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ORIGINAL ARTICLE

Non-covalent binding of azo compound to peptide chain: interactions of biebrich scarlet and naphthochrome green with four model proteins

Hong-Wen Gao · Xiang-Hu Liu · Zhi Qiu · Lu Tan

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Abstract We studied the non-specific interactions of two azo compounds: biebrich scarlet (BS) and naphthochrome green (NG), with four model proteins: bovine serum albumin, ovalbumin, poly-L-lysine and hemoglobin by UV-VIS spectrometry, fluorophotometry and circular dichroism melting technique. The optimal acidities of NG and BS for binding to proteins correspond to the physiological pHs of skin and gastro tissues. The saturation binding numbers of BS and NG on peptide chains were determined and the effects of electrolytes and temperature were investigated. These interactions were fitted by the Temkin absorption model and their thermodynamic parameters were calculated. The different bindings of BS and NG to proteins were compared from their molecular structures. We inferred that an ion-pair electrostatic interaction first fixes azo compounds to basic amino acid residues and subsequent binding involves the collective action of other non-covalent bonds: hydrogen bond, van der Waals force, and hydrophobic interaction. This combination of bonds caused a change of secondary conformation of protein from β-sheet to helix and the possible process was illustrated. The potential protein toxicity resulting from such a non-specific binding was analyzed. Besides, the interaction of BS with peptide chains was applied to protein assay.

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Introduction

Many natural and man-made organic substances exist in nature. Some, especially manufactured ones such as environmental pollutants and drugs, have serious toxic effects on human health due to their simultaneous existence with many biomacromolecules in the same medium and numerous interactions among these various molecules. Studies on such interactions may improve understanding of the toxicities of organic substances and help to elucidate the functions of proteins and DNA (Pula et al. 2005; Pelaprat 2006; Binkowski et al. 2005). Binding by any chemical substance is likely to affect the hydrolytic activity, either enhancing it (Marolia and D'Souza 1999) with potential medical significance, or inhibiting it (Pitzurra et al. 1989) if an organic contaminant or toxin is involved. The side groups of basic amino acid residues (AARs) e.g., Arg, Lys and His will form cations in acidic solution, binding organic anions by ion-pair attraction (Lee et al. 2005). Polar residues will bind other polar organic groups by hydrogen bonds; non-polar ones will interact hydrophobically with other non-polar groups, e.g., π–π electron coupling (Gao et al. 2006; Fazekas et al. 1963). Non-covalent interactions comprise ion-pair attraction, hydrogen bond, hydrophobic interaction, van der Waals force, dipole effect, etc. The non-covalent binding involved is often weak and non-specific, but a large number of non-covalent bonds may collectively alter the conformation and function of a protein (Piekarska et al. 1996). For example,

in CYP4501A, some PAHs will be oxidized to form an electrophilic epoxide and this binds to protein or DNA, potentially resulting in neoplastic transformation (Hasler 1999). Because the non-covalent interaction involved is weak, such complexes are often affected by urea, dioxane or electrolytes or by heating (Gao and Zhao 2003). The non-specific binding of a charged chromophore to protein or DNA can be determined easily (Cui et al. 2004).

In the present work, to clarify the general principles involved in the interactions of proteins with ionic azo substances, we attempted to characterize examples of such complexes and their effects on protein conformation. Using molecular spectrometry, we investigated in detail the non-covalent interactions of both anionic azo compounds BS and NG with four model proteins: BSA, OVA, PLYS and HG. BS is used for dyeing clothes and sometimes as an additive to beauty skin agents. NG is a kind of bright peach red compound, which is used as a food additive. It may enter the human body by skin absorption and food intake. It is possible for such exogenous azo compounds to cause a potential toxic action on human health. The objective of the present work is to understand the effect of non-specific binding of anionic azo compounds, BS and NG, on the structure of proteins using UV-VIS, fluorophotometry and circular dichroism (CD) melting technique. The bindings of BS and NG to peptide chains were compared by analyzing their different structures. In addition, the non-specific interaction of BS with peptide chains was applied to a protein assay.

Materials and methods

Materials

The absorption spectra of NG, BS and their protein solutions were recorded with a Model Lambda-25 spectrometer (Perkin-Elmer, Shelton, CT 06484, USA). The spectrometer was computer controlled using UV WinLab software (Version 2.85.04). A Model F-4500 fluorospectrophotometer (Hitachi High-Technologies Cooperation, Tokyo, Japan) was used to measure the fluorescence of protein solution in the presence of BS and NG. A model J-715 CD spectropolarimeter (JASCO (UK) Ltd, UK) was used to measure the secondary conformation of proteins. A weight of 0.50 g of BSA (Q/AKF 78-05-95, Shanghai Li Zhu Dong Feng Biotechnology Co. Ltd, Shanghai, China) and OVA (SigmaA5253, Beijing Biodev-Tech Scientific & Technical Co. Ltd, Beijing, China) were dissolved in 100 ml of deionized water and then diluted to 1,000 ml. The precise concentrations of proteins were determined by the UV method. A weight of 87.0 mg PLYS (26124-78-7, Sigma-Aldrich Co., USA) was dissolved in 100 ml of deionized water. The solution contained 0.870 mg/ml PLYS. HG, 0.0500 g (Q/AKF 78-06-94, Shanghai Li Zhu Dong Feng Biotechnology Co. Ltd, Shanghai, China) was dissolved in 100 ml of deionized water. This solution contained 0.500 mg/ml HG. These solutions were stored in a refrigerator freezer at less than 4°C. Both NG and BS are strong light identification reagents with different chemical structures. Two solutions of 0.500 mM NG (Koch-light Laboratories Ltd, Coinbrook Bucks, England) and 0.500 mM BS (Shanghai Chemical Reagents Company, Shanghai, China) were prepared in deionized water. They formed anions in aqueous solution so as to complex proteins. A series of Britton-Robinson (B-R) buffer solutions, pH 1.81, 2.09, 2.87, 3.76, 4.76 and 5.72 were prepared to adjust the acidity of the solution. The masking reagent, 0.1 mol/l EDTA was prepared and added into a sample to mask the metals possibly existing in the samples.

