Determination of Sorption Isotherm and Rheological Properties of Lysozyme Using a High-Resolution Humidity Scanning QCM-D Technique

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · AUGUST 2013

Impact Factor: 3.3 · DOI: 10.1021/jp404138f · Source: PubMed

2 AUTHORS:

Gesche Graf
Malmö University
15 PUBLICATIONS **123** CITATIONS
SEE PROFILE

Vitaly V. Kocherbitov
Malmö University
50 PUBLICATIONS **478** CITATIONS
SEE PROFILE

THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

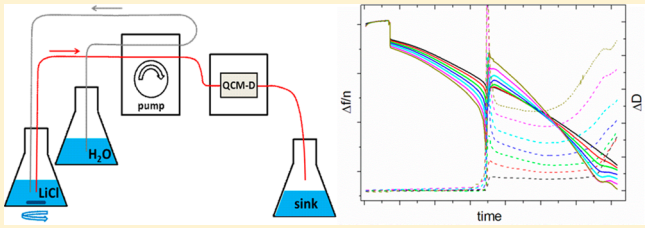
Determination of Sorption Isotherm and Rheological Properties of Lysozyme Using a High-Resolution Humidity Scanning QCM-D Technique

Gesche Graf and Vitaly Kocherbitov*

Biomedical Science, Faculty of Health and Society, Malmö University, SE-205 06 Malmö, Sweden

Supporting Information

ABSTRACT: The high-resolution humidity scanning QCM-D technique enables investigation of hydration of soft matter films using a quartz crystal microbalance with dissipation monitoring (QCM-D) equipped with a humidity module. Based on a continuous increase of relative humidity, properties of soft matter films can be investigated depending on the water content of the surrounding atmosphere. Determination of complete water sorption isotherms is possible via analysis of the overtone dependence of the resonance frequencies. Rheological properties are monitored via measurement of the dissipation. The glass transition can be identified from the change of viscoelastic properties of the film reflected in changes of the dissipation. A high-resolution water sorption isotherm of lysozyme was measured and compared with results from water sorption calorimetry. Analysis of the rheological behavior during hydration of lysozyme films revealed the presence of two separate sharp transitions at the water activities 0.67 and 0.91, which are connected to the glass transition. In previous works, only the existence of a broad glass transition has been reported so far. Combining the QCM-D data with Raman scattering data presented earlier, a new mechanism of isothermal glass transition in lysozyme is proposed.



INTRODUCTION

Understanding the hydration of soft matter is crucial for investigating the functioning of various systems. In many cases, the substances (e.g., biofilms such as mucous surfaces or skin) are in contact with humid air rather than with aqueous solutions. As the hydration, e.g., of proteins, influences their properties,[1] the functionality of any system depends on its components' ability to adsorb or desorb water from the gas phase.

In addition to analyzing the hydration behavior, rheological characterization is important to understand the properties in more detail and to help tailor the properties of surfaces to specific needs (e.g., biological active surfaces).

The quartz crystal microbalance with dissipation monitoring (QCM-D) technique is a suitable way to determine rheological data of samples in addition to measuring the mass of an adsorbate.[2] In the majority of studies, QCM-D is used to examine the adsorption of molecules from solution to a solid surface (an empty or coated sensor) from an aqueous solution that is flowed directly across the sensor. Lysozyme has been studied using QCM-D in various works using this approach including the investigation of the dependence of adsorption onto surfaces on ionic strength,[3] pH value,[4] or on the shape of the protein.[5] Rheological properties have been analyzed using the QCM-D technique for enzyme immobilization[6] or viscoelastic properties of adsorbed lysozyme films.[7]

A different approach is to use a QCM-D humidity module which separates the sensor from a solution by a membrane that is permeable only for water vapor (Figure 1A). This way a film deposited on the QCM-D sensor can be examined in controlled humidity rather than in direct contact with solution. Air of different relative humidities is achieved by the use of saturated salt solutions. Using the QCM-D humidity module, the mass of water vapor that is absorbed to a sample deposited on a sensor can be measured. Thus, it is possible to determine the water sorption isotherm of a substance which relates the amount of water taken up by a sample to the water activity (i.e., relative humidity).[8,9]

However, only a few water sorption isotherms were investigated by means of QCM-D measurements. A water sorption isotherm of lysozyme was determined using a quartz crystal microbalance/heat conduction calorimeter (QCM-D/HCC) with a stepwise change in relative humidity and compared to the D'Arcy–Watt equation.[10,11] The same method was utilized to characterize sorption behavior of polylactides (PLA).[12] Another sorption isotherm was determined for a polymeric Nafion film using QCM-D and gas of different relative humidities (controlled via saturated salt solutions).[13]

QCM-D measurements, such as the one described above, investigating hydration phenomena are most commonly carried out using a number of saturated salt solutions that set the relative humidity to specified levels.[14,15] However, a major

Received: April 26, 2013
Revised: July 15, 2013
Published: August 5, 2013

   dx.doi.org/10.1021/jp404138f | J. Phys. Chem. B 2013, 117, 10017–10026

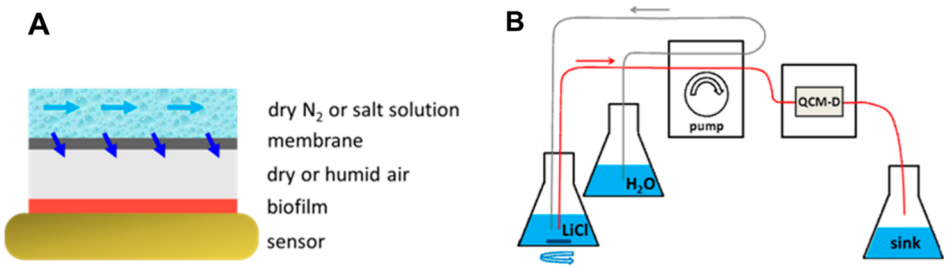


Figure 1. Schematic images of (A) q-sense humidity module in cross section[20] and (B) setup of the QCM-D scanning technique.

disadvantage of this method is the fact that no measurements with continuously increasing and decreasing relative humidity levels can be carried out, making the determination of complete water sorption isotherms impossible as only a few points on the sorption isotherm can be measured.

One aim of this work was the development of a new humidity scanning QCM-D methodology that enables hydration measurements and determination of complete water sorption isotherms with high resolution and accuracy using a QCM-D humidity module. For this purpose, an experimental setup was introduced that increases the relative humidity of the liquid pumped through the humidity module by continuously mixing a LiCl solution with increasing amounts of water (Figure 1B).

Based upon this altered setup, detailed analysis of rheological properties induced by increasing water content is possible. This includes the determination of the hydration-induced glass transition which has rarely been studied using QCM-D.[16,17] The newly developed setup was used to study lysozyme films and to determine the water sorption isotherm[18] as well as the rheological changes occurring during hydration.

MATERIALS AND METHODS

Lysozyme (from chicken egg white, ≥95%), LiCl (anhydrous, p.a., ≥99.0%), $MgCl_2$ (anhydrous, ≥98%), $Mg(NO_3)_2$ (hexahydrate, ≥99.0%), NaCl (p.a., ≥99.5%), KCl (≥99.0%), and K_2SO_4 (≥99.0%) were purchased from Sigma Aldrich (Sigma-Aldrich Co. LLC. St. Louis, MO). Lysozyme was dried under vacuum in contact with 3 Å molecular sieves for 24 h prior to use.

Ultrapure water was used from an Elgastat UHQ II, Model UHQ-PS-MK3 (Elga Ltd., High Wycombe, Bucks, UK). A Testo 605-H1 hygrometer (Testo AG, Lenzkirch, Germany) was used to confirm the activity of water (accuracy 3%) .

