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ORIGINAL CONTRIBUTION

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pH dependence of the kinetics of interfacial tension changes during protein adsorption from sessile droplets on FEP-Teflon

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Abstract Interfacial tension changes during protein adsorption at both the solid-liquid and the liquid-vapor interface were measured simultaneously by ADSA-P from sessile droplets of protein solutions on fluoroethylenepropylene-Teflon. Four globular proteins of similar size, viz. lysozyme, ribonuclease, α-lactalbumin and Ca^{2+}-free α-lactalbumin, and one larger protein, serum albumin, were adsorbed from phosphate solutions at varying pH values (pH 3–12). The kinetics of the interfacial tension changes were described using a model accounting for diffusion-controlled adsorption of protein molecules and conformational changes of already adsorbed molecules. The contribution of conformational changes to the equilibrium interfacial pressure was shown to be relatively small and constant with respect to pH when compared to the contribution of adsorption of the protein molecules. The model also yields the diffusion relaxation time and the rate constant for the conformational changes at the interface. Around the isoelectric point of a protein the calculated diffusion relaxation time was minimal, which is ascribed to the absence of an energy barrier to adsorption. Energy barriers to adsorption become larger at pH values away from the isoelectric point and can therefore become rate-limiting for the adsorption process. The rate constants for conformational changes at the liquid–vapor interface were maximal around the isoelectric point of a protein, suggesting a smaller structural stability of the adsorbed protein. At the solid–liquid interface the rate constants were smaller and independent of pH, indicating that conformational changes more readily occur at the liquid–vapor than at the solid–liquid interface.

Key words Protein adsorption – interfacial tension – solid–liquid interface – liquid-vapor interface

Introduction

Interfacial tension changes due to protein adsorption are of great influence in many industrial and biological adhesion phenomena [1, 2]. Generally, the interfacial tension of a liquid–vapor or of a solid–liquid interface decreases due to adsorption of protein segments. The kinetics of these interfacial tension changes are very complex since not only the presence of adsorbed protein molecules, but also the arrangement and conformation of the adsorbed molecules determine the interfacial tension.

There are several models available to describe the interfacial tension as a function of adsorption time. As long as the rate of adsorption is faster than the rate of arrival of proteins at the surface, interfacial tension

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changes are controlled by transport. A common model applicable in such situations is based on the Ward and Tordai equation, in which transport is solely ascribed to diffusion [3]. When it is assumed that the surface acts as a perfect sink for arriving protein molecules, the amount of molecules arriving from the bulk at an interface will equal the amount adsorbed $\Gamma(t)$, according to

$$\Gamma(t) = 2C_0\sqrt{\frac{Dt}{\pi}}, \tag{1}$$

where C_0 is the bulk concentration, D the diffusion coefficient and t the adsorption time. The corresponding change in interfacial tension $\gamma(t)$ is given by the interfacial pressure $\Pi(t)$

$$\Pi(t) = \gamma(0) - \gamma(t) = 2\nu C_0 k_B T\sqrt{\frac{Dt}{\pi}}, \tag{2}$$

where ν is the number of adsorbed segments per protein molecule, k_B the Boltzmann constant, and T the absolute temperature.

However, due to the presence of an energy barrier to adsorption not all protein molecules arriving at an interface actually adsorb [4]. The work that has to be done to overcome this energy barrier equals $\Pi\Delta A$, where A is the interfacial area occupied by the adsorbing molecule. A modified version of the Ward and Tordai equation accounting for an adsorption energy barrier has therefore been proposed [5], in which the interfacial pressure is written as

$$\Pi(t) = 2\nu C_0 k_B T\sqrt{\frac{Dt}{\pi}}\exp\left(-\frac{\Pi\Delta A}{k_B T}\right). \tag{3}$$

According to Eq. (3) plots of Π vs $t^{1/2}$ and $\ln d\Pi/dt$ vs Π should yield straight lines. In reality, several linear trajectories can be observed in such plots [6–8], which can be assigned to different stages of the adsorption process, and sometimes no linearity was found at all [9].

When transport is not the rate-limiting step in the interfacial tension changes due to adsorption, the interfacial pressure is sometimes described by the empirical equation

$$\frac{\Pi_e - \Pi(t)}{\Pi_e - \Pi(0)} = \exp(-Kt), \tag{4}$$

where Π_e is the equilibrium interfacial pressure, and K the first order rate constant for adsorption [10, 11]. Analysis of interfacial tension changes shows several linear parts in plots of $\ln(\Pi_e - \Pi(t))/(\Pi_e - \Pi(0))$ vs t, which have been related to penetration of molecules into the interfacial layer, unfolding of adsorbed molecules, and possibly rearrangements of adsorbed molecules.

