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Articles

Fluorescence Quenching of Adsorbed Hen and Human Lysozymes

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The iodide quenching of protein fluorescence was used to study the effect of surface adsorption on the conformation of human and hen lysozymes. Three different types of surfaces were used for protein adsorption: (a) hydrophobicized, uncharged DDS-silica; (b) unmodified, negatively charged silica; (c) positively charged APS-silica. The evanescent surface wave generated by total internal reflection was used to excite intrinsic fluorescence from the tryptophanyl residues of irreversibly adsorbed lysozymes. The extent of quenching of the adsorbed lysozyme fluorescence was shown to be a function of both the species of lysozyme studied (human vs hen) and the type of surface to which the protein was adsorbed. A modified Stern–Volmer quenching model, which assumes accessible and inaccessible populations of protein fluorophores, was applied to analyze the experimental results. The change in the fractional accessibility of fluorophores due to the adsorption was taken as a measure of protein conformational change. Both lysozymes appeared to exhibit smaller denaturation at the DDS-silica surface than on the other two surfaces. According to the quenching results both lysozymes were at least partially denatured upon adsorption to the unmodified, negatively charged silica surface as well as on the positively charged APS-silica surface. Human lysozyme displayed much larger changes in the denaturation parameters upon adsorption to the three surfaces than the hen lysozyme, indicating that it is less conformationally stable at interfaces. It was found that the effective quenching constant of iodide anion depended largely on the charge of the surface.

Introduction

The understanding and control of the interactions of proteins with solid surfaces are important in a number of areas of biology and medicine. In the last 20 years, there has been considerable interest in protein interactions with materials used in medical implant devices.[1-3] One area of particular interest to the contact lens industry is the interaction of tear proteins with contact lenses.[4-8] Deposition of proteins on contact lens surfaces cause a loss of visual acuity (due to the opaque nature of the adsorbed protein film), wearer discomfort, and, in some cases, acute eye diseases.[9,10] Lysozyme is a major protein constituent in tear fluids and has been shown to also be a major component in soft contact lens deposits.[11-13] Consequently, the use of lysozyme in protein adsorption studies has great practical value. Furthermore, since lysozyme is a rather simple, well-understood protein,[14,15] it can easily serve as a model for understanding the general principles that seem to govern protein adsorption.

Experimental methods used to study the structural changes that occur in proteins as they undergo adsorption at interfaces are currently very limited. Total internal reflection intrinsic fluorescence (TIRIF) spectroscopy combines well-known advantages of fluorescence spectroscopy with the surface sensitivity of internal reflection optics and it has been used in different aspects of protein adsorption.[16-19] One of the fluorescence techniques that is often used to probe protein conformational changes in solution is the technique of fluorescence quenching.[20-24] Presently, very little is done with this technique to probe

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the conformation or orientation of adsorbed proteins. In this study we have extended TIRIF to study iodide quenching of hen egg-white lysozyme and human milk lysozyme, which were irreversibly adsorbed to a set of model silica surfaces. These results are compared with those obtained for the two lysozymes in buffer solution. We then propose a simple model to explain our findings.

Materials and Methods

TIRIF Apparatus. The total internal reflection intrinsic fluorescence (TIRIF) apparatus used in these experiments was identical with that used by Hlady et al.[16] except that the γ-photon detection system was not used. Bulk solution fluorescence was measured by placing a rectangular fluorescence cuvette in place of the TIRIF cell, thus maintaining the same geometry between the excitation and the emission light path (70° angle).

Preparation of Adsorption Surfaces. The silica surface was prepared by cleaning amorphous hydrophilic silica microscope slides (ESCO Products) in hot (80 °C) chromic acid for 30 min, cooling to room temperature, and then rinsing thoroughly in ultrapure water (Milli-Q reagent water system). The slides were then desiccated for 12 h at 100 °C. Cleanliness was confirmed by the absence of hysteresis in the Wilhelmy plate water contact angle measurement.

The 3-(aminopropyl)triethoxysilane (APS) surfaces were prepared by dip-casting clean silica slides in a solution of 5% (v/v) APS (Petrarch Systems) in ethanol–water (95:5) mixture and allowing silanization to proceed for 30 min at room temperature. After the silanization the slides were rinsed several times in purified water, followed by rinsing in 100% ethanol. The slides were then desiccated under vacuum for 12 h at 60 °C.

