Reorientational Dynamics of Enzymes Adsorbed on Quartz: A Temperature-Dependent Time-Resolved TIRF Anisotropy Study

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ABSTRACT The preservation of enzyme activity and protein binding capacity upon protein adsorption at solid interfaces is important for biotechnological and medical applications. Because these properties are partly related to the protein flexibility and mobility, we have studied the internal dynamics and the whole-body reorientational rates of two enzymes, staphylococcal nuclease (SNase) and hen egg white lysozyme, over the temperature range of 20–80°C when the proteins are adsorbed at the silica/water interface and, for comparison, when they are dissolved in buffer. The data were obtained using a combination of two experimental techniques, total internal reflection fluorescence spectroscopy and time-resolved fluorescence anisotropy measurements in the frequency domain, with the protein Trp residues as intrinsic fluorescence probes. It has been found that the internal dynamics and the whole-body rotation of SNase and lysozyme are markedly reduced upon adsorption over large temperature ranges. At elevated temperatures, both protein molecules appear completely immobilized and the fractional amplitudes for the whole-body rotation, which are related to the order parameter for the local rotational freedom of the Trp residues, remain constant and do not approach zero. This behavior indicates that the angular range of the Trp reorientation within the adsorbed proteins is largely restricted even at high temperatures, in contrast to that of the dissolved proteins. The results of this study thus provide a deeper understanding of protein activity at solid surfaces.

INTRODUCTION

An important property of proteins is their structural flexibility, which occurs on very different timescales. While vibrations of chemical bonds take less than a picosecond, reorientations of protein segments may be observed within nanoseconds, and a transient complete unfolding of the protein may eventually occur within years (Creighton, 1993). The biological activity of an enzyme or the substrate binding capacity of a protein depends largely on the conformational flexibility of the molecule. The opening or closing of the active site cleft of an enzyme or the hinge-type motion of protein domains may be involved in the substrate binding step (Yguerabide et al., 1970; McCammon et al., 1976; Jensen et al., 2002). In addition, conformational flexibility is also believed to provide an entropic stabilization of the native structure of thermophilic enzymes (Fitter and Heberle, 2000).

Protein molecules are intrinsically surface active (Horbett and Brash, 1995). When a solid surface is in contact with a protein solution, spontaneous adsorption of the protein molecules at the aqueous/solid interface can almost always be observed. The coverage of materials with a protein layer may have unfavorable consequences, such as a surface-induced thrombosis when blood is exposed to an artificial surface or eye diseases caused by biofilms on contact lenses. On the other hand, there are some examples where the adsorption of proteins at solid surfaces is useful, such as the protein separation and purification by solid/liquid chromatography or the detection of proteins on biochips, which play a growing role in proteomics (Weinberger et al., 2000). In medical diagnostics, solid-phase immunoassays are used where immunoglobulins are immobilized on a substrate, which then form sandwichlike complexes with antigens. As for these immunoassays, it is essential for protein biochips, when their function is based on molecular recognition mechanisms, that the bound proteins are not denatured by surface interactions, which requires the preservation of their structure and dynamics.

Although there are many studies in the literature on conformational changes of protein molecules induced by interactions with solid surfaces, only very little is known about changes in the internal protein dynamics or about the interfacial protein mobility. Because experimentally observed surface coverages with proteins can have higher values than the so-called jamming limit of the random sequential adsorption model (Schaaf and Talbot, 1989), some translational surface diffusion of protein molecules must occur, which has indeed been found in experiments and simulations (Tilton et al., 1990; Gaspers et al., 1994; Ravichandran and Talbot, 2000). After adsorption, an interfacial structural relaxation may also include a reorientation of the protein molecules, as has been suggested for the nonspherical lysozyme (Wertz and Santore, 2002). In the fluorescence study of Buijs and Hlady (1997), an adsorption-induced increase of the steady-state fluorescence anisotropy of the human growth hormone and lysozyme has been found, which indicates reduced rotational mobilities of the proteins in the adsorbed state. Adsorption-induced changes in the internal dynamics of a protein have been reported for inhibited Savinase on Teflon

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applying time-resolved fluorescence anisotropy measurements (Maste et al., 1997).

In view of the extremely limited data available in the literature describing the internal and overall reorientational dynamics of proteins adsorbed at aqueous/solid interfaces, we have performed a detailed temperature-dependent time-resolved fluorescence anisotropy study of the reorientational dynamics of the Trp residues in staphylococcal nuclease (SNase) and hen egg white lysozyme, when these proteins are spontaneously adsorbed at the silica/water interface. Beside a favorable optical transparency, quartz glass as the solid substrate provides a polar surface, which generally alters protein conformations to a smaller extent than a hydrophobic surface. The two proteins, SNase and lysozyme, have been chosen for several reasons. Although they are similar in mass and shape with 149 and 129 amino acid residues, respectively, and carry both a net positive charge at pH = 7, they differ in their stability against thermal unfolding. The temperature of unfolding of lysozyme is in the range of 70–78°C and the Gibbs energy of unfolding is 65 kJ mol^{-1} (Privalov and Khechinashvili, 1974; van Stokkum et al., 1995; Creighton, 1993), whereas SNase is unfolding at 52–54°C and has a lower Gibbs energy of unfolding of ~20 kJ mol^{-1} (Calderon et al., 1985; Griko et al., 1988; Panick et al., 1999; Seemann et al., 2001). Thus, it is possible to study the influence of the protein stability and, in the case of SNase, the effect of conformation (folded versus unfolded) on the dynamics of the adsorbed proteins at moderate temperatures of 20–80°C. Furthermore, for lysozyme adsorbed at the silica/water interface, the effect of temperature on the degree of adsorption, on the structure of the adsorbate, and on the protein conformation has been studied recently in our group using optical and neutron reflectometry and fluorescence spectroscopy (Jackler et al., 2002; Czeslik and Winter, 2001).

