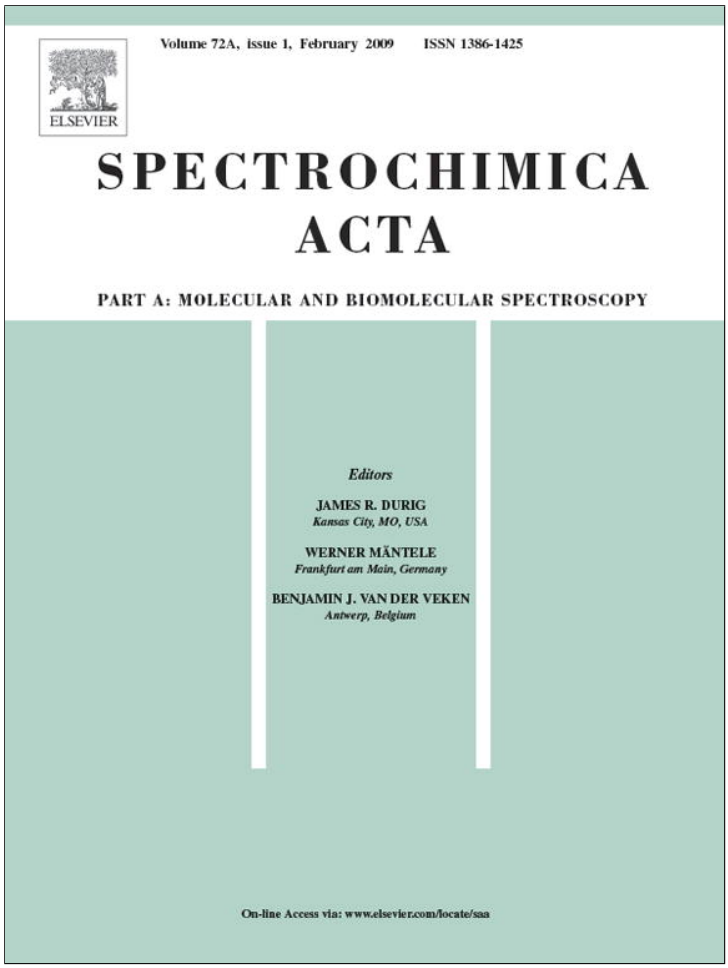

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Fluorescence spectra study the perturbations of CopC native fold by 2-*p*-toluidinynaphthalene-6-sulfonate

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ABSTRACT

2-*p*-Toluidinynaphthalene-6-sulfonate (TNS) was discovered to perturb native fold of CopC protein and to induce loss of biological activity to some extent which was dependent on TNS concentration. Hydrophobic and electrostatic interactions were revealed to account for the perturbation by comparison with some analogy. TNS, with far low concentration of 10^{-5} to 10^{-4} M, is presented as a denaturant. So TNS should be deliberated in detecting macromolecular conformation change as single evidence at higher concentration.

1. Introduction

2-*p*-Toluidinynaphthalene-6-sulfonate (TNS), a known hydrophobic probe, has been utilized in inspecting conformational change of proteins by greatly increasing its fluorescence intensity and shifting emission maximum to short wavelength when binding to hydrophobic regions [1–4]. Nevertheless, in our investigation, TNS itself was observed to cause CopC red-shift of emission maximum and loss of biological activity beyond a quantity of concentration. CopC, presented in stains of *Pseudomonas syring*ae, is a copper resistance protein [5–8], which carries out its biological function by two separated but interdependent Cu^{+} and Cu^{2+} binding sites. It is a small 102 residues protein with a β-barral structure composed of nine β-brands, which belongs to the sandwich-like family [9–11]. It is known that guanidine hydrochloride, heat, alcohol, acids and bases and heavy metal salts can unfold protein [12–14]. For TNS, few reports were involved in the aspect. Besides quenching fluorescence intensity, red-shifted emission maximum and lose biological activity of binding copper to some extent, comparison of stability of apo-type with metal-type also gave an indirect proof to perturbing CopC from TNS. Interaction mechanism was also disclosed.

