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Adsorption Kinetics, Conformation, and Mobility of the Growth Hormone and Lysozyme on Solid Surfaces, Studied with TIRF

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Abstract

Interactions of recombinant human growth hormone and lysozyme with solid surfaces are studied using total internal reflection fluorescence (TIRF) and monitoring the protein's intrinsic tryptophan fluorescence. The intensity, spectra, quenching, and polarization of the fluorescence emitted by the adsorbed proteins are monitored and related to adsorption kinetics, protein conformation, and fluorophore rotational mobility. To study the influence of electrostatic and hydrophobic interactions on the adsorption process, three sorbent surfaces are used which differ in charge and hydrophobicity. The chemical surface groups are silanol, methyl, and quaternary amine. Results indicate that adsorption of hGH is dominated by hydrophobic interactions. Lysozyme adsorption is strongly affected by the ionic strength. This effect is probably caused by an ionic strength dependent conformational state in solution which, in turn, influences the affinity for adsorption. Both proteins are more strongly bound to hydrophobic surfaces and this strong interaction is accompanied by a less compact conformation. Furthermore, it was seen that regardless of the characteristics of the sorbent surface, the rotational mobility of both proteins' tryptophans is largely reduced upon adsorption.

Keywords

protein adsorption; protein conformation; growth hormone; lysozyme; TIRF; fluorescence spectroscopy

INTRODUCTION

The interactions between proteins and solid substrates play an essential role in many biomedical applications such as purification methods, biosensors, and biocompatibility. It is well recognized that affinity-based separation, transduction events in biosensors, and materials biocompatibility are influenced by the structural properties of the adsorbed proteins. Furthermore, the adsorption characteristics of proteins can be highly influenced by changes in the protein structure (1, 2).

The present study is focused on the adsorption process of the recombinant human growth hormone (hGH) and lysozyme (LSZ), especially on adsorption-induced structural changes of these proteins. It has been found that structural changes of hGH have a pronounced effect on the retention behavior in chromatography (3), and for adsorbed LSZ a reduction in secondary structure (4, 5) and in enzymatic activity (6) have been reported. The results of

hGH adsorption are compared with those of the model protein LSZ which has well-characterized physical properties and adsorption behavior (1, 4, 7–9).

Apart from the obvious physiological function of hGH to regulate cell growth and differentiation (10) the hormone is known to regulate the metabolism of lipoproteins and it has been found that hGH treatment might reduce the risk of cardiovascular diseases (11). Recently, the application of hGH in the medical field has been largely broadened due to the production of an efficient secretion system for the recombinant hGH (12). LSZ is found in a variety of vertebrate cells and secretions, such as spleen, milk, tears, and egg white. Its natural function is to hydrolyze β glucosidic bonds in the proteoglycan layer of bacteria. Both proteins possess a relatively high structural stability. The rigidity of the single-chained lysozyme molecule is imposed by four disulfide bonds (13). For hGH, the two disulfide bridges stabilize its structure in addition to a core which almost exclusively consists of hydrophobic side chains (14). An interesting aspect of hGH is that the initial part of denaturation occurs through a molten globule state, which is known to affect the protein's tryptophan fluorescence (15).

A technique which has been successfully applied to gain information on proteins in the adsorbed state is total internal reflection fluorescence (TIRF) spectroscopy (16). The high sensitivity of fluorescence spectroscopy enables quantification of small amounts of adsorbed proteins in the time domain and thus allows the study of adsorption kinetics (17, 18). If fluorescence is emitted by an intrinsic fluorophore of the protein, like tryptophan, one can also monitor changes in the polarity of the local environment of the fluorophore and relate them to changes in the protein structure (19). Furthermore, fluorescence polarization measurements can be used to study orientation (20) and mobility (21) of adsorbed proteins.

In this study, TIRF spectroscopy is used to study adsorption kinetics, conformational changes, and rotational mobility of hGH and LSZ at solid surfaces. Tryptophan residues in adsorbed proteins are excited by an ultraviolet evanescent wave, created at the solid/liquid interface by a totally internally reflected beam. Protein adsorption kinetics is studied by following the fluorescence intensity in time while a protein solution flows through the cell. The adsorbed amount is quantified by calibrating the fluorescence signals using appropriate standards. The use of the intrinsic fluorophore avoids the necessity of labeling, which may exert a distinct effect on the adsorption pattern. Conformational changes in the protein structure are studied by interpreting the observed fluorescence spectra. In addition, the accessibility of the tryptophans to solutes is investigated by fluorescence quenching using trichloroethanol. As hGH contains only one tryptophan buried in the hydrophobic interior of the protein, its fluorescence is extremely sensitive to changes in the protein structure. Especially since the single hGH tryptophan appears to be involved in a hydrogen bond with another α -helix strand (14). By performing polarization measurements, information is obtained on the anisotropy of the emitted protein fluorescence which is related to the motion of the fluorophore and thus to the mobility of the adsorbed proteins.