In this study, total internal reflection fluorescence (TIRF) spectroscopy was applied, which is a powerful method for studying molecules, including proteins, at interfaces (Hlady et al., 1985; Morrison and Weber, 1987). Briefly, a light beam is internally reflected at the surface of a prism, which creates an evanescent wave into the adjacent medium at the point of reflection. Because the penetration depth of the evanescent wave is on the order of the wavelength of the light beam, only molecules that are in close proximity (adsorbed) to the prism surface may be excited by this wave. The TIRF experiments reported in this study served to determine the fluorescence anisotropy decay of the Trp residues of lysozyme and SNase adsorbed at the silica/water interface (there are six Trp in lysozyme and one Trp in SNase). From this decay, reorientational correlation times of the Trp emission dipoles on the nanosecond timescale were derived, which in general represent the local Trp reorientations within the protein molecules and the whole-body protein rotations (Lakowicz, 1999).

MATERIALS AND METHODS

Staphylococcal nuclease (SNase) was obtained as described before (Seemann et al., 2001). Salt free hen egg white lysozyme was purchased from Roche Diagnostics (Mannheim, Germany) and was used without further purification. The proteins had a purity of >99% that was checked by gel electrophoresis (SDS-PAGE). Protein solutions were prepared using 10 mM phosphate buffer (pH = 7.0) with concentrations of 0.08 mg mL^{-1} (lysozyme) and 0.07 mg mL^{-1} (SNase). The protein concentrations were determined from ultraviolet absorbances at 280 nm using extinction coefficients of $\varepsilon = 2.65$ mg^{-1} mL cm^{-1} for lysozyme (Steiner, 1964) and $\varepsilon = 0.93$ mg^{-1} mL cm^{-1} for SNase (Fuchs et al., 1967). The quartz glass prism used for the TIRF experiments was purchased from Aachener Quarz-Glas Technologie Heinrich (Aachen, Germany). The Dove-type prism had a size of 47 mm × 15 mm × 15 mm and angles of 70° between the largest side and the two smallest sides. The prism was initially cleaned with a mixture of 60 mL H_2SO_4 (96%), 30 mL H_2O_2 (30%) and 10 mL water for ~15 min at 90°C. A contact angle of (27 ± 5)° was found for water on the quartz prism. The TIRF sample cell consisted of the quartz prism and a quartz plate, which were separated by a silicone rubber gasket of 4-mm thickness. The interior of the cell was filled and rinsed with buffer or protein solution using two syringe needles drilled through the rubber gasket. Between protein adsorption experiments the quartz prism was cleaned by excessive rinsing with concentrated HNO_3 and deionized water. Complete removal of adsorbed protein molecules was verified by a reproducible low fluorescence background. The temperature of the TIRF sample cell was adjusted using a water jacket. The sample cell was positioned in the sample chamber of a multifrequency phase fluorometer from ISS (Champaign, IL). By the use of two mirrors, the fluorescence excitation light of 295-nm wavelength was directed perpendicular onto one of the smallest sides of the quartz prism. By internal reflection of the light beam at the quartz prism/protein solution interface, an evanescent wave into the solution was generated, by which the Trp residues of protein molecules adjacent to the quartz prism surface were excited. Fluorescence emission was recorded perpendicular to the quartz prism/protein solution interface through the prism. Fluorescence of dissolved SNase and lysozyme was measured using a standard cuvette.

Time-resolved fluorescence intensity and anisotropy measurements have been carried out in the frequency domain applying the cross-correlation technique (Gratton and Limkeman, 1983; Gratton et al., 1984). Using this technique the fluorophores are excited with intensity-modulated light yielding intensity-modulated fluorescence emission. Depending on the fluorescence lifetime of the fluorophores and the excitation modulation frequency the fluorescence emission is phase shifted and demodulated relative to the excitation. The phase shift and the demodulation are detected and denoted in the following as ''phase'' and ''modulation.'' Excitation modulation frequencies up to 250 MHz and 300 MHz were used in the intensity and anisotropy experiments, respectively. The fluorescence excitation light was generated by a mode-locked Coherent Antares 76-YAG laser whose output of 532-nm wavelength was used to pump a Coherent 700 dye laser operated with rhodamine 6G. The obtained pulse train of 590-nm wavelength was passed through a Coherent 7220 Cavity Dumper and a Spectra-Physics 390 Frequency Doubler to produce light with a wavelength of 295 nm and a fundamental repetition rate of 3.81 MHz. For the fluorescence intensity measurements, the excitation light was vertically polarized (0°) and the emission light was polarized with an angle of 54.7°. Under this so-called magic angle condition the detected fluorescence is proportional to the total fluorescence intensity of the fluorophores. The excitation light was also vertically polarized relative to the plane of reflection in the TIRF experiments. Scattered light was used for lifetime referencing in the case of the TIRF measurements (lifetime, 0 ns), whereas the fluorescence light of p-terphenyl dissolved in glycol was used in the case of the solution measurements (lifetime, 1.05 ns). Phase and modulation data of the proteins were recorded at wavelengths above 305 nm by placing a Schott WG 320-nm long pass filter in the emission path. For the fluorescence anisotropy measurements, the excitation light was vertically polarized, whereas the emission light was collected with vertical and horizontal polarization. From

these measurements, the differential phase and the modulation ratio were determined as a function of the excitation modulation frequency. All measured differential phases and modulation ratios were corrected for the polarization- and frequency-dependent detector sensitivity using p-terphenyl that was excited with horizontal polarization. Under this condition p-terphenyl (or any other fluorophore) can be used as a standard having a differential phase of 0° and a modulation ratio of 1 at all frequencies.

