Proton glass freezing in hydrated lysozyme powders

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(Received 4 June 1999; revised manuscript received 27 August 1999)

At room temperature, the dielectric relaxation of hydrated powder of the protein lysozyme is known to be due to protons migrating between ionized side chains. A recent study of this relaxation at lower temperatures suggested a behavior typical of proton glasses. An analysis of the complex dielectric susceptibility by a temperature-frequency plot presented here has revealed that ergodicity is broken due to the divergence of the longest relaxation time at 266 K, indicating specifically that this hydrated protein is a proton glass. A change in the temperature behavior of the static dielectric constant and the average relaxation frequency at 273 K indicates a further transition occurring at this temperature, whose nature remains to be investigated.
[S1063-651X(99)08412-3]

PACS number(s): 87.14.Ee, 64.70.Pf, 77.22.Gm, 78.20.Ci

I. INTRODUCTION

Hydrated powders of globular proteins provide a convenient system to correlate the stepwise and parallel onset of physical properties and biological functions, because one can easily control the main experimental variable, namely the hydration of the sample. This system has been studied for more than a decade by several experimental techniques (including dielectric relaxation), pointing to the conclusion that below the monolayer coverage the state of aggregation consists of single macromolecules hydrogen-bonded together and unable to rotate. Above the bound water, two-dimensional (2D) percolation threshold, the dielectric absorption below 10 MHz has been assigned mainly to protons produced by fluctuations of ionizable side chains and migrating as defects in the 2D water network on single macromolecules [1–6]. A look at the H-bonded network on the protein surface already shows the presence of a complex system with parallel and competitive interactions, including ionizable side chains, migrating protons, bound water, and nearby peptide backbones, and thus a good candidate for glassy behavior [2–6]. As a matter of fact, in this system a broad dielectric relaxation has been observed [7] qualitatively similar to that of ferroelectric proton or deuteron glasses [8–10], and quite similar to relaxors [11,12].

Proton and deuteron glasses can be considered as the electric counterparts of magnetic spin glasses [13,14]. The best known examples are the random mixtures $Rb_{1-x}(NH_4)_xH_2PO_4$ (RADP) of ferroelectric RbH_2PO_4 (RDP) and antiferroelectric $(NH_4)H_2PO_4$ (ADP), and their deuterated analogs. Thus in RADP the randomness lies in the cation placement and the frustration in the two competing tendencies for ordering the $OH\cdots O$ proton pseudospins which prevent either ferroelectric or antiferroelectric order. The random-bond random-field Ising model provides a description of the general properties of these glasses [15]. A typical characteristic of a proton or deuteron glass is the appearance of the broad distribution of relaxation times and the break of ergodicity at the freezing temperature T_0, where the longest relaxation time diverges [9]. In the present work we will show that this last property is displayed also in the dielectric relaxation of lysozyme hydrated powders.

II. EXPERIMENT

The powdered lysozyme samples were prepared according to the standard procedure [4,7,16], namely by dissolving the commercial Worthington $3\times$ crystallized and salt free protein. Samples were desalted by the dialysis in the cold, with N_2 bubbling through the dialysant to exclude CO_2. The pH of each powdered sample was determined at the end of its preparation. Protein powders were obtained by crystallization from appropriated pH solutions and by extensive lyophilization over P_2O_5 to obtain reproducibly low water content. Samples were prepared in groups, to minimize differences except in the parameters varied. Other details of preparations to obtain the desired pH and levels of other components (salt or substrate) are given extensively in Ref. [16].