Salt Solutions. Saturated solutions of different salts were prepared by mixing excess amounts of salt with ultrapure water and equilibration for at least 1 week. The solutions were filtered two times prior to the experiments to remove undissolved salt and impurities.

QCM-D. A q-sense QCM-D E4 with humidity module 401 and AT-cut SiO_2 sensors (5 MHz) were used (Biolin Scietific AB, Västra Frölunda, Sweden). Atmospheres with different relative humidities were achieved by flowing dry nitrogen (0% relative humidity) or salt solutions through the humidity module. The sensor was separated from gas or solutions with a Gore membrane that only enables water vapor to pass (Figure 1A).

In a typical experiment, the empty sensor was measured first under dry atmosphere at 25 °C. Afterward, a film was applied by drop-coating from an aqueous solution and the sensor was dried overnight in a desiccator with silica gel. Subsequently, the coated sensor was dried for 15 min in vacuum and then measured under dry N_2 atmosphere until a stable baseline was observed. In the next step, a LiCl solution with continuously decreasing concentration was pumped through the humidity module (see below) to achieve an increasing relative humidity. For the dilution of LiCl solution and pumping of solution through the humidity module, a pump speed of 0.1 mL/min was used. The starting volume of LiCl solution was approximately 20 mL.

In the experiment with a stepwise change in the relative humidity achieved via the use of different saturated salt solutions, the separate solutions were pumped through the humidity module until an equilibrium state was reached. A pump speed of 0.1 mL/min was used.

The obtained raw data were analyzed using Q-tools software and MATLAB. The thickness of the dry film was determined using the Sauerbrey equation[19] to calculate the mass of the dry film and eq 1 to calculate its thickness.

$$t = \frac{V}{A} = \frac{m}{A\rho} \quad (1)$$

where t is the thickness of the dry film, V the volume, A the area, m/A the Sauerbrey mass, and ρ the density. A density of 1.2 g/cm^3 was used for the lysozyme film.

Gravimetric Determination of Mass. Approximately 5 g of LiCl solution was put in each of three weighted flasks, weighed and dried at 150 °C for 24 h. The flasks were weighed again and then dried at 160 °C for 24 h. If the change in mass was more than 0.02% the flasks were dried again at 170 °C for 24 h. From the loss of mass, the mass of water and LiCl in the solution were calculated.

Conductometer. A CyberScan CON510 Bench Meter (Vermon Hills, USA) was used to measure the conductivity of the LiCl solution at the end of the experiment. A calibration with LiCl solutions of known concentration was used to calculate the concentration.

RESULTS AND DISCUSSION

Humidity Scanning QCM-D Technique. Previously published results showed that single, separate points of a sorption isotherm can be determined using QCM-D equipped with a humidity module.[16] The humidity module contains a membrane that separates the sensor from solutions that are flowed through the humidity module. The membrane is only permeable for water vapor (Figure 1A). A film deposited on the sensor takes up water from the humid atmosphere. The mass of this water can be determined from the change in the frequencies and dissipations detected by the QCM-D. Different saturated salt solutions[14] and dry nitrogen were used to achieve specific humidity levels above the thin film. Thus, the water content of the film can be calculated depending on the known

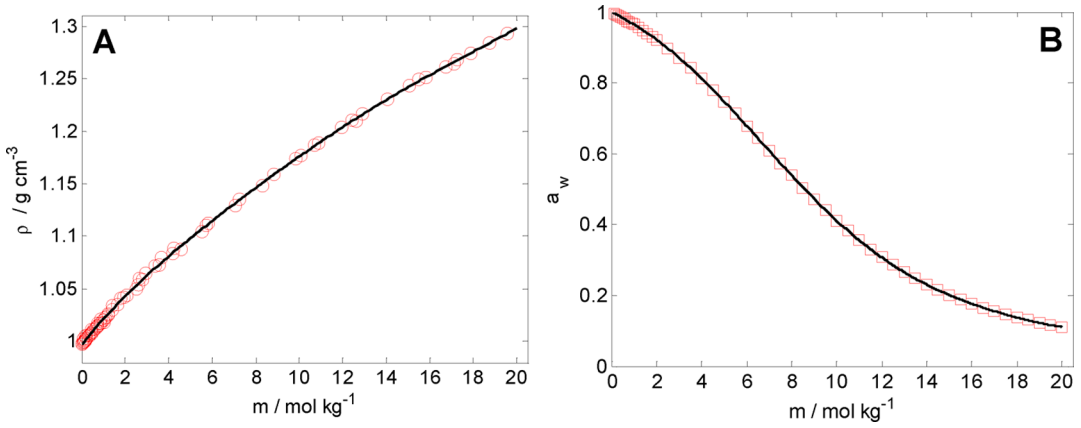


Figure 2. (A) Experimental data on the density of LiCl solutions depending on molality[21] (circles) and fit (black line). (B) Experimental data on the activity of water depending on the molality of LiCl solutions[22] (squares) and fit (black line).

relative humidity of the atmosphere (i.e., the activity of water) giving the information needed to create a water sorption isotherm. However, only a few points of the sorption isotherm can be determined in this way.[16]

In the work described here, the QCM-D setup was modified allowing a continuous increase of the humidity via dilution of a highly concentrated salt solution instead of interchanging different saturated salt solutions to enable the measurement of complete sorption isotherms.

The enhanced setup is shown in Figure 1B. Starting from a concentration close to saturation, a LiCl solution is continuously diluted with water. To achieve a homogeneous mixing, the LiCl solution is stirred during the whole experiment. The same pump is used to pump the LiCl solution through the humidity module of the QCM-D. In this way, the concentration of the LiCl solution decreases continuously, whereas the relative humidity increases.

The water activity in the LiCl solution can be calculated from the changing concentration of the LiCl solution during the experiment. Therefore, to allow an accurate determination of the water activity, the concentration of the LiCl solution needs to be calculated from the starting masses and the speed of the pump taking into account the density change occurring during the dilution of the solution. For this purpose, two equations were set up to determine the mass of salt (i.e., LiCl) and mass of water in the LiCl flask.

$$\frac{dm_s}{dt} = c_{s,in}y_{in} - c_s(t)y_{out} = -\frac{m_s(t)}{V_{sol}(t)}y_{out} \quad (2)$$

$$\frac{dm_w}{dt} = c_{w,in}y_{in} - c_w(t)y_{out} = c_{w,in}y_{in} - \frac{m_w(t)}{V_{sol}(t)}y_{out} \quad (3)$$

with m_s being mass of LiCl in grams, m_w mass of water in grams, V_{sol} volume of LiCl solution in cm^3, and y speed of pump in cm^3/s. $c_{s,in}$ and $c_{w,in}$ mark the concentration of LiCl and water that are pumped into the LiCl flask. $c_{s,in}$ is zero in the described setup. $c_s(t)$ and $c_w(t)$ mark the concentration of LiCl and water that are pumped out of the LiCl flask into the humidity module. The concentration is calculated as the ratio of mass per volume. The volume of the solution is calculated according to the following equation

$$V_{sol}(t) = \frac{m_{sol}(t)}{\rho_{sol}(t)} = \frac{m_s(t) + m_w(t)}{\rho_{sol}(t)} \quad (4)$$

where ρ_{sol} is the density of LiCl solution in g/cm^3. The resulting equations for the calculation of mass of LiCl and mass of water in the flask depending on time are as follows.

$$m_s(t) = m_s^0 - y_{out}\int \frac{m_s(t)}{V_{sol}(t)}\,dt \quad (5)$$

$$m_w(t) = m_w^0 + \int \left[c_{w,in}y_{in} - \frac{m_w(t)}{V_{sol}(t)}y_{out}\right] dt \quad (6)$$

Density values were adapted according to Abdulagatov et al.[21] by using the relation between molality and density of LiCl solutions at various concentrations, pressures, and temperatures. As in our case only data at 25 °C were of interest, a fit with a fifth-order polynomial gave good agreement with the experimental values and was used in the solution of the equations stated above (Figure 2A).

