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Adsorption of Firefly Luciferase at Interfaces Studied by Total Internal Reflection Fluorescence Spectroscopy

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The adsorption of luciferase onto silica surfaces was studied by total internal reflection fluorescence (TIRF) spectroscopy. Two model surfaces were used: hydrophilic and hydrophobic silica. Luciferase adsorbed differently on these two surfaces. Initial kinetics of luciferase adsorption onto the hydrophilic surface showed that luciferase adsorbs over an adsorption energy barrier of ≈3 kT. The quantum yield of luciferase fluorescence decreased at the hydrophilic silica surface, which indicated that the protein conformation was altered during adsorption. Luciferase adsorption onto the hydrophobic silica surface proceeded with a small adsorption energy barrier and the fluorescence efficiency of adsorbed protein remained unchanged after adsorption. The affinity of luciferase for luciferin was measured using quenching of luciferase fluorescence with luciferin. The binding constant of the adsorbed luciferase–luciferin complex at the hydrophilic silica surface was two orders of magnitude smaller than the respective binding constant in the solution. Adsorbed luciferase showed an absence of ATP-dependent visible luminescence, indicating that the adsorbed enzyme was not active at either of the two silica surfaces.

KEY WORDS: Adsorption; luciferase; luciferin; fluorescence quenching.

INTRODUCTION

The immobilization of enzymes to solid carriers is important to biotechnology, diagnostics, and sensing[1]. An enzyme is "immobilized" when its release into the solution and its surface mobility are restricted or constrained by some physical or chemical means. While covalent binding of enzymes to solid carriers is the most common immobilization method, physical adsorption of enzyme to the solid surface often precedes the formation of the covalent bond. In pure adsorption, the enzyme molecule bonds to the surface by a variety of physical forces, some of which may influence its orientation, conformation, and biological activity. An adsorbed enzyme may be desorbed by a change in ionic concentration, pH, or temperature. Exposures of adsorbed protein to other solution proteins may replace it from the surface by the mechanism of preferential adsorption. Thus, control of adsorbed enzymes is rather difficult to achieve and involves the selection of proper surface and solution conditions. The change of protein conformation at interfaces is not limited only to physically adsorbed proteins. It is possible that a covalently bound molecule of enzyme also interacts with the underlying surface in an adsorption-like manner, a process which may result in a conformational change of the enzyme and its subsequent inactivation.

This study investigates the role of the surface in the adsorption of firefly luciferase, an enzyme which cata-

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lyzes the production of yellow-green light from firefly luciferin in the presence of adenosine triphosphate (ATP), Mg^{2+}, and O_2. There has been an increased interest in firefly luciferase bioluminescence [2–15]. It stems from the fact that the emitted luminescence intensity is proportional to the amount of ATP in the system (so-called ATP-dependent luminescence). The specificity of firefly luciferase for ATP in the conversion of luciferin into luciferyl adenylate has been used to develop many different assays, some of which involve a covalently bound enzyme [16–20]. Firefly luciferase was covalently bound to alkylamine glass beads by Lee *et al.* [16], but the bound enzyme had only 0.16–0.67% of the activity of the soluble enzyme. Ugarova *et al.* [17] studied the activity and stability of immobilized firefly luciferase using several different carrier surfaces. The most active form was found when luciferase was immobilized on the polysaccharide carrier surfaces, such as Ultradex and Sepharose, i.e., on the surfaces which are known to have very weak nonspecific interactions with the bound proteins.

There is very little experimental evidence describing the interfacial properties of firefly luciferase. The present study was undertaken to fill this void and to understand the functioning of the surface-bound enzyme. A surface-sensitive spectroscopic method, total internal reflection fluorescence (TIRF) [21], was used to measure the intrinsic fluorescence emitted by adsorbed luciferase. The fluorescence emission quantum yield of adsorbed protein can be determined by analysis of the fluorescence emission of adsorbed luciferase and an independent measurement of the amount of adsorbed protein [22]. Intrinsic protein fluorescence is a sensitive probe of protein conformation, so that a change of the fluorescence quantum yield of adsorbed luciferase can be used as an indicator of protein conformation as the molecule adapts to its new environment. The purpose of this study was to elucidate the extent of the conformational changes of adsorbed luciferase at model hydrophilic and hydrophobic silica surfaces.