Dimethyldichlorosilane (DDS) surfaces were prepared via a protocol similar to that for APS surface preparation except that the reaction mixture was 10% (v/v) DDS (Petrarch Systems) in dry toluene and slides were rinsed in ethanol before rinsing with water in order to remove the residual toluene first. All of the prepared surfaces were kept covered and used within 4 days of preparation.

Buffers and Lysozyme Solutions. The buffer used for the adsorption experiments was a phosphate buffer (PBS, pH 7.4, $[KH_2PO_4] = 0.013$ M, $[Na_2HPO_4] = 0.054$ M, $[NaCl] = 0.1$ M) made from analytical grade reagents and low-conductivity water. Both the hen egg-white lysozyme (3× crystalline) and the human milk lysozyme (purified via ion exchange chromatography, salt free powder) were obtained from Calbiochem and used without further purification. The protein solution was made by dissolving 10.0 mg of lysozyme in 10.0 mL of the PBS buffer. The final protein concentration was checked spectrophotometrically by using the absorptivity, $a = 2.56$ and 2.69 L g^{-1} cm^{-1} at 280 nm for the human and hen lysozymes respectively.[27,28]

The buffer used for the fluorescence quenching experiments was identical with the PBS buffer above except that instead of $[NaCl] = 0.1$ M, the sum $[NaCl] + [KI] = 0.5$ M. The six quencher solutions had iodide concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 M, respectively.

All buffers were prepared fresh, prior to each experiment.

Lysozyme Adsorption Experiments. Ten milliliters of the 1 mg/mL protein solution was injected into the TIRIF cell and allowed to adsorb to a particular surface for 1 h. The 280-nm evanescent surface wave created by total internal reflection at the silica/buffer interface was used to excite intrinsic fluorescence of lysozyme (measured at 335 nm) by which the kinetics of adsorption were followed (D. Horsley et al., in preparation). The excitation and the emission half-bandwidth was set to 16 nm. After 1 h the cell was flushed repeatedly with 20 mL of the 0.5 M NaCl buffer solution until further flushing did not result in a further decrease in fluorescence. This ensured the removal of all of the nonadsorbed and weakly adsorbed protein and provided a baseline fluorescence signal for irreversibly adsorbed lysozyme.

Iodide Quenching Experiments. Since the quenching of fluorescence of protein tryptophanyl residues was the subject of these experiments, tryptophanyl fluorescence emission was generated by exciting at 295 nm rather then at 280 nm and by collecting the emission at 335 nm. Following the buffer flushes with the 0.5 M NaCl buffer solution, the sequence of quenching solutions of increasing iodide quencher concentrations was injected into the TIRIF cell and the fluorescence intensity was recorded. After the quenching solution with the highest iodide concentration was injected and the fluorescence signal recorded, 20 mL of the 0.5 M NaCl buffer solution was injected to determine if the fluorescence returned to the baseline value (indicating the absence of additional desorption). In all instances, the fluorescence signal recovered almost fully (>90%); the tryptophanyl fluorescence at each iodide concentration was corrected for this decrease, assuming that an equal fraction of the signal was lost due to desorption at each injection of quencher solution. The iodine quenching experiments using lysozyme dissolved in buffer solutions were performed as a reference by standard techniques.[24,25]

Experimental quenching data were analyzed by using the Stern–Volmer equation[21]

$$F_0/F = 1 + K_Q[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, and K_Q is the Stern–Volmer quenching constant.

A modified form of the Stern–Volmer equation originally proposed by Lehrer was also used to analyze the experimental data[22]

$$F_0/\Delta F = 1/(f_a K_{Qa}[Q]) + 1/f_a \quad (2)$$

where ΔF is defined as $\Delta F = F_0 - F$, f_a is the fractional accessibility of tryptophanyl residues in lysozyme, and K_{Qa} is an effective quenching constant. Three independent measurements had been made for each iodide concentration. When the modified Stern–Volmer equation (eq 2) was used, a weighted least-squares linear regression method was applied to calculate the slope and the intercept of the $F_0/\Delta F$ vs $1/[Q]$ plot.[29] This analysis takes into account the fact that each mean observation has its own parent distribution and is weighted inversely as the variance of its parent distribution. The complete analysis can be also found in ref 30, Appendix B. From the slope and the intercept one can determine f_a and K_{Qa}.

