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A TIRF Titration Study of 1-Anilinonaphthalene-8-sulfonate Binding to Silica-Adsorbed Bovine Serum Albumin

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ABSTRACT

A novel fluorescence titration method was applied to the qualitative study of conformational characteristics of a surface-adsorbed bovine serum albumin (BSA) layer. The probe, 1-anilinonaphthalene-8-sulfonate (ANS), was used as a fluorescent ligand. The selective excitation of a bound dye in the adsorbed protein layer was achieved using the evanescent surface wave created by total internal reflection at the solid/liquid interface. The same protein/ligand pair dissolved in the bulk solution was used as a reference. The apparent affinity of the ligand towards the surface-adsorbed protein is lower as compared with the dissolved protein. The results of the solution and the surface titration experiments were quantitatively compared after the titration results were normalized with respect to the intrinsic BSA fluorescence in the absence of the ligand. It was found that the protein adsorption leads to fluorescence enhancement of those bound ANS molecules which are otherwise nonfluorescent when bound to BSA in the solution. It is shown that ANS molecules, which are bound to the outer binding sites on the protein surface, can serve as a probe of protein–protein and protein–surface contacts in the adsorbed layer.

INTRODUCTION

The adsorption of proteins at solid/liquid interfaces attracts considerable interest [1,2]. In the field of biomedical engineering protein adsorption from body fluids onto surfaces remains a major concern [1,3]. Interactions between proteins and man-made surfaces are particularly important in the field of material–blood compatibility [4]. It is generally accepted that adsorption onto a solid surface can induce a conformational change of the protein and, consequently, an undesirable biological response may be started. The conformational change of a protein during adsorption is the most elusive part of the adsorption process because the structure of adsorbed protein cannot be determined directly. Consequently, the extent of the conformational change of a protein is often inferred by indirect methods. Physico-chemical events at in-

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terfaces, like adsorption, can be studied by different spectroscopic techniques using total internal reflection (TIR) [1,5,6]. A TIR counterpart of fluorescence spectroscopy, called total internal reflection fluorescence (TIRF), is a particularly suitable technique for the study of protein adsorption [1,4,7].

Many methods of fluorescence spectroscopy are successfully applied to the study of proteins in solution and in membranes [8,9]. A typical example is a fluorescence titration method which is often used to determine the number of fluorescent protein-bound ligands and the respective binding constants. In this paper a new TIRF titration method is applied to the study of ligand binding to protein which is adsorbed at the solid/liquid interface. The binding affinity of an adsorbed protein for a particular ligand may not be the same as the corresponding affinity in the bulk solution. In such a case, changes of binding affinity and number of ligands bound to the adsorbed protein might be used to examine the conformational protein change using the solution environment as a reference. In a preceding publication the fluorescence emission spectra of adsorbed bovine serum albumin (BSA) and BSA-bound 1-anilinonaphthalene-8-sulfonate (ANS) were obtained using the TIRF method [10]. It was found that the change of BSA conformation as a result of adsorption involves the microenvironment of tryptophan residues, as well as the ANS binding sites, respectively. Here, we extend the previous study by titrating irreversibly adsorbed BSA with ANS. The fluorescence quantum yield of ANS in aqueous solutions is very low but increases strongly upon binding to a hydrophobic binding site [11–14]. This characteristic of ANS is particularly suitable for the TIRF titration of surface-adsorbed protein since no fluorescence contribution from the dissolved ANS molecules is to be expected. The binding of ANS to BSA in solution can be quantitated by measuring the fluorescence of fully bound ANS in the presence of an excess of BSA. It has been found that eight to ten molecules of ANS can bind to one BSA molecule but that binding to a limited number of inner, more hydrophobic binding sites leads to the enhancement of ANS fluorescence [15].

