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The Binding of Food Dyes with Human Serum Albumin

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The binding interactions between human serum albumin (HSA) and the edible food dyes amaranth, tartrazine and sunset yellow have been studied. Intrinsic association constants and the free energy changes associated with dye-protein binding at physiological pH for amaranth and tartrazine, and at two different pH values for sunset yellow have been calculated from ultrafiltration data. The temperature dependence (20-40°C) of the intrinsic association constants at pH 7.4 for amaranth-HSA and tartrazine-HSA mixtures have been measured, from which a plot of the van't Hoff isochore exhibits a marked change in slope around 30°C indicating a possible change in protein conformation. The number of dye binding sites on HSA is reported for all the above conditions. HSA-ligand binding enthalpies have been used in conjunction with the N-B transitional binding enthalpy for HSA, to calculate the enthalpy for the N-B transition when ligands are bound with the protein.

Key words : Human serum albumin, Amaranth, Tartrazine, Sunset yellow, Food dyes, Binding constants, Conformation

INTRODUCTION

There is an extensive literature on the toxicity of artificial dyes in food (Gangolli, 1983). A high dietary intake of food dyes has been implicated in the hyperactivity of children (Feingold, 1975). The mechanism of the toxicity of azo dyes has been reviewed by Levine (1991) and while it is clear that most ingested material is metabolised anaerobically by the gut microflora, accumulation in blood plasma is a possibility (Rawson, 1943). Animal experiments have shown mutagenic activity following oral comsumption of tartrazine (Henschler and Wild, 1985) and the induction of sarcomas from subcutaneous injection (Gangolli *et al*, 1967 and 1972). In order to elucidate further the mechanism of azo dye toxicity a series of plasma protein:dye binding studies were undertaken.

Human serum albumin (HSA) is a multi-functional plasma protein. It binds organic molecules such as carboxylic acids (Chen, 1967; Peters *et al.*, 1973), azo-dyes (Klotz *et al.*, 1952) and metal cations (Janssen *et al.*, 1981).

The relative strengths of these binding processes is important when considering the use of azo-food dyes, since competition between metabolites and dye molecules can interfere with the function of HSA as a transport protein. Certain drugs bound by HSA may also be released if the protein preferentially binds food dyes.

HSA undergoes a conformational change at approximately physiological pH (Klotz *et al.*, 1952; Janssen *et al.*, 1981; Labro and Janssen, 1986; Wilting *et al.*, 1980). This is known as the N-B transition where below pH 7.4 HSA is in the N-state but above it is in the B-state as described by a simple two-state model (Janssen *et al.*, 1981). It has been shown by several authors that the N-B transition can be followed spectroscopically using bound molecules as markers (Wilting *et al.*, 1979; Jacobsen and Færch, 1980; Watanabe and Saito, 1992; Pu *et al.*, 1993), and that the transition may be effected by alterations in temperature, or by the addition of Ca^{2+} in addition to pH changes within the range 6 to 8 (Jacobsen and Færch, 1980).

The reversible temperature induced transition occurs within the range 9-38°C (Jacobsen and Færch, 1980), and since it is the tertiary, but not the secondary protein structure which is affected by temperature, pH etc. (Wallevik, 1973), the binding constants of many small molecules are often sensitive to the N-B conformational transition (Watanabe and Saito, 1992).

This is especially evident for the binding enthalpies and entropies, although since these two quantities can compensate for one another there is often little or no net change in the overall ΔG° (Janssen *et al*, 1990). In this study, amaranth-HSA and tartrazine-HSA sys-

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tems were examined over a range of temperatures in order to derive ΔH° from the integrated van't Hoff isochore. Curves were obtained from these plots, which indicate that the conformational integrity of the protein in solution is not constant over the temperature range studied, *i.e.* there is temperature dependent conformational change in the protein in the presence of these azo-dyes.

Optical methods for the measurement of HSA-bound dye molecules often rely upon either a blue or red shift in λ_{max} of the dye spectrum (Klotz *et al.*, 1946, 1952). Such changes are often not large enough to be of analytical use, and separation techniques such as ultrafiltration of dye-protein mixtures (Crawford *et al.*, 1972) may be required to determine equilibrium concentrations of free and bound dye. Estimation of the numbers of species in solution and the calculation of thermodynamic parameters by spectroscopic means (Elbourne, 1981; Klotz, 1967; Lang and Lasser, 1967; Klotz and Walker, 1948) often requires high concentrations of HSA in order to bind measurable quantities of dye (conditions under which the aggregation of HSA cannot be ruled out as a major source of error). Such difficulties can be overcome by use of ultrafiltration methods.

