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A positron annihilation lifetime study of protein hydration - evidence for a glass transition

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Abstract

Positron annihilation lifetimes have been determined for lysozyme at 298 K over the hydration range 0.007 - 0.395 h (g water / g protein) and at hydration levels of 0.15 h and 0.19 h over the temperature range 120 - 350 K. The results indicate that lysozyme undergoes a glass-like, dynamical transition with a transition temperature which decreases with increasing hydration. This behavior is similar to that found with TSDC techniques and suggests that the transitions previously observed at low hydration (0.07 - 0.2 h) at 298 K and those observed at 180 - 220 K in fully hydrated proteins are related. A second transition is observed at 160 - 170 K which may be due to protein-bound water.

Introduction

Protein-water interactions play a critical role in the stability, dynamics and function of proteins and have been extensively studied with a broad range of techniques [1]. One of the most remarkable conclusions from such studies is that very little water is required for the onset of normal (i.e. solution-like) dynamic and functional properties. The sequence of hydration events is now reasonably well described, particularly for lysozyme, although the hydration level corresponding to full hydration (i.e. monolayer surface coverage) is still a matter of some debate.

The first water molecules added to the dry protein interact with ionizable groups. Proton transfer from carboxyl groups to basic groups occurs at this point and rapidly normalizes the pK_a values of acidic and basic side chains. Hydration of ionizable groups is complete at a hydration of about 0.07 h and is followed by the addition of water to peptide groups and other polar surface groups. Judging from heat capacity and infrared spectroscopic measurements, polar group monolayer coverage is complete at a hydration of about 0.27 h . The hydration of lysozyme is completed at about 0.38 h with the condensation of water over weakly interacting non-polar surface regions [1]. Other estimates suggest that polar group monolayer coverage occurs at a somewhat higher hydration of about 0.32 h , corresponding to the fraction of non-freezing water found in protein solutions by proton NMR measurements, and that full hydration of lysozyme occurs at 0.45 - 0.5 h [2].

Studies of protein conformational dynamics have usually been confined either to measurements of dynamic properties as a function of hydration at 298 K or to measurements of protein motions as a function of temperature in fully hydrated proteins. Dry proteins are rigid and catalytically inactive. However, addition of water increases conformational flexibility and

leads to the recovery of normal internal and surface mobility and enzymatic activity. These properties develop at hydration levels well below full hydration. For example, in lysozyme enzymatic activity and surface mobility, as monitored by ESR spin labels, begin to develop at a hydration of about 0.2 h [3], while internal mobility, as measured by peptide hydrogen isotope exchange rates, begins to recover at about 0.05 - 0.07 h and is fully developed at a hydration of 0.15 h [4]. Dielectric relaxation measurements also indicate that recovery of conformational flexibility occurs in this hydration range, at least at 298 K, the temperature at which most studies of protein hydration have been performed [1]. Water thus appears to act as a plasticizer of the protein.

Studies using Mossbauer spectroscopy [5,6], neutron scattering [7], Rayleigh scattering of Mossbauer radiation (RSMR) [8] and X-ray diffraction techniques [9] indicate that fully hydrated proteins undergo a glass-like dynamical transition at about 200 K. Below this temperature the protein is frozen into a distribution of conformational states. As the temperature is increased above 200 K, segmental motions begin and transitions between conformational states are possible. Measurements of thermally stimulated depolarization currents (TSDC) in lysozyme reveal two complex depolarization bands; a "low temperature" band at 125 - 140 K and a hydration-dependent "high-temperature" band with a band peak temperature which varies from about 230 K at 0.13 h to 175 K at 0.35 h . The "low temperature" band has been assigned to dielectric relaxation of bound water while the "high temperature" band has several contributions, including proton percolation and relaxation of polar groups in the protein [10,11].

We have been using positron annihilation lifetime spectroscopy to determine free volume changes as a function of hydration in lysozyme and have recently embarked on a detailed study of the hydration and temperature dependence of o-Ps lifetimes in this protein. In this paper we present our results at 298 K over the hydration range 0.007 - 0.395 h together with results determined at hydrations of 0.15 h and 0.19 h over the temperature range 120 - 350 K.

Materials and Methods

Materials: Hen egg white lysozyme (grade 1, 1-6876) was obtained from Sigma Chemical Company (St. Louis, Mo). Molybdenum single crystals (10 mm diameter, 2 - 3 mm thick) were from AESAR/Johnson Matthey (Ward Hill, Ma). ^{22}Na was from NEN, Dupont (Wilmington, Delaware).