MATERIALS AND METHODS

BSA (monomer standard protein powder, Miles Laboratories) and ANS (bis-ANS free, Molecular Probes) were used without further purification. Other chemicals were analytical grade. Only freshly prepared solutions were used. Acetate buffer solutions (0.01 M acetate, pH 5.0) were used throughout the experiments. The concentrations of ANS and BSA stock solutions were determined from the solution absorbances and the respective extinction coefficients (BSA; $4.4 \cdot 10^4\ M^{-1}\ cm^{-1}$ at 279 nm, ANS; $6.3 \cdot 10^3\ M^{-1}\ cm^{-1}$ at 350 nm [15]). The fluorescence titration experiments were performed using the custom-built TIRF apparatus described earlier [7,16]. In this study a TIRF cell was made of a quartz hemi-cylinder which was optically coupled with a rectangular flu-

orescence cell made of fused silica [10]. The cell was closed with a stopper which had two flow lines for removing and adding solution from and to the cell. One of the inner surfaces of the fluorescence cell was used as the adsorbing surface [10]. The adsorbed molecules were selectively excited with an evanescent surface wave which was created by a totally reflected ultraviolet light beam at the cell wall/solution interface. 4 nm excitation halfwidths and 16 nm emission halfwidths were used in the case of surface titrations (8 nm halfwidths in the case of the solution titration). The incident light was perpendicularly polarized with respect to the plane of incidence. No polarizer was used in the emission light path. Fluorescence emission was collected normal to the interface through the quartz hemi-cylinder. All experiments were done at room temperature.

The experimental procedure was as follows: all inner surfaces of the TIRF cell were rigorously cleaned prior to each experiment [17]. The TIRF cell was primed with BSA buffer solution which was left to contact the inner cell surfaces for one hour. Three different BSA concentrations were used: $7 \cdot 10^{-7}$, $1.4 \cdot 10^{-6}$ and $1.4 \cdot 10^{-5}$ *M*, respectively. Nonadsorbed BSA was flushed out of the cell with 40 ml of buffer solution without allowing air to enter the cell. Following the flush-out, the interface was illuminated by the evanescent surface wave ($\lambda_{ex}=360$ nm), the ANS concentration in the cell was increased by sequential injection of ANS solution and the fluorescence was recorded ($\lambda_{em}=470$ nm). After each addition a 15 min equilibration was allowed while, at the same time, the incident light was blocked by a shutter in order to prevent overexposure of the bound dye. The surface cleanliness was a major factor influencing eventual displacement of adsorbed BSA by ANS. When clean surfaces were used no displacement of BSA was detected in the range of ANS concentrations used. The titrations were also checked for reversibility; as a rule, very small or no titration hysteresis was detected when the ANS sample was not contaminated with impurities. Preliminary experiments showed that some impurity in the ANS sample caused a strong hysteresis of the titration curve. In such a case the probe bound to adsorbed BSA fluoresced with a red-shifted emission maximum, $\lambda_{em}=495$ nm, as compared with the pure ANS sample (bulk solution BSA–ANS $\lambda_{em}=465$ nm, adsorbed BSA–ANS $\lambda_{em}=475$ nm, respectively [10]), and the TIRF excitation spectra was similar to the spectra of BSA-bound ANS dimer, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) ($\lambda_{ex}=405$ nm with a shoulder at 370 nm [18]). Bis-ANS is known to have a much stronger affinity towards BSA than ANS [18].

The blank titration experiments without protein showed no enhancement of ANS fluorescence due to its binding at the silica/buffer interface. The surface concentrations of irreversibly adsorbed BSA were determined by using I^{125}-labeled BSA in separate experiments [7]. The fluorescence emission efficiency of adsorbed BSA (relative to the fluorescence quantum yield of BSA in the bulk solution) was determined by quantitative TIRF experiments in which a 5-hydroxytryptophan methyl ester solution was used as an external