After each initial filling of the TIRF cell with protein solution at 20°C, the sample has been equilibrated for 30 min. During this time the dissolved protein molecules are spontaneously adsorbed on the quartz surface. After fluorescence data were taken, the temperature of the sample cell was increased by 10°C, the sample cell was rinsed with fresh protein solution, and the sample was equilibrated for 20 min before taking new data. All measurements have been performed at least twice. Data analysis has been carried out using the Globals WE software (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). Reduced χ^2-values in the range 2–5 were obtained when fitting the phase and modulation data for intensity analysis and values of 1–3 were obtained when fitting the differential phase and modulation ratio data for anisotropy analysis. Experimental errors derived from repeated measurements are given in the tables and the figure captions.

RESULTS AND DISCUSSION

Time-resolved fluorescence intensity

In Fig. 1, the measured phase and modulation of the Trp fluorescence of SNase and lysozyme at 20°C are plotted as a function of the frequency of the excitation intensity modulation. The data are given for the two cases when the protein molecules are dissolved in phosphate buffer solution (pH = 7.0) and when they are adsorbed at the silica/solution interface; for the latter case the TIRF sample cell was used. Under the experimental conditions, both proteins roughly form a monolayer on a silica surface with a surface concentration of 2 mg/m^2 (Jackler et al., 2002). With values of 143 nm for the penetration depth of the evanescent wave, 0.08 mg mL^{-1} for the protein solution concentration, and 2 mg m^{-2} for the protein surface coverage, one can estimate that more than 99% of all excited protein molecules are adsorbed on the quartz surface. As can already be inferred from the frequencies where corresponding phase and modulation curves intersect, the average fluorescence lifetime of lysozyme is shorter than that of SNase and there is an adsorption-induced decrease of the average lifetime for both proteins. In the case of the TIRF measurements, the observed fluorescence modulations do not approach one at low excitation modulation frequencies, which indicates that the quartz prism used contributes a long lifetime component to the signal. This component has been determined in a separate experiment without protein by filling the TIRF sample cell with pure buffer. Temperature-independent phase and modulation data were obtained, which were modeled using three discrete lifetime components with lifetimes of 293.6 ns, 5.3 ns, and 0.6 ns and respective fractional intensities of 0.774, 0.144, and 0.083. The phase and modulation data of the adsorbed proteins could be fitted using two discrete fluorescence lifetime components together with the three components for the quartz glass. The lifetimes of the quartz glass and their relative fractional intensities were fixed to the above values and were not varied in the fitting procedure. However, the total fractional intensity of the quartz glass was varied. For the dissolved proteins, the experimental phase and modulation data could also be modeled with two discrete lifetime components. In Tables 1 and 2, all obtained fluorescence lifetimes and fractional intensities are listed.

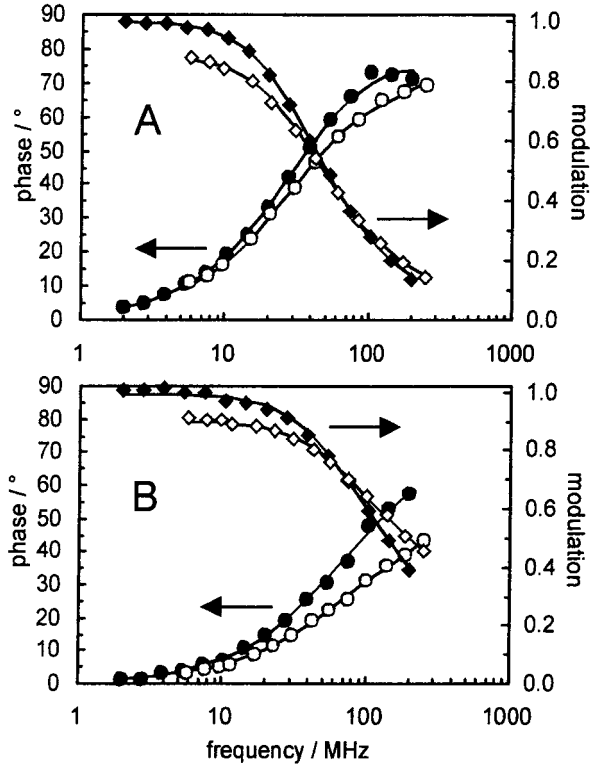

FIGURE 1 Phase and modulation of the Trp fluorescence of SNase (*A*) and lysozyme (*B*) at 20°C as a function of the excitation modulation frequency. The proteins were dissolved in buffer solution (*solid symbols*) or adsorbed at the silica/solution interface (*open symbols*). The solid lines show best fits to the experimental data.

