Sorption Equilibrium and Hydration Studies of Lysozyme: Water Activity and 360-MHz Proton NMR Measurements

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An attempt to determine lysozyme hydration by employing a proton nuclear magnetic resonance (NMR) spin–echo technique (which is applicable over the whole range of concentrations) and to correlate such measurements with the 20 °C sorption equilibrium data is made. Determinations of specific site hydration for lysozyme, as well as proton NMR transverse relaxation rates for five different types of water populations in the lysozyme–water system, are presented over the whole range of lysozyme concentrations. The proton spin–echo NMR results are consistent with a three-component analysis of the sorption isotherm up to 70% water content, above which two additional water populations are identified by 360-MHz proton NMR spin–echoes. On the basis of the proton NMR results, a major component (III) of the lysozyme sorption isotherm is assigned to the water trapped between lysozyme molecules, whose relaxation rate is increased by diffusion barriers. The trapped water population dominates the relationship between the relative vapor pressure (water activity) of hydrated lysozyme and the corresponding, transverse proton NMR relaxation rates.

The hydration of food components and the sorption of water in foods are processes of considerable importance in food science and technology (Baianu et al., 1982; Richardson et al., 1986; Asbi and Baianu, 1986). Sorption isotherms of food proteins have been intensely studied for over four decades (Bull, 1944; D'Arcy and Watt, 1970; Asbi and Baianu, 1986). Nevertheless, the underlying molecular mechanisms of water sorption still need be clarified; the currently accepted view is that the study of water sorption by proteins of low molecular weight, well-defined purity, and known structure will rapidly advance our understanding of such mechanisms. Lysozyme satisfies these criteria, and its hydration and water sorption characteristics are intensely studied (Leeder and Watt, 1972; Hsi et al., 1976; Baianu et al., 1985; Lioutas et al., 1986; Asbi and Baianu, 1986). Therefore, lysozyme can be employed as a simple model system for food proteins and proteinaceous foods to guide the investigation of the latter. Among the techniques used to study protein hydration, nuclear magnetic resonance (NMR) provides a convenient, sensitive, and nondestructive means for investigating the interactions of water with proteins over a wide range of concentrations. Most of the work by NMR, X-ray, and neutron diffraction has focused on the problem of identifying the different populations of water and their interactions with proteins (Kuntz and Kauzmann, 1974; Eisenberg and Kauzmann, 1969; England, 1972; Derbyshire, 1980; Kumosinski and Pessen, 1985; Lioutas et al., 1986). The NMR approach was recently extended to wheat proteins (Baianu et al., 1982) and wheat flour suspensions (Richardson et al., 1986).

The general consensus of such work is that water has at least two states, "bound" and free, in all studied systems. These states were distinguished primarily by their different thermodynamic properties such as phase separation at subzero temperatures (Derbyshire, 1980). Kuntz and Kauzmann (1974) reported that the properties of water affected by the presence of protein are vapor pressure, freezing point, and boiling point. They used the term binding to describe the protein–water interactions. Water binding by proteins was expressed as a modification of the kinetic properties of water molecules resulting from relatively strong interactions with charged protein residues. Water molecules near the protein interface may also rotate and translate slower than the ones closer to bulk water, hence the importance of molecular studies that can identify protein–water interactions.

A simple method for investigating the thermodynamic equilibrium properties of water interacting with proteins is the vapor pressure determination of the protein–water system at equilibrium, under isothermal conditions. This measurement yields the relative vapor pressure, p/p_0, or water activity (α_W) of the system, defined as the ratio of partial vapor pressure of the protein–water system over the partial vapor pressure of liquid water. The currently accepted technique for measuring α_W at different water contents is the isopiestic method. The isopiestic method has been extensively used to investigate water sorption by proteins (Bull, 1944; Bull and Breeze, 1968).

Sorption isotherms have been reported for a number of proteins (Bull, 1944). Such isotherms are classified as type II, sigmoidal (Brunnauer et al., 1938), and a number of theoretical models have been proposed to explain and fit experimental sorption isotherms (Brunnauer et al., 1938; D'Arcy and Watt, 1970; Asbi and Baianu, 1986). Hen egg white lysozyme is often chosen for such studies because its structure and sequence are well established (Hnojewyj and Reyerson, 1968; Leeder and Watt, 1974). The experimental sorption isotherm of lysozyme at 35 °C was found to deviate from a three-component sorption isotherm calculation (D'Arcy and Watt, 1970, 1981) at relative humidities (RH) higher than 50% because of the "high degrees of protein swelling" and eventual dissolution of the soluble proteins in the sorbed water. This explanation, however, leaves open the question of the molecular states of water in the lysozyme–water system above 50% RH. Furthermore, the distinction between tightly bound and weakly bound water has to be assumed in the analysis. A detailed discussion of the component analysis of the isotherms of lysozyme and food proteins is presented in a recent report (Asbi and Baianu, 1986).

