petitpas, A. A. Bhattacharya, S. Twine, M. East and S. Curry, *J. Biol. Chem*., **2001**, *276*, 22804-22809.
21. P. A. Zunszain, J. Ghuman, T. Komatsu, E. Tsuchida and S. Curry, *BMC Struct. Biol.*, **2003**, 3:6.
22. S. M. Sharker, A. A. Rahman and M. A. Alam, *Insight Pharmaceutical Sciences,* **2011**, *1*, 24-28.
23. R. K. Brown, J. M. Brandts, R. O'Brien and W. B. Peters, *Label-Free Biosensors,* **2009**, 223-250.
24. E. Freire**,** *Drug Discov Today,* **2008**, 13869-874.
25. J. M. Woods, M. Puri, D. Doucet, T. Derrick, S. Morar-Mitrica and D. Nesta, GE Healthcare, Application Note 28-9613-26 AA.
26. T. Wiseman, S. Williston, J. F. Brandts and L.-N. Lin, *Anal. Biochem.,* **1989**, *179*, 131-137.
27. P. L. Privalov and A. I. Dragan. *Biophys. Chem.*, **2007**, *126*, 16-24.
28. M. M. Pierce, C. S. Raman and B. T. Nall, *Methods.,* **1999**, *19*, 213-221.
29. S. Leavitt and E. Friere. *Curr. Opin. Struct. Biol*., **2001**, *11*, 560-566.
30. ITC Data Analysis in Origin. Tutorial Guide Version 7.0, January **2004**, MicroCal Inc., Northampton, MA, USA.
31. R. Lumry, and S. Rajender, *Biopolymers*, **1970**, *9*, 1125-1227.
32. R. Talhout, A. Villa, A. E. Mark and J. B. F. N. Engberts, *J. Am. Chem. Soc*., **2003**, *125*, 10570-10579.