Surface Hydrophobicity of a Low Molecular Weight Basic Trypsin Subtilisin Inhibitor from Marine Turtle Eggwhite

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Surface hydrophobicity has recently been emphasized as an important parameter for functional correlation of proteins. However, evaluations of the parameter by different experimental techniques often do not correlate well with each other. In this paper we have compared surface hydrophobicity of a basic protein with those of $\beta$-lactoglobulin, ovalbumin and lysozyme by fluorescence probe method using ANS as an external probe. Two different fluorimetric approaches to determining the surface hydrophobicity parameter, namely, the slope method and the binding parameter method, follow the same relative order. Denaturants, urea, and guanidine hydrochloride disrupted the hydrophobic clefts of the inhibitor on the surface, causing a drastic reduction of surface hydrophobicity.

Proteins contain both hydrophilic and hydrophobic side-chain residues. Because of the specific folding pattern of each protein, some hydrophobic residues may be buried in the interior while others may be exposed at the surface or in crevices. Hydrophobic interactions in proteins play a major role in dictating conformation, solubility, ligand binding, aggregating properties, etc. Extensive studies have been done to understand the relationship between protein hydrophobicity and physico-chemical properties (1-4). In this regard, many different hydrophobicity scales for proteins have been proposed by various workers (4-8). However, these early studies computed protein hydrophobicity from the sum of the side-chain hydrophobicities of constituent amino acids and hence reflected the total hydrophobicity. Since a significant part of the buried hydrophobic groups may not be felt by the constituents of the protein surroundings, a more meaningful parameter, called effective hydrophobicity or surface hydrophobicity (SH), has been proposed in order to obtain better correlation with protein functionality (9-12).

Various methods have been proposed for experimental determination of protein hydrophobicity, including binding of hydrocarbons to proteins (13), reverse phase chromatography (14), and fluorescent probe method (15). However, for any set of proteins, the relative orders of SH determined by different methods are often not same. The fluorescent probe method is widely applied for rapid, routine determinations of SH, and 1-anilino-8-naphthalene sulfonate (ANS), which is almost nonfluorescent in aqueous solution but becomes fluorescent on binding to hydrophobic sites, is a popular external fluorescent probe (12) for this purpose.

We have recently isolated and purified a protein proteinase inhibitor, named basic trypsin subtilisin inhibitor (BTSI), of molecular weight 13,800 from the eggwhite of marine turtle (Caretta caretta Linn) (16). The protein has two domains, one of which is responsible for the inhibition of trypsin while the other inhibits subtilisin. The amino acid composition (16) and sequence (17) of the protein have been determined and various physico-chemical properties have been studied (13). In this paper we report comparison of SH of BTSI with some standard proteins, namely, $\beta$-lactoglobulin ($\beta$-lg), ovalbumin, and lysozyme, determined by three different fluorimetric approaches, employing ANS as external probe in all cases. The effect of denaturants on the exposure of buried hydrophobic sites of BTSI and nature of sites are reported.

MATERIALS AND METHODS

Chemicals—BTSI was isolated and purified from marine turtle eggwhite in this laboratory according to Sil et al. (16). $\beta$-lg was isolated and purified from cow’s milk according to Aschaffenberg and Drewry (18). Lysozyme and ovalbumin were from Sigma Chemical Company. ANS, urea, and Gu-HCl were also from Sigma. All other chemicals used for preparing buffers were of analytical grade. Solutions were prepared using milli-Q water.

Protein and ANS Estimations—Concentrations of ANS and protein were determined spectrophotometrically using the molar extinction coefficients (e). For ANS $e_{295} = 5,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ was used (19). Protein concentrations were determined using $e_{280}$ of 36,000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for lysozyme (20), 24,400 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for ovalbumin (21), 7,990 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for BTSI (16), and $e_{280}$ of 17,200 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for $\beta$-lg (22). All spectrophotometric measurements were done using a Beckman Model 35 spectrophotometer.

Fluorescence Measurements—SH of BTSI and of lysozyme, ovalbumin, and $\beta$-lg was determined using ANS probe in a Perkin-Elmer Model MPF 44B spectrofluorimeter at 25°C using a 1-cm pathlength cell. ANS-protein binding parameters, energy transfer efficiency between ANS and tyrosine or tryptophan and effects of denaturants on micropolarity of ANS binding sites were studied in a...
Hitachi-3010 spectrofluorimeter using a 1-cm pathlength cell at 25°C. Details of instrument parameter settings and experimental procedure are given in the captions of the figures.