We report here the thermodynamic parameters K_{eq} and ΔG° for the association of the food dyes amaranth, sunset yellow and tartrazine with HSA using ultrafiltration to separate and determine free and bound dye. All data were fitted to the model developed by Klotz and Scatchard and described by Klotz and Walker (1948), which assumes all the binding sites are of the same class. The double reciprocal plot is a rearrangement of the Klotz plot (Chang, 1981), and takes the form:

$$\frac{[P]_T}{[SD]} = \frac{1}{K_{eq}[D_f]n} + \frac{1}{n} \qquad (1)$$

where $[P]_T$ is the total protein concentration, $[SD]$ is the concentration of dye bound to a protein site, $[D_f]$ is the concentration of free dye, K_{eq} is the intrinsic association constant for the binding process, and n is the number of binding sites on each protein molecule. The fraction of sites bound with ligands is $[SD]/[P]_T$, commonly abbreviated to r, thus a plot of $1/r$ against $1/[D_f]$ is linear. Some results are better represented using the Scatchard plot (Chang, 1981) written as:

$$\frac{r}{[D_f]} = nK_{eq} - rK_{eq} \qquad (2)$$

MATERIALS AND METHODS

Materials

The food dyes used in the study were kindly donated by Williams (Hounslow) Ltd., and were recrystallised from ethanol and desiccated under reduced pressure. The human serum albumin was used as supplied (globulin and fatty acid free) from Sigma Chemical Co., Dorset. All water used in this project was purified through Elgastat Spectrum cartridges. The dye and protein solutions were prepared in $KH_2PO_4Na_2HPO_4$ buffer solutions. The concentration of HSA protein in the presence of dye was maintained at a constant value of 1×10^{-5} mol dm^{-3} in all experiments, whereas the concentration of dye in the presence of protein was varied from a protein : dye ratio of 1 : 1 to 1 : 10.

Ultrafiltration

Ultrafiltration was performed through a Amicon model 12 cell (10 cm^3 capacity), fitted with a YM10 membrane and filter (MW cut-off, 10 kDa). Between runs, the Amicon filter and membrane were cleaned in a pepsin solution to remove adsorbed protein prior to storage in a 10% ethanol/water solution at 0 °C. The ultrafiltration cell was filled to a total volume of 10 cm^3 with mixtures of the dye and protein solutions; filtration was achieved by pressurizing the cell to 10 psi above atmospheric pressure with oxygen-free nitrogen (ex BOC). Experiments were temperature controlled by immersing the pressurized apparatus in a small thermostatted water bath. The absorbance at λ_{max} of the dye studied was measured after the first 3 cm^3 of filtrate had been collected. In other experiments, pure solutions of each dye were passed through the apparatus. The absorbance of each solution from inside and outside the cell were periodically measured and found to be identical, indicating no measurable binding of the dyes to the membrane or rejection by the filter.

Spectroscopy

Ultra-violet and visible spectra were measured using a Perkin Elmer 555 spectrophotometer (double beam, slit width 1.00 nm) fitted with a Digital Controller thermostat. All measurements were made with the sample and reference compartments thermostatted to 20.0°C. Difference spectra (data not shown) of sunset yellow-HSA mixtures (pH 6.24, 20°C) over the wavelength range 600 to 300 nm were also measured and processed in conjunction with the ultrafiltration data. These results were used to check how well the observed spectra of the dye-HSA mixtures could be reproduced. The calculated curves closely matched the experimentally determined spectra as far as the shape of the peaks were concerned, but there was an uncertainty of about ±3% in actual absorbance values. This in-

dicates the maximum uncertainty in the concentration of the bound species (The calculations from the difference spectra also assume only one form of dye-HSA complex, which is the model used for the calculation of K_{eq}).