Samples of lyophilized protein powder were hydrated isopiestically below the first water monolayer for about a day in moist air at 5°C. Specifically, three samples at two different hydration levels $h=0.28$ g H_2O/g dry weight and $h=0.30$ g H_2O/g dry weight and at two different pH3 and pH7 values will be reported here in order to ensure that the observed behavior does not depend on specific hydration or

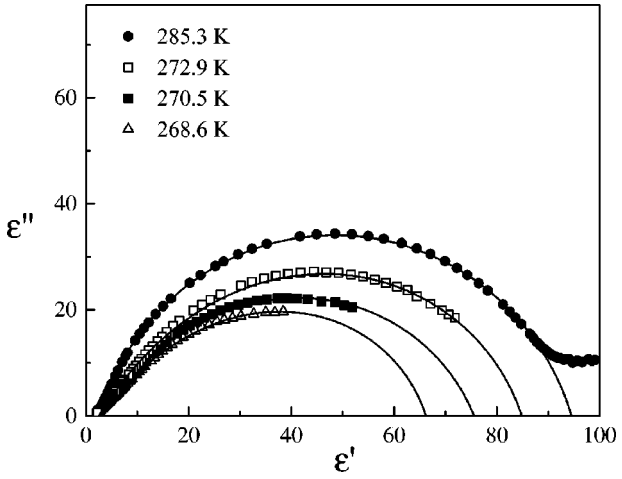

FIG. 1. Measured values of ε'' plotted vs ε', in a lysozyme sample at pH7, $h=0.28$ at four temperatures.

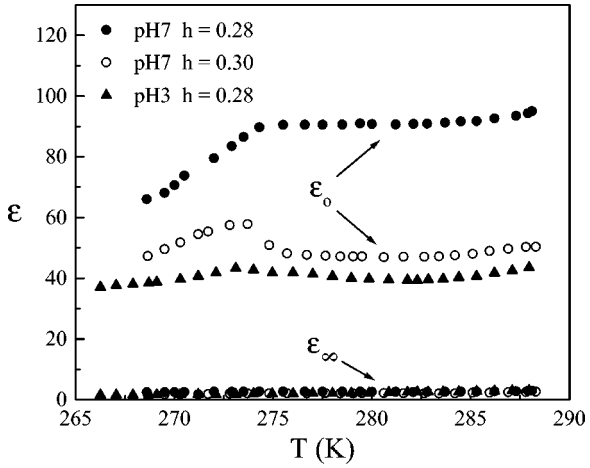

FIG. 2. Temperature dependence of ε_0 and ε_∞ in samples at various levels of pH and h.

pH values. Furthermore, these two hydration levels fall in the hydration interval above $h=0.15$ g H_2O /g dry weight where the percolative transition in water itself takes place with water molecules becoming interconnected in a cluster encompassing the whole protein surface. Actually, above $h=0.25$ g H_2O /g dry weight water starts to condense onto weakly interacting unfilled patches of protein surface. Here a long-range proton movement along percolative networks was observed in dielectric measurements [1]. On the other hand, the above hydration levels are still far away from the hydration level $h=0.38$ g H_2O /g dry weight where the monolayer of water covers the protein surface and above which the protein conductivity saturates, thus indicating a new regime.

The powdered sample was lightly compressed in the sample holder between the electrodes, which were covered by thin Teflon films. The complex dielectric constant of the protein was evaluated by considering the equivalent circuit made up of a composite capacitor in series with the aggregate capacitance of the Teflon film. Experimental details of the data acquisition system and of the raw data analysis have already been given in Ref. [7].

The samples were first quenched to about 200 K at a cooling rate of 20 K/min. Faster quenching rates did not allow reproducible results, as known in other glass-forming systems. Then the data were taken on heating the sample with the typical heating rate of 0.2 K/min. Here the measurements of the complex dielectric constant were taken at 70 frequencies in the range from 100 Hz to 1 MHz, by stabilizing the temperature during the data taking.