Changes in the hydrodynamic and kinetic properties of water at the protein surface are expected to be reflected in modified spectroscopic properties of the bound water molecules. One of the spectroscopic methods extensively used to observe such changes is nuclear magnetic resonance (NMR); NMR and, to a certain extent, laser spectroscopy have the advantage that they can provide both structural and dynamic information.

Hsi et al. (1976) studied the hydration of lysozyme crystals by 30-MHz proton NMR and concluded that there

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Table I. Water "Activity", $\alpha_w = p/p_0$, Measured at Different Concentrations of Lysozyme at 20 °C

α_w (20 °C)	lysozyme concn, % (w/w)	α_w (20 °C)	lysozyme concn, % (w/w)
0.997 ± 0.002	10.0	0.890 ± 0.005	82.0
0.996 ± 0.002	20.0	0.755 ± 0.005	87.0
0.995 ± 0.002	40.0	0.544 ± 0.005	90.0
0.994 ± 0.002	60.0	0.331 ± 0.005	93.0
0.993 ± 0.002	70.0	0.113 ± 0.005	96.3
0.946 ± 0.005	79.0	≤0.005	98.5
0.910 ± 0.005	80.4		

are three water populations in this system. One of these populations is closely associated with the protein surface and exchanges fast with the interior aqueous solution of the crystal. The water in the immediate vicinity of the lysozyme surface was found to behave like a viscous liquid, *not like a solid* (Hilton et al., 1977).

Although there have been many studies of the protein–water interactions, most sorption equilibrium measurements cover only the water activity range from 0.10 to 0.80 for concentrated protein solutions (Leeder and Watt, 1974). There has been little work done, however, at the very low, or very high, water activity ranges.

We have investigated the hydration properties of lysozyme using a proton NMR spin–echo pulse sequence that is applicable over the whole range of concentrations from solutions to powders. We are attempting to correlate the sorption isotherm with the NMR data for hydrated lysozyme, with the purpose of deriving a molecular interpretation of the complete sorption isotherm.

EXPERIMENTAL SECTION

1. Compositional Analysis. Lysozyme used in this study was obtained from Sigma Chemical Co., and it was three times crystallized, dialyzed at neutral pH, and lyophilized. The moisture content of the original sample was measured to be 3.3% (w/w), according to the AOAC (1981) method, at 25 mmHg pressure (vacuum) and 60 °C for 24 h. The sodium and potassium contents were measured by the atomic absorption method (Dean, 1960) and were found to be 77 and 3.7 ppm, respectively. Protein content was measured to be 97.0%, based on $E^{1\%} = 26.4$.

2. Vapor Equilibration and Water Activity (α_w) Measurements. Two different techniques were used for measuring α_w. For the liquid samples a modification of the method of Landrock and Proctor (1951) was employed. Small open vials of the lysozyme solutions were placed on the surface of sucrose solutions and were equilibrated at 20 ± 1 °C for 8 days. The sucrose solutions were at the bottom of a small cylindrical vial (25-mm diameter by 35-mm height) and acted as the α_w controlling agent. The α_w values of sucrose solutions were taken from Robinson and Stokes (1955). Lysozyme solutions were placed at the surface of sucrose solutions in small watch glasses, and the weight gain or loss after 8 days was measured by weighing.

The gain or loss of water per solid weight was then plotted against the α_w value at which there was no gain or loss; from such values we obtained the water activities for the lysozyme–water systems for lysozyme contents from 0 to 70% (w/w). For the solid samples the proximity equilibration cell developed by Lang et al. (1981) was used. Lysozyme powder was placed and equilibrated for 15 days at 20 ± 1 °C near the surface of saturated salt solutions; the α_w values of the saturated salt solutions were taken from Greenspan (1977). The lysozyme powder sorbed water vapor until its α_w equaled the α_w of the saturated salt solution. The α_w of the lysozyme powder at this point was taken to be equal to that of the saturated salt; the moisture content of the sample was calculated from the weight gain or loss. In this manner we are able to prepare samples for NMR measurements which have been already used for α_w determinations (Tables I and II).