MATERIALS AND METHODS

Radiolabeling of Luciferase

Crystallized and lyophilized powder firefly luciferase (*Photinus pyralis*), D-luciferin, ATP, and glycylglycine were purchased from Sigma; $MgSO_4$ was from Mallinckrodt. All other chemicals were analytical grade. Firefly luciferase was labeled with carrier-free $Na^{125}I$ (100 mCi/ml, Amersham) by chloramine-T as described by Chuang *et al.* [23]. The labeling procedure was as follows: 1 mg of firefly luciferase was dissolved into 1 ml of 0.45 *M* glycylglycine buffer. Three hundred micrograms of luciferase was added to glycylglycine buffer to make a total volume of 0.5 ml. A volume corresponding to 300 μCi of $Na^{125}I$ was added to the luciferase solution. Fifty microliters of freshly made chloramine-T solution (Kodak, 4 mg/ml in deionized water) was added to the luciferase solution and was gently mixed for 1 min. Fifty milliliters of sodium metabisulfite solution (Fisher Scientific Co., 4.8 mg/ml in deionized water) was then added and the resulting solution was mixed for 2 to 3 min in order to stop the oxidation reaction. The labeled luciferase was immediately separated from the free iodine by Sephadex G-25 column (Pharmacia) [24]. The degree of protein labeling (labeled protein/total protein) was 0.90 to 0.95.

Surface Preparation and Chemistry

Two types of surfaces were prepared for the adsorption experiments: a hydrophilic surface and a hydrophobic surface. Both surfaces were prepared by cutting a fused silica microscope quartz slide (CO grade, ESCO) into smaller (12 × 10 × 1 mm) pieces. These small slides were polished on the edges by an abrasive paper, cleaned by immersing in hot (90°C) chromic acid for 1 h, cooled to room temperature, rinsed thoroughly with purified water (Milli-Q), and dried under vacuum at 100°C for 2 h. The cleanliness of the slides was checked by the Wilhelmy plate water contact angle technique [25]. A clean fused silica slide was used as the hydrophilic surface. Hydrophobic surfaces were prepared by immersing the clean quartz slide into a solution of 10% (v/v) dimethyldichlorosilane (DDS) (Petrarch System Inc.) in dry toluene for 15 to 30 min at room temperature. After the incubation, the slides were rinsed with ethanol three to five times and then rinsed in purified water. The slides were desiccated under vacuum at 100°C for 2 h. All surfaces were used within 48 h.

Adsorption Experiments

Preferential adsorption of iodinated firefly luciferase was studied by preparing luciferase solutions of the same final concentration (0.13 mg/ml in 0.45 *M* glycylglycine buffer, pH 7.8) but with different ratios of unlabeled to labeled luciferase: 0, 1, 2, 5, and 11. Hydrophilic slides were exposed to the solution mixtures of luciferase for 14 h. After adsorption, the slides were removed and rinsed with buffer and the associated radioactivity was counted. The amount of protein on each

slide was determined from the corresponding radioactivity counts of stock luciferase solution. The adsorption isotherm was measured in a similar manner by exposing the silica slides to labeled luciferase solutions of different concentrations for 14 h at room temperature.

Adsorption Kinetics Measured by the Total Internal Reflection Fluorescence (TIRF) Spectroscopy

The firefly luciferase solution (0.2 mg/ml) was prepared in 0.45 *M* glycylglycine buffer (pH 7.8). The protein concentration was determined by UV absorbance (E_{1cm} = 0.75 ml mg^{-1} cm^{-1} at 280 nm [3]). The TIRF cell was assembled by using the hydrophilic or hydrophobic silica plate as the adsorbing surface [22]. The TIRF cell was positioned so that the collimated light struck the solid/liquid interface at a 70° angle from normal (Fig. 1). Total internal reflection of perpendicularly polarized light at the silica/buffer interface created an evanescent surface wave. The electric field amplitude of the surface wave, $E\perp$, decays exponentially with distance z normal to the interface into the buffer solution:

$$E_\perp = E_{o,\perp}\, e^{-z/d_p} \quad (1)$$

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

where $E_{o,\perp}$ is the electric field amplitude right at the interface, d_p is the depth of penetration, λ is the wavelength of the light, θ is the incident angle, and n_1 and n_2 are the refractive indices of silica and buffer solution, repsectively. The depth of penetration for the present TIRF cell configuration was 119 nm. The excitation and emission slits were 1 mm (8-nm half-width) and 2 mm (16-nm half-width), respectively. The fluorescence emission was excited at 285 nm and collected at 340 nm. The TIRF setup was calibrated prior to the adsorption experiments by following the procedure described in Ref. 22. L-5-Hydroxytryptophan methyl ester HCl (TrpOH) was used as an external standard. Adsorption was started by injecting 1 ml of the 0.2 mg/ml luciferase solution into the TIRF cell. The fluorescence signal was observed at the initial adsorption time and at other desired times. A shutter was used to protect the luciferase molecules from overexposure to UV light during the adsorption process. After 14 h of adsorption, the fluorescence signal was measured as the cell was flushed with glycylglycine buffer to remove nonadsorbed luciferase. The intensity of fluorescence due to adsorption of luciferase was converted into adsorbed mass of protein per unit area using the standard TIRF quantitation method [22]. This procedure ignores eventual changes of protein fluorescence emission efficiency due to adsorption. The quantum yield of adsorbed luciferase was determined by combining the TIRF results with the measurement of adsorbed amount of ^{125}I-labeled luciferase.

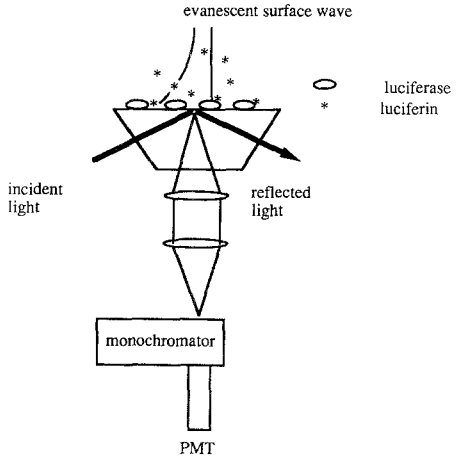

Fig. 1. A schematic picture of the TIRF cell and the optical geometry for the collection of surface fluorescence. The evanescent surface wave is not drawn to scale.

Quenching of Luciferase Fluorescence in Solution

Firefly luciferase solution was prepared in 0.45 *M* glycylglycine buffer (pH 7.8). The protein concentration was 0.2 mg/ml. This concentration was taken to be equal to 2.0 × 10^{-6} *M* by assuming that the molecular mass of protein is 100 kDa. Although this value differs from the molecular mass based on recent research on cDNA of luciferase (Mm ≈ 62 kDa [26]), it was chosen in order to compare the results of the present quenching experiments with published data [27]. Luciferin was used as a quencher. Aliquots of 10 μl of the luciferin solution (3.6 × 10^{-4} *M*) were introduced successively into a cuvette containing 1.0 ml of luciferase solution, and the intrinsic fluorescence spectra were obtained by a fluorometer operating in the photon counting mode (I.S.S., Inc., Greg 200; excitation, 285 nm; emission collected from 300 to 400 nm). A parallel quenching experiment was performed under the same conditions using the L-5-hydroxytryptophan methyl ester HCl (TrpOH) in the solution instead of luciferase. The TrpOH solution was prepared in 0.45 *M* glycylglycine buffer. The TrpOH concentration was adjusted so that the solution had the same UV absorbance (at 285 nm) as the 0.2 mg/ml luciferase solution. The quenching of TrpOH was used as correction for the inner filter effect, which occurs when the quencher absorbs at the same wavelengths as the protein and at the wavelengths of protein emission. The

fluorescence intensity was determined by integrating the background-corrected emission spectra. The fluorescence intensity of TrpOH was normalized to the fluorescence intensity of luciferase in order to correct for the difference in the emission quantum yields between TrpOH and luciferase. The quenched fluorescence of luciferase, F, was corrected in a subtractive manner, i.e., as $F = F_0 - (F_c - F_s)$, where F_0 is the fluorescence intensity of luciferase in the absence of the quencher, F_c is the normalized fluorescence intensity of TrpOH in the presence of the quencher, and F_s is the fluorescence intensity of luciferase in the presence of the quencher. One notes that, since both TrpOH and luciferase solution had the same UV absorbance, the subtractive manner of fluorescence correction also compensates for the dynamic quenching.

Quenching of Luciferase Fluorescence at the Solid/Liquid Interface

The procedure for luciferase adsorption was the same as described above. Luciferin solutions (6.0×10^{-3} *M*) were prepared in 0.45 *M* glycylglycine buffer (pH 7.8). This stock solution was then diluted with the glycylglycine buffer to produce quencher solutions with dilution factors of 0.50, 0.10, 0.05, 0.01, 0.005, and 0.001. After the nonadsorbed protein molecules were removed by the buffer and only luciferase was present at the solid/liquid interface, the luciferin solutions were introduced into the TIRF cell, starting with the least concentrated solution. The fluorescence was excited at 285 nm and emission recorded at 340 nm. After the quenching measurement with the most concentrated luciferin solution, 10 ml glycylglycine buffer solution was used to replace the quencher. This was done to determine if the intrinsic fluorescence signal will return to the original level, which was taken as a sign of no appreciable desorption of luciferase and no irreversible binding of luciferin to adsorbed luciferase. The increase in the evanescent surface wave intensity due to the increasing concentration of luciferin in the TIRF cell was very small (estimated <2%) and was neglected [22]. In the present TIRF experiment the fluorescence was collected normal to the surface, which is equivalent to the front-face geometry (Fig. 1), so that inner filter effects were absent.