RESULTS

HSA-dye binding at 20°C

Ultrafiltration of all dye-HSA mixtures, containing HSA at a constant concentration of 1×10^{-5} mol dm^{-3}, was performed at a constant temperature of 20°C. The ultrafiltration cell was filled with the dye and protein mixture to be studied in buffered media and pressurized to 10 psi with nitrogen. Absorbances of the first 3 cm^3 of filtrate collected were measured at the λ_{max} of the dye. The equilibrium constants K_{eq}, and the nũmber of binding sites on HSA, calculated from double reciprocal plots (see Fig. 1) for all the dyes studied at 20°C are shown in Table I, along with the corresponding values for ΔG°_{20} (from the relationship $\Delta G^{\circ}=-RT\ln K_{eq}$). Results from Scatchard plots (see Fig. 2) of the same data are shown in Table II.

Comparable K_{eq} values were determined for amaranth and sunset yellow with HSA (pH 7.4, 20°C) which in turn were much larger than for tartrazine. For the sunset yellow-HSA system, a reduction of around 1 pH unit leads to a twofold increase in the equilibrium constant, indicating that a stronger binding process is seen between the N-conformation of HSA and sunset yellow. The number of binding sites occupied by sunset yellow is approximately 5 (invariant with pH), whereas for tartrazine and amaranth there are only 3.

Temperature dependence of HSA-dye binding

The temperature dependence of the binding constants was determined for the amaranth-HSA and the tartrazine-HSA systems. Double reciprocal plots provided the best data from which integrated van't Hoff isochores were constructed: these exhibited a marked change in slope at around 30°C (see Fig. 3, Tables III and IV), indicating a possible change in the con-

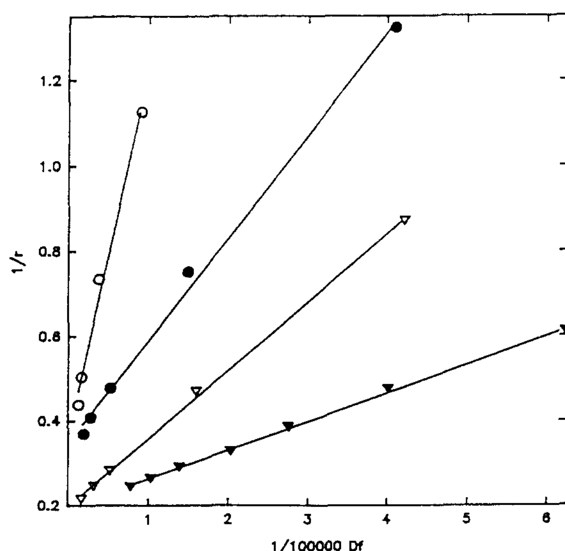

Fig. 1. Double reciprocal plots for azo dyes binding with human serum albumin at 20°C. ○: tartrazine, ●: amaranth, ▽: sunset yellow (pH 7.4), ▼: sunset yellow (pH 6.24)

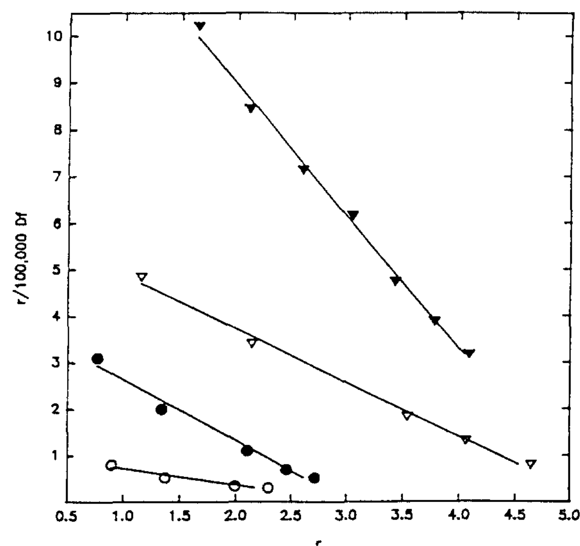

Fig. 2. Scatchard plots for azo dyes binding with human serum albumin at pH 7.4 and 20°C. ○: tartrazine, ●: amaranth, ▽: sunset yellow, ▼: sunset yellow (pH 6.24)

Table I. Thermodynamic parameters from reciprocal plots of ultrafiltration data (20°C)-n is the number of binding sites

Dye	pH	$K_{eq}/10^4$	S.D./10^4	n	S.D.	ΔG°_{20}/kj mol^{-1}
Amaranth	7.4	13.20	1.14	3.06	0.213	28.7
Tartrazine	7.4	4.11	0.50	2.80	0.262	25.9
Sunset yellow	7.4	12.30	0.47	5.06	0.167	28.5
Sunset yellow	6.24	29.20	0.78	5.09	0.100	30.7

The standard deviation (S.D.) values quoted above were calculated from five experimental data points in each case, except for tartrazine where four data points were used.

