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QCM-D Sensitivity to Protein Adsorption Reversibility

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Abstract

Using a quartz crystal microbalance with dissipative monitoring (QCM-D) we have determined the adsorption reversibility and viscoelastic properties of ribonuclease A adsorbed to hydrophobic self-assembled monolayers. Consistent with previous work with proteins unfolding on hydrophobic surfaces, high protein solution concentrations, reduced adsorption times, and low ammonium sulfate concentrations lead to increased adsorption reversibility. Measured rigidity of the protein layers normalized for adsorbed protein amounts, a quantity we term specific dissipation, correlated with adsorption reversibility of ribonuclease A. These results suggest that specific dissipation may be correlated with changes in structure of adsorbed proteins.

Keywords

protein adsorption; quartz crystal microbalance; dissipation; ribonuclease A

Introduction

The adsorption of biological molecules at solid–liquid interfaces continues to grow in importance due to its application to medical diagnostics, patterned cell cultures (Chen et al., 1997), tissue engineering (Niklason et al., 1999), biomaterials for medical implants (Kasemo, 1998), and protein purification (Oroszlan et al., 1990). Investigating the interaction mechanism is a difficult process that has been simplified by a number of researchers using well-defined, two-dimensional surfaces. A number of label-free, in situ detection methods have been developed to monitor this process via changes in refractive index at the surface and thereby quantify adsorption amounts. These detection methods include ellipsometry (Arwin, 2000), surface plasmon resonance (SPR) (Baird and Myszka, 2001; Rich and Myszka, 2002), and optical waveguide lightmode spectroscopy (OWLS) (Guemouri et al., 2000). These inspection methods are limited in their ability to directly measure adsorbed protein structure as well as adsorbed mass. Therefore, there is an ongoing need for complementary techniques to detect conformation and mass, as well as methods that can simultaneously measure adsorbed protein conformation and mass on well-defined surfaces in situ.

Quartz crystal microbalance with dissipative monitoring (QCM-D) is an experimental approach capable of measuring changes in adsorbed mass and viscoelastic properties of adsorbed material via differences in the frequency and decay of oscillation, respectively (Rodahl and Kasemo, 1996; Rodahl et al., 1995, 1996). Previous research has demonstrated these capabilities by measuring DNA adsorption and hybridization (Höök et al., 2001b) and biomolecular adsorption and cell adhesion (Rodahl et al., 1997). In each of these studies QCM-D successfully distinguished differences in adsorbed layer rigidity and adsorption mass. An additional study comparing results of QCM-D, ellipsometry, and SPR has also displayed the usefulness of QCM-D in measuring variations in coupled water, viscoelastic properties, and film thickness (Höök et al., 2001a). However, a direct correlation relating the viscoelastic properties of the adsorbed layer with protein structure has not been obtained for adsorbed proteins. In this work, we address this relationship and use the approach to investigate related changes in adsorption reversibility on hydrophobic surfaces as a function of adsorbed protein concentration (“protein loading”), mobile phase salt concentration, and adsorption time on well-defined surfaces.

Materials and Methods

Surface Preparation

The gold surfaces in these experiments were cleaned in an UV/ozone chamber for 10 min, followed by immersion in a 1:1:5 solution by volume of hydrogen peroxide (30%) (Sigma–Aldrich, St. Louis, MO), ammonium hydroxide (30%) (Aldrich), and distilled, deionized water (ddH₂O) for 20 min at 60–70°C. The surfaces were then rinsed with ethanol and ddH₂O and dried under a stream of nitrogen. Finally, the UV/ozone cleaning was repeated for 10 min. Immediately following this cleaning the surface was placed in a 10 mM 1-undecanethiol (98%) (Sigma) or 11-mercapto-1-undecanol (97%) (Aldrich) solution in anhydrous ethanol (Acros Organics, Geel, Belgium). The surfaces were then incubated for 4 h at 50°C, followed by rinsing in ethanol and ddH₂O prior to use.