Results and Discussion

All of the quenching systems studied exhibited downward curving Stern–Volmer plots when the quenching results were plotted according to eq 1 (F_0/F vs [Q], data not shown here).[30] Accordingly, the experimental results were analyzed by using the modified Stern–Volmer equation (eq 2) by plotting $F_0/\Delta F$ vs $1/[Q]$. Figures 1 and 2 show the result of iodide quenching experiments for hen egg-white and human milk lysozymes dissolved in buffer solution, respectively. Figures 4 through 8 show the modified Stern–Volmer plots for the irreversibly adsorbed layers of two lysozymes at different surfaces. Each datum is the mean value of three independent measurements while error bars represent one standard deviation. The straight lines were determined by the best fit using the weighted least-squares linear regression analysis.[29] Table I lists the parameters f_a and K_{Qa} which were determined from the best fit.

Comparison between the solution quenching of hen and human lysozyme (Figures 1 and 2) indicated different fractional accessibility of the tryptophanyl residues in these two proteins: $f_{a(hen)} = 0.37$ vs $f_{a(human)} = 0.18$, respectively.

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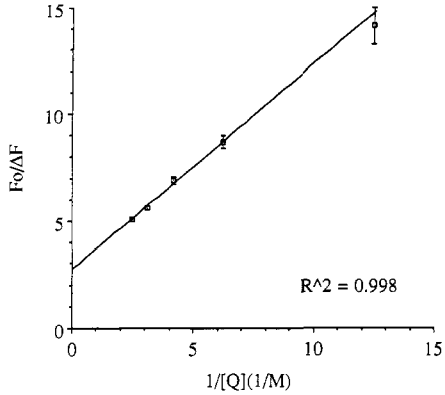

Figure 1. Modified Stern–Volmer plot for the hen egg-white lysozyme in buffer solution. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

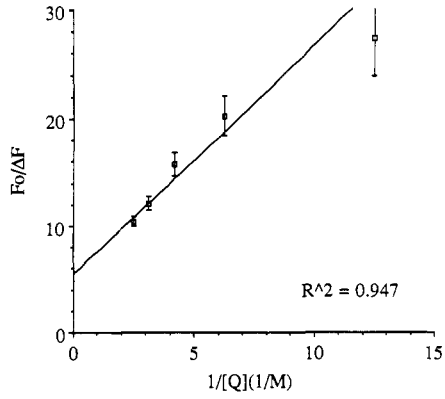

Figure 2. Modified Stern–Volmer plot for the human milk lysozyme in buffer solution. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

Table I. Stern–Volmer Quenching Parameters for Human and Hen Lysozymes

solution/surface and protein combination	f_a[a]	K_{Qa} (M^{-1})
buffer solution		
hen lysozyme	0.37 ± 0.02	2.8 ± 0.3
human lysozyme	0.18 ± 0.03	2.6 ± 0.6
DDS-silica surface		
hen lysozyme	0.34 ± 0.04	3.3 ± 0.7
human lysozyme	0.20 ± 0.04	4.2 ± 1.6
unmodified silica surface		
hen lysozyme	0.56 ± 0.10	1.7 ± 0.4
human lysozyme	0.53 ± 0.23	0.9 ± 0.4
APS-silica surface		
hen lysozyme	0.36 ± 0.03	5.7 ± 1.1
human lysozyme	0.68 ± 0.10	5.0 ± 1.6

[a] ±1 standard deviation.

