Food Research International 72 (2015) 54–61

Contents lists available at ScienceDirect

Food Research International

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

Binding of carmoisine, a food colorant, with hemoglobin: Spectroscopic and calorimetric studies

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ARTICLE INFO

Article history:
Received 22 December 2014
Received in revised form 9 March 2015
Accepted 11 March 2015
Available online 18 March 2015

Keywords:
Carmoisine
Hemoglobin
Binding
Spectroscopy
Thermodynamics

ABSTRACT

Interaction of the food colorant carmoisine with hemoglobin was studied using spectroscopic and calorimetric tools. The binding effected hypochromic changes in the Soret band of hemoglobin and induced remarkable quenching of the intrinsic fluorescence of hemoglobin. Synchronous fluorescence studies revealed that the polarity around the tryptophan residues of the protein was significantly increased in the presence of carmoisine whereas that around the tyrosine residues remained unchanged. Binding of carmoisine resulted in change of the secondary structure of hemoglobin reducing the helical composition to more than half the initial value. The binding was favored by large positive entropy changes and small but favorable enthalpy changes. The heat capacity change value and the occurrence of enthalpy–entropy compensation phenomena suggested the involvement of significant hydrophobic forces in the binding process. Detailed insights into the molecular interaction of carmoisine with hemoglobin are presented.

1. Introduction

Carmoisine (disodium-4-hydroxy-3-[(4-sulfo-1-naphthalenyl)azo]-1-naphthalenesulfonate) (Fig. 1) is a synthetic anionic azo dye which is widely used as a food colorant. Carmoisine is also known as Azorubine, Food Red 3, Azorubin S, Brillantcarmoisin O, Acid Red 14 and C.I. 14720. It usually exists as a disodium salt and hence it is highly water soluble. It is available as a red/maroon powder and is present in food products like blancmange, Swiss roll, marzipan, jams, yogurts, jellies, breadcrumbs, cheesecake mixes and oraldene mouthwash (Amin, Hameid, & Abd Elsttar, 2010). Carmoisine has been shown to exert histopathological effects on the hepatic and renal tissues of rats which were manifested by vacuolation, swelling, necrosis and pyknosis of the cells (Amin et al., 2010; Mekkawy, Ali, & El-Zawahry, 1998). Furthermore, histopathological studies have also revealed that carmoisine induces brown pigment deposition in the portal tracts and Van Küpffer cells of liver and renal tracts (Aboel-Zahab et al., 1997; Amin et al., 2010). Carmoisine has also been reported to adversely affect and alter the biochemical markers in vital organs, such as liver and kidney, at both high and low doses (Amin et al., 2010). The maximum acceptable daily intake (ADI) value of carmoisine proposed by Joint FAO/WHO Expert Committee on Food Additives is 4 mg/kg bw/day (EFSA panel on food additives and nutrient sources added to food (ANS), 2009).

Human hemoglobin (Hb) is an iron-based blood protein that carries oxygen from lungs to various respiring tissues. Important functions of Hb include dispersion of hydrogen peroxide and electron transfer to all body parts and organs. Besides, Hb is also involved in the transport of H^+, CO_2 and 2,3-bisphosphoglycerate from tissues to lungs and kidneys. Hb can also serve as a carrier for the nitric oxide molecule, which is bound to the thiol group of globin protein, releasing it along with the oxygen (Connie & Hsia, 1998). It accounts for about 92% of the red blood cells and assumes a high concentration under physiological conditions which corresponds to a volume fraction of 0.25 (Krueger & Nossal, 1988). Hb molecule consists of two α and two β subunits, non-covalently associated with erythrocytes as a tetramer. Each α-chain contains 141 amino acid residues and each β-chain contains 146 amino acid residues (http://www.rscb.org/pdb/explore/explore.do?structureId=30dq). The α and β chains comprise of seven and eight helices, respectively. The tetrameric conformation of Hb holds the key for its biological functions. Hb is also endowed with the ability to reversibly bind a number of endogenous and exogenous molecules and thus it can act as a carrier for small organic molecules (Hazra, Hossain, & Suresh Kumar, 2013; Hazra & Suresh Kumar, 2014; Hegde, Sandhya, & Seetharamappa, 2013). Thus, it is essential to undertake a detailed study of the binding of dyes to proteins as it can lend valuable insight into the toxicity of the dyes and also clarify the mechanism of biomacromolecular functioning in complex biological

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http://dx.doi.org/10.1016/j.foodres.2015.03.015
0963-9969/

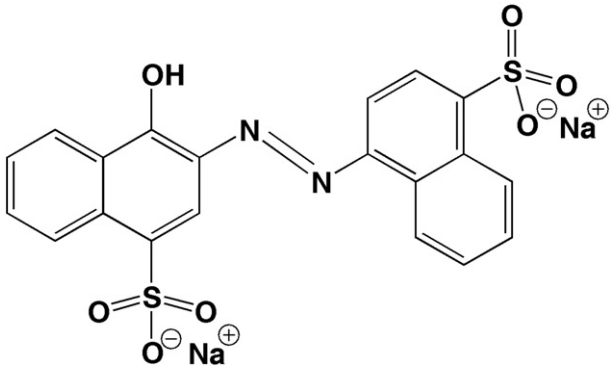

Fig. 1. Molecular structure of carmoisine.

systems (Hasni, Bourassa, & Tajmir-Riahi, 2011; Peng, Ding, Peng, & Sun, 2014). Although the interaction of some dyes with Hb has been undertaken, the effect of food colorants has been scarcely studied (Mandal, Bardhan, & Ganguly, 2010; Peng et al., 2014; Wang, Zhang, & Tang, 2010). Thus, in the present study we investigated the effect of carmoisine, the food colorant, on Hb using multifaceted spectroscopic and calorimetric tools.