It is noted that the fluorescence intensity decay of dissolved SNase at 20–40°C is almost completely described by a single exponential function. A second lifetime component is needed at higher temperatures to model the intensity decay when SNase unfolds. When the intensity decay of dissolved lysozyme is modeled assuming three instead of two discrete lifetime components, a slightly better fit of the experimental data can be obtained at 20°C only. Here, the reduced χ^2-value is decreased from 2.2 to 1.7 and lifetimes of 0.16 ns, 1.75 ns, and 5.01 ns are obtained. However, to perform a straightforward fluorescence intensity decay analysis for both proteins, dissolved and adsorbed, and at all temperatures studied, two discrete components have been used for all data.

A description of a Trp fluorescence intensity decay of a protein on the basis of exponential functions may have an

TABLE 1 Fluorescence lifetimes, τ_1 and τ_2, and associated fractional intensities, f_1 and f_2, for SNase and lysozyme adsorbed at the silica/water interface

	T/°C	f_1	τ_1/ns	f_2	τ_2/ns	f_{quartz}
SNase	20	0.083	0.72 ± 0.04	0.917	5.06 ± 0.11	0.154
	30	0.121	0.77 ± 0.04	0.879	4.70 ± 0.11	0.168
	40	0.123	0.68 ± 0.04	0.877	4.24 ± 0.11	0.200
	50	0.305	0.59 ± 0.04	0.695	3.02 ± 0.11	0.275
	60	0.326	0.45 ± 0.04	0.674	2.30 ± 0.11	0.287
	70	0.375	0.37 ± 0.04	0.625	2.00 ± 0.11	0.310
	80	0.487	0.37 ± 0.04	0.513	1.91 ± 0.11	0.347
Lysozyme	20	0.454	0.40 ± 0.06	0.546	2.16 ± 0.28	0.129
	30	0.532	0.29 ± 0.06	0.468	2.15 ± 0.28	0.162
	40	0.639	0.26 ± 0.06	0.361	1.91 ± 0.28	0.214
	50	0.662	0.18 ± 0.06	0.338	1.50 ± 0.28	0.259
	60	0.671	0.14 ± 0.06	0.329	1.26 ± 0.28	0.270
	70	0.674	0.16 ± 0.06	0.326	1.00 ± 0.28	0.241
	80	0.816	0.21 ± 0.06	0.184	1.46 ± 0.28	0.185

f_{quartz} is the total fractional intensity of the quartz glass. f_1 and f_2 are fractions of the total fractional intensity of the protein, $1 - f_{quartz}$. The listed errors reflect the maximum deviations observed over a whole temperature scan.

empirical character, because it neglects the conformational fluctuations of the protein (Alcala et al., 1987a; Alcala et al., 1987b; Gryczynski et al., 1988). On the other hand, a definite assignment of observed discrete lifetime components to Trp residues is difficult for a multi-Trp protein like lysozyme. Thus, for a comparison of the fluorescence intensity decays of dissolved and adsorbed SNase and lysozyme, it is more useful to calculate the average fluorescence lifetimes of the proteins according to

$$\langle \tau \rangle = f_1\tau_1 + f_2\tau_2, \quad (1)$$

where τ_1 and τ_2 are the fluorescence lifetimes and f_1 and f_2 are the associated fractional intensities (Tables 1 and 2). The obtained average fluorescence lifetimes are plotted in Fig. 2.

TABLE 2 Fluorescence lifetimes, τ_1 and τ_2, and associated fractional intensities, f_1 and f_2, for SNase and lysozyme dissolved in buffer solution

	T/°C	f_1	τ_1/ns	f_2	τ_2/ns
SNase	20	0.020	0.00 ± 0.06	0.980	5.42 ± 0.31
	30	0.029	0.00 ± 0.06	0.971	5.14 ± 0.31
	40	0.034	0.00 ± 0.06	0.966	4.69 ± 0.31
	50	0.066	0.03 ± 0.06	0.934	4.13 ± 0.31
	60	0.375	0.57 ± 0.06	0.625	2.84 ± 0.31
	70	0.441	0.50 ± 0.06	0.559	1.63 ± 0.31
	80	0.793	0.56 ± 0.06	0.207	1.98 ± 0.31
Lysozyme	20	0.273	0.73 ± 0.16	0.727	2.55 ± 0.40
	30	0.278	0.78 ± 0.16	0.722	2.15 ± 0.40
	40	0.397	0.77 ± 0.16	0.603	1.87 ± 0.40
	50	0.453	0.70 ± 0.16	0.547	1.56 ± 0.40
	60	0.521	0.60 ± 0.16	0.479	1.40 ± 0.40
	70	0.446	0.47 ± 0.16	0.554	1.14 ± 0.40
	80	0.383	0.32 ± 0.16	0.617	0.97 ± 0.40

The listed errors reflect the maximum deviations observed over a whole temperature scan.

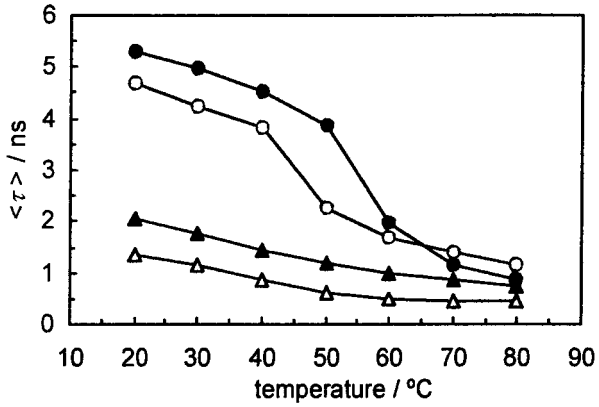

FIGURE 2 Average fluorescence lifetimes of SNase (*circles*) and lysozyme (*triangles*) as calculated from the data of Tables 1 and 2 according to Eq. 1. The solid symbols refer to dissolved proteins and the open symbols to adsorbed proteins. The average lifetimes have a maximum experimental error of ±0.1 ns.