The effective quenching constants were roughly the same ($K_{Qa(hen)} \approx K_{Qa(human)} \approx 2.7$ M^{-1}) (Table I). One should note that hen lysozyme has six tryptophanyl residues, as compared with five residues in human lysozyme, a fact that can account for different accessibility of these fluorophores. The location of tryptophanyl residues in both lysozymes can be visualized by using the protein atomic coordinates and molecular graphics, as described earlier.[31,32] It is known, however, that most of the lysozyme fluorescence comes from tryptophanyl residues located on the

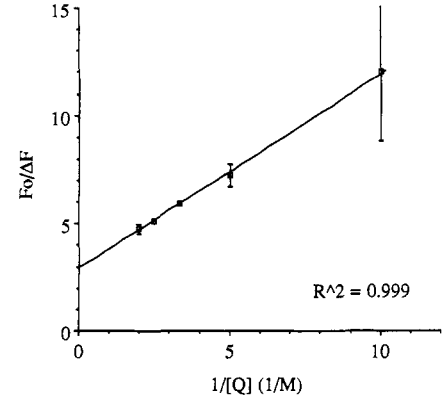

Figure 3. Modified Stern–Volmer plot for the hen egg-white lysozyme adsorbed to the DDS-silica surface. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

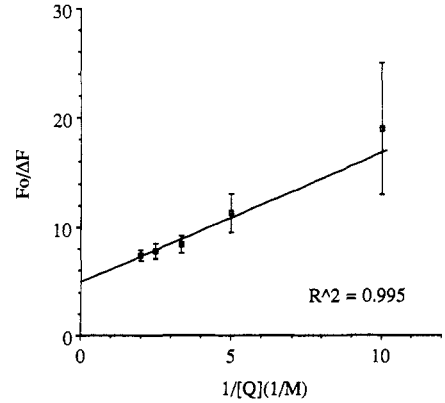

Figure 4. Modified Stern–Volmer plot for the human milk lysozyme adsorbed to the DDS-silica surface. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

surface of the active site cleft.[33-35] For example, it has been estimated that as much as 38% of the fluorescence of hen lysozyme is emitted from the active site tryptophanyl residue alone (Trp 62).[35] It is likely, therefore, that the solution fluorescence from these tryptophanyl residues will be principally affected by the presence of quencher.

When adsorbed onto the hydrophobicized, uncharged silica surface (DDS-silica surface) both lysozymes showed almost no change in the fractional accessibility of tryptophanyl residues, while the effective quenching constants increased to a different extent (Figures 3 and 4, and Table I), as compared with the reference state (i.e. lysozyme in the buffer solution). In contrast, adsorption onto the unmodified, negatively charged silica surface caused an increase of f_a for both lysozymes to approximately $f_a \approx 0.5$ and a decrease of K_{Qa} (Figures 5 and 6, Table I).

The change in the fractional accessibility can be tentatively interpreted as an extent of the conformational alteration of protein after adsorption at the particular surface. According to this simple model one can conclude that the lysozyme adsorption onto the hydrophobicized, uncharged silica surface (DDS-silica surface) does not cause a significant conformational change in both hen and human protein since there was no change of f_a (Table I). On the

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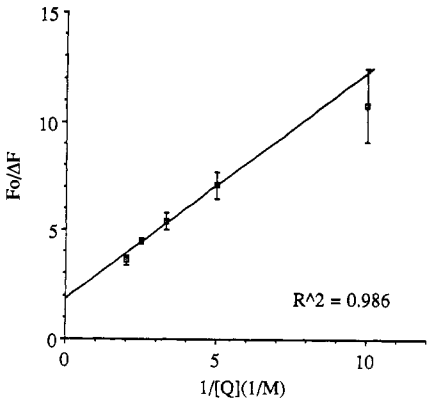

Figure 5. Modified Stern–Volmer plot for the hen egg-white lysozyme adsorbed to the unmodified silica surface. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

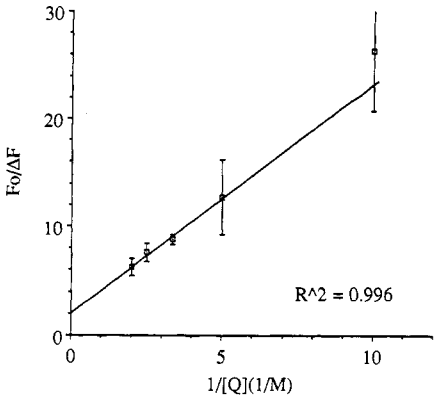

Figure 6. Modified Stern–Volmer plot for the human milk lysozyme adsorbed to the unmodified silica surface. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