To study the influence of electrostatic and hydrophobic interactions, proteins are adsorbed onto different quartz surfaces containing either silanol, methylsilyl, or quaternary aminopropyltrimethylsilyl (QAP) as surface groups. The choice of the surfaces provides a diversity in charge and hydrophobicity. To understand the relative contributions of the electrostatic and hydrophobic interactions the proteins are adsorbed under different conditions of ionic strength (10 vs. 150 mM).

MATERIALS AND METHODS

Surfaces

Three surfaces, clean silica, methylated silica, and silica modified with a quaternary aminopropyltrimethylsilyl, were prepared on $1 \times 2.5 \text{ cm}^2$ quartz slides. Prior to each experiment the quartz slides were cleaned by placing them in a radio frequency plasma discharge chamber (Plasmod, Tegal Corp.) for 15 min while oxygen with a pressure of 27 Pa was flowing through. Organic contaminants were oxidized by this treatment and a homogeneous oxide layer was produced with minimal surface damage (22). Two types of surface modifications were applied to obtain surfaces with hydrophobic methyl groups and positively charged quaternary amines. Methylated surfaces were produced by immersion of quartz slides for 30 min in a 0.3% (v/v) dichlorodimethylsilane (Hüls Inc.) in trichloroethylene (Aldrich) solution. After silanization the slides were immersed for 5 min in trichloroethylene and rinsed consecutively with ethanol and water and dried in a jet of nitrogen gas. The aminopropylsilyl groups were produced following a method described by Lin and Hlady (18). A silica slide was immersed in a 0.2% (v/v) isocyanatopropyltrimethylchlorosilane (Silar Lab. Inc.) in trichloroethylene for 1 h. After rinsing the slide with trichloroethylene, ethanol, and water, an aminopropyltrimethylsilyl surface was obtained by isocyanato moiety hydrolysis at pH 3.8 (pH adjusted by means of HCl) at 50°C for 0.5 h. The amine groups were subsequently converted into quaternary amines by immersing the slides in a 5% (v/v) solution of iodomethane (EM Science) in ethanol for 18 h. Although unreacted silanol groups can compensate the positive charge of the amines, surface characterization of this chemistry indicated a positive net charge density (18). The surfaces were characterized on their hydrophobicity by measuring the contact angle of a sessile drop of water. The resulting contact angles are given in Table 1.

Proteins and Chemicals

The recombinant hGH was kindly donated by Pharmacia & Upjohn. Each hGH sample contained 13.8 mg recombinant hGH, 2.3 mg glycine, and 14.0 mg mannitol. Hen egg white lysozyme was obtained from Sigma and used without further purification. Some of the physical properties of the proteins are listed in Table 2. Water was purified by double distillation. All experiments were performed at pH 7 by using 10 mM phosphate buffers (prepared from sodium phosphate and adjusted to pH 7 by means of NaOH). A higher ionic strength of 150 mM was obtained by adding NaCl. The proteins were dissolved in these buffer solutions and filtered through a pre-washed 0.2 μm pore filter (Acro-disc, Gelman Sci.) and stored at -20°C in amounts needed for a single experiment. Protein concentrations were determined using the extinction coefficients $E_{277} = 0.82 \text{ cm}^2 \text{ mg}^{-1}$ (15) and $E_{281.5} = 2.64 \text{ cm}^2 \text{ mg}^{-1}$ (24) for hGH and LSZ, respectively. Experiments with LSZ were performed with protein concentrations of 0.05 mg ml^{-1} while for hGH protein concentrations of 0.11 mg ml^{-1} were used. The hGH solutions contained the excipients glycine and mannitol with final concentrations of 0.3 and 0.6 mM, respectively. The purity of hGH was investigated using SDS-PAGE (Gradient 8–25 Phastgel, Pharmacia) and staining with Coomassie Blue. It has been reported that at higher hGH concentrations some aggregation might occur (3) similar, but to a lesser extent, to the pronounced self-association observed for the bovine growth hormone (25). However, for the hGH solutions used in this study only one single band with a molecular mass of 22 kDa was observed. Calibration of the TIRF instrumental sensitivity and final alignment were performed using solutions of L-5-hydroxytryptophan-HCl (TRP) (Cal-biochem) in 150 mM buffer. The fluorescence of the proteins was quenched using trichloroethanol (Aldrich) dissolved into the respective buffer solutions.

