Interaction between tns and β-lactoglobulin

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Title: Interaction between tns and β-lactoglobulin

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Interaction between tns and β-lactoglobulin

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Abstract

The major bovine milk protein β-lactoglobulin (β-LG), a member of the lipocalin superfamily, can bind a wide range of ligands and act as a transporter. In the present study, the combination of the hydrophobic molecule 2-(p-toluidino)-6-naphthalenesulfonic acid sodium salt (tns) with β-LG was analyzed using fluorescence spectroscopy and autodock modeling to discern the major binding sites of the protein and to determine the capacity of other small ligands to bind with β-LG by utilizing tns as a reference. The experimental data indicate that in a neutral pH environment, tns is located in the hydrophobic domain of the protein, 2.5 nm away from the Trp19 residues of β-LG. The binding constant of the small molecule to β-LG is $(3.30\pm0.32)\times10^6$ M$^{-1}$. An interaction model between the ligand and β-LG was developed, and autodock modeling also demonstrates that the ligand is located in the central hydrophobic calyx of β-LG within the regions covered by the Förster radius of the Trp19-ligand pair. Although the interaction between the ligand and β-LG is affected by increasing ion strength, pH change and heat treatment, the complex is maintained until the secondary structure of β-LG is destroyed. Additionally, the ligand binding stabilizes the folding of β-LG. The binding constants of sodium dodecyl sulfate (sds) and sodium dodecylbenzene sulfonate (sdfs) to β-LG were obtained using competitive ligand binding

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measurements. With a sensitive fluorescence signal and stability of complex, the ligand could be utilized as a reference to detect the binding of other small ligands to β-LG.

**Keywords**: β-lactoglobulin; 2-(p-toluidino)-6-naphthalenesulfonic acid sodium salt; autodock

1 Introduction

The major bovine milk protein β-lactoglobulin (β-LG) is a small protein with 162 amino acid residues (Mr <18,400) that fold into an 8-stranded, antiparallel β-barrel with a 3-turn α-helix on the outer surface\(^1\). As a member of the lipocalin superfamily, bovine β-LG can bind a wide range of ligands\(^2\)\(^-\)\(^5\). It has been speculated that the role of β-LG as a versatile carrier for small bioactive molecules, such as long-chain fatty acids, retinoids, steroids, cholesterol and vitamin D, is a reason for the significant quantities of β-LG found in milk. The protein’s major ligand binding site is the central calyx formed by the β-barrel, which is common to all lipocalins. Other binding sites have been identified, including the surface hydrophobic pocket in the groove between the α-helix and the β-barrel, the outer surface near tryptophan (Trp19)-arginine (Arg124), and the monomer-monomer interface of the dimer\(^6\)\(^-\)\(^8\).

Tns is weakly fluorescent in water alone. Upon binding to proteins, however, fluorescence intensity increases greatly and is accompanied by a blue shift of the maximum peak. Tns has been reported to bind many proteins, including bovine and human serum albumin, CopC, and Centrins\(^9\),\(^10\). However, there have been no reports of tns binding to β-LG. Since it is used as fluorescence probe just as ANS, maybe the binding ligand domain of β-LG, as a versatile carrier, could be explored with it. Moreover, the binding capacity of the protein to tns could be utilized as a reference for
other hydrophobic molecules. In this paper, the interaction between β-LG and tns was analyzed and the stability of β-LG-tns complex was determined. With its sensitive fluorescence signal, tns binding was used to determine the binding capacities of sodium dodecyl sulfate (sds) and sodium dodecylbenzene sulfonate (sdb) using simple spectral methods. Additionally, the major binding sites of β-LG were discerned.

2. Materials and Methods

2.1. Materials

Bovine β-LG and tns was purchased from Sigma (USA). Sds, sdb, and other chemicals utilized in the experiments were all analytical reagents.

2.2. Methods

2.2.1 Protein concentration

Protein concentrations were measured spectrophotometrically using molar extinction coefficients at 278 nm of 17600 M⁻¹ cm⁻¹[11].