2. Materials and methods

2.1. Reagents

2-*p*-Toluidinylnaphthalene-6-sulfonate and pheny-1-naphthylamine (AN) were bought from Sigma Chemical Co. and used without further purification. Sodium dodecyl sulfate (SDS) was analytical grade reagents.

2.2. Protein expression and purification

Apo–CopC protein was expressed and isolated essentially as reported with an extra gelfiltration purification step. Negatively charged *E. coli* proteins were removed by passing the lysate supernatant through a DE-52 anion exchange column in 20 mM PB buffer at pH 8. The flow-through fraction was adjusted to pH 6 with 0.2 M PB and then applied to a CM cation exchange column in 20 mM PB at pH 6. The bound proteins were eluted with a salt gradient of 0–0.2 M NaCl in 20 mM PB. The final purified proteins were detected by 15% SDS-PAGE and less than 1% Cu content was obtained by atomic absorption spectrum.

2.3. Protein concentration

The protein concentration was measured spectrophotometrically using molar extinction coefficients at 280 nm of 6970 M^{-1} cm^{-1} for CopC.

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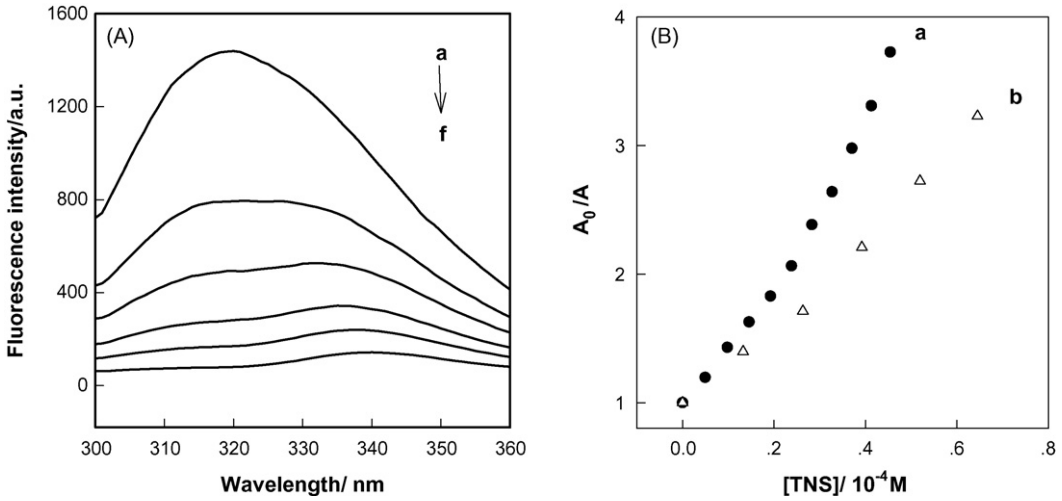

Fig. 1. (A) Emission of CopC in different concentration of TNS. (a–f) 0, 14.6, 28.3, 45.5, 61.4, 90.1 μM. 2.75 μM CopC, pH 7.4. (B) The effect of increasing TNS concentration on tryptophanyl emission. The sample's concentration was 2.75 μM CopC (a), Trp (b), pH 7.4.

2.4. Fluorescence spectra

Fluorescence spectra were recorded on an F-2500 fluorescence spectrophotometer. Fluorescence intensity was measured with a Hitachi 850 fluorescence spectrophotometer and excitation wavelength was at 280 nm. The slit widths for excitation and emission were 10 nm. A filter with a long pass of >290 nm was used to avoid secondary Raleigh scattering. The temperature of the solutions was maintained at 25 °C by a jacketed cell holder connected to an external circulating water bath (Shimadzu TB-85 or Huber).