Surface Tension of the Luciferase Solution

Kinetics of the surface tension of the luciferase solution (0.1 mg/ml in 0.45 *M* glycylglycine buffer, pH 7.8) were measured as a function of time using the Wilhelmy plate method, according to the procedure described by Wei [28].

RESULTS AND DISCUSSION

Adsorption Isotherms and Kinetics

When using iodine-labeled proteins in the adsorption experiments, one must ensure that there is no preferential interaction of either of the two protein populations with the surface. This was checked by repeating the same adsorption experiments with different ratios of unlabeled vs labeled luciferase, while keeping the total protein concentration constant. The expected surface radioactivity, calculated by assuming that the labeled luciferase adsorbs identically to the unlabeled luciferase, is given together with the experimental results in Fig. 2. The results suggested that neither labeled nor unlabeled luciferase preferentially adsorbs to the hydrophilic surface under given experimental conditions. However, in separate experiments it was found that the enzymatic activity of luciferase in solution was decreased after iodination; ^{125}I-labeled luciferase showed only about one-third the enzyme activity (measured as the intial peak of luminescence emission) of unlabeled luciferase.

The isotherms of luciferase adsorption at two different silica surfaces are given in Fig. 3. The equivalent Scatchard plots (not shown here) provided an estimate of the apparent binding constants and maximal surface coverages, Γ^{max}: in the case of the binding onto the

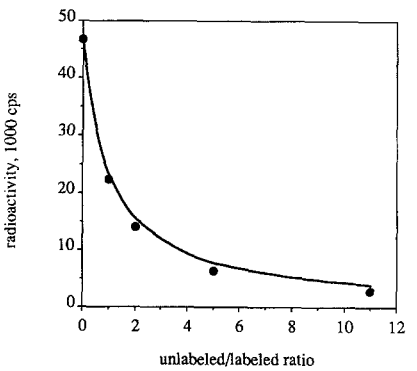

Fig. 2. Adsorbed amount of firefly luciferase as a function of the ratio of unlabeled versus labeled protein; the points are experimental results and the line is the adsorption calculated by assuming that unlabeled and labeled proteins adsorb identically. The total luciferase concentration (i.e., labeled + unlabeled protein) prior to adsorption was 0.13 mg/ml. Adsorption onto the hydrophilic silica surface was carried out for 14 h at room temperature.

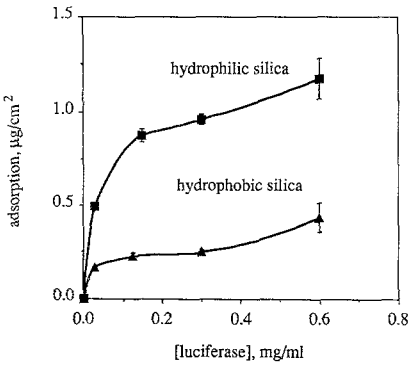

Fig. 3. The adsorbed amount of firefly luciferase on the two types of silica surfaces as a function of the solution concentration of protein. Each point represents the mean value of two separate experiments, while the error bars represent one standard deviation. Adsorption was carried out for 14 h from 0.45 *M*, pH 7.8, glycylglycine buffer at room temperature.

hydrophilic silica surface $K_{(hydrophilic)} = 0.0321$ L/g and $\Gamma^{max} = 1.062$ μg/cm², and for the binding to the hydrophobic silica surface $K_{(hydrophilic)} = 0.0151$ L/g and $\Gamma^{max} = 0.258$ μg/cm², respectively. The somewhat higher affinity in the case of the binding onto the hydrophobic surface was expected since firefly luciferase is a very hydrophobic protein: about 60% of the amino acids of luciferase are hydrophobic. It was found that the adsorbed amount of luciferase at the hydrophilic surface is much higher than at the hydrophobic surface. One hypothesis is that the high content of hydrophobic amino acids in luciferase causes an aggregation of protein at the hydrophilic surface. This hypothesis was supported by the observation that luciferase readily aggregates in the solution. Thus, if the adsorption of luciferase leads to the neutralization of electrical charges of protein and surface, lateral aggregation could be driven by the hydrophobic interactions between the adsorbed molecules. On the other hand, the adsorption of luciferase onto the hydrophobic surface may result in protein molecules at the surface which are charged so that the electrostatic repulsion opposes a dense surface packing. Accordingly, the different levels of the plateau of the adsorption isotherms (Fig. 3) may reflect a difference in the lateral interactions between adsorbed protein.