Serrien and Joos [12] proposed a model that accounts for contributions from both diffusion-controlled adsorption and subsequent conformational changes of adsorbed molecules to the decrease of the interfacial tension. In this model adsorption of native protein molecules is assumed to be reversible while adsorbed molecules may undergo conformational changes to an irreversible adsorption state with a rate constant k. Accordingly, the interfacial tension is described by

$$\gamma(t) = \gamma_e + \left[\alpha\exp\left(-\sqrt{\frac{4t}{\pi\tau_D}}\right) + \beta\right]\exp(-kt) \tag{5}$$

in which τ_D is the diffusion relaxation time and $(\alpha + \beta)$ represents the maximal change in interfacial tension $\gamma(0) - \gamma_e \cdot \alpha$ has been interpreted as the fraction of Π_e due to diffusion-controlled adsorption, and β is the fraction of Π_e due to conformational changes at the interface. Note that Eq. (5) does not account for an adsorption energy barrier and, consequently, τ_D is not related to the real protein diffusion coefficient but only to an apparent diffusion coefficient including diffusion and adsorption. Also, Eq. (5) reduces to the Ward and Tordai equation, Eq. (2), when conformational changes at the interface are ignored, i.e., when k and β are set to zero. Analogously, Eq. (5) can be reduced to resemble Eq. (4) when diffusion is not the rate-limiting factor for the interfacial tension changes to occur, i.e., when τ_D is set to zero.

Equation (5) has been used to describe interfacial tension changes during the adsorption of different types of proteins at varying concentrations, including a randomly coiled protein (casein), several globular proteins, immunoglobulin *IgG*, and a protein mixture (skimmed buttermilk) [13, 14] despite the fact that it was derived for a linear relation between γ and C and only small deviation of the interfacial tension from equilibrium. Van der Vegt et al. [14] showed that for a collection of proteins including *IgG* and the globular proteins lysozyme, ribonuclease, α-lactalbumin, calcium-depleted α-lactalbumin and serum albumin, the adsorption component α was smallest for the least hydrophobic protein ribonuclease. In a stationary end point of the adsorption process the conformational component β was found to be similar for all proteins. Furthermore, a comparison of the data for α-lactalbumin in its native and in its less stable apo state showed that the calculated rate constant k is related to the stability of the protein.

The pH of the bulk solution is known to influence the adsorption behavior and the conformational stability of protein molecules. Generally, around their isoelectric points, protein molecules show the smallest extent of lateral molecular expansion and, consequently, maximal adsorption is attained [15]. Furthermore, in some cases

aggregation of adsorbed protein molecules is observed [16]. However, minimal as well as maximal equilibrium interfacial tension changes have been found for adsorption around the isoelectric point, for instance for adsorption of BSA at liquid–vapor interfaces [9, 17, 18]. It is not known yet whether the pH only affects equilibrium interfacial tension changes or whether also the kinetics of the interfacial tension changes are influenced.

The aim of this paper is to study the influence of pH on the kinetics of interfacial tension changes during protein adsorption at the liquid–vapor and the solid–liquid interface. The model presented by Eq. (5) is employed to describe interfacial tension changes during adsorption of α-lactalbumin, calcium-depleted α-lactalbumin, ribonuclease, lysozyme, and serum albumin. Adsorption occurred from sessile protein solution droplets on FEP-Teflon, while the ADSA-P (axisymmetric drop shape analysis by profile) method was used to measure γ_{lv} and γ_{sl} simultaneously as a function of time.

Materials and methods

Proteins

Four globular proteins of similar size were used in this study (molar mass around 14 kD), including hen's egg lysozyme LSZ (Sigma L-6876), bovine pancreatic ribonuclease A RNase (Sigma R-5125), bovine milk α-lactalbumin αLA (a gift from the Netherlands Institute of Dairy Science NIZO, Ede, The Netherlands), and calcium-depleted bovine milk α-lactalbumin αLA(-Ca^{2+}) (Sigma L-6010). Also, a larger protein, bovine serum albumin BSA (Sigma A-4503), was used. Physico-chemical properties of the proteins relevant to the adsorption process are given in a related paper, where these proteins were studied at pH 7 in the concentration range of 0.001 to 5 $mg \cdot ml^{-1}$ [14].