Table II. Thermodynamic parameters from Scatchard plots of ultrafiltration data (20°C)-n is the number of binding sites

Dye	pH	$K_{eq}/10^4$	S.D./10^4	n	S.D.	ΔG°_{20} mol^{-1}
Amaranth	7.4	10.70	1.69	3.34	0.64	28.2
Tartrazine	7.4	3.58	0.63	2.99	0.60	25.5
Sunset yellow	7.4	11.30	0.72	5.38	0.40	28.4
Sunset yellow	6.24	28.40	0.90	5.16	0.19	30.6

The standard deviation (S.D.) values quoted above were calculated from five experimental data points in each case, except for tartrazine where four data points were used.

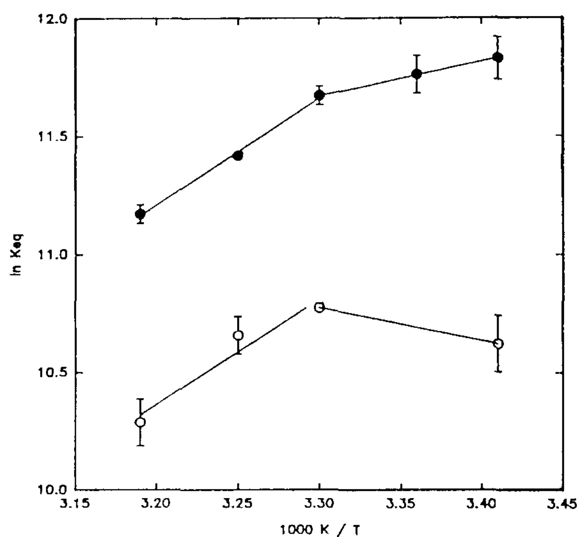

Fig. 3. Integrated van't Hoff isochores for amaranth (●) and tartrazine (○) indicating a possible temperature dependent conformational change at approximately 30°C

Table III. Temperature dependence of thermodynamic parameters obtained from reciprocal plots of ultrafiltration data (pH 7.4), including the number of binding sites (n)

Dye	T/°C	$K_{eq}/10^4$	S.D./10^4	n	S.D.	$-\Delta G^{\circ}$/kJ mol^{-1}
Amaranth	20	13.20	1.14	3.06	0.213	28.7
Amaranth	25	12.80	1.07	2.90	0.194	29.1
Amaranth	30	11.70	0.52	2.95	0.106	29.4
Amaranth	35	9.08	0.19	3.13	0.054	29.2
Amaranth	40	7.09	0.29	3.17	0.114	29.1
Tartrazine	20	4.11	0.50	2.80	0.262	25.9
Tartrazine	30	4.78	0.08	2.20	0.025	27.1
Tartrazine	35	4.25	0.32	2.14	0.115	27.7
Tartrazine	40	2.93	0.28	2.89	0.260	26.8

formation of HSA at this temperature (the thermal stability of these dyes was found to be constant over the temperature range studied). The slope of each line is proportional to the enthalpy changes in the binding process, thus for the two dyes studied the ΔH° values range from *ca.* -40 (30-40°C) to -9 kJ mol^{-1} (20-30°C) and from *ca.* -39 to +3 kJ mol^{-1} (rounding) for amaranth and tartrazine respectively. Since the integrated van't Hoff isochore requires the nature of all species in solution to be identical over a given temperature range, a marked change in protein conformation that affected the binding sites would be indicated by a distinct change in slope at $1/T_c$ (where T_c is the temperature at which the conformational change occurs). This observation in two different systems where no correlation exists in the dye molecular structures supports the proposition that only major changes in the protein structure occurred. It is also interesting to observe that the number of binding sites (3 for amaranth and tartrazine) does not vary over this temperature range.