Photometric characterization of interactions of NG and BS with peptide chains

All studies were carried out in 10.0 ml calibrated flasks containing a known volume of BSA or OVA or PLYS or HG solution, 1.0 ml of the B-R buffer (pH 2.09) and a known volume of NG solution. The solution was diluted to 10.0 ml with deionized water and mixed thoroughly. The temperature was controlled with an electrically heated thermostat in a water-cycle bath. After 5 min, the absorbances $A^0{}_{\lambda 2}$ and $A^0{}_{\lambda 1}$ of the reagent blank and $A_{\lambda 2}$ and $A_{\lambda 1}$ of the sample were measured at 545 (λ_2) and 601 nm (λ_1) against water. Using the same method, BS-protein solutions were prepared at pH 3.76 and measured at 500 and 564 nm.

CD measurement of protein solutions

The pH 2.09 buffer (1 ml) and a known volume of BSA or HG solution were added to 0, 0.50, 1.00 or 2.00 ml of 0.500 mM NG in four flasks. The solutions were diluted to 5.0 ml with deionized water. By the same method, 0, 0.500 and 3.00 ml of 0.500 mM BS were added in place of the NG to three flasks containing 1 ml of pH 3.76 B-R buffer and 0.85 ml of 0.51 mg/ml BSA. All mixtures were injected into a 0.1 cm cell and the absorbances were measured between 190 and 250 nm. Simultaneously, reagent blanks without NG and BS were measured. In addition, the NG-BSA/HG and BS-BSA/HG solutions were measured in neutral medium so as to compare the secondary conformational changes of the proteins in various pH media.

Fluorimetric measurement of protein solutions

B-R buffer (1.0 ml) and 0.85 ml of 0.51 mg/l BSA were mixed with 0–2.50 ml of 0.500 mM NG or 0–2.00 ml of

0.500 mM BS. The solutions were diluted to 5.0 ml with deionized water and the absorbances were measured at the excitation wavelength (280 nm) and the emission wavelengths (300–450 nm).

Protein assay

A known volume of a sample solution, 0.5 ml of 0.1 mol/l EDTA, 1 ml of pH 3.76 acetate buffer and 50 µl of 0.500 mmol/l BS were added in a 10-ml flask. The solution was diluted to 10.0 ml with deionized water. After 5 min, the absorbances ($A_{564\text{nm}}$ and $A_{500\text{nm}}$) were measured at 564 and 500 nm against water. With the same method, a reagent blank without any protein was prepared and the absorbances ($A^0_{564\text{nm}}$ and $A^0_{500\text{nm}}$) were measured. Thus,

$$\Delta A_{\text{r}} = \frac{A_{564\text{nm}}}{A_{500\text{nm}}} - \frac{A^0_{564\text{nm}}}{A^0_{500\text{nm}}}. \quad (1)$$

Thus, the combination of ΔA_r calculated from Eq. 1 and the corresponding standard curve was used to work out the protein content in a sample.

Results and discussion

pH dependence and spectral analysis

BSA was used as the representative protein. The interaction of BSA (M) with NG (L) is modeled as follows:

	NNG	+	BSA	$\overset{k_b}{\rightleftharpoons}$	BSA(NG)$_N$
Initiation	$C_{\text{L0}}(A^0_{\lambda 2})$		C_{M0}		0
Equilibrium	$C_{\text{L}}(A_{\lambda 1}, A_{\lambda 2})$		$C_{\text{M}\to 0}$		$C_{\text{M0}}(A_C)$

where C_{M0} and C_{L0} are, respectively, the initial concentrations of BSA and of NG or BS and C_{L} the equilibrium one of L. N is the saturation binding number of L and K_{b} the binding constant. A_c indicates the real absorbance of the protein-L complex at wavelength λ_2, which cannot be measured directly. The symbols $A_{\lambda 2}$ and $A_{\lambda 1}$ are the absorbances of the protein- L solution, respectively, measured at λ_2 and λ_1. From the absorption spectra (Fig. 1a–b) of BSA–NG and BSA–BS solutions, the interval between positive peak and negative trough increased with increased acidity of the solution. Therefore, the protein toxicity of an anionic ligand in a more acidic medium could increase due to its more obvious binding. From the dissociation constant (K_R) of side groups (R) of basic and acidic AARs: 10.53 for Lys, 6.00 for His, 12.48 for Arg, 3.65 for Asp and 4.25 for Glu, all R groups will not be with negative charge when pH is less than 3.65, while Rs of basic AARs is with positive charges. NG and BS anions will enter into BSA without any charge repulsive interaction. However, the dissociation

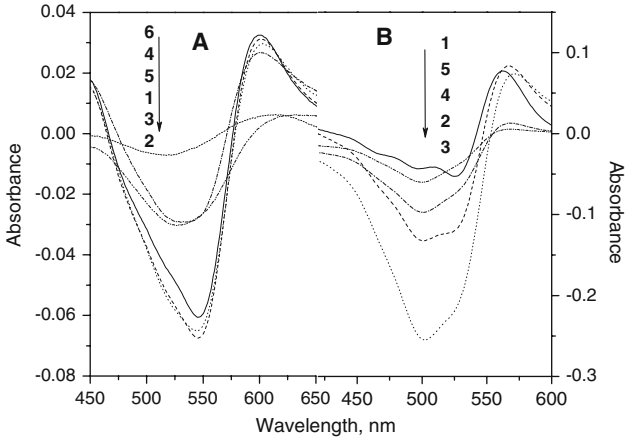

Fig. 1 The absorption spectra of the BSA-ligand solutions in various pH media. **a** It contains 0.010 mM NG and17.8 mg/l BSA, from curve 1 to 5 at pH 1.81, 2.09, 2.87, 3.76, 4.76 and 5.72. **b** The solutions contained 0.009 mM BS and 17.8 mg/l BSA from curve 1 to 6: at 1.81, 2.09, 2.87, 3.76, 4.76 and 5.72. All the absorption spectra were measured against the reagent blank