Table II. Water Activity of Dilute Sucrose and Saturated Salt Solution at 20 °C

humectant	α_w	humectant	α_w
sucrose, 0.1 m[a]	0.998	$ZnSO_4$[b]	0.890
sucrose, 0.2 m[a]	0.996	KCl[b]	0.851
sucrose 0.4 m[a]	0.993	$(NH_4)_2SO_4$[a]	0.813
Na_2HPO_4[b]	0.980	NaCl[a]	0.755
K_2SO_4[a]	0.976	KI[a]	0.699
NaCl, 1.2 m[a]	0.960	NaBr[a]	0.591
KNO_3[a]	0.946	$MgCl_2$[a]	0.331
$BaCl_2$[b]	0.910	LiCl[a]	0.113

[a] Values from Greenspan (1977) and Robinson-Stokes (1955). [b] Values determined experimentally with the isopiestic method (Lang et al., 1981) using as standards the values from Greenspan (1977).

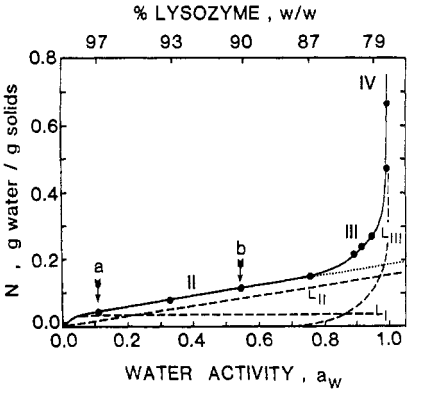

Figure 1. Sorption isotherm for lysozyme at 20 °C. Dashed curves L_I–L_{III} represent the three components in the D'Arcy–Watt theory: monolayer, weakly bound, and multilayer water, respectively. Point a is the lower limit of L_{II}, and point b is the upper limit for the BET model.

3. NMR Measurements. Proton NMR measurements were carried out at 20 °C on a Nicolet NT-360 spectrometer operating at 360.061-MHz proton resonance frequency by employing the Ostroff–Waugh (OW) pulse sequence: 90–τ–90_{90}–τ–90_{90}–τ–90_{90} ... (Ostroff and Waugh, 1966). This sequence was selected because it is very effective for line narrowing in solids (by virtually eliminating dipolar interactions) as well as in liquids, and requires the use of only a simple pulse programmer. In the limit of $\tau \rightarrow 0$, this sequence causes a spin-lock and the value of T_{2e}* thus determined approaches the value of the spin–lattice relaxation time in the rotating frame, $T_{1\rho}$ (Ostroff and Waugh, 1966). For liquids, or molecular groups that are highly mobile, the T_{2e}* value measured with the OW pulse sequence is close to the value of the transverse relaxation time, T_2, measured with the Carr–Purcell–Meiboom–Gill (CPMG) sequence. A more recent review of NMR relaxation measurements and spin–echo techniques was presented by Vold (1976).

Deuterium NMR spectra were recorded at 38.761 MHz by employing a laboratory-built, NSF-250, spectrometer; recording conditions and other experimental details are specified in the figure captions.

RESULTS

We determined the relationship between water activity (α_w) and moisture content (expressed as grams of water/lysozyme) in the range of concentrations from 0 to 98.5% lysozyme at 20 °C (Figure 1). Our data agree well with the lysozyme sorption isotherms previously reported

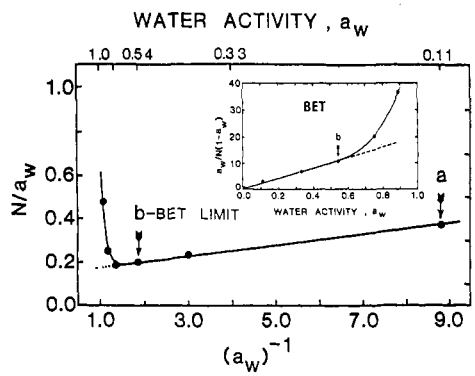

Figure 2. Linear plot of the weakly bound water component of the D'Arcy–Watt theory. The standard least-squares fit coefficient for the linear segment is 0.998. Point a is the upper limit of the BET monolayer, and point b is the upper limit of the BET model. The inset is a BET plot of the same experimental data.