2.2.2 Spectra measurement

Fluorescence spectra were recorded on an F-4500 fluorescence spectrometer, attached to a thermostatic circulating water bath maintained at a corresponding temperature. To avoid contamination from tyrosine emission in the energy transfer experiment, protein samples were excited at 295 nm. To correct the dilution, fluorescence intensity was converted to molar fluorescence intensity by dividing fluorescence intensity by the analytical concentration of bovine β-LG. The absorption spectra of tns were recorded on a 2250 UV-vis spectrophotometer.

2.2.3 Docking experiment
The crystal structure of β-LG (1BEB) was obtained from the Protein Data Bank. Molecular docking was performed using the AutoDock4.2 software package. One hundred independent docking runs were performed for tns, and results were clustered according to the root-mean square deviation (RMSD) criterion. The cluster including the most numerous conformations, not the lowest energy, was chosen. The cluster included 47 conformations, from which the docked conformation with the lowest energy was chosen.

The PyMOL molecular viewer and the MGLTools were used to render the output and to calculate the distance between tns and Trp19.

2.2.4 Light scattering measurements

The aggregation levels of β-LG induced by the addition of sodium chloride and at different pH values were monitored by synchronous scanning using the same excitation and emission wavelengths in the range of 300 to 500 nm. The light scattering signals were recorded as LS.

2.2.5 pH influence on the complex experiments

The binding of tns to β-LG was achieved by mixing them at a mole ratio of 1:1 in phosphate buffer at pH 7.0. pH levels were modified by adding either 0.5 M HCl to increase acidity or 0.1 M NaOH to increase alkalinity. The samples were kept at room temperature for at least 10 min after pH adjustment. A tns fluorescence peak at 440 nm was used to confirm the binding of tns to β-LG and to determine the influence of pH on the stability of the complex.

2.2.6 Heat treatment of complex experiments

The complex was heat treated by incubating complex-containing solution in a bath
and increasing the temperature by 1 °C per minute. The solution was kept at the detecting temperature point for 20 minutes.

2.2.7 Effects of tns binding on the stability of β-LG

β-LG solution and the mixture solution containing β-LG and tns at a ratio of 1:1 was added to gradually increasing concentrations of guanidine hydrochloride. The wavelengths corresponding to the β-LG emission maximums were detected after the β-LG and mixture solution was incubated for 20 min at every predetermined concentration of guanidine hydrochloride. The changes in wavelength corresponding to the emission maximum of the protein were regarded as the larger modification of β-LG folding in accordance with tryptophan sensitivity to environmental polarity.

2.2.8 Competitive binding experiments

β-LG was titrated by increased concentrations of tns, the mixture of tns and sds (or sdb) (1:1) and the mixture of tns and sds (or sdb) (2:1). The concentration of tns was kept the same at each parallel titration control point.

Above all experiments were executed more than 3 times.

3 Result and Discussion

3.1. The binding constant and binding sites

Tns is weakly fluorescent in water alone, with a maximum fluorescence wavelength of 500 nm that increases largely with movement from a more polar to a less polar environment; this increased intensity is accompanied by a noticeable blue shift of the maximum peak. The interaction between tns and β-LG was evaluated by monitoring the fluorescence emission spectra of β-LG and tns when adding tns to β-LG. As shown in Fig. 1, the tryptophan fluorescence of the protein gradually decreased with addition of
incremental aliquots of tns. A maximum fluorescence wavelength near 440 nm was observed for tns, and fluorescence intensity increased linearly until the concentration ratio of β-LG to tns reached approximately 1:1. An isoemissive point of 380 nm was determined. The shift of the emission maximum from longer to shorter wavelengths and the increased fluorescence intensity indicated that tns gradually transferred from the aqueous solution to the hydrophobic domain of β-LG. And a β-LG-tns complex with binding rate of 1:1 was formed.