fluorescence standard [7]. Titration of BSA with ANS in the bulk solution was performed using a front face geometry and the same fluorescence apparatus as the one used in TIRF titrations ($\lambda_{ex}=360$ nm, $\lambda_{em}=470$ nm). The binding of ANS to the BSA molecule in the bulk solution was quantitated by measuring the fluorescence of fully bound ANS in the presence of an excess of BSA ($1.4 \cdot 10^{-4}$ M) [14]. A similar procedure was not applied to the surface titration of adsorbed BSA since the BSA surface concentration could not be increased at will. In order to circumvent this experimental difficulty and to compare the surface and the solution titration results, a second set of titration experiments was performed using the intrinsic fluorescence of BSA as a normalization factor. The intrinsic fluorescence of BSA was measured ($\lambda_{ex}=285$ nm, $\lambda_{em}=340$ nm) in the absence of ANS, after which the titration experiment was executed by exciting at 285 nm with emission monitored at $\lambda_{em}=475$ nm. The same procedure was also applied to the titration of BSA in the bulk solution. The surface and the solution titration results were both normalized with respect to the intrinsic BSA fluorescence measured in the absence of the ligand so that both sets of results could be compared with each other.

RESULTS AND DISCUSSION

Table 1 shows the surface concentration of irreversibly adsorbed BSA at the silica/buffer interface and the fluorescence emission efficiency of adsorbed BSA (relative to the quantum yield of BSA in the bulk solution) as determined by measuring the adsorption of I^{125}-labeled BSA in combination with the quantitative TIRF experiments [7]. It was found that the increase of solution BSA

TABLE 1

Comparison of the surface and solution parameters which are relevant to the fluorescence titration of BSA with ANS. The silica surface BSA concentration as determined by I^{125}-labeled BSA adsorption from the $7 \cdot 10^{-7}$, $1.4 \cdot 10^{-6}$ and $1.4 \cdot 10^{-5}$ M BSA solutions, respectively [7]. The adsorbed BSA fluorescence efficiency relative to the fluorescence quantum yield of BSA in the bulk solution as calculated from the surface BSA concentrations and the quantitative TIRF adsorption measurements [7]. The apparent dissociation constant (in $pK_{0.5} = -\log K_{0.5}$) determined from Fig. 1 as [ANS] at half of maximum fluorescence intensity

	Silica surface			Solution
BSA concentration (mg m^{-2})	1.32	1.64	2.1	$3 \cdot 10^{-6}$ (M)
Adsorbed BSA fluorescence efficiency (relative to BSA in the bulk solution)	0.84	0.78	0.74	1.0
Apparent dissociation constant (in $pK_{0.5} = -\log K_{0.5}$)	5.0	5.0	4.45	5.8

concentration leads to a higher amount of adsorbed BSA, which, nevertheless fluoresces with a lower fluorescence efficiency.

Figure 1 shows the results of the first set of the fluorescence titration experiments in which λ_{ex} was 360 nm and no normalization with respect to the intrinsic BSA fluorescence was used. The comparison between the binding curves shows that the apparent dissociation constants of ANS from BSA, $K_{0.5}$, (defined as [ANS] at the half of maximum fluorescence intensity), were larger for the surface-adsorbed BSA than for the dissolved protein. The values of $pK_{0.5}$, ($pK_{0.5} = -\log K_{0.5}$) (Table 1) indicate that the binding of ANS to surface-adsorbed BSA is weaker as compared with the binding of the same ligand to BSA in solution.

Quantitative fluorescence titration of dissolved BSA with ANS showed that approximately four molecules of ANS bind to one BSA molecule in the solution. The number of bound ANS molecules ($n=3.6$) and the dissociation constant ($pK_{0.5}=5.8$), were in good agreement with the literature [14,19]. The quantitative ANS titration of adsorbed BSA could not be performed using the same methodology which was employed in the solution studies since the surface BSA concentration was fixed by the BSA adsorption isotherm [7]. Because of that, the second set of titration experiments was designed in such a way that allowed the comparison between the solution and the surface titration results, i.e. by using. $\lambda_{ex}=285$ nm and the normalization of measured ANS fluorescence with respect to the intrinsic BSA fluorescence. The intrinsic fluorescence of the adsorbed BSA, which was used as a normalization factor, was corrected for its decreased fluorescence efficiency (see Table 1). The comparison between the surface and solution titration results is given in Fig. 2 showing the normalized fluorescence as a function of free ANS concentration. It was