The proteins were used as received to prepare single protein solutions at a concentration of 0.1 $mg \cdot ml^{-1}$ in 10 mM potassium phosphate solution with pH adjusted to vary between 3 and 12. This pH range includes the isoelectric points of all proteins employed. The protein was added to a fixed volume of a 10 mM phosphate solution while, prior to addition of protein, the phosphate solution was adjusted to the desired pH by varying the ratio of KH_2PO_4, K_2HPO_4 and K_3PO_4.

Substratum

The solid surface used in this study was commercial grade fluoroethylenepropylene-Teflon, FEP-Teflon (Norton Fluorplast, The Netherlands). Surfaces were cleaned ultrasonically in ultra pure ethanol (99.0–100.0%; Merck, FRG) to yield water contact angles exceeding 106 degrees.

Interfacial tension measurements and analysis of the kinetics

Calculation of the contact angle θ and the liquid surface tension γ_{lv} was done using axisymmetric drop shape analysis by profile ADSA-P [19, 20]. Briefly, a 100 μl protein solution droplet was placed on the FEP-Teflon surface in an enclosed glass chamber containing a reservoir filled with warm water to prevent evaporation of the droplet. Measurements on one solution droplet were performed as a function of time for 7 h at room temperature. The results $\theta(t)$ and $\gamma_{lv}(t)$ were combined with the Young equation to yield the solid–liquid interfacial tension $\gamma_{sl}(t)$ at any time t during the adsorption process [21, 22] from

$$\gamma_{sl}(t) = \gamma_{sv} - \gamma_{lv}(t) \cdot \cos\theta(t) , \quad (6)$$

where γ_{sv} represents the solid–vapor interfacial tension, γ_{sv} was assumed to be 20 $mJ \cdot m^{-2}$ and not to change during the experiment. This procedure was carried out three times with separate liquid droplets and the data were averaged.

Interfacial tension data were analyzed as described before [14]. The first image taken was considered to be 5 s after the first contact between the droplet and the surface. Subsequently, Eq. (5) was used to fit the averaged data to obtain relaxation times τ_D for the diffusion-controlled decrease of the interfacial tension, rate constants k for the conformational changes at the interface, the equilibrium value γ_e, and the corresponding compounds α and β of the interfacial pressure Π_e. This analysis was carried out for the solid–liquid and the liquid–vapor interface, while setting $\gamma_{lv}(0)$ equal to the interfacial tension of the pure phosphate solution (70 $mJ \cdot m^{-2}$) and $\gamma_{sl}(0)$ to 50 $mJ \cdot m^{-2}$, i.e., the interfacial tension between solution and FEP-Teflon as measured by ADSA-P.

Results

Figure 1 illustrates the pH dependence of both the liquid–vapor and the solid–liquid interfacial tension during adsorption of *αLA* from phosphate solution droplets on FEP-Teflon. The kinetics of the interfacial tension changes of the other proteins show similar time dependences as *αLA* with a distinct influence of pH. The interfacial tension data $\gamma_{lv}(t)$ and $\gamma_{sl}(t)$ were used to fit Eq. (5), yielding Π_e, its components α and β, and the parameters τ_D and k as summarized in Figs. 2–5.

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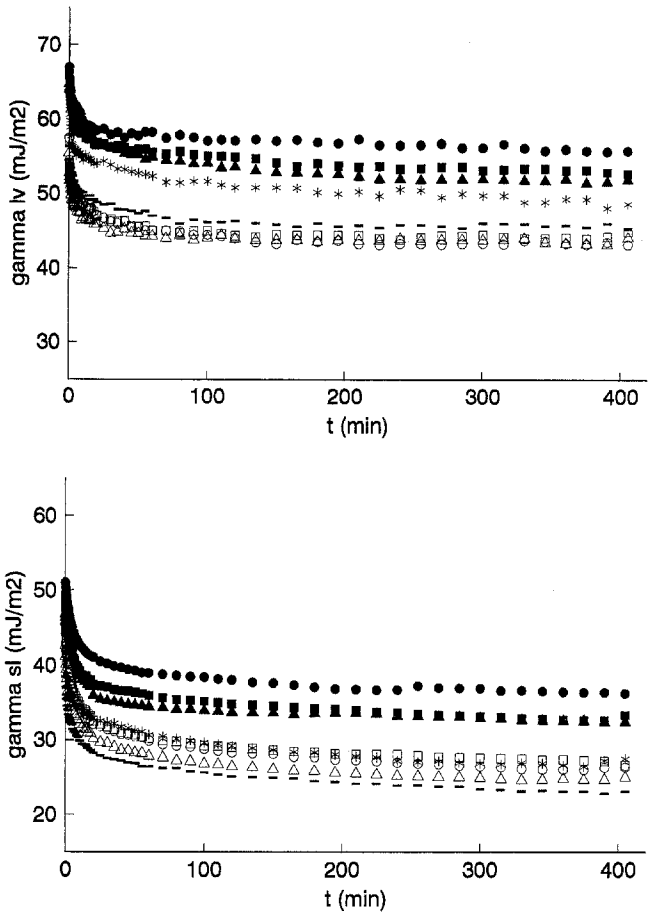

