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pH-insensitive electrostatic interaction of carmoisine with two serum proteins: A possible caution on its uses in food and pharmaceutical industry

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ABSTRACT

Here we have investigated the binding of carmoisine, a water-soluble azo food colorant, with serum proteins (HSA and BSA) by fluorescence and UV–VIS spectroscopy, circular dichroism and molecular docking studies. Results indicate that fluorescence quenching of protein has been due to site-specific binding of the dye with biomacromolecules. Site marker competitive binding and molecular docking explorations show that interaction occurs in the sub-domain IIA of HSA and the sub-domains IIA and IB in the case of BSA. Conformational investigation indicates that dye binding modifies the secondary structure of proteins and this also alters the microenvironment of the tryptophan(s). The interaction is found to be pH-insensitive which can have relevance to the toxicological profiles of the dye, and ionic strength dependence of binding can be exploited in protein purification mediated by such food colorants.

1. Introduction

Dyes constitute an important class of synthetic organic compounds with a wide range of applications in various industries like food, fabric, drinks, medicine and cosmetics. Apart from their commercial applications they can also be used as important probe for understanding the mechanism of various photophysical processes [1]. Dyes have been detected and estimated by means of various available analytical methods [2–4]. The exploration of their photophysics is one of the interesting aspects to the researchers especially when the excited state properties of these dyes are profoundly altered by the change of microenvironment [5]. Fluorescence properties of some of these dyes are considerably affected in constrained media [6] and also when these tend to interact with biopolymers like protein [7,8]. Among these dyes, azo compounds form a separate class, in which almost 60–70% of the whole dye varieties are included. Their stability in heat and wide range of pH leads to usage as coloring agents in food and cosmetics. These dyes show photoisomerization and have very low fluorescence quantum yield [9]. Carmoisine (Fig. 1a) is a well known anionic azo food dye. The presence of carmoisine has been detected by different analytical techniques [10,11]. It is found to exhibit interesting fluorescence behavior in presence of viologens (dimethyl viologen and diheptyl viologen) quenchers in neat methanol [9]. Likewise, it would be interesting to explore how carmoisine, interacts with serum proteins and other biopolymers.

The study of exogenous ligand binding of serum protein has always been more interesting mainly due to the transport properties of these macromolecules [12,13]. Quite a good number of drugs are transported in the circulatory systems in the form of complex with albumin [14]. The affinity of albumin towards small molecules, drugs can be altered by the simultaneous binding of the endo- and exogenous ligands [13]. If the target ligand binds stronger in the drug binding site, it will lead to a reduction of the drug binding. Thus this alteration of drug affinity in presence of a competitively binding small molecule can be utilized to tune the transporting function of proteins.

Acidic and basic side chains of proteins can vary their charges by protonation/deprotonation due to change of local pH. At the protein surface normally acidic residues (Glu, Asp, C-terminus) are deprotonated (i.e., becomes negatively charged) and the basic residues (Lys, Arg, His and N-terminus) are protonated (i.e., becomes positively charged). The importance of pH in the structure and function of proteins is well illustrated by the fact that about 25% of its residues contain ionizable side chains [15]. The ionizations that arise from changes in pH can generate strong electrostatic interactions, which will inevitably have a direct influence on molecular structure and binding affinity. It is well accepted that pH-effect on proteins is complex in nature. This is mainly due to the multiplicity of titrable sites, which are not only subjected to

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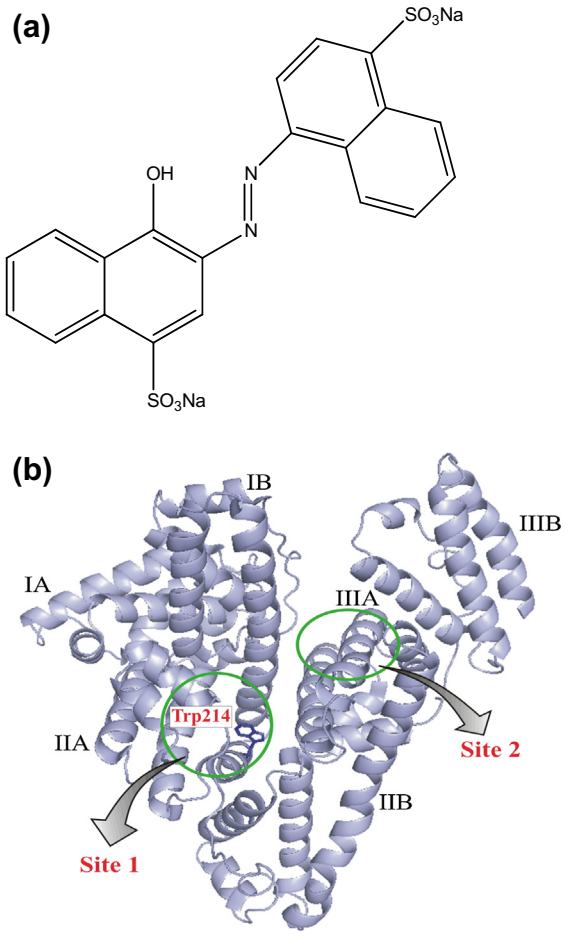

Fig. 1. Structure of (a) carmoisine and (b) crystal structure of HSA (PDB entry 1AO6).

different environments but also coupled to one another in complex ways. Therefore, considering the structural and functional aspects of protein the importance of these interactions is immense. It governs several aspects of biophysical processes and is therefore, of great significance for both applied bioengineering and more fundamental biochemical and medicinal research.