The amount of luciferase adsorbed for 14 h was calculated from the TIRF measurements using the calibration curves prepared by the extrinsic fluorescence standards. In this calculation the fluorescence emission quantum yield of adsorbed luciferase, $\phi_{surface}$, was assumed to be identical to the emission quantum yield of luciferase in the buffer solution, $\phi_{solution}$, i.e., that $\phi_{surface}/\phi_{solution} = 1$. The TIRF-measured adsorption of 0.25 μg/cm² on the hydrophobic silica surface was identical to the luciferase adsorption determined by the ^{125}I-labeled protein (0.25 μg/cm², Fig. 3), indicating that the assumption about the same fluorescence quantum yields was indeed true for this surface. In the case of the hydrophilic silica surface, the TIRF-measured adsorption amounted to 0.11 μg/cm², while the adsorption determined by the ^{125}I-labeled protein was 0.90 μg/cm² (Fig. 3). Accordingly, the emission quantum yield of adsorbed luciferase at the hydrophilic silica surface, $\phi_{surface}/\phi_{solution}$, was found to decrease to 0.12.

The fluorescence quantum yield of proteins with few tryptophanyl residues can often be used as a measure of local change in protein conformation [22]. In the case of multitryptophanyl proteins, the fluorescence intensity is often not proportional to the number or to the concentration of fluorophores. This is due to the excitation energy homotransfer, which has a higher probability when a large number of fluorophores or molecules are densely packed in a relatively small volume. Therefore, the decrease in luciferase fluorescence efficiency upon adsorption onto the hydrophilic surface could not be used to differentiate whether the observed effect is due to a conformational change in the adsorbed protein molecule, to its aggregation on the surface, or to some combination of both. The examination of the fluorescence emission spectra did not show any significant differences between protein on silica surfaces and protein in solution.

The adsorption of luciferase at the air/water interface, which may be considered as an ideal hydrophobic interface, causes the surface tension to decrease rapidly from approximately 73.0 dyne/cm ($t = 0$) to 48 dyne/cm ($t = 2$ h) (Fig. 4). This result supports the hypothesis that luciferase adsorption onto hydrophobic surfaces is

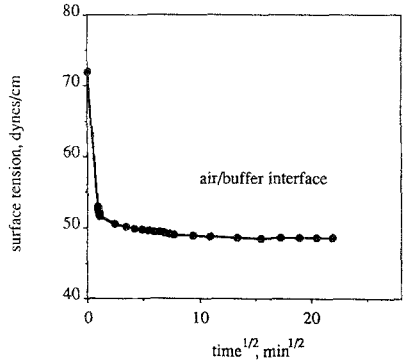

Fig. 4. Surface tension of 0.1 mg/ml firefly luciferase solution in 0.45 *M*, pH 7.8, glycylglycine buffer given as a function of time$^{1/2}$.

driven by the high effective hydrophobicity of the protein. It is instructive to compare the surface tension results with the surface tension of two other proteins, lysozyme and serum albumin [28], performed at comparable concentrations. Lysozyme lowers the surface tension of phosphate buffer solution from 73.0 to 63 dyne/cm, while serum albumin, which is known to be a very surface active protein [28], lowers the surface tension of water from 72.5 to 50 dyne/cm. The implications are that luciferase is also a protein of high surface activity.