Table IV. Temperature dependence of thermodynamic parameters obtained from Scatchard plots of ultrafiltration data (pH 7.40), including the number of binding sites (n)

Dye	T/°C	$K_{eq}/10^4$	S.D./10^4	n	S.D.	ΔG°/kJ mol^{-1}
Amaranth	20	10.70	1.69	3.34	0.643	28.2
Amaranth	25	10.90	1.20	3.09	0.413	28.7
Amaranth	30	10.90	0.85	3.03	0.288	29.2
Amaranth	35	8.78	0.35	3.18	0.152	29.1
Amaranth	40	7.18	0.37	3.16	0.195	29.1
Tartrazine	20	3.58	0.63	2.99	0.603	25.5
Tartrazine	30	4.76	0.14	2.21	0.075	27.1
Tartrazine	35	3.95	0.42	2.21	0.272	27.1
Tartrazine	40	2.98	0.27	2.86	0.287	26.8

DISCUSSION

The binding of the food dyes amaranth and tartrazine by HSA shows a distinct temperature dependence. The greatest binding affinity is seen at temperatures between 20 and 30°C; at higher temperatures the affinity rapidly decreases. These results are effectively summarized in the integrated van't Hoff isochore, which shows a marked change in slope around 30°C indicating a possible change in HSA conformation when bound with azo-dyes. The same overall change in slope is seen for two different dye-HSA systems, indicating that protein conformational changes are the cause. Thus a major temperature dependent conformational change may alter the nature of the dye binding sites, which in turn display different binding enthalpies. These results are consistent with the view that the midpoint of the N-B transition is shifted towards a more neutral pH by a rise in temperature (Wallevik, 1973), and that the binding enthalpies of many ligands are more negative when HSA is in the B-form and exhibit larger changes than the corresponding ΔG° (Janssen *et al.*, 1990).

The ΔG°s for the binding processes at 20°C and pH 7.4 for the three food dyes range from -25.9±0.3 to -28.7±0.2 kJ mol^{-1}. When the temperature is raised, ΔG° becomes slightly more negative for both amaranth and tartrazine, peaks at around 30°C and then drops slightly at 40°C. In both cases it is quite apparent that ΔG° is virtually independent of temperature, indicating enthalpy-entropy compensation (Janssen *et al.*, 1990). The Gibbs free energy change for the HSA-sunset yellow system becomes more negative as the pH is lowered from pH 7.4 to 6.24, indicating that the binding of anionic food dyes to HSA may depend to a large extent on the overall surface charge of the protein. The number of binding sites on HSA varies from three (for amaranth and tartrazine at all temperatures studied) to five (for sunset yellow at pH 6.24 and pH 7.40).

A standard enthalpy of 55±8 kJ mol^{-1} for the N-B

transition has been estimated, using bilirubin as a bound marker (Jacobsen and Færch, 1980). Since bilirubin is considered not to interfere with the N-B transition, the following energy cycle can be constructed (Janssen *et al.*, 1990):

Thus given the ΔH° (N→B) of 55 kJ mol^{-1}, and using standard enthalpy data for the binding of benzodiazepines to HSA (in N and B conformations) provided by Janssen and Dröge (1990), ΔH° (ND→BD) for each drug can be estimated. Enthalpies of about 29 to 49 kJ mol^{-1} were obtained for six of the nine related compounds; a relatively low value of 7 kJ mol^{-1} was obtained for Temazepam, whereas negative values of -38 and -16 kJ mol^{-1} were obtained for the only two compounds bearing nitro- substituents: Nitrazepam and Clonazepam respectively. A linear relationship between the association enthalpies for N+D and B+D would exist, over a given temperature range, if ΔH° (N→B) and ΔH° (ND→BD) are proportional. These results may explain why the drug binding enthalpies to HSA in the N and B states for Nitrazepam and Clonazepam were not linearly related (Janssen *et al.*, 1990). The N to B transition for HSA bound to these two compounds is enthalpically favourable.

Estimates of ΔH° (ND→BD) for the dyes amaranth and tartrazine were 6 and 19 kJ mol^{-1} respectively.

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