TIRF Spectroscopy

The basic principle of TIRF spectroscopy is the excitation of fluorophores by an exponentially decaying evanescent wave formed at an interface and the subsequent detection of emitted fluorescence. The evanescent wave is created by a totally internally reflected light beam. The schematic of the TIRF apparatus is given in Fig. 1. The excitation light source is a 300 W high pressure xenon lamp (ILC technology, R300-3). The wavelength of the excitation beam is selected by means of a monochromator (0.1 m, f/4.2, ISA Inc., H10 1200 UV) using 1 mm slits. The light is collimated by a lens (L1, focal length (f.l.) 8 cm) and passed through a polarizer. The beam, polarized vertically with respect to the incident plane, is directed normal to the face of the 70°-cut dovetail fused silica prism and focused (L2, f.l. 10 cm) on the solid/liquid interface at which the beam was totally reflected. A quartz slide is optically coupled to the prism using glycerol. A silicon rubber gasket separates the slide from a black-anodized aluminum support creating a space (0.5 mm) which can be filled with solutions. Solutions can enter and leave the cell through two rectangular holes in the aluminum support and in this way enable approximately a laminar flow of solutions along the slides. Solutions are injected into the cell using syringes, and the flow rate is controlled by a syringe pump (Sage Inst.). The fluorescence emitted from the interface is collected through the prism and collimated by a lens (L3, f.l. 3 cm) with a diameter of 2 cm. The collimated fluorescence is focused (L4, f.l. 9 cm) on the 1 mm slit of the emission monochromator (0.64 m, f/5.2, ISA Inc., HR-640) and subsequently detected by a cooled photomultiplier tube (Hamamatsu, R928). The photomultiplier output is fed into a photon counter (Stanford Research, SR 400). Both the photon counter and the emission monochromator are controlled by a PC for fluorescence emission measurements and data acquisition.

For fluorescence anisotropy measurements a polarizer is placed in the emission path (Fig. 1). Under the experimental conditions, the lens L3 collects fluorescence within an angle of 8° to the *z*-axis. This ensures that the fraction of fluorescence emitted by fluorophores with a transition dipole moment oriented parallel to the laboratory *z*-axis is negligible, making the fluorescence collection geometry suitable for anisotropy measurements (21). The polarization of the two polarizers, as determined by measuring light scattering from a 2 × 10⁻³% (w/w) polyethylenimine (average MW 750,000) solution in water, was larger than 99%. For polarization measurements the detected intensities in *x*- and *y*- directions also depend on the sensitivity of the emission monochromator for both polarizations. This sensitivity, characterized by the so-called *G*-factor equals the ratio of the sensitivity for vertically (*y*) and horizontally (*x*) polarized light (26). The *G*-factor amounted to 1.20 and was independent of the emission wavelengths used in this study.

Calibration of TIRF

Total internal reflection of excitation light at the solid/liquid interface creates an evanescent surface wave. The inset in Fig. 1 shows the coordinate system in which the excitation beam is reflected at the interface between the silica slide with a higher optical density, *n*₁, and the liquid, *n*₂. The electric field amplitude of the evanescent wave, *E*, decreases exponentially in solution (*z*-direction) (27):

$$E = E_0 e^{-z/d} \quad [1]$$

The characteristic penetration depth, *d*, is a function of the angle of incidence, *θ*, wavelength, *λ*, and refractive indices of the solid and liquid materials:

$$d = \lambda / 2\pi(n_1^2 \sin^2 \theta - n_2^2)^{1/2}. \quad [2]$$

At given excitation and emission wavelengths, the fluorescence intensity emitted by molecules in the evanescent wave region is proportional to the probability of absorption, characterized by its extinction coefficient, ε , the quantum yield, ϕ , and the concentration of molecules, c , times the intensity of the evanescent wave, I . The concentration and intensity are both a function of the distance from the interface into the cell. The observed fluorescence signal, S , can therefore be written as

$$S = f \varepsilon \phi \int_0^\infty c(z) I(z) dz, \quad [3]$$

in which f is an instrumental factor characterized by the efficiency in which the fluorescence is detected.