The kinetics of luciferase adsorption on silica surfaces are similar to the kinetics of binding to the air/buffer interface. Figure 5 shows the adsorption of firefly luciferase (from solution of $c_p = 0.2$ mg/ml) as a function of (time)$^{1/2}$ onto hydrophilic and hydrophobic silica surfaces. The amount adsorbed was calculated from surface fluorescence intensities and corrected for the change in the $\phi_{surface}/\phi_{solution}$ ratio. Luciferase adsorbed onto the hydrophilic surface at a slow adsorption rate, while a rapid increase in luciferase surface concentration was observed in the case of the hydrophobic surface. Adsorption onto the hydrophobic surface was very similar to the fast binding of the same protein to the air/buffer interface (Fig. 4). The initial part of the adsorption kinetics onto the hydrophilic silica surface suggested that the rate-limiting step in luciferase adsorption was not the transport of the protein to the surface but, rather, its slow rate of binding to the surface binding sites. The slow increase in surface fluorescence indicated that the first

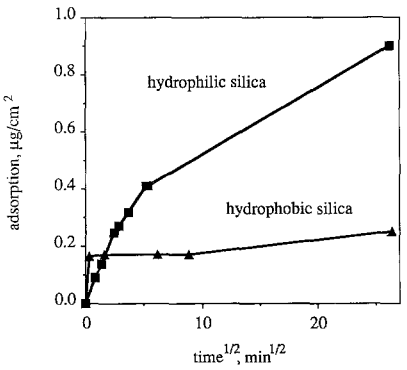

Fig. 5. Adsorption kinetics of firefly luciferase onto the two types of silica surfaces. The binding of luciferase, which was measured using the TIRF technique, is given as a function of time$^{1/2}$. The adsorbed amount was corrected for the fluorescence quantum yield decrease using the adsorption results obtained with ^{125}I-labeled luciferase. Adsorption was carried out from 0.2 mg/ml luciferase solution in 0.45 *M*, pH 7.8, glygylglycine buffer at room temperature from stagnant solution.

luciferase molecules bound to the surface already fluoresced with a decreased quantum yield; in other words, the conformational changes of the adsorbed protein molecules were immediate. In contrast, the much faster increase in luciferase surface concentration during adsorption onto the hydrophobic surface indicated efficient "sticking" of protein molecules, probably in their native form.

The adsorption kinetics results (Fig. 5) can be used to obtain an estimate of the energy barrier for the adsorption of luciferase. The adsorption energy barrier, E_a, can be found from the ratio of the experimentally measured initial adsorption rate, $k_{(exp)}$, and the maximally achievable adsorption rate, $k_{(max)}$:

$$\{k_{(exp)}/k_{(max)}\}_{t\to 0} = \exp(-E_a/kT) \qquad (3)$$

The maximum adsorption rate at the onset of adsorption is often limited by the transport of molecules from bulk solution to the surface. In the case of a surface which acts as a perfectly adsorbing barrier (i.e., an ideal adsorption sink), every protein molecule which is transported to the surface becomes adsorbed. If the solution is quiescent and diffusion is the only way by which the molecule can reach the surface, the interfacial concentration of protein per unit area is given by the classical formula [29]:

$$\Gamma_{(pot)}(t) = 2\, c_p\, (D/\pi)^{1/2} t^{1/2} \qquad (4)$$

where D is the protein diffusion coefficient (estimated as 5.0×10^{-7} cm^2/s for firefly luciferase), t is the adsorption time, and c_p is the concentration of protein in bulk solution. Using the results from Fig. 5 one can find the initial slope, $\Delta\Gamma_{(exp)}/\Delta(t^{1/2})_{t\to 0}$, and compare it with the slope from Eq. (4), which equals $c_p\,(D/\pi)^{1/2}$. Conditions under which Eq. (4) can be compared with the experiment exist only at the very beginning of the adsorption process. In such a case, the value of $[\Delta\Gamma_{(exp)}/\Delta(t^{1/2})_{t\to 0}]/[c_p\,(D/\pi)^{1/2}]$ can be used to replace $[k_{(exp)}/k_{(max)t\to 0}$ in Eq. (3) in order to estimate the value of E_a. This approximation is valid only at very low surface coverages. Once the surface is partly filled with the adsorbed protein, the assumption that the surface acts as an ideal sink fails to be correct and Eq. (4) can no longer be applied.

The energy barrier for luciferase adsorption onto the hydrophilic silica surface was $E_a \approx 3$ kT. Luciferase adsorption onto the hydrophobic surface proceeds with an apparent energy barrier of $E_a < 1$ kT. The estimates of E_a values follow the order of the apparent binding constants determined from the adsorption isotherms: the larger the apparent binding constants, the larger the energy barrier as observed from the initial kinetics measurements.