As can be seen from this figure, the average lifetime of both proteins is decreasing with increasing temperature. Although SNase shows a drastic drop in lifetime at the temperature of unfolding, such discontinuity is not observed for lysozyme. The Trp fluorescence intensity of lysozyme is dominated by only two residues, Trp-62 and Trp-108, which contribute more than 80% in the folded state and ~60% in the unfolded state to the fluorescence emission (Imoto et al., 1971). Thus, the Trp fluorescence of native lysozyme is strongly quenched, which leads to an average lifetime that is already as low as that of unfolded SNase. Eftink et al. (1991) have also measured the fluorescence intensity decay of dissolved SNase in the temperature interval of 10–60°C. For example, average lifetimes of 5.75 ns at 20°C, 3.39 ns at 50°C, and 1.74 ns at 60°C can be calculated from their data, which are in good agreement with our values of 5.31 ns, 3.86 ns, and 1.99 ns at these temperatures (Fig. 2). The average fluorescence lifetime of dissolved lysozyme at 20°C is 2.05 ns (Fig. 2), which is in reasonable agreement with a corresponding value of 1.7 ns reported by Pap et al. (1996). The average fluorescence lifetime of lysozyme and SNase is decreased due to adsorption at the silica/water interface (Fig. 2). This may indicate some partial adsorption-induced unfolding. Indeed, adsorption-induced conformational changes of lysozyme at the silica/water interface have been observed before (Horsley et al., 1991; Jackler et al., 2002). However, a shortening of the Trp fluorescence lifetime of lysozyme is also reported for the adsorption to lipid vesicles (Pap et al., 1996) and the binding of *N*-acetyl-D-glucosamine (Nishimoto et al., 1998) where major conformational changes are not expected to occur.

Time-resolved fluorescence anisotropy

As an example, the differential phase and the modulation ratio of the Trp fluorescence of SNase and lysozyme at 20°C

are plotted as a function of the excitation modulation frequency in Fig. 3. Experimental data are shown for both proteins in the dissolved and the adsorbed state. The data clearly show that the reorientational dynamics of the proteins are changing drastically when they adsorb from the solution to the silica/water interface.

For the data analysis, a sum of two exponentials could be used to describe the time-dependent anisotropy of the protein Trp fluorescence:

$$r(t) = r_0[g_1 \exp(-t/\theta_1) + g_2 \exp(-t/\theta_2)], \qquad (2)$$

where r_0 is the maximum anisotropy on the timescale of the fluorescence experiments, θ_1 and θ_2 are reorientational correlation times of the Trp residues, and g_1 and g_2 are the associated fractional amplitudes ($g_1 + g_2 = 1$). The two reorientational correlation times are generally related to the overall rotation of the protein molecule (θ_1) and to the segmental motion of the Trp residues within the protein (θ_2) (Lakowicz et al., 1983). It is interesting to note that g_1, associated with the overall protein rotation, is a measure for the local motional restriction of the Trp residues in the protein. If the protein is unfolded, the Trp residues are free to rotate and g_1 is expected to approach zero. g_1 can be related to the generalized order parameter S for the segmental Trp reorientation. The angle of the Trp displacement, α, may be derived from (Lipari and Szabo, 1982)

$$g_1 = S^2 = \left[\frac{1}{2}\cos\alpha(1+\cos\alpha)\right]^2. \qquad (3)$$

The reorientational correlation times and fractional amplitudes, as derived from fitting Eq. 2 to the measured differential phases and modulation ratios of SNase and lysozyme, are listed in Tables 3 and 4, and best fits are shown in Fig. 3 for 20°C. In the analysis of the TIRF data of the adsorbed proteins, the quartz background has been included. It was determined in a separate experiment without protein and modeled with $r_0 = 0.185$, $g_1 = 0.705$, $\theta_1 = 273.90$ ns, and $\theta_2 = 3.91$ ns. The data were fitted applying a two-component model for the adsorbed protein and the quartz prism. In this model, the parameters describing the intensity decays of both components were fixed to the fluorescence lifetimes and fractional intensities that were determined before (see Time-resolved fluorescence intensity). The above-mentioned parameters modeling the anisotropy decay of the quartz prism without protein were also used and kept constant in the fitting analysis. The maximum anisotropy, r_0, of each protein has been found to be temperature independent. Therefore, a data set that was obtained as a function temperature was fitted globally with the maximum anisotropy required to be constant.