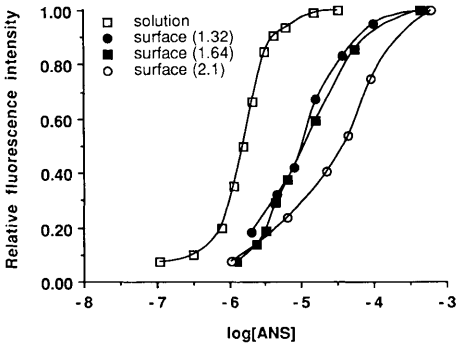

Fig. 1. The fluorescence intensity change upon the surface and the solution titrations of BSA with ANS as a function of ANS concentration in solution. The BSA concentration in the solution, $[BSA]=3\cdot10^{-6}$ M, the surface BSA concentrations at the interface as indicated (in mg m^{-2}). The fluorescence intensity maximum was set to unity for all titration experiments. $\lambda_{ex}=360$ nm, $\lambda_{em}=470$ nm.

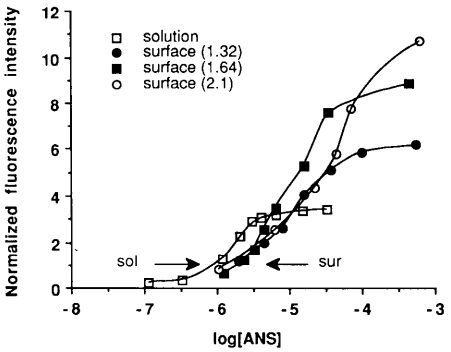

Fig. 2. The fluorescence intensity change upon the surface and the solution titrations of BSA with ANS as a function of ANS concentration in solution. The BSA concentration in the solution, $[BSA]=3\cdot10^{-6}$ M, the surface BSA concentrations at the interface as indicated (in mg m^{-2}). The fluorescence intensity was normalized with respect to the intrinsic fluorescence of BSA at 340 nm in the absence of ANS. $\lambda_{ex}=285$ nm, $\lambda_{em}=475$ nm.

assumed throughout the TIRF titration experiments that the total ANS concentrations in the TIRF cell equal the free ANS concentrations in the system. This assumption was only invalid at conditions not encountered in this work; at extremely low ANS concentration and at very high BSA surface concentration*. The normalized fluorescence intensity at the saturation binding of ANS by the solution BSA was scaled to match the number of bound ANS molecules per one BSA molecule, i.e. to 3.6, and, correspondingly the normalized fluorescence intensity of the surface titration was scaled by the same factor.

Several features in Fig. 2 which present the greatest interest are: (a) when $[ANS]<1\cdot10^{-5}$ M, the adsorbed BSA-bound ANS complex showed less fluorescence at a given ANS concentration than in the case of ANS molecules bound to the dissolved BSA, (b) when $[ANS]>1\cdot10^{-5}$ M, the fluorescence of the adsorbed BSA-bound ANS complex was still increasing with increasing ANS concentration while at the same ANS concentrations the fluorescence of ANS–BSA complex in the solution reached a plateau which indicated the saturation of approximately four ANS binding sites in BSA, and (c) binding of ANS to the three different surface-adsorbed BSA layers are shown to be almost coincident at lower ANS solution concentrations but different at higher ligand concentrations.

In addition, a common isoemissive point was found at 416 nm in the case of the titration of solution BSA and at 406 nm in the case of the adsorbed BSA (2.1 mg m^{-2}, at $[ANS]<7\cdot10^{-5}$ M), respectively (data not shown here). The

*The volume of the TIRF cell was approx. 3.5 cm^3 with 15 cm^2 surface available to BSA adsorption. In the case that $n=4$ and $[ANS]=1\cdot10^{-5}$ M, the total concentration of ANS is depleted by 0.4% by 1.65 mg m^{-2} adsorbed BSA. The free ANS concentration would be $9.96\cdot10^{-6}$ M.

existence of the isoemissivity indicates that the bound ANS molecules fluoresce with the same quantum yields as discussed earlier [14].