Fig. 1 pH dependence of the interfacial tension changes $\gamma_{lv}(t)$ and $\gamma_{sl}(t)$ for αLA adsorption from 0.1 mg·ml^{-1} solution droplets on FEP-Teflon as measured by ADSA-P. All data points are averages from three separate experiments with an approximate S.D. of 2mJ·m^{-2} for both interfaces. Symbols: *: pH 3, ○: pH 4, □: pH 4.5 △: pH 5, –: pH 6, ●: pH 7, ■: pH 9, ▲: pH 11

In Figs. 2 and 3 the equilibrium interfacial pressures Π_e for the liquid–vapor and solid–liquid interfaces are presented together with their components α and β due to diffusion-controlled adsorption and conformational changes at the interface, respectively. Note that the conformational component β is more or less independent of pH for both types of interfaces studied, whereas the adsorption component α obtains a maximal value around the isoelectric point of the protein. In Figs. 2 and 3 data for α are lacking at certain pH values when no satisfactory fit of the data to Eq. (5) could be obtained. Π_e values could, however, be directly calculated from the interfacial tension data.

A similar problem occurred for the adsorption of RNase at both interfaces, showing minimal Π_e values (up to 3 mJ·m^{-2}) for pH 8 and below, that could not be separated into an α and β component using Eq. (5). Above pH 8, i.e., in the vicinity of the isoelectric point of RNase (pH 9.4), Π_e increased (as for the other proteins towards their isoelectric points) to 10 mJ·m^{-2} on an average for both interfaces, and data could be fitted to Eq. (5), yielding a component α of about 5 mJ·m^{-2}.

The diffusion relaxation times τ_D and the rate constants k for conformational changes are presented in Figs. 4 and 5, respectively. Whereas the diffusion relaxation times are generally minimal around the isoelectric point of the proteins, the rate constants k are usually maximal. Note that for LSZ, τ_D and k are very little dependent on the solution pH. Maxima in the rate constant k as a function of pH are clearly present for the liquid–vapor interface but are virtually absent for the solid–liquid interface.

Again, for RNase, τ_D and k values could only be determined for pH values between 9 and 11, that is around the isoelectric point of the protein. On average, τ_D was 300 s for the liquid–vapor interface and 43 s for the solid–liquid interface, and k values amounted approximately 0.1×10^{-3} s^{-1} for both interfaces. Thus, for RNase around its isoelectric point, the diffusion relaxation time τ_D is larger at the liquid–vapor interface than at the solid–liquid interface, opposite to the trends found for the other proteins.

Discussion

In this study, interfacial tension changes during protein adsorption onto liquid–vapor and solid–liquid interfaces were simultaneously measured by ADSA-P and the kinetics were studied as a function of pH using Eq. (5). The model describes the decrease in interfacial tension due to diffusion-controlled adsorption and to conformational changes of adsorbed molecules at the interface.

Figures 2 and 3 show that the equilibrium interfacial pressure Π_e is maximal at or slightly above the isoelectric points of the proteins at the two interfaces. This is in agreement with the general idea that adsorbed layers are most closely packed at their isoelectric points, evidently resulting in more adsorbed segments per unit interfacial area. The component α of the total change in interfacial pressure is greatly influenced by the pH of the bulk solution, showing the largest values around or towards the isoelectric points of the proteins. This effect is most clear for the most flexible protein $\alpha LA(-Ca^{2+})$, and less clear for LSZ, which is the most rigid protein involved in this study.