constants (K_a) of HG and BS are to be pK_a between 2 and 3, so that the binding of ligand to BSA weakened at a pH less than 2 from curve 1. Rs of acidic AARs dissociate into anions when pH is more than 4.25 and impede the entry of the positive ligand into BSA. Thus, from curves 3–5, the bindings of BS and NG to BSA become weaker and weaker. Thus, there is an optimal pH for obtaining maximal binding number of the organic substance to the peptide chain; pH 2.09 optimizes the formation of complexes between BSA and NG. The wavelengths 601 and 545 nm were used in subsequent work. For the BSA–BS complex, pH 3.76 is optimal for binding and the wavelengths 500 and 564 nm were selected.

Actually, in various tissues of the human body, pH difference is very great e.g., pH 1–3 in the gastro, pH 3–4 in the vagina, pH 4–5 in the duodenum, an approximate pH of 5 on the skin and pH 7.4 in the blood. From Fig. 1a, the most sensitive binding of NG is located in the pH scope of normal gastric juice. Thus, the effect of NG on the activity of protein or enzyme in the gastro tissue should be most serious when NG is used as a food additive. From curve 5 in Fig. 1b where pH approaches that of normal skin, the appearance of both peak and valley indicates that BS could make an effect on the structure and activity of proteins in the skin tissue. However, the use of beauty skin agents, especially containing fruit acid and salicylic acid, favorable for sterilization and inflammation will cause an increase in skin acidity. Besides, it is possible for air pollution e.g., acidic rain to make the skin more acidic. Thus, the pH of the skin often approaches 4 in many cases and it is just close to the above optimal pH of BS for binding to peptide chains. BS could cause more health risk when it is used in the dyeing of clothes or as an additive to beauty skin

agents. As a result, pH 2.09 and 3.76 were selected as the experimental media of the NG–peptide chain and BS–peptide chain interactions, respectively.

Photometric characterization of complex formation

The spectral correction technique (Gao et al. 2006) is a very useful method for the composition determination of a color reaction mixed together with the excess ligand. From the above reaction, the effective fraction (η) of NG or BS bound to the peptide chain and its binding ratio (γ) to the peptide chain are calculated by the relations:

$$\eta = \frac{A_c - A_{\lambda 2}}{A^0_{\lambda 2}} + 1 \tag{2}$$

and

$$\gamma = \eta \times \frac{C_{\mathrm{L0}}}{C_{\mathrm{M0}}} \tag{3}$$

where

$$A_c = \frac{A_{\lambda 2} - \beta A_{\lambda 1}}{1 - \alpha\beta}. \tag{4}$$

Both α and β are the correction constants. In order to obtain α, the absorbance ratios of the BSA–NG and BSA–BS solutions were measured at 545 and 601 nm and at 500 and 564 nm, respectively. From the variations of these ratios with BSA concentration (Fig. 2), it is seen that the $A_{545\mathrm{nm}}/A_{601\mathrm{nm}}$ ratio of NG–BSA solutions decreases with increased BSA and approaches a constant when BSA is over 2.00 μM. This means that more and more NG molecules bind to the BSA until free NG is no longer in excess. These variations in the ratios are fitted by an exponential decay, and the constant minimum at 2.47 is α as shown in Fig. 2 for the NG–protein complex when BSA concentration is high. Using the same method, α for the BS–protein complex is 1.49 from curve 2; β for NG at pH 2.09 and for BS at pH 3.76 should correspond to the $A_{601\mathrm{nm}}/A_{545\mathrm{nm}}$ and $A_{564\mathrm{nm}}/A_{500\mathrm{nm}}$ ratios in the absence of protein.

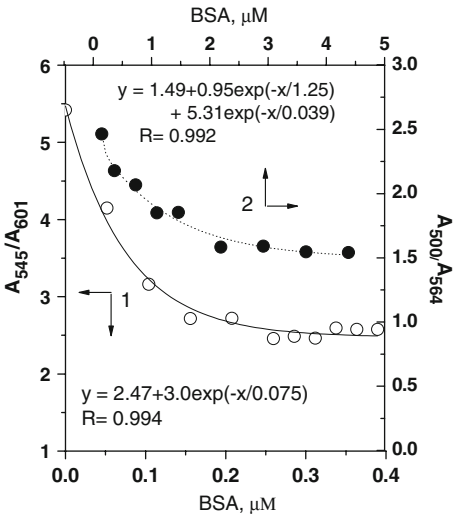

Fig. 2 Variation of the absorbance ratio ($A_{544\mathrm{nm}}/A_{601\mathrm{nm}}$) of the BSA–NG (0.0225 mM) solutions at pH 2.09 (*1*) and that of $A_{500\mathrm{nm}}/A_{564\mathrm{nm}}$ of the BSA–BS (0.025 mM) solutions at pH 3.76 (*2*)

Figure 3 indicates the variation of γ^{-1} for NG and BS in various protein solutions. With increased NG or BS concentration, γ always increases. More and more azo compounds are bound to the peptide chain. These curves were fitted by an exponential decay. We found N values of NG and BS in various proteins from the constant terms of the regressing equations (Fig. 3). N for NG in OVA, PLYS and NG is more than that for BS. The possible reason is that the ball-type NG molecules have a less steric hindrance. The Temkin isotherm equation (Jeyaprabha et al. 2005) was applied to the aggregations of BS and NG in the peptide chains.