In Fig. 4, all obtained reorientational correlation times, θ_2, of the local Trp motions are plotted as a function of temperature in a common diagram. For dissolved SNase, correlation times smaller than 0.1 ns are found at temperatures of 20–50°C, where the protein is mainly folded or starts to unfold. This finding is in agreement with a reported corresponding value of 91 ps for 20°C (Lakowicz, 1999). At

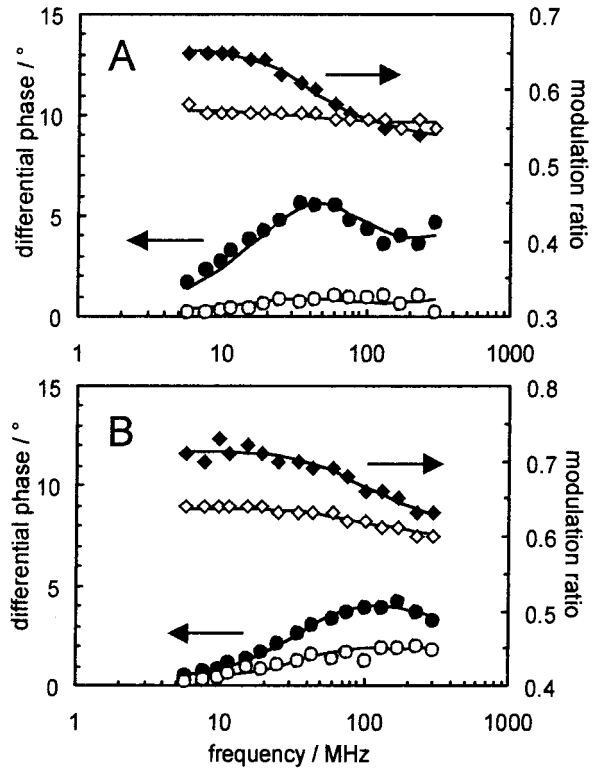

FIGURE 3 Differential phase and modulation ratio of the Trp fluorescence of SNase (*A*) and lysozyme (*B*) at 20°C as a function of the excitation modulation frequency. The proteins were dissolved in buffer solution (*solid symbols*) or adsorbed at the silica/solution interface (*open symbols*). The solid lines show best fits to the experimental data.

TABLE 3 Reorientational correlation times, θ_1 and θ_2, and associated fractional amplitudes, g_1 and g_2, of SNase and lysozyme adsorbed at the silica/water interface

	T/°C	g_1	θ_1/ns	g_2	θ_2/ns
SNase	20	0.843	76.92 ± 20	0.157	0.15 ± 0.1
	30	0.813	63.37 ± 25	0.187	0.18 ± 0.1
	40	0.751	46.73 ± 19	0.249	0.30 ± 0.1
	50	0.658	20.47 ± 6	0.342	0.29 ± 0.1
	60	0.594	34.58 ± 22	0.406	0.22 ± 0.1
	70	0.571	40.28 ± 26	0.429	0.19 ± 0.1
	80	0.557	n. r.	0.443	0.18 ± 0.1
Lysozyme	20	0.795	13.39 ± 2	0.205	0.46 ± 0.1
	30	0.625	n. r.	0.375	0.78 ± 0.1
	40	0.564	n. r.	0.436	0.77 ± 0.1
	50	0.566	n. r.	0.434	0.51 ± 0.1
	60	0.586	n. r.	0.414	0.34 ± 0.1
	70	0.576	n. r.	0.424	0.26 ± 0.1
	80	0.492	n. r.	0.508	0.26 ± 0.1

The maximum anisotropies were determined from global analyses of the temperature scans, $r_0 = 0.240$ for SNase and 0.197 for lysozyme. n. r., not resolved. The listed errors for θ_2 reflect the maximum deviations observed over a whole temperature scan.

TABLE 4 Reorientational correlation times, θ_1 and θ_2, and associated fractional amplitudes, g_1 and g_2, of dissolved SNase and lysozyme

	T/°C	g_1	θ_1/ns	g_2	θ_2/ns
SNase	20	0.790	11.41 ± 0.4	0.210	0.07 ± 0.1
	30	0.768	8.96 ± 0.4	0.232	0.01 ± 0.1
	40	0.734	7.46 ± 0.1	0.266	0.00 ± 0.1
	50	0.642	6.63 ± 0.1	0.358	0.03 ± 0.1
	60	0.194	72.39 ± 56	0.806	0.18 ± 0.1
	70	0.069	n. r.	0.931	0.18 ± 0.1
	80	0.043	n. r.	0.957	0.17 ± 0.1
Lysozyme	20	0.764	5.94 ± 0.3	0.236	0.66 ± 0.1
	30	0.827	4.17 ± 0.3	0.173	0.34 ± 0.1
	40	0.838	2.93 ± 0.3	0.162	0.18 ± 0.1
	50	0.772	2.60 ± 0.3	0.228	0.15 ± 0.1
	60	0.695	2.17 ± 0.4	0.305	0.17 ± 0.1
	70	0.534	2.22 ± 0.4	0.466	0.20 ± 0.1
	80	0.266	4.31 ± 0.8	0.734	0.17 ± 0.1

The maximum anisotropies were determined from global analyses of the temperature scans, $r_0 = 0.270$ for SNase and 0.184 for lysozyme. n. r., not resolved. The listed errors for θ_2 reflect the maximum deviations observed over a whole temperature scan.

higher temperatures than 50°C, SNase is mainly unfolded. Obviously, the thermal unfolding of SNase is accompanied by a slight increase of θ_2, which suggests that the reorientation of the Trp residue is slowed down due to hydration. For dissolved lysozyme, a relatively large correlation time of 0.66 ns is found for the local reorientation of the Trp residues at 20°C (Table 4). As already mentioned, there are two dominating fluorophores, Trp-62 and Trp-108, in lysozyme (Imoto et al., 1971). Although the indole NH group of Trp-108 is hydrogen bonded to the relatively rigid protein backbone, that of Trp-62 is hydrogen bonded to bulk water, as can be inferred from the crystal structure. Thus, the reorientational rates of both Trp residues are slowed down by