According to the present results (Fig. 2) the affinity of adsorbed BSA towards the first ANS molecules has decreased. It is assumed that the affinity decrease is due to a conformational change of the ANS binding sites in the adsorbed protein. This conformational change was inferred from the red-shift of fluorescence emission of adsorbed BSA–ANS complex [10]. As the free ANS concentration increases beyond the value of the saturation of ANS/BSA binding sites in solution, i.e. at $[ANS] > 1 \cdot 10^{-5}\ M$, the TIRF titration showed that additional ANS molecules bind to the adsorbed protein and that their fluorescence is enhanced. It has been found previously that eight to ten ANS molecules bind to one BSA molecule at $1 \cdot 10^{-3}\ M > [ANS] > 1 \cdot 10^{-4}\ M$ [20]. However, only four of the bound ANS molecules become strongly fluorescent upon binding to BSA in the solution while the other bound ANS molecules are "dark" emitters. It was suggested that the latter population of ANS molecules binds to the outer binding sites at the protein surface [20]. There, the bound ANS molecules are exposed to quenching by water molecules which act as a collisional quencher with a Stern–Volmer constant of $5\ M^{-1}$ [15]. The binding of ANS to BSA is shown schematically in Fig. 3. The present results show that the supramolecular organization of BSA molecules in the adsorbed layer effectively shields these outer site-bound ANS molecules from the quenching by water. The actual position of outer-site bound ANS molecules with respect to the surface of silica remains uncertain. It is uncertain whether the ANS molecules are shielded by two neighboring BSA molecules at the interface without contacting the solid surface (Fig. 3b), or are they sandwiched between the adsorbed BSA and the surface itself (Fig. 3c), or possibly both (Fig. 3d)? The first alternative alone (Fig. 3b) would imply that the BSA molecules at all three surface concentrations studied here are adsorbed in the form of densely populated protein "islands" on the silica surface which allow for a large number of lateral protein–protein contacts. It should be noted that the normalization of the surface fluorescence intensity with respect to the intrinsic BSA fluorescence is, effectively, also a normalization with respect to the amount of adsorbed protein. In this case the second alternative, i.e. that additional ANS molecules bind only between the protein and the surface (Fig. 3c), should bring to a full coincidence of the binding curves which should be independent on the BSA surface concentration. Such coincidence is found at $[ANS] < 4 \cdot 10^{-5}\ M$ for the two BSA surface concentrations; 1.32 and 2.1 mg m^{-2} (Fig. 2), respectively. The binding curve obtained at the BSA surface concentration of 1.64 mg m^{-2} differed slightly from the other two by showing a comparatively higher fluorescence intensity in the intermediate ANS concentration range. This probably reflects a low reproducibility of the supramolecular organization of BSA molecules in the adsorbed layer on the silica surface and cautions against oversimplification of the physical picture of an adsorbed protein layer. As shown

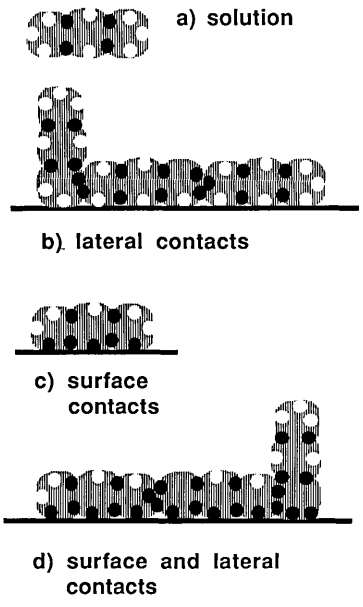

Fig. 3. Schematics of ANS binding to the dissolved and adsorbed BSA molecules at medium-to-high ANS solution concentrations: (a) only four ANS molecules emit fluorescence upon binding to the inner BSA binding sites in the solution (black circles), all other ANS molecules are bound to the outer binding sites at the BSA surface and their fluorescence is quenched by water (white circles), (b) lateral protein-protein contacts between the adsorbed protein molecules shield the ANS molecules bound to the outer protein binding sites from collisional water quenching, (c) adsorbed protein-surface contacts shield the ANS molecules bound to the outer protein binding sites from collisional water quenching, and (d) both the lateral protein-protein contacts and the adsorbed protein-surface contacts shield the outer site-bound ANS molecules.