Table III. Calculated Values of the Constants Entering the D'Arcy–Watt Three-Component Equation [$n = n_I + C\alpha_w + K_3K_4\alpha_w/(1 - K_4)\alpha_w$] for Lysozyme at 20 °C

D'Arcy–Watt param	value
N_I (also BET)	0.0236 g of water/g of lysozyme
C	0.161 g of water/g of lysozyme
K_3	9.25×10^{-3}
K_4	0.9778

in the literature for the range of α_w from 0.05 to 0.80; however, our last measured α_w value in Figure 1 is 0.997 (for 10% lysozyme).

The D'Arcy and Watt theory (1970, 1981) was specifically developed in conjunction with the analysis of such sorption isotherms. Although a multiplicity of water-binding sites is taken into account by the D'Arcy–Watt theory, the analysis of the protein sorption isotherms proceeds by considering only three populations of water: tightly bound, weakly bound, and multilayer, or trapped water. The corresponding three components of the sorption isotherm of lysozyme are shown in Figure 1 and are labeled, respectively, L_I, L_{II}, and L_{III}. For α_w values up to about 0.7 the moisture content of lysozyme is calculated to a good approximation by the simple equation

$$N = N_I + C\alpha_w \quad (1)$$

where N_I is the amount of tightly bound water and $C\alpha_w$ is the amount of weakly bound water. At α_w above 0.7 we found for 15 food proteins, including lysozyme, that an improved fit of component L_{III} can be obtained with only two adjustable parameters (Asbi and Baianu, 1986) instead of the four parameters (in addition to C), employed by the D'Arcy–Watt theory. A plot based on eq 1 is shown in Figure 2 and is compared with the BET plot (inset in Figure 2; Brunnauer et al., 1938). Above $\alpha_w \cong 0.95$, lysozyme is in solution and the three components are insufficient for describing the sorption isotherm; in this region (IV) the bulk water population needs to be considered in addition to L_I–L_{III}. The results of this analysis are summarized in Table III. We note that a discontinuity between L_{III} and L_{IV} was also found from T_1 proton NMR measurements at 30 MHz [Figure 11 in Hilton et al. (1977)] and at 10 MHz (Fullerton et al., 1986).

We have investigated by 360-MHz proton NMR the molecular dynamics of water in these four regions of the lysozyme sorption isotherm. Parts A–E in Figure 3 show the spin–echo decays of lysozyme in the range of concentrations from 1% to 96% w/w, obtained with the Ostroff–Waugh (OW) sequence for decay intervals longer than about 100 μs. The corresponding spin–echo spectra are presented in parts F–J in Figure 3 and are single

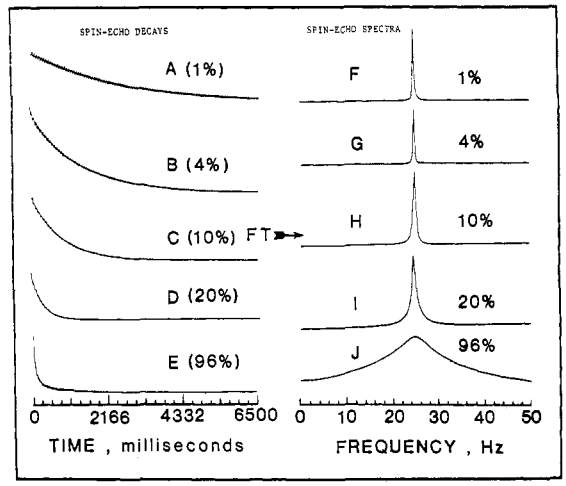

Figure 3. 360-MHZ proton NMR spin–echo decays (left) and NMR spin–echo spectra (right) for hydrated lysozyme over the entire concentration range. The pulse train consisted of 4064 (90° phase-shifted) pulses (10 μs long) following a 0° phase, initial 90° pulse, with a τ value of 800 μs between each pair of pulses for 1%, 4%, 10%, and 20% and a τ value of 200 μs for 96% lysozyme.