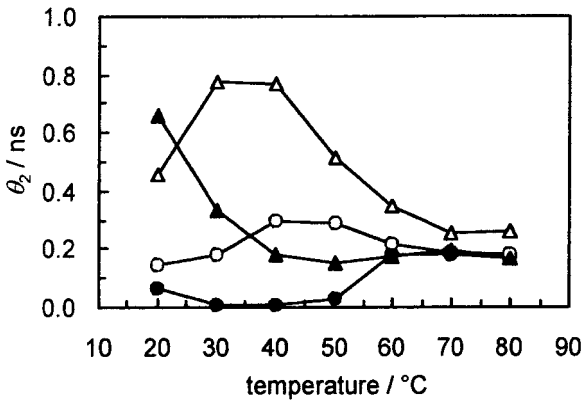

FIGURE 4 Short reorientational correlation times of the Trp residues of SNase (*circles*) and lysozyme (*triangles*) as a function of temperature. The proteins were dissolved (*solid symbols*) or adsorbed at the silica/solution interface (*open symbols*). The correlation times have a maximum experimental error of ±0.1 ns.

attractive interactions with the environment. With increasing temperature, the local reorientation of the Trp residues of dissolved lysozyme is getting faster and θ_2 reaches the same value as that of the Trp residue of dissolved SNase at high temperatures (Fig. 4).

When SNase adsorbs at the silica/water interface, the reorientational correlation time, θ_2, of the local Trp motion appears larger at temperatures of 20–50°C where the dissolved protein is folded or starts to unfold (Fig. 4), although this shift is larger than the experimental error at 30–50°C only. A possible explanation for this finding is an adsorption-induced partial unfolding of SNase, which might have a similar effect on θ_2 as the thermal unfolding of dissolved SNase described above. On the other hand, the binding of SNase to the solid silica surface has the effect of increasing the mean viscosity of the protein environment, which will lead to slower internal dynamics of the protein, and to a reduced reorientational rate of the Trp residue. Above 50°C, both adsorbed and dissolved SNase are unfolded, and the θ_2-values are equal in both states (Fig. 4). When lysozyme adsorbs at the silica/water interface, the internal dynamics of the protein are slightly enhanced at 20°C (Fig. 4). It is likely that this enhancement reflects a partial unfolding of lysozyme by which Trp-108 is exposed to water. This hydration of Trp-108 would lead to a strong red shift of the Trp fluorescence band of lysozyme. Indeed, such a red shift has been observed in a static TIRF spectroscopy study of lysozyme adsorption at the silica/water interface (Buijs and Hlady, 1997). In addition, a hydration of Trp-108 could also lead to a cleavage of the hydrogen bond between the indole NH group of Trp-108 and the protein backbone thereby increasing the reorientational rate of this residue. At higher temperatures than 20°C, the internal dynamics of lysozyme are slowed down due to adsorption at the silica/water interface, as can be inferred from the adsorption-induced increase of θ_2 at 30–60°C (Fig. 4). The observed increase of the θ_2-value of adsorbed lysozyme when heating from 20 to 30°C suggests that the viscosity of the lysozyme adsorbate is increasing with increasing temperature. This change in viscosity would be consistent with the effect of temperature on the θ_1-value of adsorbed lysozyme (see Table 3). In addition, attenuated total reflection-Fourier transform infrared spectroscopy studies indicate a gelation of a lysozyme adsorbate on a silica surface above ~40°C (Ball and Jones, 1995; Green et al., 1999).

In Fig. 5, all obtained reorientational correlation times, θ_1, of the whole-body protein rotation are summarized. Within the temperature regions where the dissolved proteins are folded, θ_1 of the dissolved proteins is decreasing with increasing temperature. When the dissolved proteins are heated above the temperatures of unfolding, θ_1 of both proteins is increasing. This behavior is consistent with an apparent increase in protein volume that is expected for protein unfolding and aggregation. When SNase is thermally unfolded, its radius of gyration is increasing from ~17

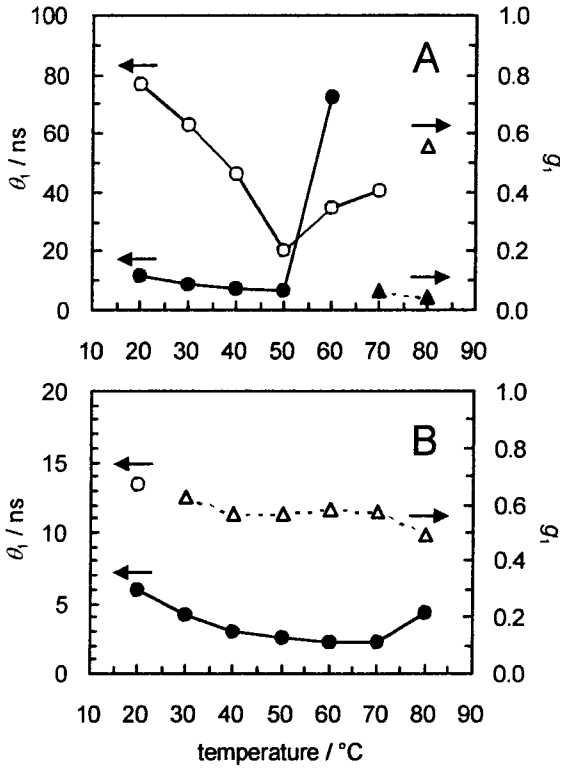

FIGURE 5 Long reorientational correlation times (θ_1, *circles*) and fractional amplitudes (g_1, *triangles*) of SNase (*A*) and lysozyme (*B*) as a function of temperature. The proteins were dissolved (*solid symbols*) or adsorbed at the silica/solution interface (*open symbols*). The maximum experimental errors of the θ_1-values are given in Tables 3 and 4, those of the g_1-values are ± 0.04 (adsorbed) and ± 0.02 (dissolved).