The two velocities y_{in} and y_{out} in eqs 5 and 6 were required as water and LiCl solution were transported through the pump in different tubes and had different (and in the case of the LiCl solution changing) densities. y_{in} (transport of pure water) was calculated from the exact change of the mass of water during the measurement using its density at 25 °C:

$$y_{in} = \frac{\Delta V}{\Delta t} = \frac{\Delta m}{\rho \Delta t} \quad (7)$$

As the density of the LiCl solution changes during the experiment, y_{out} cannot be calculated in the same simple way. Therefore, starting with the value of y_{in} for both, y_{in} as well as y_{out}, eqs 5 and 6 were solved numerically. As a result, the composition of the LiCl solution with m_s and m_w is calculated depending on time. This yields the density of the LiCl solution as a function of time. Integration of this function $\rho(t)$ enables the determination of the velocity y_{out} according to eqs 8 and 9 using the mass of the LiCl solution pumped through the humidity module (Δm).

$$\int dm = \Delta m = y_{out}\int \rho(t)\,dt \quad (8)$$

$$y_{out} = \frac{\Delta m}{\int \rho(t)\,dt} \quad (9)$$

Subsequently, eqs 5 and 6 are solved again with now two different speeds y_{in} and y_{out}. Repeating the calculation of y_{out} according to eqs 8 and 9, a more accurate value is determined.

dx.doi.org/10.1021/jp404138f | J. Phys. Chem. B 2013, 117, 10017−10026

Three iterations are needed until no significant change in y_{out} occurs.

A following numerical solution of eqs 5 and 6 using the two determined values for the speed of the pump yields the change for the mass of LiCl and water depending on time (Figure 3).

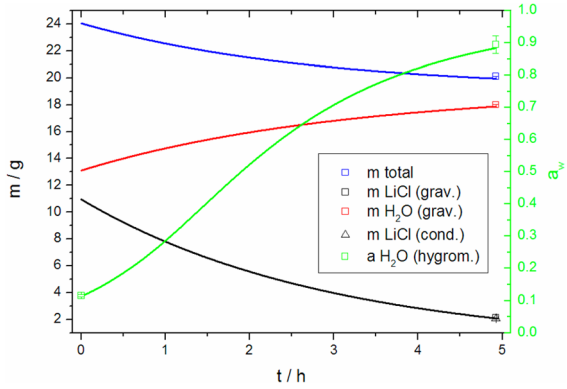


Figure 3. Calculated (solid lines) and experimental values (squares and triangle) for the masses of LiCl and water and the activity of water. Blue, total mass of solution; red, mass of water; black, mass of LiCl; and green, activity.

Using these data for the masses of LiCl and water depending on time, the LiCl concentration and, thus, the water activity can be calculated depending on time. For this purpose, experimental data on the relation between molality and activity of LiCl solutions[22] (Figure 2B) were fitted and this fit was subsequently used to calculate the activity depending on the time of the experiment. Experimental results and calculations for one representative measurement are shown in Figure 3.

Comparison of calculated and experimental results at the end of a measurement shows that the calculated mass of LiCl is in good agreement with values determined gravimetrically and conductometrically. Calculated values for masses of LiCl and water and total mass of solution show an error of below 1% compared to the experimental values.

The standard deviation for the gravimetric determination of the LiCl concentration is 0.1%. The water activity calculated based on these values can be expected to have the same accuracy. Additionally, the water activity was verified with a hygrometer showing an agreement between calculated and experimentally determined activity as well.

The scan rate for the change of activity depending on time is not constant during the measurement but ranges from 0.1/h at the start of the experiment to 0.25/h (Figure S1 in the Supporting Information). At high water activity above $a_w = 0.9$ the scan rate decreases to 0.05/h. The mean value for the scan rate of water activity for the experiment presented in Figure 3 is 0.16/h.

Data Analysis. QCM-D experiments are based on the measurement of the resonance frequency of an oscillating quartz crystal. The resonance frequency changes upon addition of a layer of material onto the crystal. In the case of rigid, evenly distributed layers with masses that are small compared to the mass of the crystal, the size of this change is connected to the mass of the applied material according to the Sauerbrey equation[2,19]

$$\Delta m = -\frac{C}{n}\Delta f \tag{10}$$

with Δm the change in areal mass, Δf change in frequency, n overtone number, and C mass sensitivity constant.

The second parameter that is examined using QCM-D is the dissipation D. It is a measure of the energy of the oscillating crystal that dissipates from the system during one oscillation cycle

$$D = \frac{E_{lost}}{2\pi E_{stored}} \tag{11}$$

with E_{lost} being the energy dissipated during one oscillation cycle and E_{stored} the energy stored in the system.[2,23]

Γ is the bandwidth of the resonance peak and is connected to the dissipation D according to eq 12:

$$\Gamma = \frac{Df}{2} \tag{12}$$

Changes of viscoelastic properties of the substance deposited on the quartz crystal can be examined via monitoring the dissipation as the viscoelastic properties directly influence the oscillation behavior of the crystal supporting the additional layer of the material.

For the analysis of the experimental data in this work, the mass of the dry film on the sensor $\bar{m}_{dry}$ was determined. This was done according to the following equation based on the Sauerbrey equation[19]

$$\bar{m}_{dry} = -\overline{\frac{C}{n}\Delta f^{(n)}} = -\overline{\frac{C}{n}(f^{(n)}_{dry} - f^{(n)}_{empty})} \tag{13}$$

where $\bar{m}_{dry}$ is the mass of the dry film and $f^{(n)}$ is the frequency of the nth overtone of the empty sensor or sensor with dry film.

As the dry films are rigid, no overtone dependence was observed supporting the applicability of the Sauerbrey equation in this case. After the examined films took up water from the gas phase, a dependence of the normalized frequency on the overtone number was observed (see below). This is an effect described for viscoelastic films.[24]

An equation describing the frequency change normalized to overtone number for single viscoelastic films in air is[25]

$$\frac{\Delta f}{n} = -\frac{2{f_0}^2 m_f}{Z_q}\left(1 + \frac{1}{3}\frac{{Z_q}^2}{{Z_f}^2}\left(\frac{m_f}{m_q}n\pi\right)^2\right) \tag{14}$$

where Δf is the change in resonance frequency, n is the overtone number, f_0 is the fundamental frequency, Z_q and Z_f are the acoustic impedances of the quartz and film, m_q and m_f are masses of quartz and film, m_w is the mass of water, and $\bar{m}_{dry}$ is the mass of dry sample film. All masses m refer to mass per unit surface.

According to eq 14, the change in frequency depends on the overtone number and should increase with increasing overtone number. Assuming an overtone zero, this equation gives a frequency shift $\Delta f/n$ proportional to the mass of the film m_f. Thus, extrapolation to overtone zero results in a frequency change proportional to the mass of the sample film.