The component β, constituting only a small part of the total change in interfacial pressure, is hardly influenced by

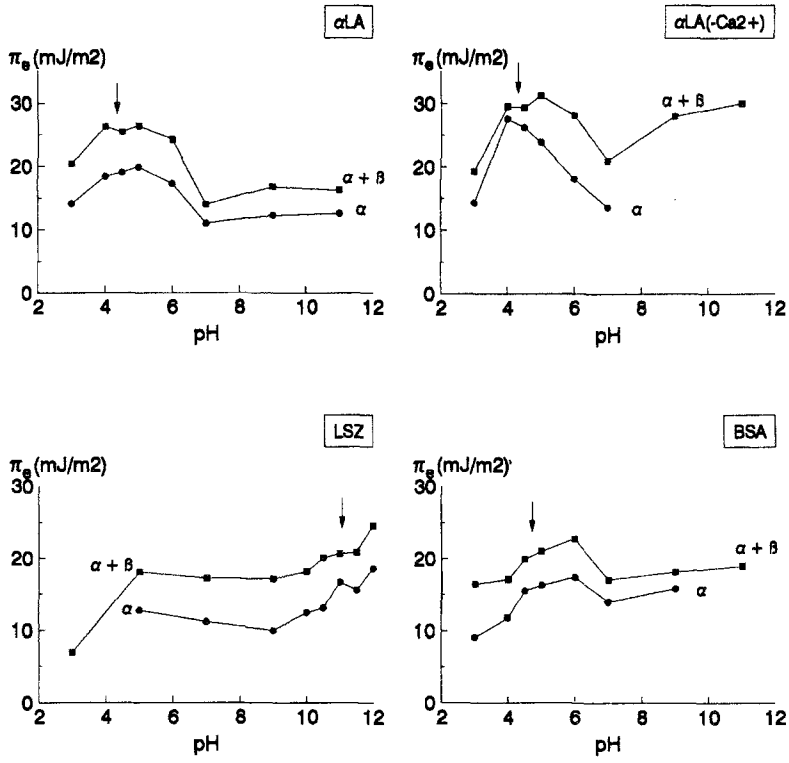

Fig. 2 pH dependence of the equilibrium interfacial pressure Π_e for adsorption of different proteins at the liquid-vapor interface. Π_e (■) was calculated from the sum of its components α (●) and β due to diffusion-controlled adsorption and conformational changes at the interface, respectively. The arrows indicate the isoelectric points of the proteins. The uncertainties in α and β amount 0.9 and 0.6 mJ · m^{-2} on an average, respectively, due to the quality of the fit

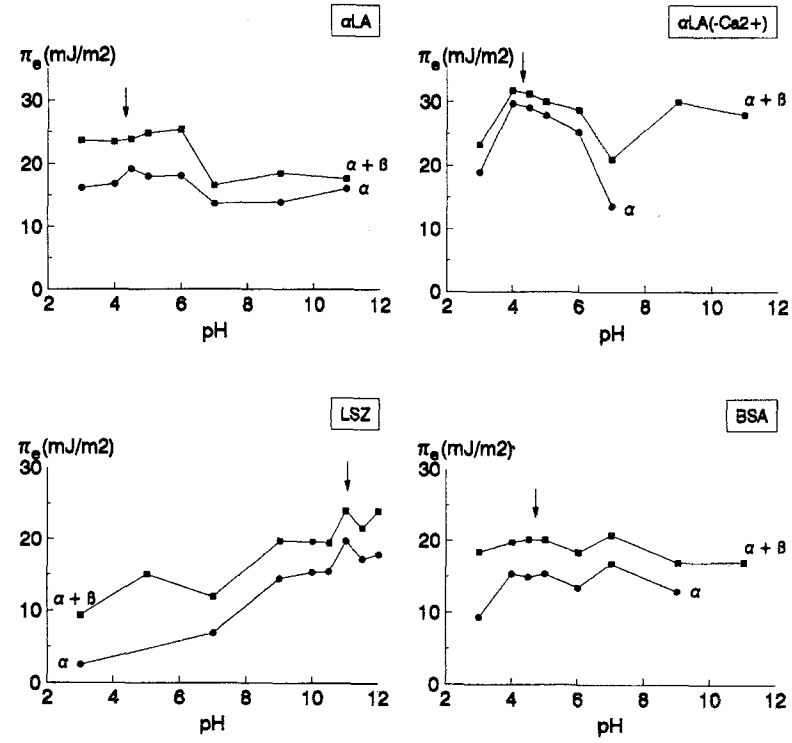

Fig. 3 pH dependence of the equilibrium interfacial pressure Π_e for adsorption of different proteins at the solid–liquid interface. Π_e (■) was calculated from the sum of its components α (●) and β due to diffusion-controlled adsorption and conformational changes at the interface, respectively. The arrows indicate the isoelectric points of the proteins. The uncertainties in α and β amount 0.9 and 0.6 mJ · m^{-2} on an average, respectively due to the quality of the fit