$$\frac{\gamma}{N} = -\frac{RT}{\Delta Q}\ln(K_{\mathrm{b}}C_{\mathrm{L}}) \tag{5}$$

where

$$C_{\mathrm{L}} = (1 - \eta)C_{\mathrm{L0}} \tag{6}$$

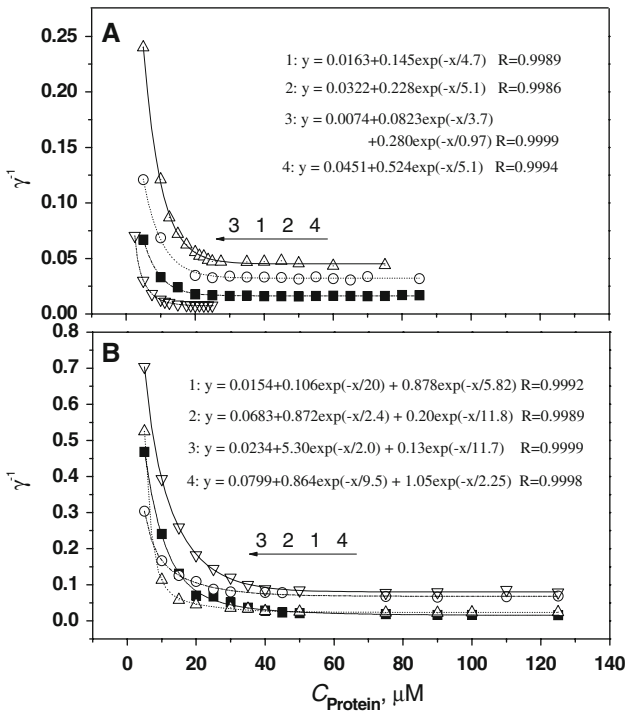

Fig. 3 Variation of γ^{-1} of the ligand–protein solutions. **a** NG solutions containing 0.0092 mg/ml BSA (curve *1*), 0.0167 mg/ml OVA (*2*), 0.087 mg/ml PLYS (*3*) and 0.01078 mg/ml HG (*4*) at pH 2.09. **b** BS solutions containing 0.0554 mg/ml BSA (curve *1*), 0.0556 mg/ml OVA (*2*), 0.0087 mg/ml PLYS (*3*) and 0.0431 mg/ml HG (*4*)

$\Delta Q = Q^s - Q^0$, where Q^s and Q^0 are respectively the adsorption free enthalpies at saturation ($\gamma = N$) and the initial state ($\gamma = 0$), R is the gas constant (8.314 J mol^{-1} K^{-1}) and T the temperature (293.15 K). C_L for each solution is calculated from Eq. 6. By regression of plots of γ/N versus $\ln(C_L)$, all γ/N versus $\ln(C_L)$ plots are shown in Fig. 4. They all show good linear relationships, indicating that the binding of NG and BS to peptide chains obeys the Temkin adsorption model. The bindings of NG and BS to peptide chains correspond to the heterogeneous chemical adsorption in the monolayer. Both K_b and ΔQ are calculated as shown in Table 1. The interactions of BS and NG with peptide chains are all non-covalent because the $-\Delta Q$ values are much less than 60 kcal/mol (Patrick 1995). Moreover, the interactions are spontaneous and exothermic in both cases. The K_b and $-\Delta Q$ values obtained with the BS solutions containing BSA, PLYS and HG are less than those with the NG solutions, so binding of BS to BSA, PLYS and HG is weaker than to NG. In contrast, K_b and $-\Delta Q$ for BS is more than that for NG in OVA solution. For example, the fraction of α-helix in HG is only 3.7% at pH 2.09 from Table 2, which is much less than 26.9% at pH 3.76. HG gives much less steric hindrance at pH 2.09 than at pH 3.76, so the small NG molecules can be inserted into the HG cavity without obstruction. Finally, NG binding results in a marked increase (11.6-fold) in the α-helix fraction from 3.7% in the absence of NG to 42.9% in 0.200 mM; but BS binding causes only a 1.4-fold increase from 26.9% in the absence of BS to 38.7% in 0.30 mM. Therefore, the NG–HG binding is more stable. The effective binding rates (φ) of BS and NG to peptide chains can be calculated by $\varphi = N/N_{BAAR}$, where N_{BAAR} is the number of basic AARs in the protein (Table 1). By comparison, good correlations between N for NG and BS, and N_{BAAR} for the proteins were found. As an example, N for NG to PLYS and HG approaches their N_{BAAR}. This shows that almost all the basic AARs were positively charged in PLYS and in HG and bound NG by ion-pair attraction. Thus, there is too little steric hindrance in the interactions of NG with PLYS and HG to make φ more than 90%. From the above analyses, it is seen that ion-pair attraction induces the anionic organic compound to be inserted into the cavities of the protein, i.e., electrostatic attraction has a position-fixing role in the interaction of small organic anions with peptide chains. After these molecules are positioned on the peptide chain, other non-covalent bonds

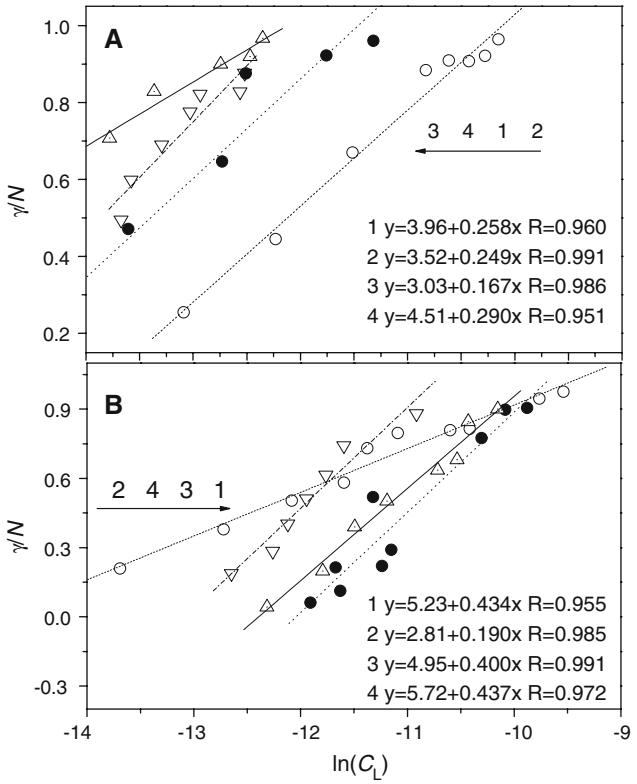

Fig. 4 Plots of γ/N versus $\ln(C_L)$ for the ligand–protein solutions. **a** NG solutions containing BSA (curve *1*), OVA (*2*), PLYS (*3*) and HG (*4*). **b** BS solutions containing BSA (curve *1*), OVA (*2*), PLYS (*3*) and HG (*4*)