For the use of eq 14 in the data analysis, some simplifications were carried out which were described in detail by Znamenskaya et al.[16]

The resulting equation used for the extrapolation procedure is as follows:

dx.doi.org/10.1021/jp404138f | J. Phys. Chem. B 2013, 117, 10017−10026

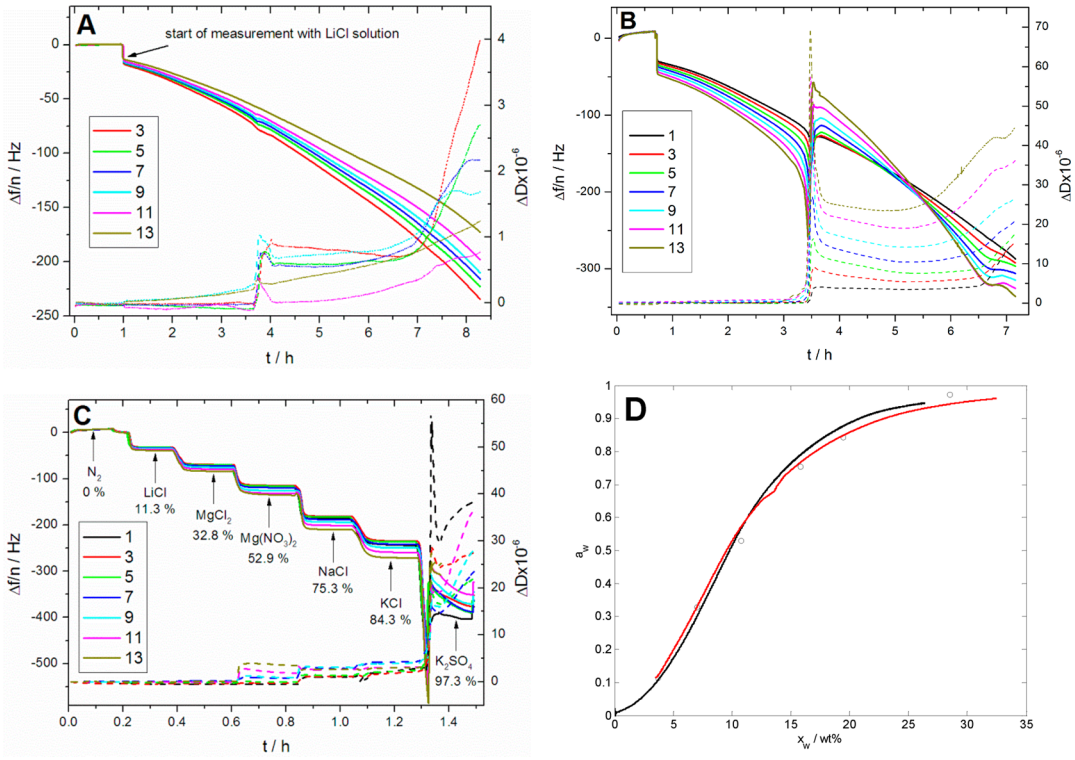


Figure 4. (A–C) Frequency and dissipation change during QCM-D measurements of lysozyme films depending on time. Solid lines represent frequency, dashed lines represent dissipation. The experiments were conducted at 25 °C with film thicknesses of (A) 73 nm, (B) 107 nm, and (C) 150 nm of lysozyme on SiO_2 sensors. (D) Sorption isotherm of lysozyme extrapolated from the frequency change during the scanning QCM-D measurement (red) and determined from water sorption calorimetry (black). The open circles represent data determined by stepwise QCM-D using saturated salt solutions to control the activity of water. The experiments were conducted with a film thickness of 73 nm (scanning QCM-D) and 150 nm (QCM-D with saturated salt solutions) at 25 °C on SiO_2 sensors.

$$\frac{m_w^{s(n)}}{\bar{m}_{dry}} = \frac{m_w}{\bar{m}_{dry}} + b\frac{m_f^3}{\bar{m}_{dry}}n^2 \quad (15)$$

$m_w^{s(n)}$ is the formal mass of water absorbed by the sample film calculated according to the Sauerbrey equation for every single overtone and m_w is the real mass of the water absorbed by the film.

For the analysis of the data in this work, the mass of water that was taken up by the investigated film was calculated according to the formula

$$m_w^{s(n)} = -\frac{C}{n}(f_{wet}^{(n)} - f_{dry}^{(n)}) \quad (16)$$

In the following step, the ratio r_w of mass of water (per overtone) to mass of dry film was determined.

$$r_w^{(n)} = \frac{m_w^{s(n)}}{\bar{m}_{dry}} \quad (17)$$

Extrapolation of the dependence of $r_w^{(n)}$ on n^2 to overtone zero yields the true mass of the film m_f ($m_f = m_w + \bar{m}_{dry}$).

$$r_w^0 = \lim_{n\to 0}\frac{m_w^{s(n)}}{\bar{m}_{dry}} \quad (18)$$

The dissipation measured during the QCM-D experiment is connected to the viscoelastic properties of the investigated film.[23,26] They can be characterized by the ratio G'/G''. G' is the storage modulus and describes elastic properties of a sample, whereas G'', the loss modulus, describes its viscous properties. This ratio can be derived from the change of the frequency during the QCM-D measurement for single viscoelastic films in air.[25] The full derivation of the G'/G'' ratio was described elsewhere.[16] The resulting equation is

$$\frac{G'}{G''} = -\frac{\Delta f + \frac{2f_0 n m_f}{Z_q}}{\Delta\Gamma} = \frac{-\Delta f + n\left(\frac{\Delta f}{n}\right)_{extrap}}{\Delta\Gamma} \quad (19)$$

$(\Delta f/n)_{extrap}$ is the normalized frequency change extrapolated to overtone zero according to eq 14. f_0 is the fundamental frequency, m_f mass of the film, and Z_q the acoustic or mechanical impedance of quartz.

Study of the Hydration of Lysozyme. We applied the humidity scanning QCM-D method to study hydration of lysozyme. Lysozyme is a globular protein that is often used as model protein to study hydration phenomena. Using a combination of several techniques, different aspects of lysozyme hydration have been studied.[1,18,27,28]

The change of resonance frequency relative to the dry film depending on time is shown in Figure 4A,B for different film thicknesses (and Figure S2 in the Supporting Information). Dependence of the frequency change depending on the calculated water activity is shown in Figure S2 in the Supporting Information. Starting from the dry lysozyme film, a continuous decrease in the frequencies can be observed that is connected to an increasing mass of water caused by the continuously increasing humidity of the air above the lysozyme film. After approximately 3.5 h a distinct drop in the frequencies can be observed. This coincides with a stepwise increase in the

dx.doi.org/10.1021/jp404138f | J. Phys. Chem. B 2013, 117, 10017–10026

dissipation. A further increase in the values of the dissipation starts after 7 h of measurement.

For comparison, a QCM-D measurement of a lysozyme film using the stepwise change of relative humidity with different saturated salt solutions is shown in Figure 4C. In this experiment, the frequency decreases in steps correlating with the increase in relative humidity. The dissipation starts to increase at 52.9% relative humidity and shows the largest change at the highest relative humidity.

Additionally, at high humidities, the overtones start to deviate more in both kinds of experiments. The dependence of frequencies on overtone number suggests a significant deviation from the properties assumed in the Sauerbrey equation and indicates viscoelastic properties of the film.[29,30] Therefore, for further analysis of the film properties and evaluation of the film mass, eq 14 that assumes viscoelastic behavior was used.

An additional reason for the overtone dependence of frequencies might be found in the roughness of the investigated layer. In QCM-D experiments the effect of roughness is expected to be most pronounced in experiments in liquid as it is caused by long-range hydrodynamic interactions in the presence of water.[31,32] However, as the masses determined for the dry lysozyme film show a standard deviation of up to 10% for higher film thickness, an influence of the roughness of the film surface is not negligible for thick films in air. Therefore, the thinnest film was used for the calculation of the water sorption isotherm shown in Figure 4D.