![Graph showing emission spectra of β-LG and bound tns.](image)

Given that there are $n$ tns-binding sites that are independent and identical in the tns-protein complex, the binding equation is as follows:

$$P + nTNS \leftrightarrow TNS_nP$$  \hspace{1cm} (1.1)

Assuming $F$ is fluorescence intensity of every titration dot at 440 nm, $F_{\text{max}}$ is
maximum fluorescence intensity, and \( F_0 \) is the initial value at 440 nm, the increase in fluorescence intensity was the result of tns binding to the protein. The following equations can be obtained:

\[
[P]_b = \frac{(F - F_0)}{(F_{\text{max}} - F_0)} [P]_t \quad (1.2)
\]

\[
[A]_b = n[P]_b \quad (1.3)
\]

\([P]_b\) and \([P]_t\) are the bound and total concentrations of the protein, respectively. \([A]_b\) is the bound concentration of tns. The binding constant \( K \) can be given as follows:

\[
K = \frac{[P]_b}{[P]_t[A]_f} \quad (1.4)
\]

Because \([A]_f = [A]_t - n[P]_b\) \quad (1.5)

\([A]_f\) and \([A]_t\) are the free and total concentrations of tns, respectively.

Finally, the equation can be expressed as mass balance equations in (1.6)-(1.7)

\[
\frac{1}{K} = \frac{(F_{\text{max}} - F)}{(F - F_0)} ([A]_t - n[P]_b) \quad (1.6)
\]

\[
\frac{[A]_b}{[P]_b} = \frac{(F - F_0)}{[P]_b K (F_{\text{max}} - F)} + n \quad (1.7)
\]

Because \([P]_b\) is the only unknown in formula (1.2) and can be given, \(1/K\) was obtained by the linear slope of the plot of \([A]_t/[P]_b\) versus \((F-F_0)/[P]_b/(F_{\text{max}}-F)\) and \(n\) was equal to an intercept. Based on these calculations, the value of \( K \) is \((3.30 \pm 0.32) \times 10^6 \) M\(^{-1}\) and the value of \( n \) is 0.9, which indicates a strong binding tns site per molecule of \(\beta\)-LG.
3.2. Distance from tryptophan residue to tns

Forster’s theory of non-radiative energy transfer was used to determine the distance between the tryptophan residue and the tns binding site. According to Forster’s theory, the energy transfer effect is not only related to the distance between the donor (tryptophan residue) and acceptor but is also influenced by the critical energy transfer distance $R_0$:

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \quad (2.1)$$

where $r$ is the distance between the acceptor and the donor, and $R_0$ is Forster radius at which 50% of the excitation energy is transferred to the acceptor. It is defined by the following equation:

$$R_0^6 = 8.79 \times 10^{-5} k^2 Q n^4 J(\lambda) \quad \text{ (in Å}^6) \quad (2.2)$$

In Eq.(2.2), $k^2$ is the orientation factor, which is related to the geometry of the donor-acceptor of transition dipole. Forster radius reported for $k^2$ typical value is $2/3^{[12]}$. $n$ the refractive index of the medium, and $Q$ the fluorescence quantum yield of the donor in
the absence of the acceptor. Q can be calculated as 0.152 using the absolute quantum yield (0.14) of Trp in aqueous solution as a standard value with the following equation:

\[
\frac{Q_2}{Q_1} = \frac{F_2}{A_2} \frac{A_1}{F_1}
\]  

(2.3)

Where the subscripts 2 and 1 denote β-LG and trp, respectively. F denotes the integrated fluorescence intensity of dilute solution, and A the absorbance at the excitation wavelength. Compared with \(Q_{\text{H2O}}\) in water (0.013), the quantum yield of tns in β-LG increased about 12 times.

The spectra overlap integral \(J\) between the donor emission spectrum and the acceptor

\[
J(\lambda) = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}{\int F(\lambda)d\lambda}
\]  

(2.4)

Where \(F(\lambda)\) is the fluorescence intensity of the donor at wavelength and \(\epsilon(\lambda)\) is the molar absorption coefficient of the acceptor at wavelength \(\lambda\). The spectral overlap of the tns UV absorption spectrum and the fluorescence emission spectrum of β-LG in experimental conditions is illustrated in Fig. 3. It was then calculated that \(J = 9.89 \times 10^{13} \text{ M}^{-1} \cdot \text{cm}^{-1} \cdot \text{nm}^4\).