The QCM-D Technique

The QCM-D technique, described in detail elsewhere (Rodahl et al., 1995), utilizes AT-cut quartz crystals coated with Au(1 1 1). For sufficiently rigid films, measured changes in resonant frequency are associated with changes in adsorbed mass per area according to the Sauerbrey relation (Sauerbrey, 1959)

$$\Delta m = -\frac{C}{n}\Delta f_n \quad (1)$$

where C is the mass sensitivity constant ($C = 17.7 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ at 5 MHz) and n is the frequency overtone number ($n = 1, 3, \dots$). This relation holds so long as the adsorbed mass is small compared to the crystal, is sufficiently thin and has limited viscoelastic coupling with the surrounding medium. In solution, one may expect adsorbed viscoelastic layers to underestimate the adsorbed mass (Höök et al., 2001a). However, when applying the Voigt–Kelvin model (Voinova et al., 2002), similar thin adsorbed protein layers have displayed

<10% difference from the Sauerbrey predictions and measured experimental values (Vörös, 2004).

The energy applied during the pulsed, forced oscillation of the QCM-D experiment dissipates at a rate related to the properties of the crystal and the adsorbed layer. For the adsorption of molecules from solution, a rigid film displays little viscoelastic coupling and has a longer decay time, while a non-rigid surface demonstrates significant viscoelastic coupling and a considerable decrease in decay time. In the case of proteins, changes in dissipation upon adsorption have been predominantly attributed to intra-layer processes such as those related to changes in layer density (Vörös, 2004), with minimal contributions from changes in surface roughness and slip at the surface (Höök et al., 1998; Rodahl et al., 1997). The dissipation for the system is related to the crystal's electrical equivalent parameters (Rodahl et al., 1997) and the ratio of dissipated and stored energy (Vogt et al., 2004) according to

$$D = \frac{R}{2\pi fL} = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}} \quad (2)$$

where R and L are the electrically equivalent resistance and inductance, respectively, and $E_{\text{dissipated}}$ (E_{stored}) is the energy dissipated (stored) during one period of oscillation. Regardless of rigidity, adsorption of more mass decreases the frequency of oscillation.

Experimental Procedure

All measurements were performed under static adsorption conditions after loading the gold sample surfaces (Q-Sense AB, Göteborg, Sweden) into an axial flow QCM-D chamber (Q-AFC 302, Q-Sense AB). Each buffer solution used was first sonicated under vacuum to minimize bubble formation on the sample surface. Following adequate time for system equilibration in protein-free buffer solution of PBS (pH 7.4) (Sigma), protein solutions (Ribonuclease A, Fisher Bioreagents, Fair Lawn, NJ) of the desired concentrations were loaded into the sample loop (2.5 mL) and then injected (0.5 mL) for the desired length of adsorption. At the end of adsorption the sample loop was again filled with protein-free buffer solution (4.0 mL) and then injected (1.0 mL) to the sample chamber to remove weakly and unadsorbed protein molecules. The solutions and cell were temperature-controlled at $25.4 \pm 0.1^\circ\text{C}$.

Results and Discussion

Sample Experiment and Important Measured Variables

Figure 1 displays a sample trace for $n=3$ during the 20-min adsorption of 1.0 mg mL^{-1} ribonuclease A (RNase) on a methyl-terminated self-assembled monolayer (SAM). Measurements at the $n=3, 5$, and 7 overtones are used for analysis but only the $n=3$ overtone is displayed for graphical simplicity. The change in frequency at the end of adsorption, $f_{\text{adsorption}}$, and change in frequency due to irreversible adsorption, $f_{\text{irreversible}}$, are noted in the figure. The change in frequency due to reversible adsorption, $f_{\text{reversible}}$, is

the difference of these two values. The change in dissipation due to adsorption, $D_{\text{adsorption}}$, is also noted in the figure. Rapid transients during the initial adsorption and rinsing steps can occur due to the large volume passing through the measurement chamber relative to its actual size. As shown in the second rinse portion of Figure 1 at 32 min as well as in preequilibration rinses (data not shown) such transients do not result in a contribution to f . For high concentration adsorption experiments, increased viscosity during protein introduction could contribute to apparent changes to frequency and dissipation. Rheological measurements (data not shown) confirmed a difference in viscosity between protein-free and concentrated protein solutions of 0.01 cP. For the high protein concentration condition, this viscosity difference results in a calculated increase in measured frequency and dissipation change of 3.2 Hz and 1.29×10^{-6} , respectively, according to Stockbridge's well-known relation due to coupling of the crystal with a Newtonian fluid (Stockbridge, 1966). All high concentration results shown here include this correction.