3. Results and discussions

3.1. TNS perturbation of CopC native fold

3.1.1. Emission quenched and red-shifted

Single tryptophan residue (83 site) of CopC, sandwiched between the two sheets, is under a low-polar hydrophobic microenvironment [10]. This is consistent with the emission maximum of CopC near 318 nm. In order to detect the accessibility to the hydrophobic domain of CopC, TNS was employed. The protein emission is strongly quenched with the addition of TNS and it was found that the emission maximum of CopC observably shifted towards longer wavelength upon addition of TNS beyond 3 equiv. (Fig. 1A). Fig. 1B shows the effect of increasing TNS concentration on the fluorescence intensity of CopC. Owing to the emission maximum of CopC shifting with the addition of the quencher, the most current F_0/F ratio–quencher concentration plot is substituted by areas of the emission A_0/A ratio–quencher concentration plot [15]. The fluorescence spectrum of CopC excited at a wavelength of 280 nm, at which both tryptophanyl and TNS absorb, for comparison, the TNS concentration – dependent quenching of tryptophan is also displayed. It is evident that the extent of fluorescence quenching produced by increasing TNS concentration on CopC is much higher than that observed for the tryptophan. It allows one to consider that the occurrence of the interaction between TNS and CopC account for the higher quenching except the absorption of TNS and collisional quenching.

In general the position of fluorescence peak of tryptophanyl in protein is considered to move towards a longer wavelength when the residue in a protein is exposed to a polar medium. So the red-shifted emission maximum of CopC along with higher fluorescence quenching can be taken as an indicator of the occurrence of the perturbations of CopC native fold like that caused by urea [16,17] so that interior hydrophobic residues including tryptophanyl gradual exposure to polar aqueous solution. That is, TNS beyond a quantity of concentration induced the denaturation of CopC.

3.1.2. Loss of biological activity

The loss of native fold often implies a loss of protein biological activity. As a copper resistance protein, binding copper is specific for CopC, which is exhibited by significant quenching fluorescence intensity of the protein until the ratio of $[Cu^{2+}]/[apo–CopC] = 1.0$ [18]. The binding copper would be affected if the unfolding of CopC occurred according to the result from thermal and urea denaturation. No interaction of TNS to copper was displayed in the control of experiment. So the capacity of copper-binding was inspected by copper titrating CopC in the presence of increasing concentration of TNS. Fig. 2 shows normalized fluorescence titration curves of copper to CopC at the concentration of 0, 15, 37, 76 μM TNS, respectively. As could be seen that quenching fluorescence gradually decreases with increased concentration of TNS. Quenching fluorescence by copper is regard as standard in the absence of TNS, the percentage capacity of binding copper of CopC in different concentration of TNS can be obtained by the formula of

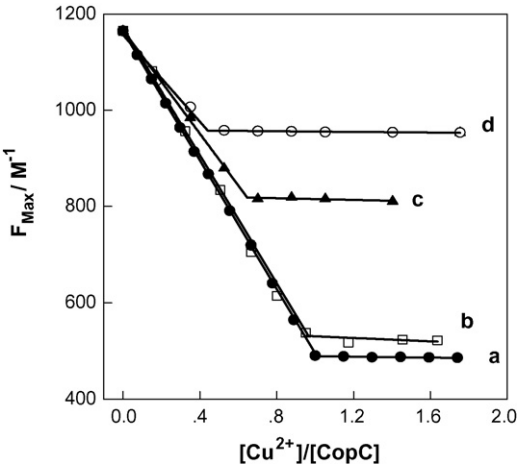

Fig. 2. Normalized fluorescence titration curves of adding Copper into CopC (2.75 μM) in presence of TNS, 0 μM (a) 15 μM (b) 37 μM (c) 76 μM (d).