Transfer efficiency \(E\) is typically measured using the relative fluorescence intensity of the donor in the absence \((F_D)\) and presence \((F_{D,A})\) of the acceptor consistent with the values in Fig. 1B. \(r\) was then calculated as 2.5 nm.

\[
E = 1 - \frac{F_{DA}}{F_D}
\]  

(2.5)
3.3. Interacting forces between β-LG and tns using thermodynamic parameters

According to previously published reports, binding mode can be discerned using thermodynamic parameters that rely on temperatures \[^{[13,14]}\]. When temperature changes are very small, the enthalpy and entropy changes are often regarded as constant. \(\Delta G^\theta\) can be obtained using Eq. (3.1), while \(\Delta H^\theta\) and \(\Delta S^\theta\) can be calculated using the Vant–Hoff Eq. (3.2).

\[
\Delta G^\theta = -RT \ln K^\theta
\]  
\[
\Delta G^\theta = \Delta H^\theta - T\Delta S^\theta
\]

In Eq. (3.1), \(K^\theta\) corresponds to the binding constant at a specific temperature. The values of \(\Delta H^\theta\), \(\Delta G^\theta\) and \(\Delta S^\theta\) are the standard enthalpy change, free energy change and entropy change, respectively.

Results are shown in Table 1. \(\Delta G^\theta < 0, \Delta S^\theta > 0\) indicates that tns binding to the hydrophobic patch of β-LG releases the water molecule previously located in the patch; additionally, this process is spontaneous and free energy change decreases. With entropy change \(\Delta S^\theta > 0\) and enthalpy change \(\Delta H^\theta < 0\), it can be deduced that the acting force for
the binding reaction between tns and β-LG is mainly hydrophobic interaction, though

electrostatic interaction also serves a function\textsuperscript{[10,15]}.

Table 1 The binding constants and thermodynamic parameters at different temperature

<table>
<thead>
<tr>
<th>T/ K</th>
<th>$K_b^{0}(10^6M^{-1})$</th>
<th>$\Delta G^{0}(kJ\cdot mol^{-1})$</th>
<th>$\Delta H^{0}(kJ\cdot mol^{-1})$</th>
<th>$\Delta S^{0}(J\cdot mol^{-1})$</th>
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<td>295</td>
<td>3.30 ± 0.32</td>
<td>-36.81 ± 0.21</td>
<td>-17.08 ± 0.33</td>
<td>66.90 ± 1.09</td>
</tr>
<tr>
<td>305</td>
<td>2.66 ± 0.34</td>
<td>-37.50 ± 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>315</td>
<td>2.12 ± 0.54</td>
<td>-38.15 ± 0.62</td>
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3.4 Docking modelling

Molecular Docking modelling was also used to identify the binding of tns to β-LG. The one hundred docking conformations for tns were divided into groups using the Cluster module in ADT. The cluster including the most numerous conformations (47) was chosen. AutoDock was also used to make RMSD calculations to find the best binding mode of the conformation of this cluster. The interaction modes of tns and β-LG are depicted in Fig. 4.

The docking results show that the tns molecule indulges the central calyx formed by the β-barrel of β-LG (Fig. 4A). The size of the calyx is large enough to accommodate tns. The molecule is located within the regions covered by the Förster radius of the Trp19-tns pair and is 23.68 Å. The representative build derived from the best conformation with minimal binding energy is -7.95 kcal·mol\textsuperscript{-1}. The interaction models are shown in Fig. 4B.

The model demonstrates that Asn88 and Asn90 anchor the tns molecule by forming hydrogen bonds with the sulfonic group. The naphthyl ring and benzene ring of the ligand are located in the pocket surrounded by hydrophobic amino acids, such as Ile, Leu,
Val, Phe and Glu. Phe105 forms an aromatic-aromatic interaction with the benzene ring of the ligand. In addition, electrostatic interaction is found between the ligand and Leu87, Met107, Glu89 and Leu87 residues.