For thin polymer films (<40 nm) similar to that observed here, the Sauerbrey relation has been successfully applied (Vogt et al., 2004). Further, overtone traces for $n=5$ and $n=7$ are consistent at low and moderate loadings (data not shown). Slight overtone dependence at high loadings does not alter the qualitative trends of the data.

Effect of Protein Loading, Adsorption Time, and Mobile Phase Salt Concentration

To evaluate the effects of bulk solution protein concentration, adsorption time, and mobile phase salt concentration we have defined the percent of adsorption reversibility as the following:

$$\text{Reversibility}(\%) = \frac{\Delta f_{\text{reversible}}}{\Delta f_{\text{adsorption}}} \times 100 \quad (3)$$

This value corresponds to the fraction of molecules adsorbing on the surface that desorb upon returning to protein-free buffer solutions. The observed irreversible adsorption for each condition is consistent with previous studies indicated conformational changes (Benedek et al., 1984) and reduced enzymatic recoveries (Kato et al., 1985) for proteins encountering strongly hydrophobic reversed-phase chromatography surfaces. Additional studies using lysozyme, another model stable protein, have exhibited unfolding on a variety of hydrophobic surfaces under relatively mild conditions as well (McNay and Fernandez, 1999; Sane et al., 1999; Sethuraman and Belfort, 2005; Sethuraman et al., 2004). Further biophysical techniques including circular dichroism (CD) and differential scanning calorimetry (DSC) support such observations (Giacomelli and Norde, 2001).

Our results indicate a dramatic increase in reversibility with increased bulk solution concentrations as shown in Figure 2. Although this result differs from reports based on other surface techniques including TIRF and ellipsometry, it is consistent with similar QCM-D studies. One possible cause of this increased apparent adsorption may be that QCM-D senses interactions between the primary adsorbed layer and proteins in free solution that loosely associate with the adsorbed layer. This would be suggested by continued increases in measured adsorption beyond monolayer coverage. Ribonuclease A has molecular

dimensions of 3.8 nm×2.8 nm×2.2 nm. These dimensions result in a packing density of approximately 2.1–3.7 mgcm⁻², depending on adsorption orientation. As shown in Table I, these adsorption values suggest that only at high concentrations are increased intermolecular interactions of adsorbed species likely to occur. Our limited experimental conditions do not show a clear plateau at a monolayer that might help exclude the possibility of QCM-D sensing interactions with loosely bound molecules in solution. However, the loading-dependent result is consistent with the hypothesis that increased loading results in protein-protein crowding that inhibits unfolding (Fogle et al., 2006; Maste et al., 1997).

Similarly, our observed decreases in reversibility with time, also shown in Figure 2, are also consistent with the observation that protein molecules gradually unfold with increased exposure time on hydrophobic surfaces. Such unfolding has resulted in an increased apparent footprint of the protein (Wertz and Santore, 1999), increased solvent accessibility (McNay and Fernandez, 2001), and decreased density of adsorbed protein molecules (Vörös, 2004).

Ammonium sulfate has been shown to help stabilize the native conformation of proteins in solution (Von Hippel and Wong, 1964). In contrast, our current work suggests that for both varying bulk solution concentrations (data not shown) and adsorption times (Fig. 2), added ammonium sulfate decreases the reversibility of the adsorption process relative to low salt conditions. Other experiments and a thermodynamic framework developed in our group focusing in the linear portion of the isotherm offers a resolution of this difference, suggesting that salt affects native and unfolded protein adsorption differently (Xiao et al., 2006). Further studies of proteins adsorbed to hydrophobic interaction chromatography surfaces (Fogle et al., 2006) apply this hypothesis to high loading conditions as studied here and again show that even a salt that is stabilizing to proteins in solution, ammonium sulfate, can be destabilizing upon adsorption.