Figure 1 shows Cole-Cole diagrams at four different temperatures obtained on the sample of powdered lysozyme at pH7, which was hydrated at 0.28 g H_2O /g dry weight ($h=0.28$). Solid lines are fits to the so-called inverse Havriliak-Negami (HN) expression,

$$\varepsilon^*(\omega)=\varepsilon_0-(\varepsilon_0-\varepsilon_\infty)\left\{\frac{(i\omega\tau_{\mathrm{HN}})^{1-\alpha}}{1+(i\omega\tau_{\mathrm{HN}})^{1-\alpha}}\right\}^{\beta}. \qquad (1)$$

Here τ_{HN} is the characteristic relaxation time, ε_0 is the static dielectric constant, ε_∞ is the high-frequency dielectric constant, and α and β are parameters associated with the shape and width of the distribution of relaxation times. The temperature dependence of ε_0 and ε_∞ is shown in Fig. 2 for three lysozyme samples at various levels of hydration and pH values. In all cases, it was found that below 273 K the static dielectric constant ε_0 decreases. In Fig. 3, a measuring frequency is plotted as a function of inverse temperature at which the temperature-dependent $\varepsilon''(T)$ reaches a maximum value. This frequency plays the role of an effective characteristic relaxation frequency. Above 273 K, the data for all three lysozyme samples can be described by a Vogel-Fulcher (VF) expression,

$$f=f_0\exp[-U/(T-T_0)], \qquad (2)$$

with $T_0=268\pm2$ K (see Table I). Below 273 K an Arrhenius activated behavior $f=f_0\exp[-E/T]$ appears to be valid.

In order to extract direct information on the temperature variation of the relaxation spectrum, we analyzed the dielectric dispersion in hydrated lysozyme samples above 273 K using the temperature-frequency plot applied recently to various glassy and relaxor systems [9–12,17]. A detailed de-

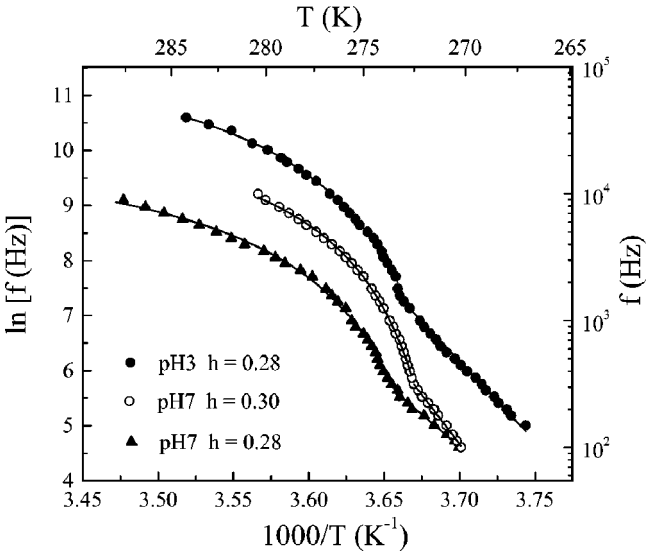

FIG. 3. Measuring frequency as a function of inverse temperature, defined as the temperature of the maximum of $\varepsilon''(T)$, in the same samples as in Fig. 2.

TABLE I. Fitted parameters of lysozyme powders as a function of pH and hydration level (h) in g H_2O /g dry weight.

pH	h hydration	T_0 (K)	U (k_bK)	f_0 (Hz)	$T_0(\delta\rightarrow 1)$ (K)	$U(\delta\rightarrow 1)$ (k_bK)	$f_0(\delta\rightarrow 1)$ (Hz)
7	0.28	268 ± 2	28 ± 10	$(3.3\pm 1.9)\times 10^4$	266 ± 2	23 ± 10	$(3.0\pm 1.5)\times 10^3$
7	0.30	268 ± 2	22 ± 5	$(5.9\pm 2.8)\times 10^4$	266 ± 2	44 ± 10	$(1.1\pm 0.6)\times 10^4$
3	0.28	267 ± 2	32 ± 10	$(2.5\pm 1.6)\times 10^5$	260 ± 2	90 ± 10	$(3.0\pm 2.4)\times 10^4$