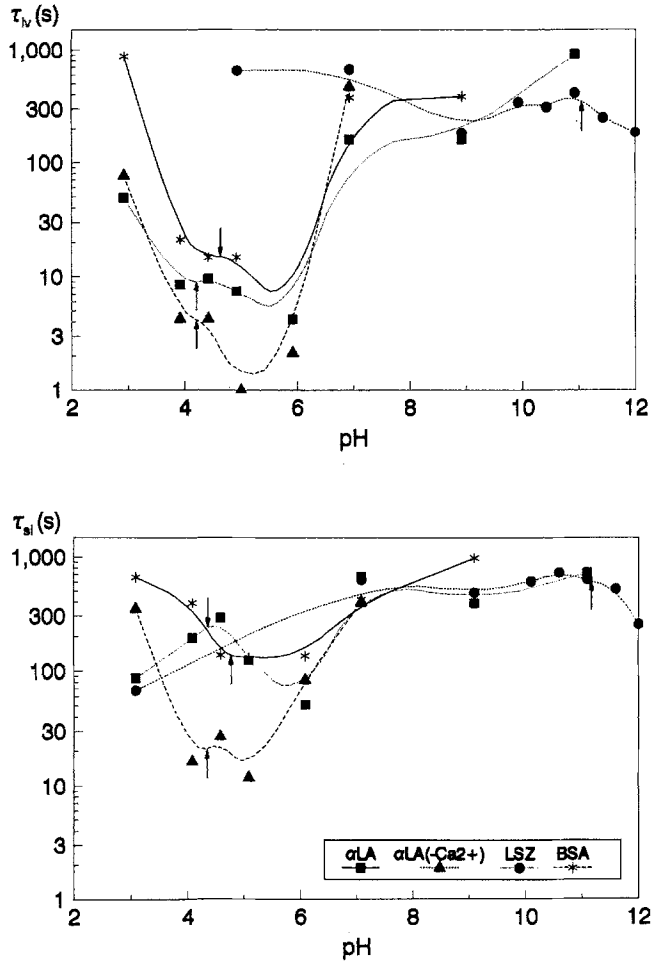

Fig. 4 pH dependence of the diffusion relaxation times $\tau_D(s)$ for adsorption of different proteins at the liquid–vapor and the solid–liquid interface from solution droplets on FEP-Teflon. The arrows indicate the isoelectric points of the proteins. The uncertainty in τ_D amounts 40% on an average due to the quality of the fit