Water Sorption Isotherm of Lysozyme. Analysis of the measured frequencies shows a linear dependence of the frequency change on the square of the overtone number (Figure S3 in the Supporting Information). The slope of the linear fit has a positive value up to high relative humidity. This supports the applicability of eq 14, which predicts an increasing frequency change for increasing overtone numbers in viscoelastic films. The slope changes at the highest examined water activity (scanning QCM-D a_w = 0.96, stepwise QCM-D a_w = 0.97, Figure S3 in the Supporting Information) to a negative value indicating a change of the properties of the lysozyme film in this region. This indicates a change of the film properties to more liquidlike.[16] The same effect of the change of the slope at high relative humidity was observed also for pig gastric mucin films studied with QCM-D.[16]

The differing behavior of the slope around the water activity of 0.7 for the scanning QCM-D experiment is connected to the drop in the frequency observed at this point (Figure 4). The nature of this effect is discussed further below. As this drop is more pronounced for thicker films, the thinnest investigated film (Figure 4A) was used for the calculation of the water sorption isotherm.

Extrapolating the data according to eq 18 gives the mass of the wet lysozyme film depending on the time of the measurement and on the relative humidity of the air above the film. From this information the water content of the film can be calculated and plotted against the activity of water to give the water sorption isotherm of lysozyme at 25 °C (Figure 4D). For comparison, Figure 4D also includes water sorption QCM-D data of a lysozyme film obtained by using saturated salt solutions (Figure 4C) as well as a water sorption isotherm measured by means of water sorption calorimetry.[8]

Differences in water content x_w between water sorption isotherms determined by water sorption calorimetry and scanning QCM-D at the water activities 0.11, 0.50, and 0.80 are 0.35, 0.93, and 1 wt %, respectively, which shows a good agreement between both methods regarding also the different experimental conditions under which the results were obtained. Calorimetric measurements of sorption isotherms take several days and are performed in bulk, whereas the QCM-D measurements are carried out using a film and take only several hours. The reason for the differences between these sorption isotherms might be the diffusion of water. At low water content lysozyme is in the glassy state (see discussion below) and diffusion of water is slow. Therefore, the amount of water absorbed in the QCM-D experiment was slightly lower than in the sorption calorimetric experiment, as the equilibration time is much longer in sorption calorimetry. Above the glass transition, the kinetics of water diffusion in the lysozyme film is probably not the limiting factor. The slightly higher amount of water absorbed in the case of QCM-D compared to the longer calorimetric experiments can be related to relaxation processes in lysozyme. These processes create more efficient packing of the protein molecules, which decreases the amount of absorbed water in longer experiments.

The agreement between the data obtained by the two different QCM-D techniques is even better than with the water sorption calorimetric measurement. This validates the accuracy of scanning method, including eqs 2−9. It also shows that the speed of water activity change in the scanning experiments is slow enough to be as close to the equilibrium state as in the experiments with stepwise water activity changes. A more pronounced difference in the water content is observed only at very high water content, which can be explained by the fact that in the measurement with stepwise change in water activity no equilibrium state was obtained at the last humidity point (97.3%).

Rheological Analysis of Lysozyme Films. A major advantage of the QCM-D technique is the possibility to get insights into the rheological characteristics of a sample via measuring the dissipation. The change of the dissipation occurring during a measurement gives important information about changes in the rheological properties of the investigated sample. Figure 5 shows the dependence of the dissipation on

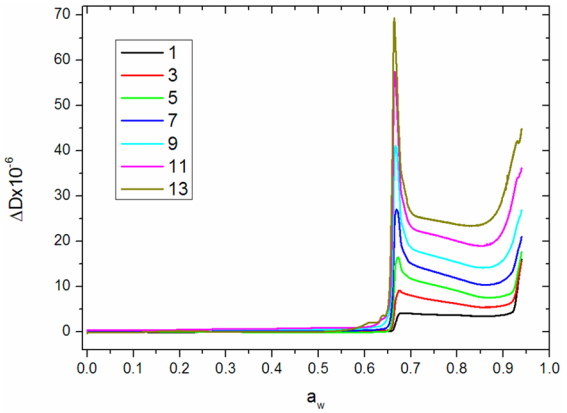


Figure 5. Dependence of the dissipation change for each overtone on water activity for a lysozyme film (thickness 107 nm) at 25 °C.

the water activity for a lysozyme film in the measurement shown in Figure 4B. Up to a water activity of approximately 0.65 the dissipation is close to zero and shows no change. Subsequently, a sharp increase can be observed. Above a_w = 0.9 another increase in the dissipation is detected. Water activities at the onset of the observed transitions and the corresponding

dx.doi.org/10.1021/jp404138f | J. Phys. Chem. B 2013, 117, 10017−10026

water content of the film are summarized in Table 1. Dissipation data for other measurements are provided in Figure S4 in the Supporting Information. They show the same dependence on water activity.

Table 1. Activities of Water for Transitions in Lysozyme Films Determined by Scanning QCM-D and Calorimetry

	QCM-D[a]			sorption calorimetry[8,18]	
	a_w	x_w/wt %	x_w[b]/wt %	a_w	x_w/wt %
first transition	0.67	13	13	0.6	11
second transition	0.91	24	22	0.9	21

[a]Mean value determined from onset of transition in the plot of change of dissipation against water activity at 25 °C (five measurements with lysozyme films, thicknesses ranging from 73 to 472 nm). [b]Values determined from the water sorption isotherm determined by sorption calorimetry.

Values of the water content of the film at the transitions observed by scanning QCM-D can also be determined using the sorption calorimetric isotherm and the activity values from QCM-D (Table 1). For the second transitions the results are in better agreement with the results obtained by other methods as the isotherm is closer to the equilibrium value due to longer equilibration times in sorption calorimetry (see above).

Analysis of the QCM-D data using eq 19 to calculate the ratio of storage to loss modulus G'/G'' shows the dependence of the viscoelasticity of the film on the water content (Figure 6A and Figure S5 in the Supporting Information). Up to a water activity of approximately 0.65, the ratio G'/G'' has a high value and then exhibits a fast drop to around zero. Above a_w = 0.9 a second step in the ratio G'/G'' occurs.

In the case of the QCM-D experiment with stepwise change in relative humidity, the analysis of the G'/G'' ratio yields a stepwise decrease in the ratio as well (Figure 6B). During the step from 52.9 to 75.3% relative humidity, the ratio falls below 1 and then drops to 0 at the highest relative humidity. These changes agree with the ones observed in the scanning QCM-D experiment. However, the scanning technique enables more precise determination of the water activity connected to the changes, whereas in the other case only a range can be determined.

The changes observed in the G'/G'' ratio correlate with the ones determined from change in dissipation. Moreover, a drop in the frequency can be observed at the same time as the increases in the dissipation (Figure 4B). This is also reflected in the water sorption isotherm that is calculated based on the frequency change. Figure 7 shows water sorption isotherms

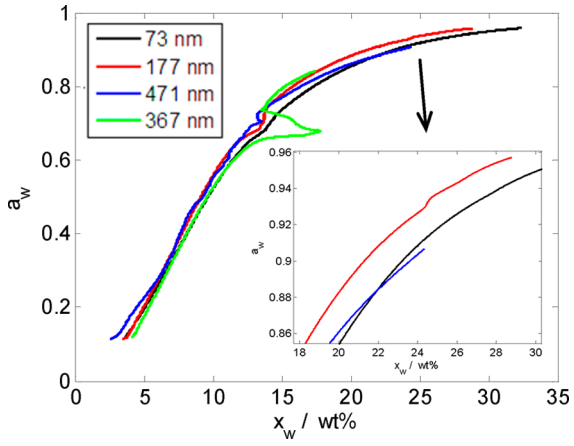


Figure 7. Water sorption isotherms calculated from the third overtone for lysozyme films of different thicknesses at 25 °C.

calculated from the third overtones for measurements with different lysozyme film thicknesses. A shift of the curves to the right around a_w = 0.7 indicates a rise of the water content in the film. This becomes more pronounced for larger film thicknesses; however, no dependence of onset of the transition on the film thickness was observed (Figure S6 in the Supporting Information) in the analysis of the dissipation.