To relate the measured fluorescence intensity to the surface concentration of the fluorophore a calibration procedure is required. The fluorescence intensity depends on properties of both the fluorophore and the instrumental setup, which can be calibrated using a standard fluorescence solution (28). The instrumental properties were calibrated by monitoring the fluorescence intensities of a number of TRP solutions with different concentrations. Once the fluorophore concentration is increased beyond the point for which a plateau in the amount of adsorbed TRP molecules is reached and where scattered light is entirely absorbed by solution molecules, the fluorescence contribution from non-adsorbed TRP increases linearly with its concentration. This linear increase is used as a measure for the instrumental sensitivity (28).

To convert the fluorescence signal from the adsorbed proteins into surface concentrations the protein fluorescence is normalized to the increment in fluorescence signal per increment in concentration (in units of absorption) of TRP. The fluorescence of TRP emanates from molecules in solution which are excited by the evanescent wave and can be derived by integration of Eq. [3]. The size of an adsorbed protein is two orders of magnitude less than the penetration depth and therefore the protein concentration profile can be considered as a quantity equal to the surface concentration, Γ_p , positioned at $z = 0$. After rewriting the normalized fluorescence intensity the surface concentration can be expressed as

$$\Gamma_p = \frac{S_p d}{2\varepsilon_p \Delta S_t / \Delta(c_t \varepsilon_t)} \frac{\phi_t}{\phi_p} \quad [4]$$

where the subscripts p and t refer to quantities of the protein and TRP standards, respectively. The penetration depth as given in Eq. [2] was calculated by using $n_1 = 1.515$, $n_2 = 1.333$, and $\theta = 70^\circ$. The only unknown parameters are the quantum yields. However, the ratio between the quantum yields can be established by measuring the fluorescence intensities of the protein and TRP solutions using the TIRF instrumentation but then in an L-shaped configuration and replacing the TIRF cell by a cuvet. A linear relation between fluorescence and low concentrations of the fluorophore is obtained as shown in Fig. 2 for both proteins and the TRP solutions. The quantum yield of the proteins were identical for both ionic strengths of 10 and 150 mM.

Although the adsorbed amounts obtained using this calibration procedure are similar to those found with other methods one should consider that this procedure includes some uncertainties. The quantum yield of fluorophores is sensitive to its environment and therefore, the fluorescence intensity in solution can differ from that in the adsorbed state. Furthermore, a diminished rotational mobility in the adsorbed state can cause an anisotropy in the fluorescence signal while the detection efficiency of the parallel and perpendicular components differ. Nevertheless, for the purpose of comparing the amounts of proteins adsorbed, the procedure described above was applied throughout this study.

Experimental Procedure

Before a measurement was started, the emission and excitation monochromators were calibrated by using the 365 nm line of a low pressure Hg lamp. Surfaces were prepared/cleaned within a few hours before use and mounted together with the silicon rubber gasket and the dovetail prism to the aluminum support, which in turn was placed in the TIRF setup. A final alignment was performed, and the sensitivity of the setup was calibrated using three TRP solutions with increasing concentrations. After the cell was rinsed with water to remove the TRP, the cell was filled with the buffer solution which is used throughout the experiment and the background fluorescence for the different types of measurements was recorded. Protein solutions were flown through the cell at a flow rate of 0.2 ml min^{-1} . The adsorption kinetics were followed by monitoring the fluorescence signal at an emission wavelength of 340 nm for 20 min (5 s per point for LSZ and 8 s per point for hGH). The anisotropy of the signal is obtained by measuring the fluorescence signals at both the x - and y -polarization. Then, a fluorescence emission spectrum is recorded at 0.5 nm wavelength intervals and 5 s measuring time per point. The fluorescence signal during desorption is followed for 8 min while a buffer solution is flowing through the cell (1 ml min^{-1}). Finally, quenching is measured by gently injecting increasing concentrations of TCE. Between each fluorescence measurement in the presence of a TCE solution, the cell is gently rinsed with a pure buffer solution to monitor additional desorption. The additional desorption never exceeded 5% of the adsorbed amount, and it was accounted for by using the average of the nonquenched fluorescence signals as measured before and after monitoring the quenched signal.

RESULTS AND DISCUSSION

Adsorption Kinetics

The adsorption kinetics of hGH and LSZ onto the three different surfaces at ionic strengths of 10 and 150 mM are shown in Fig. 3. All adsorption curves are averages of at least duplicated experiments with a deviation between the steady-state values of adsorption of less than 0.1 mg m^{-2} for hGH and 0.2 mg m^{-2} for Lysozyme.