Human serum albumin (HSA) and bovine serum albumin (BSA) are two extensively studied proteins in literature [13,14]. The X-ray crystallographic structure of HSA (Fig. 1b) is known to be organized into three homologous domains (I–III), that are composed of two sub-domains (A and B) [16]. It has only one tryptophan residue (Trp 214) which is located in the hydrophobic pocket in sub-domain IIA [16]. BSA has almost 88% sequence homology with HSA [1] and there are two tryptophan residues (Trp 134 and Trp 213) of which Trp 134 is located on the surface of the molecule in sub-domain IB and Trp 213 resides in the hydrophobic pocket/fold in sub-domain IIA [13]. The binding pockets for small exogenous ligands of these two serum proteins are well identified. Azo dyes are found to have binding interactions with serum protein resulting in secondary structural modification of the bio-molecule. But the effect of alteration of local charge and environment, due to the change of pH of the solution, on the dye–protein interaction is not explored very well. For this, in the present work we attempt to explore the interaction of carmoisine, a negatively charged food colorant, with HSA and BSA as function of pH and ionic strength of the medium. In this study we have used general optical spectroscopic techniques like UV–VIS, fluorescence, synchronous fluorescence and circular dichroism (CD) spectroscopy.

2. Materials and methods

2.1. Materials

HSA (>98%) and BSA (~99%), hemin (>98%, HPLC), ibuprofen (>98%, GC), carmoisine (molecular weight 502.44, 98%) are purchased from Sigma–Aldrich and warfarin (analytical grade) is purchased from TCI Chemicals, Japan. All other chemicals are of analytical reagent grade. Ultra pure water is used throughout the study. All solutions are prepared in 5 mM sodium phosphate buffer of pH 4.8 (±0.1), 5.5 (±0.1), 6.3 (±0.1), and 7.4 (±0.1). The pH measurements are carried out with a pre-calibrated EUTECH pH 510 ion pH-meter.

2.2. UV–vis absorption spectra

The UV-VIS absorption spectra are obtained by scanning the solution on a Shimadzu UV-2450 absorption spectrophotometer against solvent reference in the wavelength range 200–650 nm. For the determination of association constant from UV-VIS absorbance data, the concentration of carmoisine is kept at 15 μM and that of proteins is varied up to 22.5 μM.

2.3. Fluorescence measurements

The steady state fluorescence spectra are recorded on a Jobin Yvon - Spex Fluorolog-3 spectrofluorimeter equipped with temperature controlled water cooled cuvette holder. Each sample is kept for 8–10 min before each measurement to ensure thermal equilibrium. Quartz cuvette having 1-cm path length is used. The steady-state fluorescence measurements have been carried out at three temperatures, namely, 288, 300 and 308 K. In order to monitor the intrinsic fluorescence of protein from Trp residues only, the samples are excited at 295 nm and the emission spectra are recorded from 310 to 470 nm. The excitation and emission slits are kept at 5 nm and 2 nm, respectively, in steady state fluorescence measurements. The concentration of HSA and BSA has been kept at 2 μM and 3 μM, respectively. Concentrations of carmoisine are 0, 0.2, 0.42, 0.64, 0.84, 1.06, 1.27, 1.7, 2.11, 2.7, 3.18 and 4 μM with HSA, and 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.4, 3, 3.75, 4.5, 5.25, 6, and 7.5 μM with BSA.

Fluorescence lifetimes are measured by means of a single photon counting apparatus (Horiba, Jobin Yvon, IBH Ltd., Glasgow, Scotland) equipped with a LED excitation source (λ_{exc} = 295 nm) with a peak emission at 349 nm and the signals are collected at the magic angle 54.7° using a Hamamatsu microchannel plate photomultiplier tube (R3809U). Data have been analyzed as sum of the exponential components with pre-exponential factors (α_i) normalized to unity, using iterative reconvolution with IBH DAS-6 software. The perfectness of fit is judged in terms of a χ^2 value and weighted residuals.

The synchronous fluorescence spectra (SFS) are obtained by simultaneously scanning the excitation and emission monochromators. Both the excitation and emission slits are kept at 5 nm for this measurement. When the $\Delta\lambda$ value between excitation and emission wavelength is set at some value, the synchronous fluorescence spectra can provide characteristic information of the local environment near some specific chromophore. Any alteration in SFS will correspond to binding and consequent change of micro-environment of the concerned chromophore near the binding site. We have reproduced our results by repeating the SFS experiments for five times.

2.4. Circular dichroism spectra

Circular dichroism (CD) spectra are recorded on a Jasco-810 automatic recording spectropolarimeter at 300 K under constant