The decrease in the ratio G'/G'' above a_w = 0.9 that is connected to the increase in dissipation at this relative humidity is also reflected in the sorption isotherm. The inset in Figure 7 shows a segment of the sorption isotherm at high water activity. At a_w = 0.91 ($x_w \approx$ 24 wt %) a small shift to the right can be observed.

The values of water activities related to the observed transitions can be determined either from the dependence of dissipation on activity (Figure 5) or from the change of the ratio G'/G'' plotted against the activity (Figure 6). In the case of the dissipation, a small dependence of the onset value of the transition on the overtone number is observable which seems to be less pronounced in the case of the ratio G'/G''. Using the dissipation is a more straightforward approach based only the

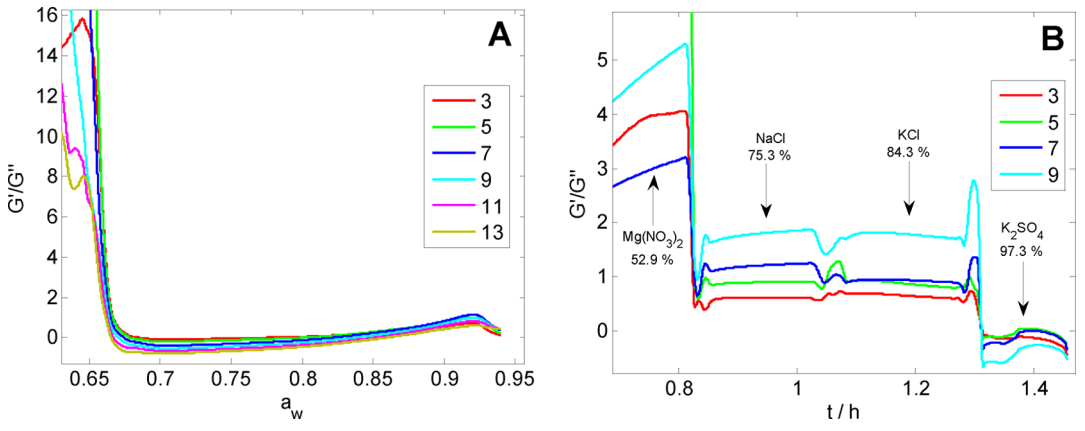


Figure 6. Ratio G'/G'' for lysozyme films measured at 25 °C calculated according to eq 19. (A) Humidity scanning QCM-D, film thickness 107 nm, (B) stepwise QCM-D, film thickness 150 nm.

dx.doi.org/10.1021/jp404138f | J. Phys. Chem. B 2013, 117, 10017−10026

measured dissipation, whereas the ratio G'/G'' involves calculation using values of dissipation, frequency, and an extrapolation according to eq 14. The values presented in this work are determined from the dissipation.

After analysis of the rheological properties of the lysozyme films, it can be inferred that two separate transitions occur in the studied hydration range. From the change of the dissipation during these transitions it can be concluded that the viscoelastic properties of the lysozyme film change.

The two observed transitions are both associated with a drop in frequency and a stepwise increase in dissipation or drop in the G'/G'' ratio, respectively. In the sorption isotherm a temporary increase of the mass of water in the lysozyme film is visible.

At relative humidity above 90%, a similar behavior was observed for amorphous cellulose in water sorption experiments.[33] It can often be observed when a material releases water during a recrystallization process.

Nature of the Two Transitions. Hydration of lysozyme was investigated using different techniques such as differential scanning calorimetry (DSC), sorption calorimetry, rheology, or light scattering.[27,34,35]

Sorption calorimetric measurements were used to measure the water sorption isotherm[8] and to create a phase diagram of lysozyme and water (some transitions are listed in Table 1) via determining the partial molar enthalpy of mixing.[18] At 25 °C this system was found to show three different regimes of sorption: from 0 to 10 wt %, 10−20 wt %, and above 20 wt % of water. Also, a transition at 35 wt % was observed upon dehydration of lysozyme.

Yang and Rupley[36] used calorimetric measurements to study the change in heat capacity depending on hydration of lysozyme at 25 °C. Four different regions with linear response to composition were found. The most important finding of this work is a linear increase of the apparent heat capacity in a broad composition range between 0.07 and 0.26 g of water per gram of dry lysozyme (which corresponds to 7 and 21 wt % of water).[1,36]

Rheological changes occurring in highly hydrated lysozyme samples have been monitored via changes in the Young's modulus (water content higher than 0.3 g of water per gram of dry lysozyme (23 wt % of water)) by Morozov and Gevorkian.[37] With decreasing temperature a steplike increase of the modulus was observed in the range from 237 to 251 K. Subsequently, a large increase in the modulus was observed between 240 and 130 K.

Gregory[1] summarized results obtained by several experimental methods and concluded that the transition observed at low hydration (x_w around 11%) at 298 K and the one in the range of 180−220 K for fully hydrated lysozyme are a manifestation of the same hydration-dependent phenomenon.

Raman scattering experiments investigating the hydration of lysozyme have been carried out between 0 and 44 wt % of water.[38] Monitored marker bands showed that structural changes start at 7−10 wt % of water. Following the intensity change of the Amide-I spectral band depending on water content, the breakup of intermolecular β-sheet structures was observed over a broad concentration range. The inflection point was found at 16 wt %; however, the changes level off only above 20 wt % of water. At 35 wt % of water the native structure of lysozyme is formed.

Dielectric and neutron scattering measurements were carried out using hydrated lysozyme powders.[39] A strong dependence of the measured relaxation times on the degree of hydration of lysozyme was observed, showing a decrease of 6 orders of magnitude of the relaxation time (approximately 10^{-5} to 10^{1} s) for a decrease in hydration from ca. 0.37 to 0.05 g of water per grams of lysozyme (27 to 5 wt % of water) at 193 K. Analysis of the temperature dependence showed even faster relaxation times for higher temperatures.

The molecular-level interpretation of the two transitions observed via humidity scanning QCM-D in this work is not completely clear. However, based on the results and interpretations published on hydration of lysozyme or proteins in general, two options concerning these transitions can be considered. First, the two transitions can be manifestations of the same type of molecular motions appearing on different time scales at different water contents. Indeed, apart from the true glass transitions that are usually seen on the time scale of 10^{2}−10^{3} s, so-called dynamical glass transitions can be seen at higher temperatures or water contents if much smaller times (higher frequencies) are probed. In this case, the transitions registered in slow and fast time scales originate from the same relaxation phenomena or the same molecular motions, but probed at different time scales at different temperatures or water contents. In scanning QCM-D experiments, two time scales are present: the "laboratory" time scale 10^{2}−10^{3} s and the time scale of the oscillations of the sensor (10^{-7} s). Because of the plasticizing effect of water, the processes seen on the slow time scale at 10 wt % of water can become very fast at 20 wt % of water.

Alternatively, the two transitions can correspond to different types of molecular motions. The heat capacity data[36] and the Raman data[38] measured on the laboratory time scale indicate that strong changes in structure and dynamics of the lysozyme−water system occur in the range between the two transitions. Therefore, it seems more probable that the two observed transitions are actually two transitions taking place on the same time scale. They can be interpreted as two processes in a very broad glass (and structural) transition that has been described to consist of several relaxation processes.[35,40] A proposition for the interpretation of the hydration-induced transitions observed for the lysozyme films at isothermal conditions is given below.

In the hydration range from 0 to 10 wt % of water, lysozyme is in a glassy state. The film behaves like a solid material and the structure of lysozyme does not change during water sorption as also indicated by the values of dissipation which are close to zero in this range.

