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Study on the interaction of the toxic food additive carmoisine with serum albumins: A microcalorimetric investigation

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HIGHLIGHTS

- Carmoisine binds to both the serum albumins with affinity of the order of $10^6\ M^{-1}$.
- The binding was favored by negative enthalpy and positive entropy changes.
- The binding was dominated by hydrophobic forces.
- Carmoisine enhanced the thermal stability of both the proteins remarkably.

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ABSTRACT

The interaction of the synthetic azo dye and food colorant carmoisine with human and bovine serum albumins was studied by microcalorimetric techniques. A complete thermodynamic profile of the interaction was obtained from isothermal titration calorimetry studies. The equilibrium constant of the complexation process was of the order of $10^6\ M^{-1}$ and the binding stoichiometry was found to be 1:1 with both the serum albumins. The binding was driven by negative standard molar enthalpy and positive standard molar entropy contributions. The binding affinity was lower at higher salt concentrations in both cases but the same was dominated by mostly non-electrostatic forces at all salt concentrations. The polyelectrolytic forces contributed only 5–8% of the total standard molar Gibbs energy change. The standard molar enthalpy change enhanced whereas the standard molar entropic contribution decreased with rise in temperature but they compensated each other to keep the standard molar Gibbs energy change almost invariant. The negative standard molar heat capacity values suggested the involvement of a significant hydrophobic contribution in the complexation process. Besides, enthalpy–entropy compensation phenomenon was also observed in both the systems. The thermal stability of the serum proteins was found to be remarkably enhanced on binding to carmoisine.

1. Introduction

The carcinogenic effect of azo dyes on human life is well documented in the literature [1–3]. The influence of azo dyes has been linked to human bladder cancer, splenic sacromas, hepatocarcinomas and nuclear anomalies in animal models [3–5]. Carmoisine (Fig. 1) is one such water soluble red azo dye used widely as food colorant in blancmange, Swiss rolls, jams, jellies, yoghurts, breadcrumbs, oraldene mouthwash and cheese cake mixes. This dye is also known in different names like Azorubine, Azorubin S, Food Red 3, Brillantcarmoisin O, Acid Red 14 and C.I. 14720 and usually exists as a disodium salt [6]. The toxicological effects of carmoisine are well documented based on the reports by Mekkawy et al. They showed the histopathological effects of carmoisine on the hepatic and renal tissues of rats [7]. Besides this, Amin and coworkers reported that carmoisine adversely affected and altered the biochemical markers in vital organs like liver and kidney even at low doses of intake [6]. Thus, carmoisine can cause potential toxicity during its transmission and metabolism in the human body. Therefore, the toxicity of such synthetic organic carcinogens, introduced into the blood stream through food intake and other means, is a real cause for concern in the modern times [8].

Serum albumins, the multifunctional and most abundant transport proteins in the blood plasma, are the major constituents of the circulatory system. They perform many critical physiological

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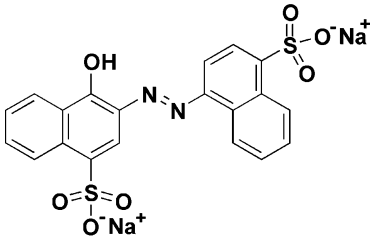

Fig. 1. Chemical structure of carmoisine.

functions relating to the maintenance of colloid osmotic blood pressure, transportation and disposition of endogeneous and exogeneous compounds [9–11] etc. Serum proteins can also regulate ligand sequestration and transportation which may impact many biological functions like membrane penetration, metabolism, half life and several other pharmacokinetic properties [12]. The binding affinity of toxic substances to serum albumins can have physiological relevance, as it can serve as a tool to control their free and active concentrations, and influence the duration and intensity of their side effects [13]. The available free concentration for toxic action can be effectively regulated by high binding to serum proteins [13,14]. Human serum albumin (HSA) and bovine serum albumin (BSA) are the two most abundant multifunctional proteins in the human and bovine blood. These two serum proteins have structural similarity to the extent of about 76% [15,16]. The two major ligand binding regions in these serum proteins were identified as sudlows site I in the subdomain IIA and site II in the subdomain IIIA [17,18]. The majority of the small molecule ligands have been reported to bind in these regions [12,15,19–22].

Binding of carmoisine to serum albumins has been studied recently by Halder and coworkers using spectroscopy and molecular docking as tools [8]. The authors concluded that carmoisine binds to the sub-domain IIA of HSA, and the sub-domains IIA and IB of BSA. Thermodynamic analysis of this interaction is essential for not only understanding the energetics aspects of the interaction, but for correlating the structural aspects with the energetics in order to ascertain, in a better way, the toxicological aspects of the dye. At present microcalorimetry is the only technique which allows direct determination of the basic physical forces that guide the binding of a ligand to a protein by measuring the heat effects [15]. Therefore, in the present work we undertook elucidation of the thermodynamics of the interaction of carmoisine with HSA and BSA from microcalorimetry experiments.