Å–42 Å (Panick et al., 1998), whereas a corresponding increase from 16 Å to 27 Å is reported for the thermal unfolding of lysozyme at pH = 7 (Arai and Hirai, 1999). Thus, assuming spherical shapes, the protein volumes are increasing by factors of 15 and 5, respectively. At 70 and 80°C, the anisotropy decay of dissolved SNase can be described with a single correlation time θ_2 and a very small residual anisotropy $r_0 g_1$. The fractional amplitude g_1 approaches zero at these temperatures indicating a nearly completely unfolded conformation (Fig. 5 *A*), which is in agreement with Fourier transform infrared spectroscopy data (Panick et al., 1998).

When SNase adsorbs at the silica/water interface, its overall reorientational correlation time θ_1 is increased by a factor of ~7 at 20–40°C (Fig. 5 *A*). A plot of θ_1 of adsorbed SNase as a function of η/T can be fitted by a straight line through the origin at 20–40°C suggesting that the shape and volume of the adsorbed SNase molecules are not changing significantly in this temperature region. Thus, the change in the correlation time is proportional to the change in apparent viscosity sensed by the protein molecules. This apparent viscosity within the protein adsorbate may be regarded as a quantitative measure for attractive protein-interface interactions. For comparison, when lysozyme adsorbs at the silica/water interface, the reorientational correlation time and thus the apparent viscosity is changing by a factor of only ~2 at 20°C (Fig. 5 *B*). Obviously, SNase is adsorbed more strongly at the silica/water interface than lysozyme. This result is in good agreement with the general finding that proteins with a small Gibbs energy of unfolding show a large affinity for interfaces (Arai and Norde, 1990; Jackler et al., 2002). An additional interpretation of the adsorption-induced increase of θ_1 of SNase and lysozyme would be a protein self-association, which is very likely considering the very high protein concentration within the protein adsorbates (Ball and Ramsden, 2000). At higher temperatures than 20°C, the overall reorientational correlation time of adsorbed lysozyme is very large and cannot be resolved on the nanosecond timescale of the performed fluorescence experiments. Thus, the rotation of adsorbed lysozyme molecules appears to be completely frozen, which in fact has to be attributed to protein aggregation and gelation (Green et al., 1999; Ball and Jones, 1995). In contrast, the rotation of adsorbed SNase molecules can be observed up to 70°C suggesting that SNase is characterized by a smaller tendency for self-association than lysozyme. Above 50°C, where SNase is mainly unfolded, the overall reorientational correlation time of adsorbed SNase is increasing with increasing temperature and is not resolvable anymore at 80°C (Fig. 5 *A*) which has to be attributed to aggregation of unfolded adsorbed protein molecules.

At 20°C, the obtained fractional amplitudes g_1 of dissolved and adsorbed SNase and lysozyme have a value of ~0.8 (Tables 3 and 4). Using Eq. 3, this value corresponds to an angle of $\alpha = 22°$ for the displacement of the Trp residues. In Fig. 5, fractional amplitudes g_1 are given where the overall reorientational correlation times of the proteins were too large to be resolvable on the nanosecond timescale of the fluorescence experiments. In these cases, the anisotropy decay is determined by the internal protein dynamics only. It is interesting to note that both proteins show a g_1-value of ~0.6 when they are completely immobilized on the silica surface, which is observed for SNase at 80°C and for lysozyme at 30–80°C. Using Eq. 3, an angle of 33° for the displacement of the Trp residues can be calculated from a g_1-value of 0.6. This result is somewhat surprising, because at high temperatures one expects the Trp residues of an unfolded protein to rotate freely, as found for dissolved SNase at 80°C with $g_1 = 0.043$ and $\alpha = 72°$. Thus, although there are adsorption-induced conformational changes and thermal unfolding, the reorientation of the Trp residues of SNase and lysozyme molecules remains largely restricted on the silica surface at all temperatures studied.

CONCLUSIONS

Pronounced changes in the overall rotational diffusion and the internal dynamics of SNase and lysozyme have been detected when these proteins adsorb from the solution on a plane silica

surface. The internal dynamics of the proteins, as probed by the Trp residues, are generally slowed down by surface interactions over the temperature regions where the dissolved proteins are folded. The overall rotational diffusion is also reduced at the silica/water interface. This reduction is affected by both protein-interface binding strength and protein self-association. In the case of SNase, which has a relatively low Gibbs energy of unfolding, the overall rotational diffusion is probably determined by protein-interface interactions, whereas the rotational diffusion of lysozyme molecules at the silica/water interface is likely to be prevented by self-association within the adsorbate. In addition to the reduced rates of Trp reorientation, the angular ranges of Trp reorientation within the protein molecules remain strongly restricted even at high temperatures, when SNase and lysozyme are adsorbed at the silica/water interface.

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