Table 1 Determination of characteristic factors of the protein–BS and protein–NG complexes

Protein	N_{BAAR}	L	N	ΔQ (kJ/mol)	$K_b \times 10^5$	Complex	φ (%)
BSA	102	BS	65	−5.62	1.71	BSA·BS_{65}	64
		NG	61	−9.45	46.3	BSA·NG_{61}	60
OVA	42	BS	15	−12.8	26.5	OVA·BS_{15}	36
		NG	31	−9.79	13.8	OVA·NG_{31}	74
PLYS	145	BS	43	−6.09	2.37	PLYS·BS_{43}	30
		NG	135	−14.6	758	PLYS·NG_{135}	93
HG	24	BS	13	−5.58	4.84	HG·BS_{13}	54
		NG	22	−8.40	56.8	HG·NG_{22}	92

Table 2 Change of the secondary structure of proteins in BSA and HG solutions

Protein	Factor	NG (mM)				BS (mM)		
		0	0.025	0.05	0.1	0	0.025	0.15
BSA	α-helix (%)	21.1	31.1	38.6	33.9	39.2	51.6	43.7
	β-sheet (%)	47.5	37.8	24.1	0	30.1	0	0
	Turn (%)	0	2.6	10.5	32.1	4.1	20.6	29.3
	Random coil (%)	31.4	28.4	26.8	34	26.6	27.8	27
HG	α-helix (%)	3.7	2.6	21.3	42.9	26.9	38.3	38.7
	β-sheet (%)	50.9	52.2	34.7	0	34.2	14.2	0
	Turn (%)	3.6	3.8	7.6	26.6	7.4	15.7	31.2
	Random coil (%)	41.8	41.4	36.3	30.5	31.5	31.9	30.1

will form, such as hydrogen bond and hydrophobic interaction (e.g., π–π interaction), and will act in concert. In fact, the charge number of ligand often plays an allocation role. From Table 1, the φ values for NG are more than 60%. In contrast, the distance between the two SO_3^- groups in BS is great and they can bind two basic AARs. Thus, φ is less than 64%. Besides ion pair binding, there are other potential binding in NG, such as one –OH and three =O groups for forming hydrogen bonds with the peptide chain, and three hydrophobic groups for interacting hydrophobically with non-polar residues. BS contains one –OH and three $-SO_3^-$ groups and some naphthol and phenyl groups. Plane sketch of BS as a representative bound to the peptide chain is shown in Fig. 6a.

Variation of protein secondary conformation

The specific conformation of a protein with a special function results from covalent and non-covalent interactions among its amino acid residues. When an organic compound is added to a protein solution, the internal non-covalent interactions of the peptide chain will be disrupted, possibly changing the original conformation. CD spectrometry is often used to evaluate the secondary structure of a protein, such as the fractions of β-sheet, α-helix and β-turn. In the present work, the CD spectra of BSA and HG were measured (Fig. 5). The fractions of β-sheet, α-helix, β-turn and random forms were calculated from curves in Fig. 5 and given in Table 2. With the addition of BS or NG, the CD spectra of BSA and HG changed obviously, which also can be found in Table 2. From the data in Table 2, the secondary conformation of BSA hardly changes in the absence of BS and NG at pHs between 3.76 and about 7. However, the β-pleated sheet fraction of BSA increases from 39.2% at pH 3.76 to 47.5% at pH 2.09 in the absence of BS and NG, and the α-helix content decreases from 30.1 to 21.1%. The secondary structure of HG changes markedly from 45.1% α-helix at about pH 7 to 26.9% at pH 3.76 and down to only 3.7% at pH 2.09 in the absence of BS and NG, and from 25% β-sheet at about pH 7 to 34.2% at pH 3.76 and 50.9% at pH 2.09. Thus, acidity affects the structure of HG more markedly than that of BSA. Increased acidity protonates the basic AARs and disrupts the original hydrogen bonds within the peptide chain, especially in HG, which has a higher α-helix content. The addition of BS or NG causes no obvious secondary conformation change in either protein in neutral solution. This indicates that BS and NG do not interact with the peptide chain in a neutral medium because the basic AARs are not sufficiently protonated. The β-sheet fractions of BSA and HG decrease rapidly at pH 3.76 from 47.5 and 34.2% in the absence of BS to 0 and 14.2% in 0.050 mM BS. In contrast, the α-helix fractions increase from 39.2 and 26.9 to 51.6 and 38.3%, respectively. Moreover, the β-turn fractions increase from 4.1 and 7.4 to 29.3 and 31.2%, respectively. Similarly, the β-sheet contents of BSA and HG decrease at pH 2.09 from 47.5 and 50.9% in the absence of NG to 0 and 0 in 0.10 mM NG, the α-helix fractions increase from 21.1 and 3.7 to 33.9 and 42.9% and the β-turn fractions increase from 0 and 3.6 to 32.1 and 26.6%. Therefore, addition of BS or NG transforms some β-sheet into helix and turn. We illustrated in