scription of this method was already given elsewhere [9,10]. Here a natural assumption is adopted that the distribution of relaxation times $g(z)$ is limited between lower and upper cutoffs z_1 and z_2, respectively, where $z_i=\ln(\omega_a\tau_i)$ with ω_a as an arbitrary unit frequency. By scanning the reduced dielectric constant δ,

$$\delta=\frac{\varepsilon'(\omega,T)-\varepsilon_\infty}{\varepsilon_0-\varepsilon_\infty}=\int_{z_1}^{z_2}\frac{g(z)dz}{1+(\omega/\omega_a)^2\exp(2z)}, \qquad (3)$$

between the values 1 and 0, the filter in the second part of Eq. (3) is scanning the distribution of relaxation times $g(z)$ by shifting its position in ω space [9], thus probing various segments of the relaxation spectrum. Note that δ is now playing the role of an experimentally adjustable parameter. In practice, this is achieved by finding, within the set of dielectric data at a given temperature T, a frequency $\omega=2\pi f$ at which the prescribed value of δ is reached. Obviously, prior knowledge about the temperature behavior of the two required parameters ε_0 and ε_∞ is needed. These parameters (shown in Fig. 2) were determined by fitting the data to Eq. (1).

Characteristic temperature-frequency plots in the (T,f) plane for each fixed value of the reduced dielectric constant δ are shown in Fig. 4 for the sample at pH7 and $h=0.28$. The bending of the data above 273 K indicates a divergent behavior of all relaxation times in the relaxation spectrum. This implies that even at small values of δ all relaxation frequencies in the (T,f) plane can be effectively described by a Vogel-Fulcher law. Here the parameters f_0, U, and T_0 were determined by fitting each curve in Fig. 4 by Eq. (2).

Figure 5 shows the temperature dependence of the Vogel-Fulcher (VF) temperature T_0 as a function of a reduced dielectric constant δ. By extrapolating $\delta\rightarrow 1$ and $\delta\rightarrow 0$, one can determine the VF temperature T_0 at $\omega\rightarrow 0$ and $\omega\rightarrow\infty$, respectively, to give $T_0(\delta=1)=266\pm 2$ K and $T_0(\delta=0)=256\pm 2$ K in the case of the sample of pH7 and $h=0.28$. These two freezing temperatures $T_0(\delta=1)$ and $T_0(\delta=0)$ actually correspond to the VF temperatures of the relaxation spectrum cutoffs z_1 and z_2, respectively. In contrast to deuteron glasses [9,10], where only the longest relaxation time diverges, Fig. 5 shows that both lower and upper cutoffs z_1 and z_2 diverge, i.e., different parts of the relaxation spectrum diverge at different freezing temperatures.

It should be noted that a similar analysis has been carried out also in a sample hydrated at $h=0.30$ g H_2O /g dry weight at pH7, and in a sample hydrated at $h=0.28$ g H_2O /g dry weight at pH3. The results of the analysis of these samples are collected in Table I. The values in the first three columns are determined from data shown in Fig. 3 and thus refer to the average, characteristic relaxation frequency, while the values in the last three columns are limiting values obtained in the limit $\delta\rightarrow 1$ (cf. Figs. 4 and 5).

III. DISCUSSION

The analysis of the data by the temperature-frequency plot shows that hydrated lysozyme powders show a dielectric relaxation which is typical of the glassy behavior observed in ferroelectric proton glasses, magnetic spin glasses, and relax-