2. Materials and methods

2.1. Materials

Hemoglobin and carmoisine were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Hemoglobin was purified as reported previously (Hazra & Suresh Kumar, 2014). All the samples were dissolved in 10 mM citrate-phosphate buffer, pH 7.2, containing 5 mM Na_2HPO_4 prepared in deionized and triple distilled water and filtered through Millipore membrane filters of pore size of 0.22 μM. The concentration of Hb was determined by absorbance measurements using molar absorption coefficient (ε) value of 1,79,000 M^{-1} cm^{-1} at 405 nm (Antonini & Brunori, 1971). The concentration of carmoisine was determined by weighing. Buffer salts, chemicals and other reagents used in this study were of analytical grade obtained from Sigma-Aldrich. The dye solutions were freshly prepared each day and stored in the dark to avoid any light induced photochemical changes.

2.2. Absorbance studies

Absorbance spectral studies were conducted on a Jasco V-660 spectrophotometer (Jasco International Co., Hachioji, Japan) at 298.15 ± 0.10 K. Protein–dye titrations were performed in matched quartz cuvettes (Hellma, Germany) of 1 cm path length. At first a known concentration of Hb solution was kept in the sample cuvette and then small aliquots of known concentration of carmoisine solution were added into the sample and reference cuvettes. After each addition, the solution was thoroughly mixed and allowed to re-equilibrate for at least 10 min. before noting the absorbance at the desired wavelength.

2.3. Fluorescence studies

Steady state fluorescence was performed on either a Shimadzu RF-5301 PC (Shimadzu Corporation, Kyoto, Japan) or a Hitachi F4010 (Hitachi Ltd., Tokyo, Japan) spectrofluorimeter in fluorescence free quartz cuvettes of 1 cm path length keeping emission and excitation slit widths of 5 and 10 nm, respectively. The sample temperature was maintained at 298.15 ± 0.10 K using an Eyela UniCool U55 water bath (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Intrinsic fluorescence of Hb and carmoisine was measured by exciting at 295 and 324 nm, respectively. Temperature dependent fluorescence spectral studies were performed on the Hitachi unit. Synchronous fluorescence spectra were measured keeping Δλ fixed at 15 and 60 nm, respectively. Correction of the fluorescence intensities for the absorption of exciting light and reabsorption of the emitted light was applied to eliminate the inner filter effect according to the procedure described in details earlier (Hazra & Suresh Kumar, 2014; Wang et al., 2010). Three-dimensional fluorescence spectroscopy experiments were performed on a PerkinElmer LS55 spectrofluorimeter (PerkinElmer, Inc., USA) as described previously (Hazra & Suresh Kumar, 2014).

2.4. FT-IR studies

FT-IR measurements were performed on a Bruker FTIR, TENSOR 27 spectrometer. In a typical experiment, a spectrum of the buffer solution was recorded at first followed by the spectra of Hb and Hb–carmoisine complexes. The buffer spectrum was subtracted from the sample spectra to obtain the corrected IR spectra of Hb and Hb–carmoisine complex.

2.5. Circular dichroism studies

The secondary and tertiary structural changes induced in Hb upon binding with carmoisine were measured on a Jasco J815 spectropolarimeter. A Peltier cell holder and temperature controller PFD 425L/15 was used to maintain the cuvette temperature at 298.15 ± 0.10 K. The scan speed, bandwidth and sensitivity were maintained at 20 nm/min, 1.0 nm and 100 mdeg, respectively. The far UV and Soret band CD spectra were recorded in 1 mm and 10 mm pathlength cuvettes, respectively. Ten successive accumulations were taken and averaged out in order to improve the signal-to-noise ratio and smoothed within permissible limits by the Jasco software of the unit.

2.6. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on a MicroCal VP-ITC unit (MicroCal, Inc., Northampton, MA, USA). The samples were extensively degassed on the Thermovac accessory of the unit prior to titration to avoid the formation of bubbles during the course of the experiment. Aliquots of Hb solution were injected from the rotating syringe (290 rpm) into the calorimeter cell containing carmoisine solution (1.4235 mL). Corresponding control experiments to determine the heat of dilution of Hb were performed by injecting identical volumes of the same concentration of the protein into the buffer alone. The initial delay before the first injection was 60 s. The duration of each injection was fixed at 10 s and the delay time between successive injections was 240 s. This time gap enabled the heat signal to return to the base line after each binding event. The area under each heat burst spike was determined by integration using the Origin 7.0 software to obtain a measure of the heat associated with the injection. The heat associated with the protein-buffer mixing was subtracted from the corresponding heat of carmoisine–Hb reaction to obtain the actual heat of carmoisine–Hb binding reaction. The corrected injection heats were finally plotted as a function of the molar ratio (χ) of Hb/carmoisine and fitted to "one set of sites" model to obtain the equilibrium constant (K_a), the binding stoichiometry (N) and the standard molar enthalpy change (ΔH^0). The standard molar Gibbs energy change (ΔG^0) was calculated using the following standard relationship

$$\Delta G^0 = -\mathrm{RT}\ln K_a \tag{1}$$

where R is the gas constant ($R = 8.314472\ \mathrm{J\cdot K^{-1}\cdot mol^{-1}}$) and T is the thermodynamic temperature. The standard molar entropic contribution ($T\Delta S^0$) to the binding reaction, where ΔS^0 is the standard molar entropy change, was calculated using the relationship

$$T\Delta S^0 = \Delta H^0 - \Delta G^0. \tag{2}$$