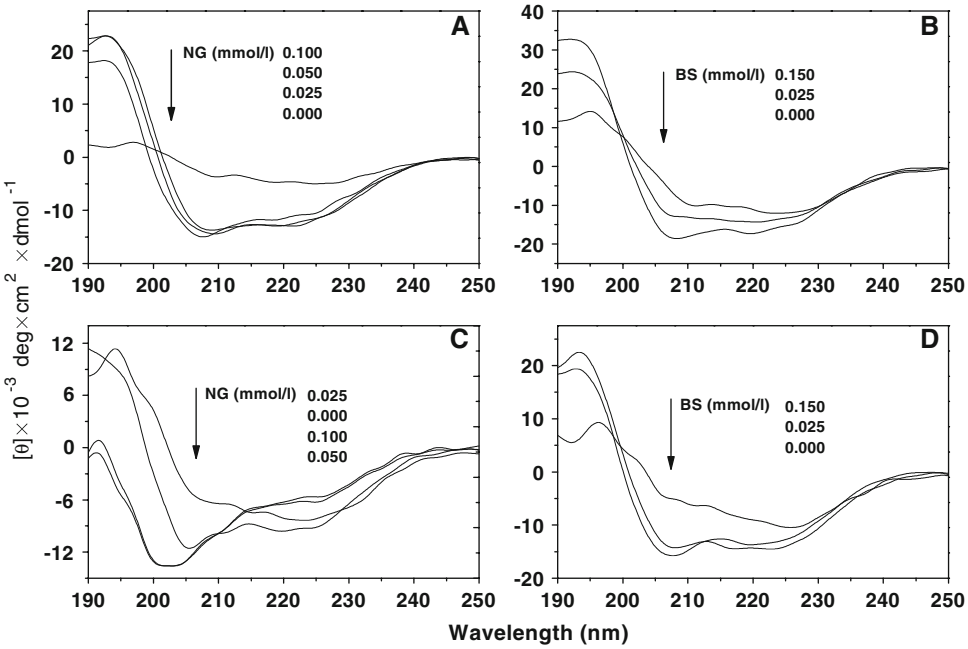

Fig. 5 The CD spectra of the BSA–NG (**a**), BSA–BS (**b**), HG–NG (**c**) and HG–BS (**d**). The concentration of BSA is 0.0867 mg/ml, and that of HG is 0.17 mg/ml. NG concentration is 0, 0.025, 0.050 and 0.100 mmol/l while BS is 0, 0.025 and 0.150 mmol/l

Fig. 6b how these interactions may affect changes from β-sheet to helix and turn. With BS as the representative, on a single peptide chain (chain 1), BS can bridge between C_{N1} and C_{H1} by binding their side groups (R) via ion-pair attraction if both C_{N1} and C_{H1} are basic AARs. Thus, both K–N and H–K sheets would rotate inversely around C_{K1}. This might result in the break of original H-bonds between chains 1 and 2 and formation of the H-bond between C=O (M1) and NH (I1). Therefore, the β-sheet was changed into turn. If both Rs were located in chain 1 and 2, respectively, such as C_{B2} and C_{H1} (Fig. 6b), the ion-pair attraction made both of them close the two $-SO^{3-}$ groups of BS by rotating inversely. Thus, the original H-bonds between chains 1 and 2 would be destroyed and the H-bonds between C=O (C2) and –NH (F1) and –N=N group of BS be formed. Therefore, the β-sheet was changed into a helix shape. Without doubt, the conformational change of proteins resulting from the non-specific bindings of BS and NG influenced the activity and function of proteins so they could cause the potential toxic action and health risk in some acidic physiological tissues, e.g., skin and gastro.

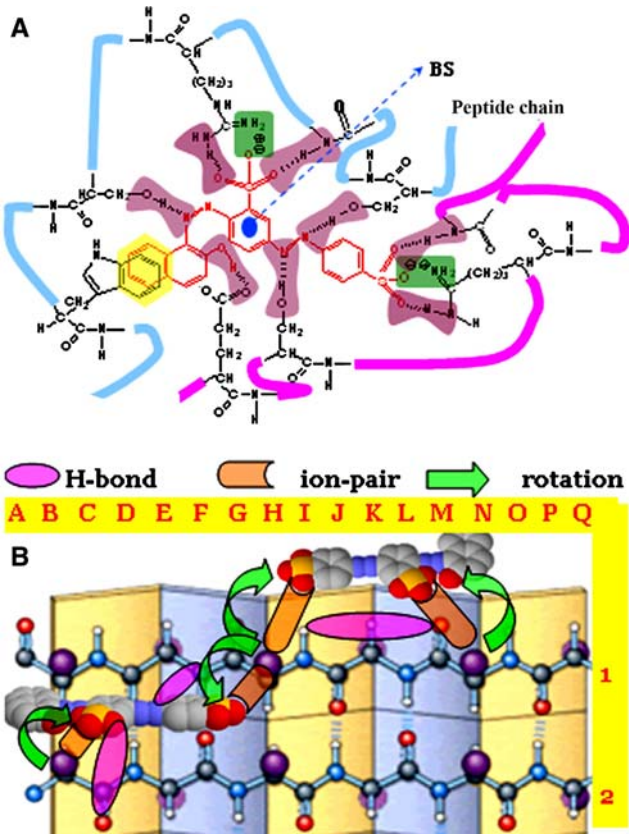

Fig. 6 **a** Cartoon illustrating the binding of BS as a representative to amino acid residues through collective action of many non-covalent bonds e.g., electrostatic attraction, H- bonds and hydrophobic interaction. **b** Cartoon illustrating how the secondary structure of BSA may be changed in the presence of BS and NG