Lorentzians up to about 30% lysozyme. The relaxation rates determined from the spin–echo decays are denoted by R_{2e}*, where the subscript e stands for a single exponential. For lysozyme concentrations higher than about 30% w/w the slow-relaxing component was also found to be a single exponential and the corresponding relaxation rate is also denoted by R_{2e}*. The fast decay, lasting for a few milliseconds in Figure 3E, corresponds to fast-relaxing groups that have a 300-Hz broad, *Gaussian* spin–echo spectrum in the 96% lysozyme sample. For the same sample, the slow-relaxing component corresponds to a much narrower (14-Hz) Lorentzian peak in the NMR spin–echo spectrum (Figure 3J).

The narrow, single Lorentzian component is assigned to water protons in fast exchange with a small fraction of (exchangeable) lysozyme protons. The latter was minimized by repeated exchange of lysozyme protons with deuterons from D_2O. Because of this exchange methodology that reduces greatly the effects of intermolecular dipolar interactions (Baianu et al., 1978, 1984) and the cross-relaxation, the proton NMR measurements could not be carried out at low frequency (e.g., 30 MHz) for our deuterium-exchanged samples, for reasons of sensitivity. Since the protein activity can also affect significantly the interpretation of NMR relaxation measurements (Kumosinski and Pessen, 1985), we have carried out our measurements *in the absence of salt and at neutral* pD. The results in Figure 4 show that both ^1H and ^2H NMR transverse relaxation rates increase linearly with concentration up to about 10% lysozyme, indicating that lysozyme activity and cross-relaxation (Edzes and Samulski, 1978) have little effect on R_{2e}* in this concentration range (Kumosinski and Pessen, 1985). At lysozyme concentrations higher than 10% w/w the R_{2e}* dependence on concentration (Figure 5) deviates markedly from the line expected from Figure 4. Since 34-MHz ^2H NMR transverse rleaxation rates have a similar dependence on concentration (data not shown) to that presented in Figure 4, this behavior cannot be caused by cross-relaxation. For the analysis of the spin–echo NMR data it is convenient to plot the excess transverse relaxation rate, R_{2exc}, rather than the relaxation rate R_{2e}* = $R_{2sample}$ of the hydrated lysozyme samples; R_{2exc} is defined as $R_{2sample} - R_{2,HDO}$, where $R_{2,HDO}$ is the transverse relaxation rate R_{2e}* of a 98% D_2O sample at 20 °C. R_{2exc} is plotted in Figure 5 as a function of

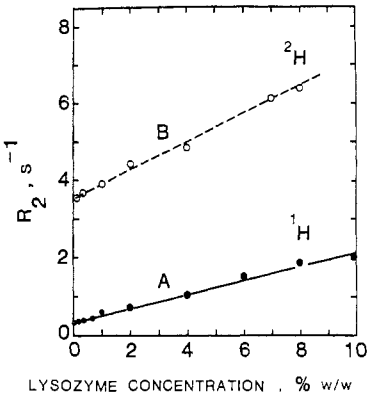

Figure 4. Dependence of measured relaxation rates, R_{2e}*, on the concentration of lysozyme solutions in D_2O, for the range of 0–10% at 20 °C: (A) Proton NMR relaxation rates, R_{2e}*, measured using the O–W sequence at 360 MHz. (B) Deuterium NMR line widths determined at half-height (multiplied by π), of the D_2O peak in lysozyme solutions (averages of four sets of measurements, carried out at 38.76 MHz).

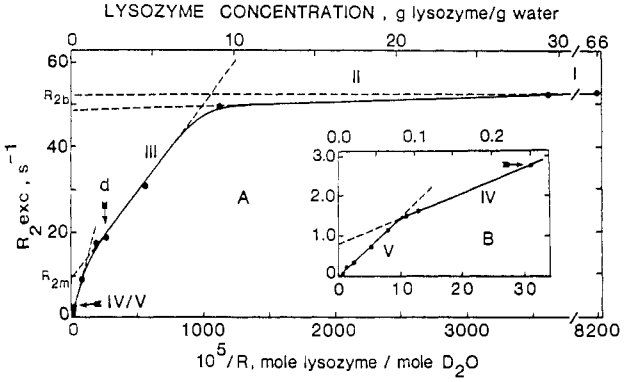

Figure 5. Dependence of the excess proton NMR spin–spin relaxation rates, R_{2exc}, upon the concentration of lysozyme at 20 °C: (A) Over the entire concentration range. Regions I–IV correspond to tightly bound, weakly bound, trapped (multilayer), and lysozyme-dimer water, respectively, as determined from the sorption isotherm; point d represents a sample at $0.994\alpha_w$, the upper limit of fit for the D'Arcy–Watt theory. (B) Over the concentration range from 0 to 100 mol ratio of lysozyme to water. Regions IV and V correspond to lysozyme-dimer water and water in lysozyme-monomer solutions, respectively.

lysozyme concentration, $1/r$, expressed in moles of lysozyme/mole of D_2O.