contrary, the adsorption onto the unmodified, negatively charged silica surface brings a change of f_a for both lysozymes, indicating that the silica surface does induce conformational changes in both proteins. The difference in the hen lysozyme conformation at the DDS-silica surface and at the unmodified silica surface was supported by variable angle ESCA studies, which showed that the thickness of the adsorbed lysozyme layer was less on the silica surface than on the DDS surface.[31,36] Namely, a denatured protein is expected to spread out on the surface and thereby decrease its adsorbed layer thickness. The interaction of the positively charged amino acid residues (Lys, Arg), which are present on the surface of the lysozymes,[32] with the negative groups on the silica surface may be responsible for "spreading" the lysozyme molecule out on the surface. A similar ESCA study and comparison were not available for human lysozyme. The fractional accessibility of silica-adsorbed human lysozyme changes more than the fractional accessibility of hen lysozyme at the same surface; for example, f_a changes from 0.18 to 0.53 for human vs from 0.37 to 0.56 for hen lysozyme, respectively (Table I). A larger fraction of the human lysozyme tryptophanyl residues which become accessible after adsorption indicated that more extensive conformational changes were taking place in the silica-adsorbed human lysozyme as compared to the hen lysozyme molecule.

It is also interesting to compare how the effective quenching constant changes for the two lysozymes at these two surfaces. The K_{Qa} parameter reflects how easily iodide ion can find its way to the accessible tryptophanyl population. In an analogy to the association binding constant, the large K_{Qa} values indicate that iodide ion "associates" on average more readily with the tryptophanyl excited state. The penetration of iodide ion into an optimal quenching position is largely affected by the electrostatic repulsive forces, because of the charged nature of this quencher. It is known that a negative charge on the protein will be effective in preventing iodide ion from accessing neighboring tryptophanyl residue(s).[26] By taking the buffer solution as a reference state, one notes from Table I that the absolute value of the effective quenching constant change was always larger for human lysozyme than for hen lysozyme (i.e., $|\Delta K_{Qa(human)}| > |\Delta K_{Qa(hen)}|$). In the case of lysozyme adsorption onto hydrophobicized, uncharged DDS-silica, the value of K_{Qa} increases approximately 18% for hen and more than 3 times as much (approximately 62%) for human lysozyme, respectively. Although the total fractional accessibility and the total electric charge (protein + surface) in the case of both proteins remained unchanged, it is the human protein that was more affected by the adsorption onto the DDS-silica surface than the hen protein. The larger increase of $K_{Qa(human)}$ indicates that the same accessible fraction of fluorophores in the two lysozymes was more easily quenched in the case of the human protein because of the adsorption.

In the case of lysozyme adsorption onto unmodified, negatively charged silica surfaces, the interpretation of the effective quenching constant change becomes complicated by the presence of negative charges on the silica surface and by the unknown compensation of these negative charges by the positive charges of lysozymes. An overall decrease of the K_{Qa} values was presumably the result of the presence of negative surface charges, which were repelling iodide anions, rather than the result of some specific conformational change of adsorbed lysozymes. Human lysozyme at silica surfaces showed a larger change in the fractional accessibility of its tryptophanyl residues as well as a larger decrease of K_{Qa} (by approximately 65%) from the respective buffer solution values as compared with hen lysozyme. It is also known that human lysozyme is more susceptible to thermal denaturation than hen lysozyme.[30]

It is interesting to see how the experimental results of lysozyme quenching at the modified, positively charged silica surface (APS-silica surface) fit in this simplified model, in which the adsorption-induced conformational alterations are to be inferred from the fluorescence quenching. Namely, the positively charged surface of APS-silica should, in principle, increase K_{Qa} values for both lysozymes (due to the fact that electrostatic attraction forces make more iodide ions available for quenching at the surface) while the difference between the conformational stability of the two proteins should be reflected as a change of the parameter f_a. The experimental evidence for overall larger stability of hen lysozyme can be found in Figures 7 and 8 and in Table I; the K_{Qa} values of both lysozymes approximately doubled and, while $f_{a(hen)}$ remains unchanged, the $f_{a(human)}$ increased from $f_{a(human)} = 0.18$ (buffer solution) to the largest fractional accessibility found in this study, $f_{a(human)} = 0.68$ (APS-silica surface). This is a clear indication that human lysozyme is also conformationally more unstable when adsorbed onto positively charged surfaces than the hen lysozyme.