in Fig. 2 the surface titration results become dependent on BSA surface concentration only at higher ANS concentration. The increase of fluorescence intensity followed the increase of the BSA surface concentrations at $[ANS] > 1 \cdot 10^{-4}$ *M*. Although the fluorescence quantum yield of bound ANS is not constant at these conditions as indicated by the lack of isoemissivity, this trend indicates the existence of ANS binding sites between the lateral BSA–BSA contacts and suggests that at higher BSA surface concentrations the protein molecules are organized in some sort of surface aggregates. The existence of two-dimensional aggregates of adsorbed BSA has been found by others [21]. Because the observed BSA layer was not in contact with protein in solution, any conclusion about the organization of the adsorbed layer may not be pertinent to a physical picture of the same layer during the adsorption process. Some artifacts can be also due to the extensive flush-out of nonadsorbed BSA from the TIRF cell which might cause desorption of less tightly adsorbed protein from the surface.

The excitation at 285 nm in the titration experiments caused an intrinsic BSA fluorescence emission which decreased as an increased number of ANS molecules was bound to the protein. This is due to the radiationless fluorescence energy heterotransfer from BSA tryptophan residues to bound ANS ligands. A necessary condition for this energy mechanism to be operative is extensive overlap between the emission band of the donor and the absorption band of the acceptor [8]. This condition is met by the ANS molecules bound to BSA: the ANS absorption band ($^{max}\lambda_{abs}=360$ nm) overlaps with the emission band of intrinsic protein fluorescence which is centered at $\lambda_{em}=342$ nm in the case of solution BSA, and at $\lambda_{em}=335$ nm in the case of surface-adsorbed BSA, respectively. The 285 nm excitation will excite tyrosinyl BSA residues as well. In order to avoid seeing a contribution from tyrosine fluorescence emission, the intrinsic BSA fluorescence emission decrease was monitored at 365 nm. Figure 4 shows the fractional decrease of intrinsic BSA fluorescence due to the ANS binding; the quenching of dissolved BSA and surface-adsorbed BSA fluorescence was quite different. In order to achieve the same quenching effect in the case of adsorbed BSA the concentration of ANS in solution needed to be increased almost 10-fold. Part of this effect is certainly due to the different spectral overlaps between the ANS absorption band and the BSA emission in the solution and at the surface, respectively. The characteristic distance for energy transfer between tryptophanyl BSA residues and bound ANS ligands in solution, $R_{o(solution)}$, equals 2.9 nm, as calculated from spectroscopic data according to the Förster mechanism of energy transfer [22]. The value of $R_{o(surface)}=2.7$ nm was estimated from $R_{o(solution)}$ by applying corrections for a different spectral overlap due to blue-shifted BSA fluorescence [10] and a 25% decrease in the fluorescence quantum yield of the adsorbed BSA [7]. The same average orientation of donor–acceptor pair in the solution and at the silica

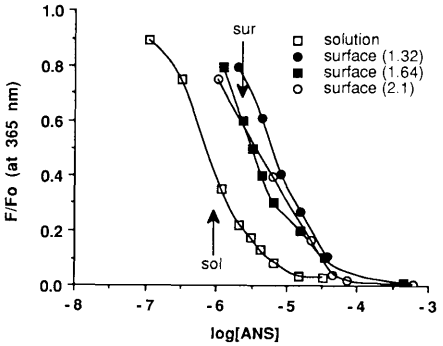

Fig. 4. The intrinsic BSA fluorescence intensity change upon the surface and the solution titrations of BSA with ANS as a function of ANS concentration in solution. The BSA concentration in the solution, $[BSA]=3\cdot10^{-6}$ M, the surface BSA concentrations at the interface as indicted (in mg m^{-2}). $\lambda_{ex}=285$ nm, $\lambda_{em}=365$ nm.