3.5. Stability of the β-LG-tns complex

3.5.1 pH effect

Firstly, the effect of pH on the stability of the β-LG-tns complex was assessed. The pH values were selected to be within a range between 2.0 and 9.0. The β-LG-tns complex was first formed and then exposed to specific pH values. The maximum emission wavelengths of tns in β-LG solution concentrated near 440 nm and when detected was taken to indicate the existence of β-LG-tns complex at selected pH values. The notable tns fluorescence peak clearly shows the binding of tns to β-LG at all pH values between 2.0 and 9.0 (Fig. 5). The β-LG-tns complex appeared to dissociate above pH 9.0, as the
fluorescence peak disappeared. The fluorescence intensity of tns from the β-LG-tns complex at pH values below 5.0 (2.0, 3.0 and 4.0) was greater than at pH values above 5.0 (6.0, 7.0, 8.0 and 9.0). The lowest fluorescence intensity of tns was observed at pH 5.0. pH 5.0 is close to the isoelectric point (pI = 5.2) of β-LG, where octamers are formed by the oligomerization of the protein dimers [8,16]. Light scattering of the β-LG-tns complex, which provides information on the oligomerization extent of the protein, also confirmed that maximum aggregation occurs at pH 5.0 (Insert in Fig. 5). It has been reported that self-aggregation can contribute to internal fluorescence intensity quenching of Trp in the calyx of the protein [7]; this may similarly affect quenching of the binding tns in the calyx. It is more likely that aggregation causes changes in binding of β-LG to tns. Using a method similar to Section 3.1, the binding constant and site of the interaction of tns and β-LG at different pH levels was determined (Table 2). The results further indicate that aggregation decreased tns binding. The higher fluorescence intensity of tns observed at lower pH is probably due to the dissociation of dimers into monomers, which increased binding. Moreover, at acidic pH levels (lower than 6.5) the EF loop over the entrance of the calyx is in close conformation and the protein is more compact [17]. A tighter packing of β-LG around tns, which would be a more hydrophobic environment, could improve the interaction between tns and β-LG. Although monomers increase as |pH-pI| increases [18], at alkaline pH above 7.5 (at pH 8.0) the EF loop is in open conformation and there is a loosening in the interior packing of β-LG. Moreover, partial unfolding of β-LG at pH 9.0 could occur [8] and contribute to decreased binding of the protein to the ligand. In summary, although the fluorescence intensity of tns is pH-dependent, the interaction between β-LG and tns does not seem to be affected by the EF loop until an irreversible
base-induced unfolding transition occurs and leads to the loss of structural integrity of the
protein above pH 9.0\textsuperscript{[19]}.

![Graph showing light scattering intensity (LS) at 440 nm at different pH values.]

**Table 2 Binding constants of tns to β-LG at different pH**

<table>
<thead>
<tr>
<th>pH</th>
<th>Binding constants K ($10^6$ M$^{-1}$)</th>
<th>Binding sites n</th>
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<tr>
<td>pH=2</td>
<td>3.76 ± 0.22</td>
<td>1.37 ± 0.03</td>
</tr>
<tr>
<td>pH=5</td>
<td>3.02 ± 0.18</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>pH=8</td>
<td>2.71 ± 0.25</td>
<td>0.71 ± 0.02</td>
</tr>
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3.5.2 *The influence of ionic strength on binding*