Fluorophotometric identification of target amino acid residues

Proteins contain Trp, Phe and Tyr residues and its intrinsic fluorescence intensity depends on the degree of exposure of these residues to the polar, aqueous solvent and their proximity to specific quenching groups such as protonated carboxyl, protonated imidazole and deprotonated ε-amino groups (Dockal et al. 2000). Two models have been proposed for the quenching of protein fluorescence, static quenching and synthetic dynamic quenching (Behera et al. 1999). The quenching effect of an organic substance on protein fluorescence is often studied using the Stern–Volmer equation:

$$\lg\left(\frac{I_0 - I}{I}\right) = \lg k + n \lg C_L \tag{7}$$

where I_0 is the fluorescence intensity of protein without BS or NG, I is the fluorescence intensity in the presence of BS or NG, k is the binding constant of ligand with only Trp, Phe and Tyr residues and n indicates the number of BS or NG binding sites. In static quenching, the quencher will bind to Trp, Phe and Tyr residues and alter the secondary structure of the protein. The intrinsic fluorescence will be influenced directly by the microenvironments of these residues. Some organic substances interact with peptide chains and affect the conformation so as to influence these microenvironments. A change in the intrinsic fluorescence of a protein may provide information about the change in protein conformation. From the fluorescence spectra of the BSA solutions in the presence of NG or BS as shown in Fig. 7 (a and b), the small molecules quenched the intrinsic protein fluorescence through energy transfer. The quenching constant was calculated to be over 1.0×10^{12}, which is

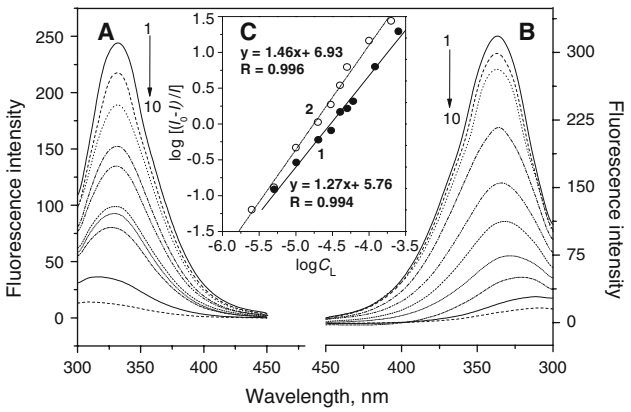

Fig. 7 The fluorescence spectra of the BSA–NG (**a**) and BSA–BS (**b**) solutions containing 0.085 mg/ml BSA. From 1 to 10: NG concentrations are 0, 0.005, 0.010, 0.02, 0.030, 0.040, 0.050, 0.060, 0.12 and 0.25 mM and BS concentrations are 0, 0.0025, 0.005, 0.010, 0.020, 0.030, 0.040, 0.050, 0.10 and 0.20 mM. **c** Plots of lg[(I0-I)/I] versus lgC_L for the above NG (**a**) and BS (**b**) solutions: 1-NG and 2-BS

much greater than the highest dynamic quenching constant, 2.0×10^{10}. Thus, interactions between BS or NG and the protein result in static quenching of the intrinsic protein fluorescence, especially in Trp residues. Moreover, BS has a greater quenching effect on BSA fluorescence than NG. For example, the quenching efficiency is 86% with 0.050 mM BS, but about 62% with 0.050 mM NG. Again, this indicates a stronger interaction of BS with BSA. From the linear regression equations as shown in Fig. 7c, n and k for NG and BS were calculated to be 1.27 and 1.46, and 5.8×10^5 and 8.6×10^6, respectively. Thus, about two NG and two BS bind Trp residues in BSA by hydrogen bond and π–π interactions. There are only two Trp residues in BSA (Trp_{134} and Trp_{212}). It seems highly likely that NG and BS bind to these two Trp residues. In the BSA, there were three hydrophobic cavities and all hydrophobic were present there (Yi et al. 2003) and their hydrophobic environment was favorable for azo compounds to enter there. When BS and NG interacted with BSA, they entered these cavities and acted with Trp and other amino acid residues through electrostatic attraction, hydrogen bonds and hydrophobic interactions.

Effects of electrolytes and temperature

Sodium chloride was added to BSA–BS and BSA–NG solutions to investigate the effect of electrolytes on the non-covalent interactions. With increasing sodium chloride concentration, γ for BS increases slightly, but that for NG decreases rapidly (Fig. 8). In sodium chloride solutions over 0.1 M, γ approaches only half its value as that in the absence of sodium chloride. This is due to the Debye-Huckel screening, where the Debye length is inversely proportional to the square root of the ionic strength of solution (Quinn et al. 1998). BS has more potential polar binding points and these points are distributed uniformly. It is difficult for π–π interactions to occur between BS molecules. Thus, γ hardly changes with increasing ionic strength. Therefore, BS–peptide chain binding is stronger than NG–peptide chain binding, as described above.

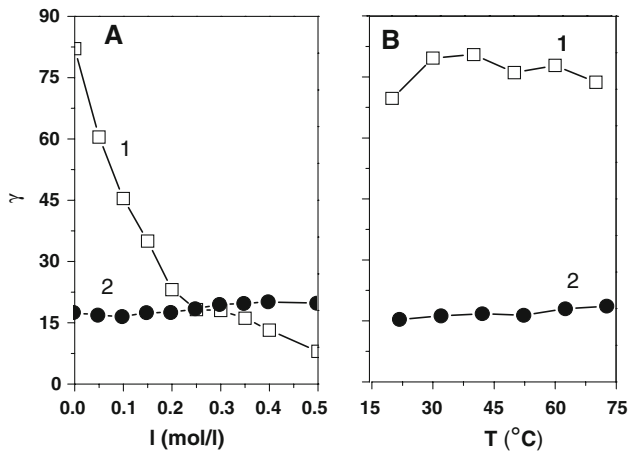

Fig. 8 Effects of electrolyte concentration (**a**) and temperature (**b**) on γ for solutions: (*1*) 0.0225 mM NG and 0.0174 mg/ml BSA and (*2*) 0.0250 mM BS and 0.0554 mg/ml BSA

High temperature has two opposing effects on non-covalent interactions. On the one hand, the peptide chain will expand with heating, which could weaken its three-dimensional conformation in such a way as to favor the insertion of organic molecules. On the other hand, such expansion will increase the distance between peptide chains. Thus, it will redistribute the effective binding points on the peptide chain and lead to desorption of small organic substances. The balance between these mechanisms decides the effect of temperature on the non-covalent interactions. In BS γ changes slightly with heating (Fig. 8). This is because the polar binding points of BS occupy a high fraction of the molecule and are hardly disrupted by temperature increase. In the same way, the hydrophobic binding of NG to the peptide chain may be weakened because molecular movements are accelerated at higher temperature, but its polar binding to the peptide chain will not be affected seriously.