RESULTS AND DISCUSSION

Fluorescence Emission Spectra—Fluorescence emission spectra of protein-ANS conjugate for BTSI along with the standard proteins β-lactoglobulin, ovalbumin, and lysozyme are shown in Fig. 1. BTSI has an emission maximum at 472 nm while β-lg, ova, and lysozyme have λmax at 477, 470, and 466 nm respectively. For the same concentration of protein and ANS, β-lg show maximum intensity followed by ova, lysozyme, and BTSI in order.

Surface Hydrophobicity of BTSI—Fluorescence intensity of ANS-BTSI conjugate increased with increasing concentration of BTSI for a given concentration of ANS. Kato and Nakai (9) expressed SH of protein in terms of the initial slope of the intensity vs. protein concentration plot. Since fluorescence intensity is measured in an arbitrary unit, initial slope determined from data of various instruments may not be comparable. So we have defined the quantity "relative fluorescence" (Fr) as

\[ F_r = \frac{F - F_0}{F_0} \]

where F is the fluorescence intensity of the protein-ANS conjugate and F0 that of ANS alone. Figure 2 shows the plot of relative fluorescence of BTSI-ANS system as a function of BTSI concentration. Surface hydrophobicity has been calculated as 1.14 from the initial slope of the regression line in Fig. 2. Since ANS is a relatively bulky molecule, it is unlikely to penetrate into the buried hydrophobic groups of BTSI and thus it reflects outer "surface hydrophobicity." We have compared the surface hydrophobicity of BTSI with those of some standard globular proteins, namely, β-lg, ovalbumin, and lysozyme, determined in the same way as BTSI. The values are reported in Table I. Relative

**Table I.** Surface hydrophobicity, protein-ANS binding parameters, and percentage of energy transfer tyrosine or tryptophan to ANS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>n</th>
<th>Kc (M)</th>
<th>SH</th>
<th>Td</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTSI</td>
<td>0.6</td>
<td>2.65 x 10^-4</td>
<td>1.14</td>
<td>21.2</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2.98</td>
<td>1.35 x 10^-4</td>
<td>2.26</td>
<td>26.2</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.9</td>
<td>1.40 x 10^-4</td>
<td>12.6</td>
<td>23.5</td>
</tr>
<tr>
<td>β-lg</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Excitation and emission wavelengths for fluorescence measurements for all proteins were 370 and 470 nm, respectively, and the excitation and emission band passes were 5 nm each. Tyrosine for BTSI and tryptophan for ovalbumin and lysozyme. n is the number of ANS binding sites per molecule of protein. Td is the percentage of energy transfer from tyrosine/tryptophan to ANS. ND = not determined.

![Fig. 1. Fluorescence emission spectra of protein-ANS conjugate.](image)

![Fig. 2. Plot of relative fluorescence vs. concentration of BTSI.](image)

![Fig. 3. Spectrophotometric titration of BTSI with ANS. ΔF is the fluorescence intensity of the ANS-BTSI conjugate minus that of ANS at same concentration as in ANS-BTSI conjugate. To 2.0 ml of 13.1 μM BTSI in 0.05 M phosphate buffer, pH 8.0, aliquots of 0.20 mg/ml ANS in the same 0.05 M phosphate buffer were added and the solutions were incubated for 5 min. After each addition before reading in Hitachi 3010 fluorimeter. Excitation and emission wavelength of 370 and 470 nm with 5 nm each for excitation and emission band pass.](image)

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Fig. 4. (a) Plot of inverse of fluorescence intensity ($F$) vs. inverse of total ANS concentration ($I_o$). The BTSI concentration was 13.1 $\mu$M and ANS concentration was varied from 0-50 $\mu$M. From the least square regression according to Eq. 4, the value of $F_{\text{max}}$ was calculated from the intercept to be 3.98 and the $K_c$ value was calculated from the slope and the intercept to be $2.52 \times 10^{-4}$ M. (b) Plot of $P_0$ \((1-F/F_{\text{max}})\) vs. $L_o$ \((F_{\text{max}}/F-1)\), where $P_0$ is total protein concentration, $L_o$ is total ANS concentration. $F_{\text{max}}$ was obtained from (a) and other data are from Fig. 3. The values of $n$ and $K_c$ were obtained from the slope and the intercept using least square regression according to Eq. 2.

Fig. 5. Dependence of percentage of energy transferred from tyrosine residues of BTSI to ANS on BTSI concentration. Cuvette contained 2.0 ml 13.1 $\mu$M BTSI solution in 0.05 M phosphate buffer, pH 8.0, plus 20 $\mu$l of 0.4 mg/ml ANS in phosphate buffer, pH 8.0, for measurement of $F$. For measurement of $F_o$, ANS was replaced by buffer. Solutions were excited at 280 nm and emission measured at 310 nm with 5 nm band pass for both excitation and emission.