INTERPRETATION

The amount of water at the BET monolayer, b, in Figure 2 is $N_I \cong 2.4\%$ or 19 mol of H_2O/mol of lysozyme, which implies that at an α_w of 0.11 not all charged, or polar, groups have a bound water molecule, unless there is sharing of water molecules bridged between the binding sites. In this monolayer, or L_I, region, R_{2e}* is constant (R_{2b}, for varying moisture contents less than about 3%) and is, presumably, characteristic of tightly bound water in lysozyme powders. The value of R_{2b} is about 54 s^{-1} and is substantially smaller than the value reported at very low frequency (10 MHz) by employing the 90°–180° sequence (Fullerton et al., 1986) or at low frequency (30 MHz; Hilton et al., 1977).

In region L_{II}, a total of ~148 mol of H_2O is bound to 1 mol of lysozyme, corresponding to $N_I + C = 18.5\%$ extrapolated at $\alpha_w = 1.00$. This total amount of bound water agrees remarkably well with the amount of unfreezable water in lysozyme crystals determined by 30-MHz ^1H NMR (Hsi et al., 1976). An analysis of our 360-MHz ^1H NMR relaxation data consistent with the three-component D'Arcy–Watt theory of sorption isotherms is now proposed. Region I in Figure 5 corresponds to component L_I in Figure 1, while region II in Figure 5 corresponds to $L_I + L_{II}$ in Figure 1. Region III in Figure 5 corresponds to $L_I + L_{II} + L_{III}$ in Figure 1. The limits of regions I–III in Figure 5 toward low concentrations correspond, respectively, to 19, 150, and 2000 mol of D_2O/mol of lysozyme, consistent with the numbers derived from the D'Arcy–Watt analysis of the lysozyme sorption isotherm.

For comparison with the sorption isotherm, we are considering the proton NMR relaxation data in the first hydration region (I), which is the BET monolayer zone. As expected, the relaxation rate R_{2e}* is almost constant in region I (Figure 5), which contains tightly bound water up to a total of $N_I = 2.4\%$ or 19 mol of H_2O/mol of lysozyme and a few additional moles of H_2O/mole of lysozyme that are weakly bound. The slope of R_{2e}* in region II was employed to determine the maximum total number of water molecules bound to lysozyme at weakly binding sites such as polar residues, as described by the equation

$$R_{2obsd} = (1 - N_I/r)R_{2w} + (N_I/r)R_{2b} \quad (2)$$

or by

$$R_{2obsd} = r^{-1}N_{II}R_{2w} + (r - N_{II})R_{2b}r^{-1}$$

where R_{2obsd} is the measured R_{2e}* or $R_{2sample}$ in region II, $1/r$ is the lysozyme concentration, R_{2b} is the value of R_{2e}* for tightly bound water in the BET monolayer containing N_I water molecules, and R_{2w} is the value of R_{2e}* for water bound to the weak binding sites; R_{2w} is obtained by extrapolating the linear region II to $r^{-1} = 0$ (see Figure 5). In region III, eq 2 has to be modified to include two additional types of water: free, or bulk, water with a characteristic value R_{2free} and a fraction of trapped, or multilayer, water (D'Arcy and Watt, 1981) with a characteristic R_{2m} aproximated by the extrapolated value at $r^{-1} = 0$. The inset in Figure 4 shows that, above $\alpha_w = 0.994$, there are two additional, distinct regions, IV and V, with region V containing only lysozyme solutions, while region IV has a lower slope and extrapolated value $R_{2d} > R_{2f}$, of 0.82 s^{-1} ($R_{2f} = 0.11$ s^{-1} is the measured R_{2e}* for 1% HDO). Region IV may, therefore, be associated with discrete aggregates of lysozyme such as dimers. It is interesting that the theory of D'Arcy and Watt breaks down above $\alpha_w = 0.993$; the breakpoint corresponds to the end of region III in the proton NMR relaxation data shown in Figure 5.