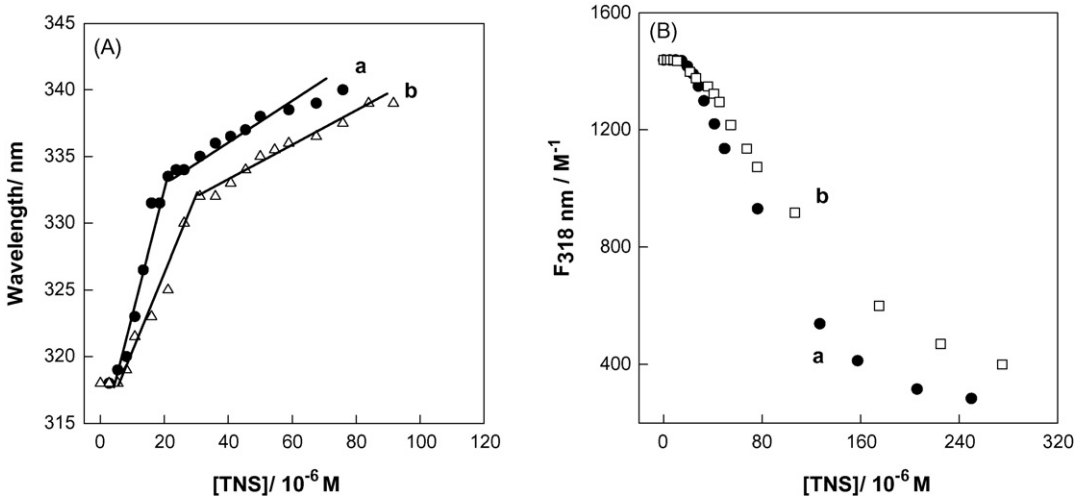

Fig. 3. TNS-induced red-shift (A) and normalized fluorescence titration curves (B) in CopC (a) and Cu(I)–CopC–Cu(II) (b). Both sample's concentrations were 2.75 μM.

$(F_0 - F_{b(c,d)})/(F_0 - F_a)$. F_0 indicates fluorescence intensity of CopC in the absence of copper; F_a, $F_{b(c,d)}$ are fluorescence intensities of the inflexions in curve a, curve b(c, d), respectively, indicating quenching fluorescence of CopC by copper in the absence and presence of different concentration of TNS. The date reveals that CopC kept binding copper capacity of 93.8%, 51.3%, 30.7% corresponding to 15, 37, 76 μM TNS. Together with red-shifted emission maximum and strong quenching fluorescence, loss of biological activity again proves that TNS perturbed native fold of CopC and greater perturbation appeared at higher concentration of TNS.

3.1.3. Binding copper effect

In order to prove the result of the denaturation, another experiment was performed. As is known that the second or tertiary structures can be stabilized via binding metal in urea (or guanidine hydrochloride)-induced denaturation [19]. The higher thermodynamic stability of metal-form of CopC than apo–CopC was also proved in urea-induced unfolding experiment [20,21]. If TNS-induced denaturation occurred, the similar result should be expected. Comparison between apo–CopC and Cu(I)–CopC–Cu(II) was shown in Fig. 3A. It is visual that the emission maximum of both proteins shifted towards longer wavelengths with increasing concentration of TNS and the emission maximum of apo–CopC is more red-shifted than Cu(I)–CopC–Cu(II) at the same condition. Choosing the emission of CopC at 340 nm as a parameter, 67 μM TNS is required for apo–CopC while 84 μM for metal–CopC. That is, Cu(I)–CopC–Cu(II) is more stable than apo–CopC in TNS solution. After emission of CopC at different concentration of TNS being normalized, fluorescence intensity changes of both proteins at 318 nm also are showed in Fig. 3B. TNS leads higher quenching for CopC than for Cu(I)–CopC–Cu(II), which is in accordance with results from urea unfolding. Structure stability of proteins from binding metal maybe involves in disrupting the covalent bonds between the metals and ligands during the process of unfolding; conversely, greater stability of Cu(I)–CopC–Cu(II) could provide an indirect or complementary proof to the perturbation of CopC from TNS.