Fig. 6. Effect of denaturants on surface hydrophobicity. (a) The plot of surface hydrophobicity vs. concentration of guanidine hydrochloride. (b) The plot of surface hydrophobicity vs. concentration of urea. For both cases, excitation wavelength was 370 nm and emission wavelength was 470 nm with a band pass of 5 nm. Readings were corrected for blank contributions from urea and Gu-HCl at each concentration.

The magnitudes of surface hydrophobicity of $\beta$-lg, ovalbumin, and lysozyme are similar to those reported by other workers (9, 10). While $\beta$-lg is classified as a protein having high SH, ovalbumin, and lysozyme belong to the class of low SH proteins. Clearly, BTSI is a member of the latter class, with lowest SH among the four proteins.

**ANS-BTSI Binding Parameters**—It has recently been proposed that binding parameters, i.e., the number of binding sites and the binding constant, may be used as a measure of the relative SH of proteins (12). The interaction of ANS with BTSI has been studied in some detail. Fluorescence intensity of ANS-protein conjugate depends upon the number of ANS binding sites as well as the quantum yield. Binding of ANS to BTSI has been studied by fluorimetric titration of BTSI with ANS, and the titration data are shown in Fig. 3. Fluorescence intensity increases almost linearly up to about 20 $\mu$M ANS concentration, above which it slowly tends to level off. Scatchard analysis of the data leads to complex nonlinear curves, and hence the alternative approach of Cogan et al. (23) was employed. According to Cogan et al. (23),

\[ P_0\alpha = \frac{1}{n} [L_o\alpha/(1-\alpha) - K_o]. \]  

Here, $P_0$ is total protein concentration, $L_o$ is total ANS concentration, $n$ is the number of binding sites, $K_o$ is the dissociation constant of protein-ANS conjugate, and $\alpha$ is the fraction of binding sites remaining free. This can be written as:

\[ \alpha = (F_{\text{max}} - F)/F_{\text{max}}, \]

where $F_{\text{max}}$ is fluorescence intensity when all protein molecules are saturated by ANS. Since it was not possible experimentally to reach a plateau of fluorescence intensity by increasing the concentration of BTSI (vide Fig. 4), and since BTSI tends to aggregate at higher concentration, the method of Wang and Edelman (24) was used to obtain $F_{\text{max}}$. According to Wang and Edelman (24), for varying ligand concentration,
The unfolding of a protein can change its SH. We have therefore studied the SH of BTSI as a function of increasing concentration of denaturant Gu-HCl and urea. The results are presented in Fig. 6. In the presence of Gu-HCl, SH increased up to a Gu-HCl concentration of 0.5 M and then decreased, falling below its value in the absence of denaturant at 6 M Gu-HCl. A similar trend was observed in presence of urea, where a maximum was observed at 2 M urea and minimum at 8 M urea. The initial rise in SH with increase of denaturant concentration can be ascribed to the availability of newly exposed sites on the surface of BTSI. The subsequent decrease in SH could be due to two reasons: (i) aggregation of BTSI due to extensive unfolding and exposure of hydrophobic groups; (ii) destruction of the ANS holding capacity of the hydrophobic pockets due to unfolding. The average hydrophobicity of BTSI calculated according to Bigelow (5) based on the amino-acid composition of BTSI is around 1,100 cal/residue and with a molecular weight of BTSI of about 13,500, this protein lies close to the class of associating proteins. However urea and Gu-HCl are known to be good solubilizers as well as being denaturants, and aggregation in the presence of a high concentration of urea or Gu-HCl is less likely (25-27). In fact, no aggregation of BTSI in 8 M urea or 6 M Gu-HCl could be detected by the fluorimetric method of Zettlmeissl et al. (28), where aggregation would have resulted in an enhanced emission at 320 and 400 nm following excitation at the same wavelength.

Disruption of the ANS binding hydrophobic pockets (clefts) should change the microenvironment polarity of bound ANS, which should be accompanied by a shift in the ANS emission maximum (λmax). Indeed an appreciable change in the λmax was observed with increase in the concentration of urea or Gu-HCl. In 0.4 M Gu-HCl, λmax is close to 470 nm. On increasing the Gu-HCl concentration to 6 M, λmax shifted to 484.5 nm. This red shift is indicative of the increased polarity of the ANS microenvironment (29, 30).

Fluorescence quantum yield of ANS is known to be reduced as its environment polarity is increased (19, 20). This, as well as reduction of binding affinity (i.e., higher Kd) of ANS to hydrophobic sites of BTSI, accounts for lower fluorescence and hence SH.

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REFERENCES

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