DISCUSSION

Our proton spin–echo and deuterium NMR results are consistent with the presence of five distinct water populations in hydrated lysozyme, as determined from the sorption isotherms. These are not all present at low water contents; for example, at $\alpha_w = 0.54$ only two populations are present, one associated with tightly binding sites (charged residues) in lysozyme ($R_{2b} \simeq 54$ s^{-1} or $T_{2b} \simeq 18.5$ ms) and the other (region II) associated with weaker binding sites ($R_{2w} \simeq 49$ s^{-1} or $T_{2w} \simeq 20.5$ ms), which extends to $\alpha_w = 1.00$. These two populations of water were also observed by proton NMR at 30 MHz (Hsi et al., 1976) in frozen lysozyme crystals that contained 0.23 g of H_2O/g of lysozyme. The first water population that corresponds to region I in Figures 2 and 5 was determined to be 0.03 g of H_2O/g of lysozyme (Hsi et al., 1976) and is in good agreement with our value of 0.024 determined for the BET monolayer. The second population of water below 0 °C was found to contain 0.20 g of H_2O/g of lysozyme, which is still in reasonable agreement with the value of 0.185 g of H_2O/g of lysozyme that we obtained for the total fraction of water bound to lysozyme at the limit of region

II (Figure 2). Apparently, the trapped water in region III corresponds to component L_{III}, which freezes below 0 °C and was also reported by Hsi et al. (1976). The existence of distinct water populations L_I, L_{III}, and L_{IV} is also strongly supported by 30-MHz proton NMR data for lysozyme powders [Figures 8–11 in Hilton et al. (1977)].

Although the original D'Arcy and Watt theory (1970) contains a nonzero component L_{III} in both regions II and I (based on thermodynamic considerations), we find that this component is negligible below $\alpha_w = 0.54$ (region II) for hydrated lysozyme at 20 °C. The proton NMR R_{2e}* value for the trapped or multilayer water population is $R_{2m} = 8.8\ s^{-1}$, as estimated from Figure 5, and is closer to the value of R_{2d} at the high-concentration limit of lysozyme solutions ($0.82\ s^{-1}$) than to the value of R_{2w} for the water bound weakly to lysozyme ($49\ s^{-1}$).

The increase in the value of the R_{2e}* from 0.82 to $8.8\ s^{-1}$ for the trapped water can be explained by the presence of a water molecule diffusion term in R_{2m} compared with R_{2d} or R_{2f} (for the bulk HDO). This would imply that trapped water is locally quite similar to the bulk, liquid water, and therefore the water in component L_{III} is dissimilar thermodynamically to the bound water; this is consistent with the fact that the BET theory breaks down above $\alpha_w = 0.54$ for hydrated lysozyme at 20 °C (arrow at point b in the inset to Figure 2). It is also interesting that at high water contents the limit of the D'Arcy and Watt theory can be precisely determined from our linear plots (Asbi and Baianu, 1986); for hydrated lysozyme at 20 °C, this theory breaks down at a value of $\alpha_w = 0.993$ (at 70% lysozyme), which indicates that for α_w values higher than 0.994 water is no longer trapped, and only water bound to lysozyme modifies the chemical potential of the system, as one would expect for solutions.

We have followed up these studies by ^2H and ^{17}O NMR to determine the correlation time (s) of water associated with specific sites in lysozyme (Lioutas et al., 1986).

Such measurements for the quadrupolar nuclei ^2H and ^{17}O at high magnetic fields are very sensitive to water motions and binding to proteins (Lioutas et al., 1986) and provide additional, detailed information concerning protein hydration; the approach is also extended to more complex food systems such as wheat flour (Richardson et al., 1986), myofibrillar proteins (Baianu et al., 1986), wheat and corn starch (Mora-Gutierrez and Baianu, 1985), chemically modified starch and polysaccharide mixtures (Mora-Gutierrez and Baianu, 1985, 1986a), maltodextrins (Mora-Gutierrez and Baianu, 1985) and wheat gliadins (Mora-Gutierrez and Baianu, submitted for publication in *J. Agric. Food Chem.*).

The value of the slow T_{2e}* relaxation component of water in hydrated lysozyme powders is in surprisingly good agreement with the short correlation times determined by ^{17}O NMR (Lioutas et al., 1986); in solutions there are few water molecules with a correlation time equal to that associated with the tumbling of the lysozyme molecule.

Registry No. Lysozyme, 9001-63-2.

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