3.2. Interaction mechanism of TNS to CopC

3.2.1. Hydrophobic interaction

Since TNS is a hydrophobic probe, it can be assumed that hydrophobic interaction play a key role in denaturation. In order to test it, AN, an analogy with no sulfonate ion, was introduced to perform similar experiment. The fluorescence of CopC is observed to be quenched and to shift gradually towards longer wavelength with the increasing concentration of AN. AN, almost neutral at pH 7.0, obviously reacted with CopC chiefly by hydrophobic interaction. This implies that hydrophobic interaction is crucial in the reaction. However, difference still appeared between TNS and AN in interactions to CopC. Compared with AN, TNS brings greater red-shift under the same conditions, it is required 67 μM to produce the emission of CopC at 340 nm while AN arrived to 330 nm at the concentration of value. TNS is obviously superior to AN in denaturing CopC. It is doubtful that TNS has an additional methyl which enhances hydrophobic property of the molecule. But, whether sulfonate ion with charge that gives additional electrostatic interaction to strengthen reaction between TNS and CopC was then exploited.

3.2.2. Electrostatic interaction

It has been reported that the sulfonate anion could bind to cationic groups of water-soluble proteins through ion pair formation [22–24]. Hence, protein–TNS interaction is rather dependent on electrostatic forces under the condition. The perturbation of TNS to CopC at different pH that confers different net charges on the protein molecule is shown in Fig. 4. It can be seen that the perturbation of TNS to CopC is increased with lowering pH, higher stability at

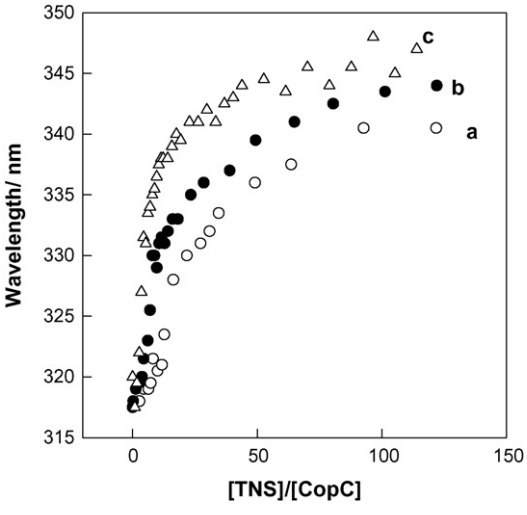

Fig. 4. The pH effect on the emission change of CopC in increasing concentration of TNS. pH 11 (a); pH 8.5 (b); pH 7.5 (c).

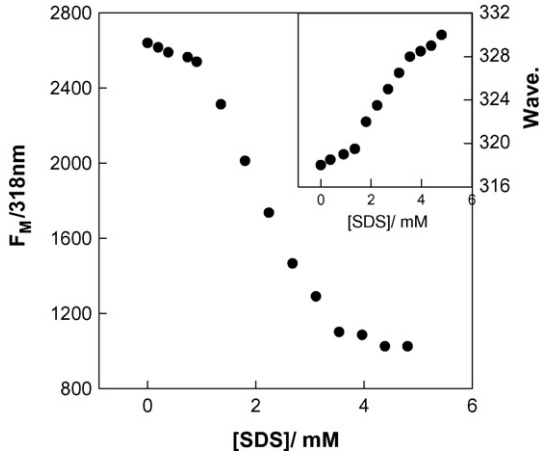

Fig. 5. The changes in fluorescence intensity of CopC as a function of SDS concentration. Insert is the changes in emission maximum. 2.75 μM CopC, pH 7.4.