Application to protein assay

Calibration graphs and LOD

From the above work, the BS–peptide chain complexation is more sensitive than the NG–peptide chain one. Moreover, it will not be influenced in a high concentration electrolyte medium. As a result, BS was selected as the chromogenic reagent for protein assay by the light-absorption ratio variation approach (LARVA) (Liang et al. 2007), which increases the spectrophotometric sensitivity for over ten times. Three standard series of each protein were prepared according to the measurement method. The absorbance of each solution was measured and ΔA_r was calculated from Eq. 1. Their regression equations are given in Table 3. The LOD of BSA, OVA and HG, defined as the blank values plus three times the standard deviation of ten replicated blanks was also calculated and is given in Table 3. The less the BS added, the higher the sensitivity that was obtained. However, a too low BS could cause an obvious error in measurement because of the noise of instrument in the background. The LOD of series 2 is lower and the difference between the slopes of BSA and HG is small. Thus, 2.50 mmol/l BS can be added so as to determine the total concentration of proteins mixture in body liquid, e.g., blood and cell liquid. The OVA standard curves are suitable for analysis of food. The final linear equation may be corrected averagely as $\Delta A_r = 0.0769 C_{M0}$ for protein assay. The corresponding LOD of protein is 0.12 mg/l. For OVA, the LOD is 0.74 mg/l.

Table 3 The linear regression equations and LODs of BSA, OVA and HG with BS as the chromogenic reagent in the presence of EDTA

BS (μmol/l)	Protein, C_{M0} (μg/ml)		Regression equation	R^a	σ^b	LOD[c] (μg/ml)
1.25	BSA	0–1.84	$\Delta A_r = 0.0872C_{M0} + 0.0065$	0.9887	0.0078	0.27
2.50			$\Delta A_r = 0.0884C_{M0} - 0.0023$	0.9972	0.0036	0.12
3.75			$\Delta A_r = 0.0806C_{M0} - 0.0026$	0.9907	0.0036	0.13
1.25	OVA	0–3.34	$\Delta A_r = 0.0194C_{M0} + 0.0063$	0.9808	0.0078	1
2.50			$\Delta A_r = 0.0145C_{M0} + 0.0004$	0.9654	0.0036	0.74
3.75			$\Delta A_r = 0.0093C_{M0} + 0.0010$	0.9752	0.0036	1.16
1.25	HG	0–0.862	$\Delta A_r = 0.1224C_{M0} + 0.0073$	0.9694	0.0078	0.23
2.50			$\Delta A_r = 0.0655C_{M0} + 0.0004$	0.9787	0.0036	0.16
3.75			$\Delta A_r = 0.0444C_{M0} - 0.0020$	0.9782	0.0036	0.29

[a] Linear correlation coefficient

[b] Standard deviation of ten replicated reagent blanks

[c] LOD = $3\sigma/p$

Effect of foreign substances

The normal masking reagent, EDTA, was used to mask most of the metals possibly existing in various complicated samples. The experimental result indicated that none of the following species affected the direct determination of 0.45 mg/l BSA (error less than 10%): 0.5 mg/l Cu^{2+} and Al^{3+}; 1 mg/l Pb^{2+}, Cr^{3+}, Fe^{3+}, Mg^{2+} and Ca^{2+}; 2 mg/l Zn^{2+} and vitamins mixture; 3 mg/l Mn^{2+}, 10 mg/l amylum, citrate, glucose and sucrose and 50 mg/l K^+ and PO_4^{3-}. Therefore, the recommended method was highly selective and fitted to the analysis of food, body liquid and water.

Analysis of samples

In the present work, the *Escherichia coli* was broken down with an ultrasonic cell disruptor, and then the solution with *Escherichia coli* was centrifuged to separate the cell residue from the solution. The purified solution as a sample was analyzed and the protein mixture was determined. A kind of commercial milk was selected as the other sample for protein assay. The two samples were analyzed according to the measurement procedure. The recovery of the first sample was 86% by this method and the second sample contained 31 g/l protein, which was near 30.0 g/l marked on the packing box. Besides, the colorimetry with coomassie brilliant blue (CBB) (Lopez et al. 1993] was used to examine the accuracy of this method and the protein contents above were confirmed. Therefore, the recommended method was accurate.

Conclusions

The covalent bond complexation with specific residues of protein is studied increasingly, such as in DNA repair (Liao et al. 2006), identification of enzyme activity point (Brazeau et al. 2004) and in the development of medicines (Wang et al. 2006). However, the non-specific non-covalent interaction exists most extensively in cells and organisms. Though a non-covalent bond is often weaker than a covalent bond, the union of non-covalent poly-bonds will produce a stronger aggregation. Recently, more and more biochemists are interested in protein–ligand [Hoare et al., 2005; Futaki et al. 2004] and protein–protein and protein–DNA (Hasche and Voss 2005; Nelson et al. 2002) interactions because a lot of significant biological functions depend on such interactions. In the present work, the ion-pair electrostatic interaction first fixes an anionic azo substance at the basic AARs of protein, and then the co-action of the other non-covalent bonds formed between it and the peptide chain binds it with hydrophobic interaction, van der Waals force, dipole effect and hydrogen bond. It is confirmed that the union of non-covalent bonds results in the conformational change of protein. Thus, it is possible that the potential toxic action of anionic azo substance exists in the acidic physiological tissues. This work provided a useful approach for characterization of such non-specific interactions occurring in cells and organisms. It is helpful in the research of protein toxicity of exogenous chemical substances e.g., azo pollutant and drug molecule species and identification of their health risk mechanism.

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