The influence of salt on fluorescence intensity of tns in the presence of β-LG was
also studied, with NaCl used as a reagent (Fig. 6). To exclude the dilution effect and clearly determine the effect of salt, relative fluorescence intensity was calculated by dividing the fluorescence intensity of tns (440 nm) by the fluorescence intensity of the β-LG (340 nm). Initially, relative fluorescence intensity decreased sharply as the concentration of NaCl increased from 0-0.25 M. Then, the decline slowed up to 1.2 M, which was the maximum concentration of NaCl used in this experiment. The addition of salt visibly affected the binding of tns to β-LG. In the first phase, it could be assumed that salt disturbed the electrostatic force between tns and β-LG as Cl⁻ competed with tns to bind the protein and thus reduce the number of bound tns molecules, resulting in the sharp decrease of tns fluorescence intensity. Because binding tns caused internal fluorescence quenching in β-LG, the protein increased in fluorescence intensity upon setting free the molecule (Curve b in insert of Fig. 6). This also indicates that the electrostatic interaction between tns and the protein is important. As the concentration of salt increased, β-LG aggregated increasingly from monomer to dimer\cite{20}. Dissolving protein in aqueous solution is hydrophilic groups of protein and water forming water film. Because salt has a greater affinity to water than the protein, adding salt weakens and eliminates the hydration layer around the protein. At the same time, the increased ionic strength neutralizes a large amount of the protein surface charge and leads to aggregation. The oligomerization could also be affirmed by increasing LS intensity as additional NaCl is added. The slowly decreasing fluorescence intensity of tns and β-LG in the second phase should be attributed to the auto-association of the monomers influencing the binding of tns and β-LG. Moreover, it is possible that the conformation of the protein changed at higher salt concentrations, which contributed to declining relative
fluorescence intensity of tns to β-LG.

![Graph showing fluorescence intensity changes](image)

**Fig. 6 NaCl effect on emission of tns to β-LG**
Insert: β-LG-tns with the addition of 0 (a), 0.25 and 1.18 M NaCl (b) in 0.01M phosphate buffer, pH 7.0. $c_{tns}:c_{β-LG}=1:1$, $[β-LG]=25$ µM

3.5.3 Thermal stability

The β-LG-tns complex was heated to 90 °C at neutral pH to test its stability under heat treatment. Fig. 7 displays the fluorescence intensity changes of tns and β-LG as the temperature increased. For comparison, we also present a normalization to address fluorescence intensity change by $F_0/F$. The fluorescence intensity of tns decreased with increasing temperature until the emission maximum completely disappeared at 60°C. Compared with β-LG, tns fluorescence decreased more rapidly and the difference in fluorescence intensity between the two increased as temperature increased. Thus, it could be determined that the binding capacity of tns to β-LG gradually weakened until the complex completed dissociation at 60 °C; this result was confirmed by the disappearance of the tns fluorescence peak. Based on Curve b, it should be inferred that β-LG changed its conformation (unfolded) largely above 70 °C and that the change had a transition
midpoint near 60 °C. It has been reported that all helical conformations and up to a fifth of β sheets melt above 60 °C \cite{20}. Although tns binding could affect fold stability, tns located in the central calyx formed by the β-barrel dropped from the hydrophobic domain, suggesting that part of the β-sheet structures were significantly destroyed. Thus, tns fluorescence could be utilized to discern a large β-fold change in β-LG.

![Graph](https://mc06.manuscriptcentral.com/cjc-pubs)

**Fig. 7** Fluorescence intensity change of tns (a) and β-LG (b) over temperature in 0.01M phosphate buffer, pH 7.0. Experimental conditions: β-LG 10 µM and tns 10 µM.

3.6 *Tns binding effect on the stability of β-LG.*

The maximum fluorescence emission of β-LG, located near 340 nm due to the Try19 residue embedded inside the hydrophobic barrel, can be used as a signal to detect unfolding of the protein. In the absence and presence of tns, increases in guanidine hydrochloride (GuHCl) concentration led the fluorescence peaks of β-LG to gradually shift towards longer wavelengths and finally reach 351 nm due to the exposure of tryptophan residue to water; this result suggested the unfolding of β-LG (Fig. 8). Compared with β-LG, tns binding stabilized the protein fold with a midpoint transition at a guanidine hydrochloride concentration of approximately 3.9 M. In the absence of tns,
this transition midpoint occurred at a guanidine hydrochloride concentration of approximately 2.3 M. This may also be a supplemental indication that it is more likely for tns binding to occur inside the protein than on the surface, such that the binding enhances the β-barrel fold.