Protein Sample Preparation: Lysozyme (20 mg/ml) was dialyzed against deionized water and lyophilized. The lyophilized protein was stored in a vacuum desiccator over phosphorus pentoxide at room temperature. The hydration level of the lyophilized protein was determined from the weight loss of a sample after drying at 104°C for 24 hours. Hydrated samples were prepared by isopiestic equilibration of the lyophilized protein over solutions of sulfuric acid of appropriate concentration to establish the desired level of water in the sample. Hydration levels were determined gravimetrically and were corrected for the residual water bound to the lyophilized protein:

$$h = h_{\text{app}}(1 + h_e) + h_e \quad (1)$$

where h_{app} is the uncorrected hydration determined after isopiestic equilibration and h_e is the hydration level of the lyophilized protein. Hydrated proteins (0.007 h to 0.394 h) were packed into two Teflon cells to a density of between 0.7 and 0.9 g cm^{-3} to give disks 2 mm thick and 8 mm in diameter. The positron source consisted of about 50 μCi of ^{22}Na heat-sealed between two sheets of Teflon-coated Kapton (100FN099 Dupont Co., Wilmington, Delaware) or between two sheets of 7 μm aluminum foil which was sandwiched between the protein disks.

Positron Annihilation Lifetime Spectroscopy: Positron annihilation lifetime data were collected with a fast-fast coincidence system (EG&G Ortec, Oak Ridge, TN) using RCA 8850 or Burle 8575 photomultiplier tubes and BC 418 1" truncated cone plastic scintillators (Bicron, Newbury, Ohio). The instrument resolution function, measured from the prompt spectrum of a ^{60}Co source,

had a FWHM of about 280 ps. Molybdenum single crystals were employed as a reference for which an annihilation rate of 9.1 ns^{-1} was assumed. Sample and reference decay curves had total counts of at least 1.0×10^6 . The data employed for analysis comprised 704 channels with a channel width of 50 ps starting 77 channels (3.85 ns) to the left of the peak.

Source Corrections: Positron implantation in aluminum source foils was determined by Monte Carlo calculation based on the graphical algebra of Schrader [12,13], together with experimental measurements on aluminum multilayers. The resulting source corrections for the sample and reference were $\lambda = 6.024 \text{ ns}^{-1}$, $\alpha = 0.23$ and $\lambda = 6.024 \text{ ns}^{-1}$, $\alpha = 0.293$, respectively. Analysis of decay curves for Teflon and hexane samples with these source corrections gave lifetime parameters in excellent agreement with published results. Experimental measurements with Teflon-coated Kapton foils at 25°C gave the following corrections: $\lambda_1 = 4.80 \text{ ns}^{-1}$, $\lambda_2 = 2.53 \text{ ns}^{-1}$, $\lambda_3 = 0.740 \text{ ns}^{-1}$, $\lambda_4 = 0.238 \text{ ns}^{-1}$, $\alpha_1 = 0.095$, $\alpha_2 = 0.193$, $\alpha_3 = 0.004$ and $\alpha_4 = 0.006$ for the sample and $\lambda_1 = 4.80 \text{ ns}^{-1}$, $\lambda_2 = 2.53 \text{ ns}^{-1}$, $\lambda_3 = 0.740 \text{ ns}^{-1}$, $\lambda_4 = 0.238 \text{ ns}^{-1}$, $\alpha_1 = 0.124$, $\alpha_2 = 0.251$, $\alpha_3 = 0.006$ and $\alpha_4 = 0.010$ for the reference. The source terms are in very good agreement with those determined by Zhou et al [14]. Analysis of decay curves for water samples with these source corrections gave lifetime parameters in excellent agreement with published results. Analysis of protein decay curves obtained with aluminum and Teflon-coated Kapton foil sources also are in excellent agreement. Source corrections employed at different temperatures were corrected for the temperature dependence of the annihilation parameters (i.e. the decrease in the lifetime of the small long-lived component in Teflon-coated Kapton with decreasing temperature).

Analysis of Decay Curves: Sample decay curves are represented by:

$$y(t) = N_s \int_0^{\infty} \lambda \alpha(\lambda) I(t) * \exp(-\lambda t) d\lambda \quad (2)$$

where N_s is the total number of annihilation events observed, $I(t)$ is the instrument resolution function and $\alpha(\lambda)$ is the annihilation rate probability density function (pdf). * denotes a convolution. The fraction of positrons annihilating with rates between λ and $\lambda + d\lambda$ is $\alpha(\lambda)d\lambda$. Annihilation rate pdfs were determined with the program CONTIN (PALS2) [15,16] and were sampled at 50 grid points over the range $0.1 < \lambda < 15.0 \text{ ns}^{-1}$. The solutions were constrained to be positive over this range and a second order regularizer was employed to penalize deviations from smoothness in the solutions. Sample and reference decay curves were corrected for annihilation in the source materials and for zero-time shift errors as described previously [17].