2. Experimental

2.1. Materials

Human serum albumin (0.99 mass fraction purity, essentially fatty acid and globulin free), bovine serum albumin (0.99 mass fraction purity, essentially fatty acid and globulin free) and carmoisine (0.98 mass fraction purity) were purchased from Sigma-Aldrich corporation (St. Louis, MO, USA). The sample provenance and purity values are given in Table 1. All the samples were dissolved in 10 mM citrate-phosphate buffer, pH 7.0, containing 5 mM Na_2HPO_4 prepared in triple distilled and deionized water and filtered through membrane filters of pore size 0.22 μM (Millipore India Pvt. Ltd. Bangalore, India). The pH was adjusted using citric acid and was measured on a Sartorious bench pH meter. The concentration of HSA and BSA solutions were determined by absorbance measurements at 280 nm using molar extinction coefficient (ε) values of 36,600 and 43,824 $M^{-1}\,cm^{-1}$, respectively [15]. Buffer salts, chemicals and reagents used in this study were of analytical grade obtained from Sigma. All the solutions for calorimetry experiments were degassed on the Thermovac unit of MicroCal.

Table 1
List of provenance and purity values (in terms of mass fraction) of different samples.

Sample	Provenance	Mass fraction purity[a]
Bovine serum albumin	Sigma-Aldrich	0.99
Human serum albumin	Sigma-Aldrich	0.99
Carmoisine	Sigma-Aldrich	0.98

[a] The mass fraction purities are based on information provided by the supplier Sigma-Aldrich. The purities of BSA and HSA were estimated by agarose gel electrophoresis and the purity of carmoisine was independently verified by HPLC.

2.2. Isothermal titration calorimetry experiments

All isothermal titration calorimetry experiments were performed using a MicroCal VP-ITC unit (MicroCal, Inc., Northampton, MA, USA) at 293.15 K [23]. The experimental procedure adopted was as follows. Aliquots of protein solutions were injected from the rotating syringe (290 rpm) of the calorimeter unit into the sample chamber containing carmoisine solutions (1.4235 mL). The volume and number of injections were predetermined and controlled by the ITC unit software. The parameters set for the titration were as follows: duration 10 s, time lag between successive injections 240 s. Each protein injection to the dye generated a heat burst spike resulting from the heat generated due to the binding. As the binding progressed, the strength of the heat bursts diminished and ultimately spikes with constant height were produced indicating the onset of saturation of the binding sites. Corresponding control experiments to determine the heat of dilution of the proteins were performed by injecting identical volumes of the same concentration of HSA and BSA into the buffer alone. The area under each heat burst curve was determined by integration using the Origin software (OriginLabs, Northampton, USA) to derive the heat associated with the injections. The heat generated in protein-buffer mixing was subtracted from the corresponding heat associated with the protein injection to carmoisine to obtain the heat of carmoisine-protein binding. The resulting corrected injection heats were plotted as a function of the molar ratio (χ) of protein/carmoisine and fitted to a model for single binding sites using Levenberg–Marquardt non-linear least squares curve fitting algorithm, inbuilt with the software of the unit. The association constant K, stoichiometry N, and standard molar enthalpy change ΔH^0 were obtained using the following relations:

$$K = \frac{\theta}{(1-\theta)[X]} \tag{1}$$

where θ = fraction of sites occupied by carmoisine X, and $[X]$ = concentration of free carmoisine. The total concentration of carmoisine (free and bound), X_t is obtained by the relation

$$X_t = [X] + n\theta M_t \tag{2}$$

where M_t is the bulk concentration of the protein in the active cell volume V_{cell}. The total heat content Q of the solution in V_{cell} is

$$Q = n\theta M_t \Delta H^0 V_{cell} \tag{3}$$

Considering the volume change ΔV_i for the injection i, the heat released, ΔQ_i from the i^{th} injection is given by

$$\Delta Q_i = Q_i + \frac{\Delta V_i}{V_{cell}}\left[\frac{Q_i + Q_{(i-1)}}{2}\right] - Q_{(i-1)} \tag{4}$$

Standard molar Gibbs energy change and standard molar entropy change can be obtained from the relation

$$\Delta G^0 = -RT\ln K = \Delta H^0 - T\Delta S^0 \tag{5}$$