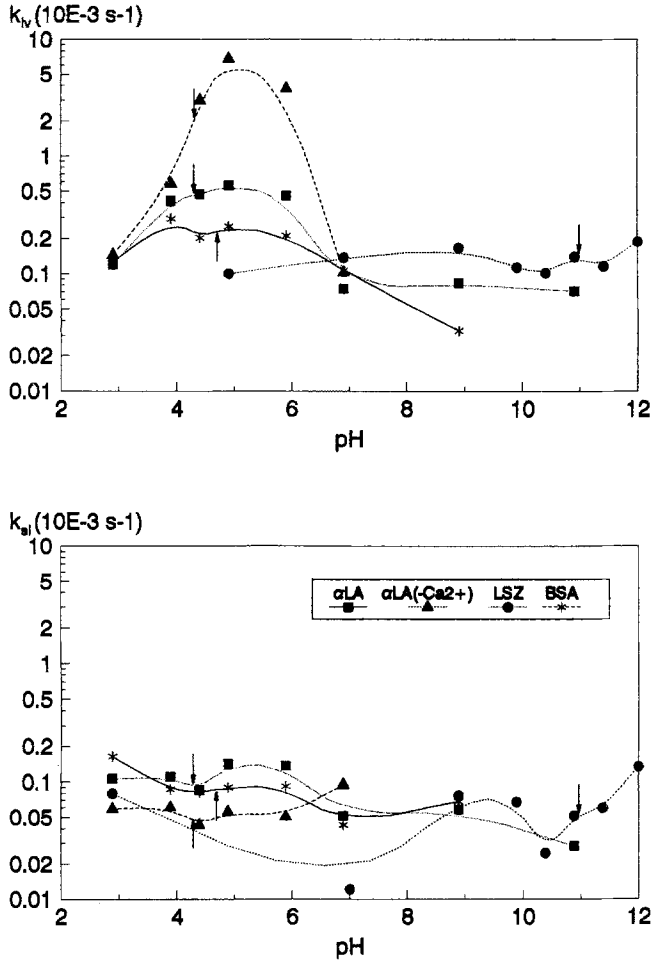

Fig. 5 pH dependence of the rate constants $k(s^{-1})$ for conformational changes of adsorbed proteins at the liquid–vapor and the solid–liquid interface from solution droplets on FEP-Teflon. The arrows indicate the isoelectric points of the proteins. The uncertainty in k amounts to 20% on an average due to the quality of the fit

pH and is more or less similar for all proteins used in this study. This supports the hypothesis that the effect of conformational changes of adsorbed proteins has only a minor influence on the equilibrium interfacial pressure. Apparently, the presence rather than the conformational state of an adsorbed protein controls the interfacial tension. This explains why other methods to assess the interfacial tension of adsorbed protein layers on solid surfaces, in which the conformational state of the adsorbed proteins is not preserved (e.g., in contact angle measurements on dried adsorbed protein layers), yield results more or less comparable with the results of the in situ technique ADSA-P [23].

The diffusion relaxation times show a minimum at or just above the isoelectric point of a protein (see Fig. 4). Strictly speaking, the word diffusion relaxation time is misleading because Eq. (5) refers to protein molecules that have traveled by diffusion towards the interface, and which have subsequently successfully adsorbed. Therefore, the minimum in the diffusion relaxation time of proteins around their isoelectric points points to a smaller energy barrier to adsorption at this pH. Neglect of the energy barrier to adsorption as a flaw in Eq. (5) also became evident in the present study because our data points taken far away from the isoelectric point of $\alpha LA(-Ca^{2+})$ (pH 9 and 11), LSZ (pH 5), RNase (pH 3, 5, 7, 8), and BSA (pH 11) could not be satisfactorily fitted by Eq. (5).

In addition, τ_D is generally larger at the solid-liquid interface than at the liquid–vapor interface. This was also observed for adsorption of these proteins from solutions with various protein concentrations [14], pointing to the

existence of a greater energy barrier to adsorption at the solid–liquid interface than at the liquid–vapor interface. A greater reaction component (or smaller sticking coefficient) at the solid–liquid interface as compared to the liquid–vapor interface might be due to stronger repulsive electrostatic forces between the adsorbing proteins and the FEP-Teflon. Electrostatic interactions are obviously absent for adsorption to the liquid–vapor interface. Since for RNase, τ_D is smaller at the solid–liquid interface, the above clearly does not hold for this protein. Possibly, this is due to the fact that the hydrophobicity of RNase is greater than that of the other proteins studied, indicating a major role of non-electrostatic interactions in the adsorption process.

In Fig. 5 the rate constants k for conformational changes at the liquid–vapor and the solid–liquid interface are presented. At the liquid–vapor interface there is a major difference in the pH dependence of the rate constants k for αLA in its native as compared to its apo state. In the apo state, αLA shows a much clearer maximum of k around the isoelectric point than in the native state, indicating that the stability of the protein molecule is reflected in the rate constant k. This is supported by the observation that for LSZ, the most rigid protein employed in this study, rate constant values k are low and essentially independent of pH. Generally, k values are larger and more influenced by pH for the liquid–vapor interface than for the solid–liquid interface, indicating that conformational changes more readily occur at the liquid–vapor interface. Interestingly, the present study points out that adsorbed proteins are less stable around their isoelectric points, as also observed for BSA adsorption on silica particles [24].

In conclusion, this paper demonstrates in a simultaneous study at the liquid–vapor and solid–liquid interface that protein adsorption at the solid–liquid interface is a process that differs from adsorption at a liquid–vapor interface, both with respect to its kinetics as a function of pH, as well as with respect to the conformational changes of the adsorbed proteins as induced by the interfaces.

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