Fluorescence Quenching with Luciferin

The adsorption results from the previous section result in the following hypotheses: luciferase is a hydrophobic protein and easily undergoes aggregation in solution. When it adsorbs to the hydrophilic surface, it does so with a low "sticking" coefficient (high E_a); it becomes conformationally unstable (decrease in the $\phi_{surface}/\phi_{solution}$ ratio) and/or possibly aggregates at the interface (high Γ^{max}). When it adsorbs to the hydrophobic surface, it does so efficiently (low E_a) and probably retains at least parts of its native conformation (no change in the $\phi_{surface}/\phi_{solution}$ ratio). One way to test these hypotheses is to investigate the accessibility of luciferase binding sites to luciferin (LH_2) when the only protein present in the system is in the adsorbed state at the silica/buffer interface. It is known that the binding of luciferin quenches the intrinsic fluorescence of luciferase [27]. The quenching constant is expected to reflect the accessibility of the binding site and/or the binding affinity of luciferase toward luciferin and whether or not that has changed upon adsorption.

Figure 6 shows the modified Stern–Volmer plots for luciferase fluorescence quenching in the bulk solution. Figure 7 shows the same plot for quenching at the two types of silica/buffer interfaces. Several features of these plots deserve additional comments. Standard fluorescence quenching plots (F_o/F vs $[LH_2]$) showed an upward curvature which could not be corrected by replotting the data in the form which would allow differentiation between static and dynamic quenching, i.e., as $[(F_o/F) - 1)]/[LH_2]$ vs $1/[LH_2]$. Hence, modified Stern–Volmer plots were used in order to compare the results of the present study with the results from the literature [27]. The downward curvature of the modified Stern–Volmer plots implied that there were at least two different luciferin binding sites, each with a significantly different binding constant. This conclusion is consistent with the stoichiometric relation between luciferin and the luciferase used [30]. The quenching constants (K_{QS}) and accessible fractions of tryptophanyl residues (f_a) were determined from the slopes of the modified Stern–Volmer plots using only the high $1/[LH_2]$ region (i.e., data obtained at very low luciferin concentrations). It was assumed that in this quencher concentration range, the luciferase fluorescence was quenched entirely due to the binding of luciferin to the luciferase high-affinity binding site. In such a case the quenching constant is equivalent to the binding constant of quencher to the substrate [31, 32]. Calculation of the constants in the low $1/[LH_2]$ region was not attempted because of the possibility that dynamic quenching in solution at high luciferin concentrations was not entirely corrected by subtraction of the quenched TrpOH fluorescence. Dynamic quenching of surface-bound fluorophores is not expected to be the same as quenching of flurophores in solution. This is due to spatial restrictions between the quencher and the surface-

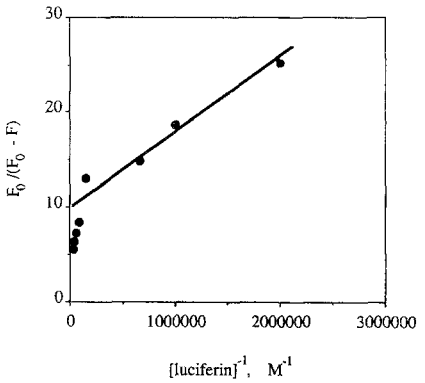

Fig. 6. Modified Stern–Volmer plot for the quenching of firefly luciferase fluorescence with luciferin in the bulk solution. The concentration of luciferase was 0.2 mg/ml (2.0×10^{-6} *M*) in 0.45 *M*, pH 7.8 glycylglycine buffer. The quenched fluorescence, *F*, was calculated as $F = F_0 - (F_c - F_s)$, where F_0 is the fluorescence intensity of luciferase in the absence of the quencher, F_c is the corrected fluorescence intensity of TrpOH in the presence of the quencher, and F_s is the fluoresence intensity of luciferase in the presence of the quencher.

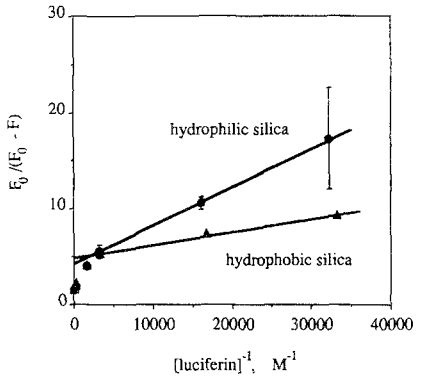

Fig. 7. Modified Stern–Volmer plot for the quenching of fluorescence of adsorbed firefly luciferase with luciferin. Luciferase was adsorbed onto the two types of silica surfaces from the 0.2 mg/ml solution prepared in 0.45 *M*, pH 7.8, glycylglycine buffer. Any nonadsorbed protein has been removed from the TIRF cell by the buffer flush. *F* is the quenched fluorescence and F_0 is the fluorescence intensity of adsorbed luciferase in the absence of the quencher, both measured in the TIRF optical geometry. No corrections for the inner filter effect have been applied. In the case of hydrophilic silica, each point represents the mean value of two separate experiments, while the error bars represent one standard deviation. Similar experimental errors are expected in the case of hydrophobic silica.