surface, and no change of the absorption spectrum of ANS when bound to adsorbed BSA were assumed, respectively. The average distance between donor and acceptor can be calculated from:

$$E = R_o^6/(R_o^6 + r^6)$$

where E equals $1 - F/F_o$ [8]. When the first ANS ligand binds to dissolved BSA, i.e. at $[ANS] = 9 \cdot 10^{-7}$ M (indicated by "sol" in Fig. 2), the average distance, $r_{(solution)}$, calculated from $F/F_o = 0.4$ (indicated by "sol" in Fig. 4), was found to be 2.7 nm. For the binding of the first ANS ligand to the surface adsorbed protein the ANS concentration has to be increased to approximately $[ANS] = 2.5 \cdot 10^{-6}$ M (indicated by "sur" in Fig. 2). In this case the F/F_o parameter amounted to approximately 0.7 (indicated by "sur" in Fig. 4) and the average distance was calculated to be, $r_{(surface)} = 3.0$ nm. As a rule, similar calculations performed at different levels of ANS fluorescence intensity always resulted in a $r_{(solution)} < r_{(surface)}$. This increase in distance between the donors and acceptors can be interpreted as due to conformational changes in BSA upon adsorption. Assuming that the four inner binding sites of BSA are occupied first in the surface titration as in the case of the solution titration, the present results point to the partial unfolding of adsorbed BSA; the average ANS binding site(s) distance from emitting tryptophan residue(s) has increased by 10% due to the adsorption of BSA.

Throughout this study the steady-state protein–ligand fluorescence was used in the analysis of particular characteristics of the adsorbed protein/dye layer. A more informative study of the adsorbed protein–ligand dynamics can be made by measuring the fluorescence lifetimes of BSA tryptophan residues and ANS ligands as a function of ANS binding. Such a study of adsorbed BSA-bound ANS fluorescence lifetime has been initiated at the Utah laboratory. The first results indicate that the overall ANS lifetime decreases at high ANS concentrations, possibly reflecting an increased fluorescence contribution from ANS molecules bound in the outer protein binding sites (P. Suci and V. Hlady, unpublished results).

CONCLUSIONS

It was found that the apparent binding affinity of surface-adsorbed BSA towards the ANS ligand is lower as compared with the solution protein (Fig. 1 and Table 1). However, only when properly corrected and normalized with respect to intrinsic protein fluorescence, the respective results of the protein fluorescence titrations in the solution and at the solid/liquid interface could be compared in a quantitative way (Fig. 2). This comparison revealed that at higher ANS concentrations the number of BSA-bound ANS ligands, as detected via their enhanced fluorescence, was considerably larger in the case of adsorbed protein than in the case of dissolved protein. In the latter case only

the four ANS molecules which bind to the inner BSA binding sites could be detected by fluorescence. According to the present results, the ANS molecules bound to the outer binding sites on protein surface were "dark" emitters in the solution but were effectively shielded from the quenching by water molecules in the case when the protein was adsorbed at the interface. The fact that this ligand population is bound on the protein surface can be used for a qualitative analysis of supramolecular organization in the adsorbed protein layer. The other difference between the protein–ligand interactions in the solution and at the solid/liquid interface was the weaker quenching of adsorbed BSA intrinsic fluorescence by the bound ANS ligands. The energy transfer calculations showed an increased distance between BSA tryptophan residues and bound ANS at the interface.

In conclusion, the new TIRF titration method showed a potential for the study of the conformational change of adsorbed proteins. The present availability of various fluorescent probes, by which one can probe many different properties of adsorbed proteins, warrants further use of this technique.

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