Results

Positron annihilation lifetime pdfs consist of three peaks which can be attributed to the annihilation of p-Ps, free positrons and o-Ps species. o-Ps lifetimes are shown as a function of hydration in Figure 1. The dry protein has an average o-Ps lifetime of about 1.6 - 1.7 ns. This decreases initially as water is added to the dry protein, but at a hydration of about 0.12 h the o-Ps lifetime begins to increase, reaching a value of about 1.8 - 1.9 ns at 0.395 h (Note that lifetime parameters given in a preliminary report of this work [18] were incorrect due to errors in the source corrections employed). Cavity volume pdfs obtained by transformation of the annihilation rate pdfs [19] suggest that cavity volumes are distributed over a range of values from $25 \text{ \AA}^3$ to $150 \text{ \AA}^3$. The average cavity volume in the dry protein is about $65 \text{ \AA}^3$. This shifts to about $90 \text{ \AA}^3$ on hydration. The o-Ps fraction is 0.17 - 0.18 in the dry protein and decreases as the hydration is increased beyond 0.1 h to about 0.14 at 0.2 h where it remains constant up to full hydration. These changes are remarkably similar to those reported for polyamides [20] and are consistent with the existence of a glass transition. At low hydrations the glass transition temperature, T_g , is higher than the measurement temperature and the protein is rigid.

Figure 1: The mean o-Ps lifetime for lysozyme as a function of hydration at 298 K.

As the hydration level (plasticizer content) of the protein is increased, T_g decreases. When sufficient water is present for T_g to have decreased below the measurement temperature (i.e. $> 0.12 \text{ h}$ at 298 K), the protein becomes flexible. In the flexible protein, Ps increases the size of the cavities in which it is localized as a consequence of exchange repulsion between Ps and closed shell electrons of the surrounding medium. This together with an intrinsic expansion of available free volume in the flexible state lead to a rapid increase in o-Ps lifetime.

These results suggest that lysozyme at a hydration of 0.12 h undergoes a glass-like transition at 298 K. As noted earlier, X-ray diffraction and various spectroscopic studies provide evidence for a glass transition at 180 - 220 K in fully hydrated proteins. We have therefore begun an examination of the temperature dependence of o-Ps lifetimes in partially hydrated lysozyme samples in an attempt to define the hydration dependence of the glass transition temperature. The results obtained at hydration levels of 0.15 h and 0.19 h are shown in Figure 2. The curves display two transitions; a sharp "low temperature" transition at 160 - 170 K and a hydration-dependent "high temperature" transition characterized by a change in volume expansion from essentially zero below the transition to about $0.6 \text{ Å}^3\text{K}^{-1}$ above the transition. Increasing the hydration from 0.15 h to 0.19 h shifts the "high temperature" transition from about 240 K to 220 K.

These transition temperatures together with those determined with other techniques are shown as a function of hydration in Figure 3. The decrease in transition temperature with increasing hydration (plasticizer content), so characteristic of glass transitions in synthetic polymers, is clearly seen. Water plasticizes proteins by providing alternate, mobile hydrogen bonding donors and acceptors for peptide groups. This decreases barriers between conformational states and allows conformational rearrangements to occur. The efficiency of water as a plasticizer suggests that the amount of buried water is significantly greater than that seen by X-ray crystallography.

Proteins are heterogenous systems so the glass transition is likely to be quite broad. Hydrogen isotope exchange studies indicate that exchange rates are broadly distributed and provide evidence for a number of dynamically distinct protein regions [21]. The slowest exchanging peptide proteins are located in rigid, well-packed non-polar regions (so called "knots") of the protein characterized by low Debye-Waller factors. These regions are embedded

Figure 2: The temperature dependence of the average o-Ps lifetime in lysozyme at hydration levels of 0.19 $\frac{\text{g water}}{\text{g protein}}$ (filled symbols) and 0.15 $\frac{\text{g water}}{\text{g protein}}$ (open symbols). Data obtained with aluminum source foils are indicated by triangles.

Figure 3: The hydration dependence of denaturation and glass transition temperatures for lysozyme. Data for the denaturation temperature determined by differential scanning calorimetry (open circles) are taken from ref. 22. TSDC results are from ref. 10 (open squares) and ref. 11 (open triangles). The range of transition temperatures reported with the other techniques discussed in the text are indicated by the open boxes. Results of the present work are represented by the filled circles.

in a more mobile, water-permeable matrix. Analysis of the temperature dependence of the Debye-Waller factors in ribonuclease A [9] suggests that main-chain atoms located in exchange-stable regions do not undergo the 200 K glass transition observed for main-chain atoms in the surrounding matrix. As the temperature is increased above the glass transition temperature only the matrix regions become flexible and undergo segmental motions. The exchange-stable "knot" regions remain glass-like. Disruption of the exchange-stable regions only occurs at much higher temperatures and leads to denaturation of the protein [21]. The hydration dependence of the denaturation temperature for lysozyme is shown in Figure 3. This figure defines three major dynamic states of lysozyme; (i) the rigid, glass-like native state, (ii) the flexible native state characterized by a mobile matrix tethered to glass-like "knot" regions and (iii) the denatured state in which both "knot" and matrix regions are highly mobile. Other states are known to exist in proteins (i.e. the cold-denatured state and molten globule state) so the diagram is as yet incomplete. However, a reasonably consistent picture of the influence of hydration and temperature on protein dynamics is emerging.

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