alkaline pH, moderate near p*I* value and lower below p*I*. Therefore, the interaction between TNS and CopC could be deduced to depend on electrostatic factors [25], governed, in turn, by the relation of pH to the protein's p*I*, to overall protein titration charge, and to the number of positive charges on the protein molecule. In the lower pH regions, where the protein molecule amino acid side chains are cationic, CopC strengthens the interaction to TNS with negative sulfonate ion. Near the isoelectric point, the electrostatic contributions are poor according to the theory of Alonso and Dill [26], the perturbation is due only to the hydrophobic effect. In alkaline pH well above CopC's p*I*, increasing negative charge of the protein strengthens electrostatic repulsion so that the perturbation of TNS to CopC decreases. The experimental results are consistent with the assumption that the sulfonate ion plays a role in denaturalizing CopC.

Excluding GuHCl and urea, SDS is a prevalent denaturant with amphipathy among small organic molecules that can induce denaturation. Although TNS and SDS are different structures, they both have hydrophobic groups and hydrophilic sulfonate ion. Since TNS-induced denaturation is from its amphipathy, SDS was compared with TNS to further clarify the interaction of TNS to CopC.

As can be seen in Fig. 5, fluorescence emission maximum of CopC suffers a red-shift from 318 to 330 nm in the range of 1–4.0 mM of SDS. Simultaneously the fluorescence intensity of CopC at 318 nm also decreases with increase in SDS concentration. Beyond 4.0 mM SDS the fluorescence maximum and intensity have hardly changed with increase in SDS concentration. The isotherms in Fig. 4 exhibit a typical sigmoidal shape which has been suggested for protein systems as a description of a cooperative binding process [27,28].

The critical micelle concentration of SDS is 8 mM in water and it increases in protein solution [29], at which (critical aggregation concentration) the protein unfolding process begins [30]. From Fig. 5, it can be seen that CopC can not be denaturalized below critical aggregation concentration and the SDS-induced denaturation of CopC is from forming aggregation. This may mean the hydrophobic interaction of SDS works chiefly in forming aggregation. Upon forming aggregation, it is expected that large amounts of bound ionic surfactants both break the intrachain hydrophobic bonding and provide an electrostatic repulsion favoring an extended structure [31], which makes the Trp residue be located at a more polar microenvironment, and hence a higher λ_{max}. It is evident that TNS with the aromatic rings can not form micelle. How had it interacted to CopC? It was found in early literature that an interaction exists between amide and aromatic groups and *N*-methylamides in nonaqueous solvents in the presence of aromatic compounds had formed complex [32,33] reflected by the nuclear magnetic resonance and infrared spectra change. And the adsorption behaviors of 1-naphthylamine in aqueous solutions on nonpolar adsorbents were verified to result from its greater electronic density of the aromatic ring [34]. Maybe all these provide a partial explanation for TNS-induced conformational change of CopC. TNS interact directly to residues on the surfaces of CopC by hydrophobic aromatic rings, and increasing TNS molecules gradually disturb the intrachain hydrophobic bonding to break native fold. And an electrostatic repulsion from sulfonate ion provides a positive effect. Compared with SDS, the denaturation concentration of TNS is far low at around 10^{-5} M for CopC (unfolding process begins at 1 mM for SDS). This shows TNS denaturation is more effective than SDS owing to being not demanded to achieve critical aggregation concentration; the interaction from greater electronic density of the aromatic ring to residues of proteins is stronger.

In summary, TNS induces conformational change of CopC and the loss of biological activity partially or completely, that is, it denaturalizes CopC. Compared with urea or guanidine hydrochloride, far low concentration of TNS (in the range of 10^{-5} to 10^{-4} M) can achieve denaturation. It is turned out that hydrophobic interaction play a key role in the interaction between TNS to CopC and electrostatic interaction also function. As a fluorescence probe, TNS has been utilized in many fields. Since TNS itself can bring great conformational change, it should be employed deliberatively at higher concentration.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.saa.2008.07.025.

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