In general, the initial adsorption rate is limited by the protein flux toward the surface and is further affected by interactions between the protein and sorbent surface which determine whether the protein adsorbs or not. The interactions considered to be the major driving force for protein adsorption are electrostatic and hydrophobic interactions and structural changes in the protein (9). For one type of protein the flux toward the surfaces is identical for all measurements which means that differences in the initial adsorption rate reflects differences in protein/surface interaction. After longer adsorption times equilibrium in amounts adsorbed are reached, generally close to monolayer coverages of the sorbent surface. These amounts adsorbed depend on spatial distribution, lateral interactions, and conformation and orientation of the adsorbed proteins.

Figures 3a and 3b show that for hGH the steady state adsorption values are rather low and a closely packed surface monolayer is not expected which implies that the adsorption is mainly determined by protein/surface interactions and less by lateral protein/protein interactions. Furthermore, it is observed that the hGH adsorption curves at 10 mM resembles those at 150 mM ionic strength which indicates that electrostatic interactions have a minor influence on the adsorption behavior. This is supported by the observation that electrostatic attraction between the negatively charged hGH molecule and the positively charged QAP surface yields lower initial adsorption rates than those for adsorption on the negatively charged methylated surface. On the contrary, the effect of dehydration of the hydrophobic sorbent surfaces has a pronounced effect on the adsorption; both the initial adsorption rates and the final amounts adsorbed increase with increasing surface hydrophobicity.

For LSZ adsorption (Figs. 3c and 3d) the amounts adsorbed are higher than those for hGH. Adsorbed amounts around 1 mg m^{-2} are generally found for LSZ (1,4) and it is expected that these plateau values correspond to a complete, or near complete, coverage of the sorbent surface. Thus, it can be assumed that at low ionic strength the methylated and QAP surfaces are fully covered by a monolayer of LSZ molecules. On silica, however, a relatively high initial adsorption rate and adsorbed amount are found at low ionic strength. High adsorption rates can be explained by the electrostatic attraction between the oppositely charged LSZ molecule and silica surface. However, it does not explain amounts adsorbed beyond the level of a monolayer. It is known that in solution higher concentrations of lysozyme form viscoelastic gels when the solution is heated to about 70°C . This irreversible process is called heat-set gelation. It has been reported that gelation of adsorbed layers of lysozyme occurs at much lower temperatures (7). Such a gelation process might explain the relative high adsorbed amount on silica at 10 mM ionic strength.

At the higher ionic strength both the initial adsorption rates and the amounts adsorbed of LSZ are smaller than those at low ionic strength indicating that electrostatic interactions play an important role in the adsorption process. That screening of electrostatic attraction at higher ionic strength reduces the adsorption rates on the negatively charged silica and methylated surfaces can be understood. However, LSZ adsorption on the positively charged QAP surface is also faster at low ionic strength, implying that an additional driving force for adsorption exists which is related to electrostatic interactions other than that between LSZ and the sorbent surface. A possible explanation is that the low ionic strength affects the internal coherence of LSZ thereby changing the affinity for the sorbent surface. It should be noted that LSZ concentrations of 0.05 mg ml^{-1} can be below the plateau region of the adsorption isotherms (1). As LSZ adsorbs with a fairly high affinity, a small variation in adsorption energy can result in a relatively strong change in the amount adsorbed. Performing the experiments with protein concentrations which can be a little below the plateau region of the adsorption isotherm might also explain why duplicated measurements of LSZ adsorption exhibits a higher variation in amounts adsorbed than those of hGH adsorption.

In Fig. 4, the various desorption curves for hGH and LSZ are shown. The desorption pattern for hGH shows that in all experiments an initial decrease of about 0.1 mg m^{-2} occurs in the first minute. After this first initial desorption, the amount adsorbed on the hydrophobic methylated surface remains almost the same, while hGH is more or less completely depleted from the silica surface. The QAP surface with intermediate hydrophobicity shows an intermediate desorption pattern. This supports the indication that hydrophobic interactions are the major driving force for hGH adsorption. The question remains why are some hGH molecules easily removed from the sorbent surface while others are not. It is unlikely that the quick initial decrease in signal is caused by removal of the bulk proteins because even for the lowest amounts adsorbed the contribution from the bulk proteins to the total signal is

less than 3%. Apparently, a fraction of the hGH molecules is weakly bound and readily desorbs when a buffer solution is applied. It is not known which adsorbed state results in this weak binding. It is possible that part of the hGH molecules are adsorbed onto unsilanized parts of the silica surface whose dimensions are comparable to the size of the hGH molecule. However, the initial desorption already exceeds the amount adsorbed on the silica surface. A more likely possibility is that hGH molecules can adsorb in two different states where one state is, for example, adsorbed in aggregates or in a different conformation. Proteins, adsorbed in two states with different mobility has earlier been reported by Tilton *et al.* (29).