![Graph](image)

**Fig. 8** Effect of GuHCl concentration on the $\lambda_{\text{max}}$ of β-LG tryptophan fluorescence emission in the absence (a) and presence (b) of tns. Both protein and tns concentration were 10.0 µM in phosphate buffer at pH 7.0

3.7 *Sds (or sdbs) competitive binding to β-LG with tns*

Sodium dodecyl sulfate (sds) is located in the central calyx of β-LG under neutral conditions, according to a previous report[22]. To further discern the tns binding site on the protein, β-LG was added to mixtures containing sds and tns (Fig. 9). The binding of tns to β-LG decreased obviously in the presence of sds, as observed by its weakening fluorescence intensity. Low concentrations of sds had little effect on fluorescence intensity of the molecule, which suggests that sds competed to bind to β-LG by occupying some of the calyx.

Assuming that the decreases in fluorescence quench at any given [TNS]/[β-LG] is
attributed to sds binding to β-LG, the binding constant of β-LG-sds can be determined.

The reaction occurred with (6.1):

\[
P + nSDS \leftrightarrow SDS_nP
\]  

(6.1)

The following formulas (6.2-6.4) can be given

\[
\frac{[A]'}{[P]'} = \frac{1}{[P]} \frac{+ n}{K'}
\]

(6.2)

\[
[P] = \frac{F_{(b,c)} - F_0}{F_{\text{max}} - F_0} [P]'
\]

(6.3)

Where \([P]_b\), \([P]_b'\) and \([P]_f\) are the bound concentrations of β-LG to tns, sds and free protein, respectively. \([A]_t\) represents the total concentration of sds. \(F(b, c)\) represents the fluorescence intensity of tns when sds is present. \(F_{\text{max}}\) is identical to that in equation in (1.2). \(K'\) is the binding constant of β-LG to sds. Because the binding constant and site number of tns to β-LG are known, the free concentration of β-LG([P]_f ) can be determined.

Fig. 9 Titration curve of tns (a), the mixture of tns and sds with the ratio of 1:1 (b) and the mixture with the ratio of 2:1 (c), in 0.01M phosphate buffer, pH 7.0. Experimental conditions: β-LG 10 µM and tns 10 µM.

Fig.10 Titration curve of tns (a), the mixture of tns and sds with the ratio of 1:1 (b) and the mixture with the ratio of 2:1 (c), in 0.01M phosphate buffer, pH 7.0. Experimental conditions: β-LG 10 µM and tns 10 µM.
According to mass balance equations for protein, tns and sds and results of part (3.1)
\[
[P]_b = [P]_i - [P]_b - [P]_f
\]  
(6.4)

Using equation (6.2) the binding constant of complex β-LG-sds could be calculated as 
\((5.88\pm0.07)\times10^6 \text{ M}^{-1}\) and \(n\) as 1.06 (Fig. 11A). This result is consistent with data 
\((K_d=(0.23\pm0.03) \mu\text{M} \text{ or } K=4.35\pm0.07)\times10^6 \text{ M}^{-1}\) published by Lamiot et al.\textsuperscript{[23]}

The mixtures containing sodium dodecyl benzene sulphonate (s dbs) and tns titrating to the β-LG are shown in Fig 10 b, c. Tns binding to β-LG also decreased in the presence of s dbs, and a binding constant of \((4.81\pm0.01)\times10^6 \text{ M}^{-1}\) and binding sites of 0.97 were calculated (Fig. 11B).

![Graphs A and B](https://mc06.manuscriptcentral.com/cjc-pubs)

**Fig.11 Plot of \([A]_t'/[P]_b\) versus 1/\([P]_f\) from sds(A) and sdbs(B) to β-LG**

**4. Conclusions**

We report a detailed study of the interaction of tns with β-LG, as investigated using spectroscopic methods and autodock research. Tns probably binds to the central hydrophobic cavity of β-LG constituted by β-sheets and forms a 1:1 complex. The β-LG-tns complex can be formed over a wide range of pH levels. Binding tns stabilizes the secondary structure of β-LG. With sensitive fluorescence signal and complex stability, tns could be utilized as a reference to detect the binding of other small ligands to β-LG. Because the β-sheet of the protein should be largely destroyed once the complex
dissociates, tns could also be used to survey β-sheet changes in β-LG.

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**References**


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