bound fluorophore molecules. Hence, it is questionable whether any accurate corrections for the dynamic quenching can be made in the case of adsorbed luciferase. No attempt was made to determine the dynamic portion of the observed quenching by fluorescence lifetime measurements. The Stern–Volmer quenching constants, K_{QS}, and accessible fluorophore fractions, f_a, are listed in Table I. One notes that K_{QS} changes by a factor of 100 upon adsorption of luciferase onto the hydrophilic silica surface. This dramatic difference between the solution and the surface quenching constants indicated that the binding affinity and/or the accessibility of the luciferin binding site had drastically changed upon adsorption. The changes are not so dramatic in the case of the hydrophobic silica surface: K_{QS} decreases by a factor of 26 from the respective solution value. Dement'eva *et al.* [27] investigated the binding of luciferin to firefly luciferase in solution using the same fluorescence quenching technique in the 0–20 μM luciferin concentration range. From the modified Stern–Volmer plot they reported an average binding constant, $K \approx 10^5\ M^{-1}$, for the range of solution pH values. However, they did not correct for dynamic quenching.

The differences between the two silica surfaces are not so illustrative in the case of the observed f_a values: f_a increases from 0.1 (solution) to 0.2 (hydrophobic silica) and to 0.24 (hydrophilic silica). It is uncertain, however, whether protein-bound luciferin quenches the same population of luciferase tryptophanyl residues at these two surfaces. The fluorescence quantum yield of luciferase was decreased upon adsorption at the hydrophobic silica surface. A possible conformational change of adsorbed protein at this surface might have changed the average distance between tryptophanyl residues and the bound quencher. Hence, the f_a values can not be interpreted unambiguously.

The luciferin quenching experiments were followed by the assay of light emission, in which luciferin was added together with ATP, Mg^{2+}, and O_2 to the adsorbed luciferase. No ATP-dependent visible luminescence was observed from the bound enzyme on either of the two surfaces, indicating that adsorption had resulted in inactivation of the enzyme.

Table I. Fluorescence Quenching Parameters for Firefly Luciferase in the Adsorbed State and in the Solution

System	f_a	K_{SQ}/M^{-1}
Luciferase in 0.45 *M* glycylglycine buffer solution	0.10	1.0×10^6
Luciferase adsorbed on hydrophobic silica surface	0.20	3.8×10^4
Luciferase adsorbed on hydrophilic silica surface	0.24	1.0×10^4

CONCLUSIONS

This study showed that the interfacial behavior of luciferase is determined largely by its hydrophobic nature. Luciferase adsorption onto hydrophobic silica surfaces and to air/buffer interfaces proceeds very fast as compared with its binding to hydrophilic silica. The initial slopes of the adsorption kinetics provided an estimate of the adsorption energy barrier, which was found to be larger for hydrophilic ($\approx$3 kT) than for hydrophobic (<1 kT) surfaces. The adsorption isotherm indicated that the enzyme, although it adsorbs relatively slowly, does aggregate on the hydrophilic silica surface. The plateau of the adsorption isotherm obtained on the hydrophilic silica surface was several times higher than the respective adsorption plateau found in the case of adsorption onto the hydrophobic surface. Comparison between the calibrated fluorescence intensity from adsorbed luciferase and the adsorbed amount as determined with ^{125}I-labeled luciferase showed that the fluorescence emission quantum yield of adsorbed protein is unchanged at the hydrophobic surface but decreased in the case of the hydrophilic surface. Further support for the change of enzyme conformation and/or aggregation at hydrophilic surfaces was found from fluorescence quenching of intrinsic luciferase fluorescence with luciferin. The luciferin binding constant to the high-affinity luciferase binding site decreased approximately 100 times upon protein adsorption to the hydrophilic silica surface, as compared with the respective binding affinity of the luciferase–luciferin complex in the solution. As judged from the absence of any ATP-dependent luminescence, luciferase was not enzymatically active upon adsorption at either of the two silica surfaces.

The luciferase used here was obtained from firefly lanterns using classical extraction, purification, and stabilization procedures (Sigma Chemical Co.). We are currently extending this study using pure firefly luciferase prepared by recombinant techniques (Amgen, Inc).

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