The desorption patterns for LSZ follow a more gradual decrease without a fast initial desorption. If desorption is compared for the different surfaces it shows that in general desorption rates decrease with increasing surface hydrophobicity. This effect is similar to that observed for hGH and is independent of ionic strength. This suggests that although adsorption is largely influenced by electrostatic interactions, the strength of adsorption is dominated by hydrophobic interactions. Another observation is that the general pattern for LSZ desorption is different for both ionic strengths regardless of the sorbent surface indicating a difference in the adsorbed state as function of the ionic strength.

Conformational Changes

Fluorescence emission spectra of tryptophan residues in proteins are very sensitive to the polarity of the local surrounding of the fluorophores which generally results in a red-shift of the emission spectra if the tryptophan residue is exposed to water molecules in solution. A less uniform distribution of the protein structure is generally represented by a broadening of the spectrum, because a larger variety in the local surrounding of the fluorophores gives rise to diverse shifts in emission wavelengths. Figure 5 shows fluorescence emission spectra of hGH and LSZ adsorbed on a methylated silica compared to the spectra of solution proteins. Additional information on the conformation of the protein structure is obtained from quenching experiments. As quenching occurs upon molecular contact, it reveals the permeability of the protein for TCE, which is a polar, uncharged quencher. Furthermore it should be noted that quenching is enhanced if the lifetime of the excited state increases, which generally occurs if proteins are denatured. Collisional or dynamic quenching of fluorescence is described by the Stern–Volmer equation

$$F_0/F = 1 + K[Q], \quad [5]$$

where F and F_0 are the fluorescence intensities in the absence and presence of quencher, respectively, Q the concentration of the quencher, and K the Stern–Volmer quenching constant. Quenching of adsorbed proteins was measured after the desorption step which means that only the more tightly bound proteins are still adsorbed. No data could be collected for hGH on silica surfaces because all proteins desorbed. The results of the quenching measurements are shown as Stern–Volmer plots in Fig. 6. The Stern–Volmer plot for LSZ in solution at 150 mM ionic strength shows a downward curvature. This is generally observed if two or more populations of the fluorophore are present and can for example occur if the tryptophans in the interior of the protein are less accessible for the quencher than the tryptophans on the protein surface. Therefore, the LSZ quenching curve in solution at 150 mM is fitted with the assumption of a two state model in which the fluorophores are divided in a quenched part and a part which is inaccessible for TCE. Accordingly, the results are fitted to a modification of Eq. [5], incorporating the two states of the tryptophans. The result of this fit yields the fraction of tryptophans which is quenched, together with the quenching constant.

The peak wavelength, spectral bandwidth, and quenching constants of hGH and LSZ for the various conditions are collected in Table 3. For the peak wavelength and the spectral bandwidth the result from a single experiment deviates less than 2 nm from the average value. Although the deviation is relatively large for the quenching results of hGH (Fig. 6a) it is clear that upon adsorption the permeability of the hGH structure increases strongly, especially for adsorption on the hydrophobic methylated surface. It is then expected that the fluorescence shifts to higher wavelengths as it will be more accessible to polar water molecules. For adsorption on the QAP surface with intermediate hydrophobicity a slight increase in fluorescence wavelength is observed. However, the opposite effect is observed for hGH adsorption on silica and methylated surfaces which indicates that the direct environment of the tryptophan becomes less polar. The fluorescence of hGH in solution occurs at a relatively high wavelength (338 nm) compared to other proteins with a single tryptophan located in the hydrophobic interior of the protein (26). This might be caused by the polarity which arises from the H bond with another α -helix strand in which the tryptophan is involved (14). Distortion of the protein structure can position the tryptophan between less polar part of the protein, thereby reducing the fluorescence wavelength. An alternative explanation is that upon adsorption the fluorescence lifetime of tryptophan reduces and the spectra shift to blue because of the reduced dielectric relaxation around the tryptophans. These reduced lifetimes imply that the actual amounts adsorbed are higher than those obtained with TIRF. Although the precise effect of a change in the local environment of the tryptophans is still unclear, it can be stated that the conformation of hGH changes upon adsorption and that these changes are larger on more hydrophobic surfaces. Furthermore, the bandwidths of the hGH fluorescence spectra are broadened a little upon adsorption which indicates a more diverse distribution of the hGH structures.