In the concentration range from approximately 10 to 21 wt % the properties of the lysozyme film are changing. The first transition observed at 12−13 wt % of water (activity of water 0.67) in the humidity scanning QCM-D experiment is close to the onset of a broad glass transition reported earlier for the lysozyme−water system.[18,27] The drop of the G'/G'' ratio found at this point is consistent with the viscoelastic changes expected at the glass transition, i.e., a change from a rigid glassy state to a more fluid state.

The temporary uptake of a high amount of water found at the onset of the glass transition can be explained by a structural relaxation process. During drying of the lysozyme film on the QCM-D sensor, stress caused by deformation of the dried molecules accumulates. A change in the structure of lysozyme molecules and their packing between dry and hydrated states has been observed before.[8] The stressed system is prone to absorb higher amounts water since small and mobile water molecules can ease the stresses. Then at the point of the first

transition a structural rearrangement of the lysozyme molecules occurs. As a result, the system obtains a more ordered structure and part of the water is released during this process. The magnitude of this effect is dependent on the history of the sample and therefore is different for every lysozyme film, as can be seen in the extent of the frequency drop in Figure 4.

Between the first and the second transition the dissipation and the G'/G'' ratio have rather constant values. Raman measurements of hydrated lysozyme samples showed a decrease of intermolecular β-sheet structures in the hydration range between 10 and 20 wt % of water caused by adsorption of water molecules at the interface between lysozyme molecules.[38] Yang and Rupley[36] found a continuous increase in heat capacity in a similar range between 7 and 21 wt %. These facts indicate that the broad glass transition in lysozyme involves both dynamical and structural changes. Moreover, this can be the reason for the broadness of the glass transition in native proteins. One should note that in denatured lysozyme the glass transition is less broad,[28] which can be explained by difference in the structure.

The second transition occurs at the water activity of 0.91 (Table 1) which corresponds to 22−24 wt % of water (depending on the use of the sorption calorimetric or QCM-D isotherm for determination of the water content). The rheological analysis showed a transition starting at a_w = 0.91 and another drop in the G'/G'' ratio (Figures 5 and 6A). Similarly to the first transition, in the water sorption isotherm this transition is reflected in an increase of the water content of the film followed by a subsequent release of water (Figure 7).

Raman measurements showed changes of the Amide-I band occurring in the range from 10 to above 20 wt % of water, which is associated with the decrease in intermolecular β-sheets. The inflection point of the intensity of the Amide-I spectral band depending on water content was found at 16 wt %. This value coincides with monolayer coverage of the interface between lysozyme molecules with water.[38] It correlates quite well with the transition observed in the scanning QCM-D experiment and could indicate that in this concentration region an amount of water higher than monolayer coverage causes a breakup of physical bonds between lysozyme molecules. As a result, the lysozyme molecules start to move with respect to each other causing the increase in the dissipation and the observed drop in the G'/G'' ratio. Thus, the broadness of the glass transition in lysozyme can be caused by interplay between hydration induced structural and dynamical changes.

An obvious advantage of the humidity scanning QCM-D technique is demonstrated by the described phenomena, which were not observed in such detail in any measurements carried out previously. The frequency and dissipation change can be monitored in a continuous way with high resolution for the activity of water. Thus, a more precise determination of the transitions points and characterization of the glass transition are possible. In addition, two sharp transitions are observed by the humidity scanning QCM-D technique in contrast to other methods which usually show only one diffuse broad glass transition. Interpretation of the two transitions in combination with Raman measurements provides new insights into processes and mechanisms related to the glass transition on a molecular level.

CONCLUSIONS

A new method for hydration measurements using quartz crystal microbalance with dissipation monitoring (QCM-D) was introduced. This technique was used to determine the water sorption isotherm of lysozyme films deposited on silica sensors as well as the rheological properties that change during hydration-induced transitions of the films. The following was concluded:

- humidity scanning QCM-D technique enables measurement of complete water sorption isotherms in a continuous way with high resolution of the activity;
- high-resolution sorption isotherm can be obtained in a much shorter time compared to sorption calorimetry using very small amounts of substance (ca. 20 μg);
- water sorption isotherms and rheological changes of soft matter can be determined via analysis of the overtone dependence of frequency and dissipation;
- water sorption isotherm of lysozyme shows agreement between data from QCM-D experiments with stepwise increasing a_w as well as with water sorption calorimetric data;
- analysis of rheological behavior during hydration of lysozyme films reveals the presence of two transitions connected to the glass transition;
- humidity scanning QCM-D enables the observation of separate sharp transitions during a broad glass transition which has not been reported for any other method before;
- a new mechanism of hydration-induced isothermal glass transition in lysozyme is proposed; according to this mechanism, the dynamical changes are accompanied by structural changes, which causes apparent broadness of the glass transition.

ASSOCIATED CONTENT

Supporting Information

Additional data and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +4640 6657946. E-mail: vitaly.kocherbitov@mah.se.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge financial support from the Biofilms Research Center for Biointerfaces at Malmö University. We thank Y. Znamenskaya for help with the QCM-D technique as well as T. Arnebrant for fruitful discussions. We thank J. Wojtasz for carrying out one of the measurements.

REFERENCES

(1) Gregory, R. B., Ed.; In *Protein-solvent interactions*; Marcel Dekker: New York, 1995.

(2) Dixon, M. C. Quartz Crystal Microbalance with Dissipation Monitoring: Enabling Real-Time Characterization of Biological Materials and their Interactions. *J. Biomol. Tech.* **2008**, *19*, 151−158.

(3) Lundin, M.; Elofsson, U. M.; Blomberg, E.; Rutland, M. W. Adsorption of Lysozyme, B-Casein and their Layer-by-Layer Formation on Hydrophilic Surfaces: Effect of Ionic Strength. *Colloids Surf., B* **2010**, *77*, 1−11.

(4) Nezu, T.; Masuyama, T.; Sasaki, K.; Saitoh, S.; Taira, M.; Araki, Y. Effect of pH and Addition of Salt on the Adsorption Behavior of Lysozyme on Gold, Silica, and Titania Surfaces Observed by Quartz Crystal Microbalance with Dissipation Monitoring. *Dent. Mater. J.* **2008**, *27*, 573−580.

(5) Nezu, T.; Taira, M.; Saitoh, S.; Sasaki, K.; Araki, Y. Viscoelastic Adlayers of Collagen and Lysozyme Studied using Quartz Crystal