Relating “Specific Dissipation” Measurements to Adsorption Reversibility and Protein Structure

To compare the viscoelastic properties of adsorbed molecules we have defined the specific dissipation (which we term specific $\bar{D}$, or $\bar{D}$) as the following:

$$\bar{D} = \frac{\Delta D}{\Delta f} \quad (4)$$

where f has been normalized based on overtone number. Plots of $\bar{D}$ versus f have been used to analyze changes as proteins adsorb, but the specific dissipation parameter above allows a ready comparison of changes before and after adsorption/rinsing. This value is a measurement of the dissipation per adsorbed molecule on the surface. Höök et al. (2001a) attributed large $\bar{D}$ values to high amounts of associated water and expanded molecule conformations extending far from the surface. In their work a protein was adsorbed in an expanded, flexible conformation, and then cross-linked via chemical oxidation. Cross-linking reduced the associated solvent molecules as measured by a two-fold reduction in $\bar{D}$.

by QCM-D, and the adsorbed protein layer thickness was reduced fourfold as measured by ellipsometry. Cross-linking led to a four- and a half-fold decrease in $\bar{D}$.

Similarly, adsorption of water vapor into dry polymer films has been shown to increase polymer film thickness with a concomitant increase in associated water and $\bar{D}$ (Vogt et al., 2004). These studies both support the idea that decreases in $\bar{D}$ arise from compaction and dehydration of polymer films. Our hypothesis is that unfolding and lateral extension of an adsorbed protein results in a similar process.

Our results indicate a strong correlation between adsorption reversibility and $\bar{D}$ measurements, supporting the idea that $\bar{D}$ is directly related to protein structure on the SAM. In all cases we observe greater reversibility in situations with larger values of $\bar{D}$ at the end of adsorption. Interestingly, when the contributions to the total $\bar{D}$ are separated into reversible and irreversible components as in (Eq. 5) there is a distinct difference in $\bar{D}$ of the reversibly and irreversibly adsorbed species

$$\bar{D}_{\text{reversible}} = \frac{\bar{D}_{\text{adsorption}}\Delta f_{\text{adsorption}} - \bar{D}_{\text{irreversible}}\Delta f_{\text{irreversible}}}{\Delta f_{\text{reversible}}} \quad (5)$$

These results, summarized in Figure 3a, are based on the 20-min adsorption experiment summarized in Figure 2. Accordingly, the cluster of points with 40–50% reversibility is from 10 mg mL⁻¹ experiments and that with 20–30% reversibility is from 0.1 and 1 mg mL⁻¹ experiments. Standard deviations for reversibility values are included in Figure 2. Combined with irreversible adsorption data from methyl-terminated and hydroxyl-terminated surfaces (Fig. 3b), these results suggest two possible explanations for the observed behavior.

A first possible explanation for these results in the presence of two conformational states on the surface, native and unfolded, with a gradually increasing proportion of native molecules as the loading increases. Fogle et al. (2006) have observed a similar loading dependent stability on hydrophobic interaction chromatography surfaces. This hypothesis is not completely consistent with the observed specific dissipation values when separated into reversibly and irreversibly bound contributions. For two conformational states, one should observe a single range of specific dissipation values for irreversibly and reversibly adsorbed protein, respectively. These would appear as two vertical clusters in Figure 3a; a low specific dissipation cluster corresponding to irreversibly adsorbed protein, presumably unfolded, and a high specific dissipation cluster corresponding to reversibly adsorbed protein, presumably native or near native state. Instead, our observations show one cluster for the irreversibly adsorbed protein and a range of values for the reversibly bound material. The range of dissipation values for reversibly bound material suggests more than one conformational state is present in the reversibly bound protein. This could arise from a continuous mixture of native and unfolded states or a continuum of partially unfolded states with varying dissipation values.