From adsorption and desorption experiments it was concluded that hGH adsorption was dominated by hydrophobic interactions and apparently these strong interactions result in a more expanded structure of the hGH molecule. However, the fluorescence wavelengths suggest that the tryptophan is not fully exposed to the solution and will still be buried inside the protein structure or exposed to the methylated surface. It is possible that the hGH structure, adsorbed on the methylated surface, can be described by a molten globule state which consists of a secondary structure close to that of the native state and a somewhat unfolded tertiary structure offering additional hydrophobic surface for interaction with the sorbent (15).

LSZ contains six tryptophans per protein of which at least three are located at the exterior of the protein around the active site (13). As a result of the six tryptophans, each with a different local environment, a relatively broad fluorescence spectrum is obtained. A remarkable difference is observed for the fluorescence data of LSZ in solution with respect to the ionic strength. All differences indicate that the structure of LSZ is more compact at low ionic strength compared to that at high ionic strength. As was already indicated, the affinity of LSZ for all sorbent surfaces was higher at low ionic strength. Apparently, this higher affinity at low ionic strength corresponds to a more compact structure of the LSZ molecule. In the adsorbed state the difference in fluorescence wavelength and bandwidth between ionic strength are diminished which suggest that the structural differences caused by the ionic strength are smaller than those in solution. Nevertheless, quenching results of adsorbed LSZ still show a difference between the two ionic strengths.

Upon adsorption, an increase in the bandwidth is observed, especially when LSZ is adsorbed on the hydrophobic surface. The quenching constants for LSZ show that an increase in the TCE accessibility is only observed if adsorbed on the methylated surface. Apparently, the LSZ structure changes under influence of hydrophobic interactions. This is in line with the observation from the desorption measurements where it was seen that LSZ is more strongly

bound to the methylated surface. For the silica and QAP surface a reduction rather than an increase in the accessibility is observed which easily can be explained by taking into account that accessibility of the tryptophans can be blocked in the adsorbed state. As the solvent accessible tryptophans are located in the active site, this result suggest that this part of LSZ is oriented towards the sorbent surface.

Rotational Mobility

In a two-dimensional system the fluorescence anisotropy, r , is given by $(I_y - I_x)/(I_y + I_x)$ (21). The maximum anisotropy is limited by angular distribution of the fluorophores and the angular displacement, α , of the dipole between excitation and emission and is given by (21)

$$r_0 = \cos^2 \alpha - 1/2, \quad [6]$$

which leads to the maximum anisotropy of 0.5 for the two-dimensional array of fluorophores. For tryptophan, however, the anisotropy is further limited by two perpendicular-oriented excited states of tryptophan which at an excitation wavelength of 295 nm result in a maximum anisotropy in solution of around 0.2 and correspond to an average displacement angle of 35° (26). By using Eq. [6] this angle yields a maximum value for the anisotropy of adsorbed molecules of 0.17. The measured anisotropy is related to molecular rotation of a molecule and is reduced if the lifetime of the fluorophore increases. The values for the anisotropies of the fluorescence signal are given in Table 3. For the proteins in solution it can be seen that the anisotropy values are well below the maximum value of 0.3. This implies that their correlation time for rotational diffusion is in the same order of magnitude as the fluorescence lifetime, which is generally on the order of a few nanoseconds. The anisotropy of hGH is larger than that of LSZ, which is expected because the rotational mobility decreases with increasing dimensions of the molecule. In the adsorbed state, all anisotropy values are close to the maximum value implying that the correlation times for rotational diffusion are much smaller than the fluorescence lifetimes or both have decreased to some extent. Therefore, it can be concluded that protein/sorbent interaction largely reduces the rotational mobility of the tryptophans and thus of the proteins.

CONCLUSION

It is demonstrated that TIRF spectroscopy, utilizing the fluorescence of the intrinsic tryptophans of proteins, offers a valuable tool for simultaneously studying protein adsorption and different aspects of the adsorbed state.

The adsorption of hGH is dominated by hydrophobic interactions and these interactions result in a more expanded hGH structure. LSZ adsorption is affected by electrostatic interactions which probably originate from a difference in structure in solution at different ionic strengths, thereby changing the affinity of LSZ for adsorption. The strength of the LSZ/sorbent interaction is stronger at the hydrophobic methylated surface and result in a small conformational change in the LSZ structure. Furthermore, it is shown that the rotational mobility of both proteins is reduced upon adsorption, regardless of the sorbent surface.