Microbalance with Dissipation Monitoring. *Int. J. Biol. Macromol.* **2010**, *46*, 396−403.
(6) Fogel, R.; Limson, J. L. Probing Fundamental Film Parameters of Immobilized Enzymes-Towards Enhanced Biosensor Performance. Part I-QCM-D Mass and Rheological Measurements. *Enzyme Microb. Technol.* **2011**, *49*, 146−152.
(7) Paul, S.; Paul, D.; Basova, T.; Ray, A. K. Studies of Adsorption and Viscoelastic Properties of Proteins Onto Liquid Crystal Phthalocyanine Surface using Quartz Crystal Microbalance with Dissipation Technique. *J. Phys. Chem. C* **2008**, *112*, 11822−11830.
(8) Kocherbitov, V.; Arnebrant, T. Hydration of Lysozyme: The Protein-Protein Interface and the Enthalpy-Entropy Compensation. *Langmuir* **2010**, *26*, 3918−3922.
(9) Towns, J. K. Moisture Content in Proteins: Its Effects and Measurement. *J. Chromatogr. A* **1995**, *705*, 115−127.
(10) Smith, A. L.; Shirazi, H. M.; Mulligan, S. R. Water Sorption Isotherms and Enthalpies of Water Sorption by Lysozyme using the Quartz Crystal Microbalance/Heat Conduction Calorimeter. *Biochim. Biophys. Acta (BBA): Protein Struct. Mol. Enzymol.* **2002**, *1594*, 150−159.
(11) D'Arcy, R. L.; Watt, I. C. Analysis of Sorption Isotherms of Non-Homogeneous Sorbents. *Trans. Faraday Soc.* **1970**, *66*, 1236−1245.
(12) Du, A.; Koo, D.; Ziegler, M.; Cairncross, R. A. The Effect of Heat Treatment on Water Sorption in Polylactide and Polylactide Composites Via Changes in Glass-Transition Temperature and Crystallization Kinetics. *J. Polym. Sci., Part B: Polym. Phys.* **2011**, *49*, 873−881.
(13) Krtil, P.; Trojanek, A.; Samec, Z. Kinetics of Water Sorption in NafionThin Films - Quartz Crystal Microbalance Study. *J. Phys. Chem. B* **2001**, *105*, 7979−7983.
(14) Greenspan, L. Humidity Fixed Points of Binary Saturated Aqueous Solutions. *J. Res. Natl. Stand. A* **1977**, *81A*, 89−96.
(15) Peresin, M.; Kammiovirta, K.; Setälä, H.; Tammelin, T. Structural Features and Water Interactions of Etherified Xylan Thin Films. *Jo. Polym. Environ.* **2012**, *20*, 895−904.
(16) Znamenskaya, Y.; Sotres, J.; Gavryushov, S.; Engblom, J.; Arnebrant, T.; Kocherbitov, V. Water Sorption and Glass Transition of Pig Gastric Mucin Studied by QCM-D. *J. Phys. Chem. B* **2013**, *117*, 2554−2563.
(17) Ohlsson, G.; Langhammer, C.; Zoric, I.; Kasemo, B. A Nanocell for Quartz Crystal Microbalance and Quartz Crystal Microbalance with Dissipation-Monitoring Sensing. *Rev. Sci. Instrum.* **2009**, *80*, 083905/1−083905/10.
(18) Kocherbitov, V.; Arnebrant, T.; Söderman, O. Lysozyme-Water Interactions Studied by Sorption Calorimetry. *J. Phys. Chem. B* **2004**, *108*, 19036−19042.
(19) Sauerbrey, G. Verwendung Von Schwingquarzen Zur Wagung Dunner Schichten Und Zur Mikrowagung. *Z. Phys.* **1959**, *155*, 206−222.
(20) Adapted from q-sense Application note: Analysing vapor uptake and release with QCM-D.
(21) Abdulagatov, I. M.; Azizov, N. D. Densities and Apparent Molar Volumes of Concentrated Aqueous LiCl Solutions at High Temperatures and High Pressures. *Chem. Geol.* **2006**, *230*, 22−41.
(22) Robinson, R. A. The Water Activities of Lithium Chloride Solutions Up to High Concentrations at 25 Degree. *Trans. Faraday Soc.* **1945**, *41*, 756−758.
(23) Cooper, M. A.; Singleton, V. T. A Survey of the 2001 to 2005 Quartz Crystal Microbalance Biosensor Literature: Applications of Acoustic Physics to the Analysis of Biomolecular Interactions. *J. Mol. Recognit.* **2007**, *20*, 154−184.
(24) Wolff, O.; Seydel, E.; Johannsmann, D. Viscoelastic Properties of Thin Films Studied with Quartz Crystal Resonators. *Faraday Discuss.* **1997**, *107*, 91−104.
(25) Johannsmann, D. Viscoelastic Analysis of Organic Thin Films on Quartz Resonators. *Macromol. Chem. Phys.* **1999**, *200*, 501−516.
(26) Rodahl, M.; Hook, F.; Krozer, A.; Brzezinski, P.; Kasemo, B. Quartz Crystal Microbalance Setup for Frequency and Q-Factor Measurements in Gaseous and Liquid Environments. *Rev. Sci. Instrum.* **1995**, *66*, 3924−3930.
(27) Khodadadi, S.; Malkovskiy, A.; Kisliuk, A.; Sokolov, A. P. A Broad Glass Transition in Hydrated Proteins. *Biochim. Biophys. Acta* **2010**, *1804*, 15−19.
(28) Kocherbitov, V.; Arnebrant, T. Hydration of Thermally Denatured Lysozyme Studied by Sorption Calorimetry and Differential Scanning Calorimetry. *J. Phys. Chem. B* **2006**, *110*, 10144−10150.
(29) Höök, F.; Kasemo, B.; Nylander, T.; Fant, C.; Sott, K.; Elwing, H. Variations in Coupled Water, Viscoelastic Properties, and Film Thickness of a Mefp-1 Protein Film during Adsorption and Cross-Linking:A Quartz Crystal Microbalance with Dissipation Monitoring, Ellipsometry, and Surface Plasmon Resonance Study. *Anal. Chem.* **2001**, *73*, 5796−5804.
(30) Zhu, D.; Fang, J.; Wu, B.; Du, X. Viscoelastic Response and Profile of Adsorbed Molecules Probed by Quartz Crystal Microbalance. *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.* **2008**, *77*, 031605/1−031605/7.
(31) Johannsmann, D.; Reviakine, I.; Rojas, E.; Gallego, M. Effect of Sample Heterogeneity on the Interpretation of QCM(-D) Data: Comparison of Combined Quartz Crystal Microbalance/Atomic Force Microscopy Measurements with Finite Element Method Modeling. *Anal. Chem. (Washington, DC, U.S.)* **2008**, *80*, 8891−8899.
(32) Daikhin, L.; Gileadi, E.; Katz, G.; Tsionsky, V.; Urbakh, M.; Zagidulin, D. Influence of Roughness on the Admittance of the Quartz Crystal Microbalance Immersed in Liquids. *Anal. Chem.* **2002**, *74*, 554−561.
(33) Kocherbitov, V.; Ulvenlund, S.; Kober, M.; Jarring, K.; Arnebrant, T. Hydration of Microcrystalline Cellulose and Milled Cellulose Studied by Sorption Calorimetry. *J. Phys. Chem. B* **2008**, *112*, 3728−3734.
(34) Bell, L. N.; Hageman, M. J.; Muraoka, L. M. Thermally Induced Denaturation of Lyophilized Bovine Somatotropin and Lysozyme as Impacted by Moisture and Excipients. *J. Pharm. Sci.* **1995**, *84*, 707−12.
(35) Sartor, G.; Mayer, E.; Johari, G. P. Calorimetric Studies of the Kinetic Unfreezing of Molecular Motions in Hydrated Lysozyme, Hemoglobin, and Myoglobin. *Biophys. J.* **1994**, *66*, 249−258.
(36) Yang, P.; Rupley, J. A. Protein-Water Interactions. Heat Capacity of the Lysozyme-Water System. *Biochemistry* **1979**, *18*, 2654−61.
(37) Morozov, V. N.; Gevorkian, S. G. Low-Temperature Glass Transition in Proteins. *Biopolymers* **1985**, *24*, 1785−1799.
(38) Kocherbitov, V.; Latynis, J.; Misiunas, A.; Barauskas, J.; Niaura, G. Hydration of Lysozyme Studied by Raman Spectroscopy. *J Phys Chem B* **2013**, DOI: 10.1021/jp4017954.
(39) Khodadadi, S.; Pawlus, S.; Sokolov, A. P. Influence of Hydration on Protein Dynamics: Combining Dielectric and Neutron Scattering Spectroscopy Data. *J. Phys. Chem. B* **2008**, *112*, 14273−14280.
(40) Jansson, H.; Swenson, J. The Protein Glass Transition as Measured by Dielectric Spectroscopy and Differential Scanning Calorimetry. *Biochim. Biophys. Acta (BBA): Proteins Proteomics* **2010**, *1804*, 20−26.