The technique of fluorescence quenching is relatively simple to execute in the study of irreversibly adsorbed

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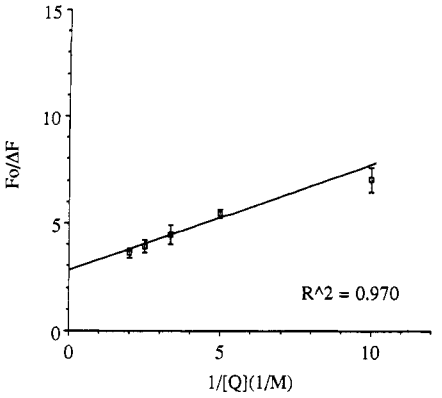

Figure 7. Modified Stern–Volmer plot for the hen egg-white lysozyme adsorbed to the APS-silica surface. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

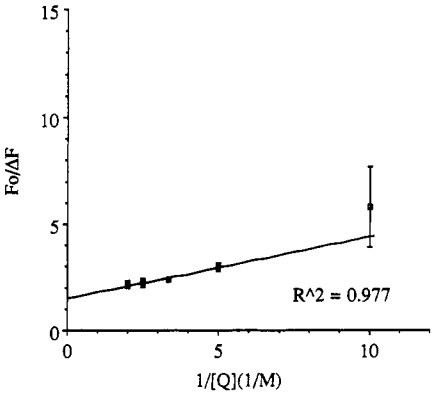

Figure 8. Modified Stern–Volmer plot for the human milk lysozyme adsorbed to the APS-silica surface. The value of the correlation coefficient, R^2, is indicated. For explanation of other symbols see text.

protein layers. However, as discussed in ref 21 protein fluorescence quenching data are easily overanalyzed. The use of modified Stern–Volmer plots is straightforward only in the case in which a protein contains one fluorophore population accessible to quencher and one totally inaccessible, as proposed by Lehrer in his derivation of eq 2.[22] The presence of the surface presents an additional level of complexity: it can specifically interact with a quencher. It is also hypothetically possible to have a grossly denatured protein which is adsorbed at the surface in such an orientation that all of the fluorophores are exposed toward the surface and effectively shielded from the quencher. The proposed simple model for the analysis of conformational alterations of adsorbed protein from the fluorescence quenching data should be, therefore, taken as a first approximation in the conformational analysis, which should be supplemented by different quenchers and different methods of fluorescence spectroscopy. For example, the conformational change of hen egg-white lysozyme adsorbed onto the chromatographic hydrophobic silica gel had been inferred from the 8-nm red shifts of the lysozyme fluorescence emission maxima.[37] We have recently used a combination of total internal reflection fluorescence and phase-resolved fluorescence spectroscopy in the study of surface-induced quenching of rhodamine-labeled bovine serum albumin at silica–solution interface.[38] It is our long range goal to apply the same technique to adsorbed proteins by using their intrinsic fluorescence.

Conclusions

Total internal reflection intrinsic fluorescence (TIRIF) spectroscopy was used to study the effect of surfaces on the conformation of irreversibly adsorbed human and hen lysozyme by using the iodide fluorescence quenching technique. Three different surfaces were used: (a) hydrophobicized, uncharged DDS-silica; (b) unmodified, negatively charged silica; (c) positively charged APS-silica. The quenching of the adsorbed lysozyme fluorescence was shown to be a function of both the species of lysozyme studied (human vs hen) and the type of surface to which the protein was adsorbed. A modified Stern–Volmer quenching model, which assumes accessible and inaccessible populations of protein fluorophores, was applied to analyze the experimental results. The change in the fractional accessibility of fluorophores due to the adsorption was taken, in a first approximation, to represent a measure of protein conformational change. We conclude that both lysozymes appeared to experience smaller denaturation at the DDS-silica surface than on the other two surfaces. According to the present results both lysozymes were at least partially denatured upon adsorption to the unmodified, negatively charged silica surface as well as on the positively charged APS-silica surface. Human lysozyme displayed much larger changes in the denaturation parameters upon adsorption of the three surfaces than those for hen lysozyme, indicating that it is less conformationally stable at different types of interfaces.

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Registry No. APS, 919-30-2; DDS, 75-78-5; silica, 7631-86-9; lysozyme, 9001-63-2.

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