A second possible explanation that could account for these results is the presence of a continuous distribution of adsorbed protein states, each having a progressively larger

effective footprint area as the degree of unfolding of an individual increases. Based on studies of fibrinogen, Wertz concluded that that fibrinogen likely forms unfolded conformations on the surface having a wide size distribution (Wertz and Santore, 2002). Differences in the footprint size between proteins on the hydroxyl- and methyl-terminated surfaces could explain the differences in amounts adsorbed as observed in Figure 3b. On the methyl-terminated surface, highly unfolded states would be expected with much larger footprints. On the hydroxyl-terminated surface, unfolded states might be more compact, possessing small footprints, leading to the increased amounts of protein adsorbed when approaching monolayer capacity. Additionally, if one were to consider any of the unfolded states, regardless of the extent of unfolding, as a minimal contribution to the specific dissipation, this series of conditions would account for the single cluster of points representing the irreversibly adsorbed protein contribution to the total specific dissipation.

Conclusions

The adsorption of ribonuclease A at high bulk solution concentrations and low ammonium sulfate concentrations leads to increased adsorption reversibility. Further, by reducing the length of adsorption time the reversibility of adsorption increases as well. These differences in adsorption reversibility support previous results from the literature suggesting changes in adsorbed protein structure for irreversibly adsorbed proteins. QCM-D measurements also display a correlation between adsorption reversibility and a measurement we have termed specific dissipation ($\bar{D}$). This surface rigidity measurement, normalized for adsorbed protein amounts, indicates that reversibly adsorbed material has highly dissipative, quite possibly native structure, while irreversibly adsorbed material has minimally dissipative, presumably denatured structure. The sensitivity of dissipation to loading, reversibility, and correspondence with trends observed in parallel studies raises the possibility that specific dissipation may be correlated with change in structure of adsorbed proteins.

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Figure 1.

Adsorption of 1.0 mg mL^{-1} ribonuclease A on a methyl-terminated self-assembled monolayer for the $n=3$ overtone. Sample injection (0.5 mL) occurs shortly after $t=1 \text{ min}$ and proceeds for 20 min . The change in frequency at the end of adsorption, $f_{\text{adsorption}}$, and change in frequency due to irreversible adsorption, $f_{\text{irreversible}}$, are noted in the figure. Irreversible adsorption is measured after rinsing the sample chamber with protein-free solution ($1.0 \text{ mL} \times 2$). The change in frequency due to reversible adsorption, $f_{\text{reversible}}$, is the difference of total and irreversible adsorption two values. The change in dissipation due to adsorption, $D_{\text{adsorption}}$, is also noted in the figure.

Figure 2. Adsorption reversibility as a function of surface exposure time for 10 mg mL⁻¹ (○) and 1 mg mL⁻¹ (×) bulk solution concentrations in low salt (0 M ammonium sulfate) and 1 mg mL⁻¹ (■) high salt (1 M ammonium sulfate) solutions on a methyl-terminated surface. Reported error bars are ± 1 standard deviation.

Figure 3.

a: Percent reversibility of adsorption as a function of specific dissipation ($\bar{D}$) for the reversibly bound (○) and irreversibly bound (●) material following protein adsorption. $\bar{D}$ at the end of adsorption and after rinsing are used to calculate $\bar{D}$ for the reversibly bound material according to (Eq. 5). Each data point is based on a 20-min adsorption period for varying bulk solution concentrations (0.1–10 mg mL^{-1}) in PBS solution. **b:** Amount of irreversible protein adsorption on methyl-terminated (▲) and hydroxyl-terminated (△) surfaces following a 20-min adsorption period. Reported error bars are ± 1 standard

deviation. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table I.

Comparison of adsorption masses on surfaces of varying hydrophobicity after 20 min of surface exposure.

Surface	Bulk solution concentration (mgmL ⁻¹)	Reversible adsorption (mgcm ⁻²)	Irreversible adsorption (mgcm ⁻²)	Total adsorption (mgcm ⁻²)
Methyl-terminated (-CH ₃)	0.1	0.18±0.06	0.67±0.23	0.85±0.24
	1.0	0.34±0.02	0.95±0.16	1.29±0.16
	10	0.75±0.16	1.51±0.07	2.26±0.17
Hydroxyl Terminated (-OH)	0.1	0.20±0.01	0.85±0.02	1.05±0.02
	1.0	0.45±0.09	1.30±0.25	1.75±0.27
	10	0.87±0.26	2.52±0.26	3.39±0.37

Reported errors are ±1 standard deviation.