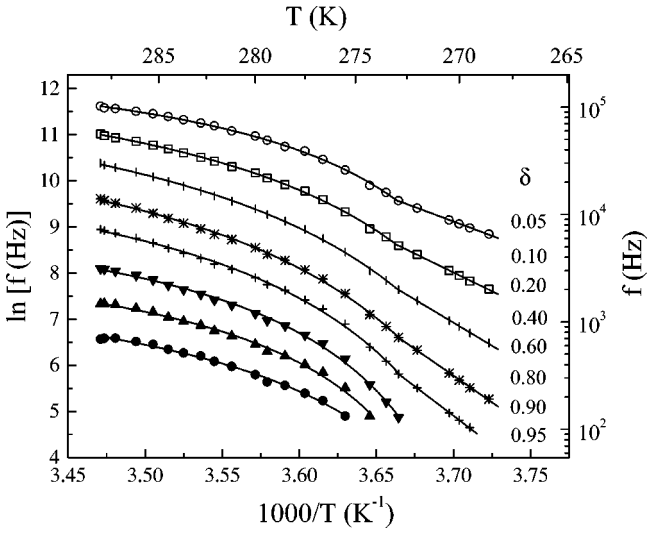

FIG. 4. Temperature-frequency plots for several values of reduced dielectric constant δ in the sample at pH7 and $h=0.28$. Solid lines are fits obtained with a generic Vogel-Fulcher expression.

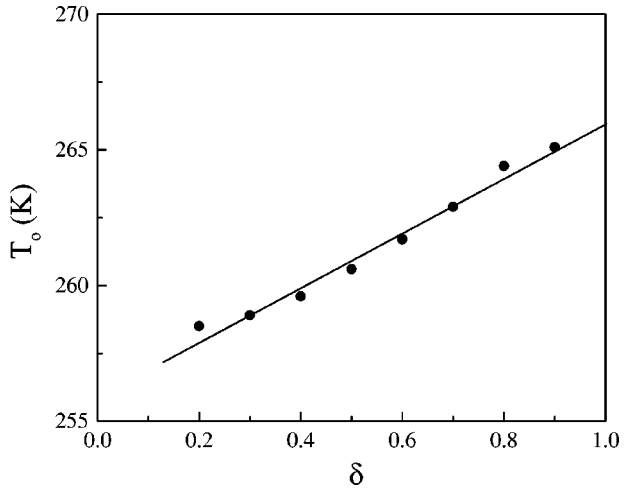

FIG. 5. Vogel-Fulcher temperature T_0 as a function of the reduced dielectric constant δ in the sample at pH7 and $h=0.28$.

ors. Dielectric relaxation exhibits a broad distribution of relaxation times whose polydispersity increases with decreasing temperature. The relaxation spectrum shows a distribution of freezing temperatures $T_0(\delta)$ at which the system becomes effectively frozen. The divergence of the longest relaxation time at $T_0(\delta=1)=266\pm2$ K effectively breaks ergodicity, thus separating an ergodic from a nonergodic glassy state. As already suggested [7], in ferroelectric proton glasses this behavior originates in the acidic proton subsystem from an interplay of site disorder and frustrated interactions, while in hydrated lysozyme powders the network of $OH\cdots O$ bonds is two-dimensional and the potential barriers are weak and distributed broadly, thus displacing the freezing transition to temperatures higher than in ferroelectric H-bonded crystals.

We note that before the system can reach the nonergodic phase, a transition is taking place near 273 ± 0.5 K, and this calls for an explanation. This temperature is close to the melting temperature of H_2O. However, it seems that its origin cannot be trivially connected to the formation of bulk ice in the sample because this last event would raise the ε_0 value, instead of being lowered below this temperature (see Fig. 2). Moreover, a three-dimensional H-bonded network including clusters of hydrated protein molecules cannot be excluded at hydration levels close to the monolayer coverage near $h\sim0.38$. Yet, disregarding the nature of the system observed below 273 K, our previous analysis and conclusions for the region above this temperature remain unaffected. The temperature region below 273 K deserves further study at frequencies below 100 Hz.

In conclusion, the analysis by the temperature-frequency plot of dielectric data of hydrated lysozyme powders below room temperature confirms the attempted ergodic to nonergodic proton glass freezing [7], similar to the behavior observed in deuteron spin glasses [9]. We believe that, on the grounds of all previous work on this subject [1,4,5,7], in spite of the fact that we have considered only one particular globular protein, all the conclusions reported here have a wider validity, because the hydrated surface is quite similar in all globular proteins in the temperature range considered in this work.

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