In general, it can be concluded that hydrophobic interactions are a major contribution to strong protein/sorbent interactions and that these strong interactions are accompanied by a change in the protein structure. It remains difficult, however, to make a distinction between cause and effect; strong interactions may lead to conformational changes in the protein but

conformational changes, such as exposing hydrophobic parts of the protein to the sorbent surface, may lead to stronger interactions. The initial stage of hGH adsorption was clearly dominated by hydrophobic interactions which might suggest that hydrophobic interactions induce structural changes. For LSZ, initial adsorption seems to be less influenced by the sorbent hydrophobicity which indicates that structural changes induce a stronger interaction.

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FIG. 1.

Schematic drawing of the TIRF setup. The inset shows the coordinate system. The solid/liquid interface is within the xy -plane. The excitation beam is polarized normal to the plane of incidence (xz -plane) and is incident at the solid/liquid interface at an angle, θ , measured from the normal to the interface.

FIG. 2.
Quantum yield for hGH (●), LSZ (○), and TRP (◆).

FIG. 3.

Adsorption kinetics of (a) hGH at ionic strength 10 mM, (b) hGH at 150 mM, (c) LSZ at 10 mM, and (d) LSZ at 150 mM. The different sorbent surfaces are indicated as silica (○), QAP (□), and methylated silica (●).

FIG. 4. Desorption kinetics of (a) hGH at ionic strength 10 mM, (b) hGH at 150 mM, (c) LSZ at 10 mM, and (d) LSZ at 150 mM. Buffer flow started at $t = 50$ min, as indicated by the vertical dotted lines. The different sorbent surfaces are indicated as silica (○), QAP (□), and methylated silica (●).

FIG. 5. Fluorescence spectrum of hGH (a) and LSZ (b), in solution (×) and after 0.5 h of adsorption on a methylated surface (+), pH 7, and 10 mM ionic strength.

FIG. 6. Stern–Volmer plots of quenching of hGH (a) and LSZ (b) by TCE. Quenching results for proteins in solution or adsorbed on the different surfaces are as indicated; solution (◆ or ◇), silica (● or ○), QAP (▲ or △), and methylated silica (■ or □). Ionic strengths are indicated by using filled symbols and solid lines for 10 mM and open symbols and dotted lines for 150 mM.

TABLE 1

Chemical Surface Groups and Contact Angles of the Sorbent Surfaces

Surface	Chemical groups	Contact angle
Silica	—OH	<7°
Methyl	—CH ₃	90–94°
QAP	—(CH ₂) ₃ [–] N ⁺ (CH ₃) ₃ I [–]	52–56°

TABLE 2

Some Physical Properties of hGH and LSZ

	hGH	LSZ
MW	22,000	14,300
i.e.p.	5.1 (23)	11.1
Dimensions (nm ³)	$5.5 \times 3.5 \times 3.5$	$4.5 \times 3.0 \times 3.0$
Number of tryptophans	1	6

TABLE 3

Peak Wavelength (λ_m), Spectral Bandwidths, Stern–Volmer Quenching Constants (K), and Anisotropy Values for hGH and LSZ in Solution and Adsorbed to Sorbent Surfaces at Ionic Strengths of 10 and 150 mM

Ionic strength	hGH				Lysozyme			
	λ_m (nm)	Bandwidth(nm)	K (M ⁻¹)	Anisotropy	λ_m (nm)	Bandwidth(nm)	K (M ⁻¹)	Anisotropy
Solution								
10 mM	337.4	53.6	7.5	0.141	341.9	60.8	6.4	0.080
150 mM	338.1	53.7	7.8	0.144	346.4	62.1	22.7 ^a	0.087
Silica								
10 mM	334.0	54.4	–	0.21	346.5	62.3	3.6	0.15
150 mM	332.2	54.3	–	0.15	345.7	62.7	5.1	0.16
QAP								
10 mM	339.5	56.5	34.6	0.17	343.6	62.0	4.5	0.15
150 mM	339.1	55.3	28.9	0.19	342.3	63.0	6.0	0.14
Methyl								
10 mM	333.8	55.5	49.6	0.15	345.9	64.4	10.1	0.15
150 mM	331.2	57.9	45.7	0.18	344.9	64.0	21.3	0.13

^aResult of fitting a two-state population of fluorophores which yielded this quenching constant for the 60% of the tryptophans which are accessible for TCE.