Glassy dynamics and enzymatic activity of lysozyme

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There has been much interest in the analogies between dynamic processes in proteins and in other complex systems such as viscous liquids and glasses. We have investigated the dynamics of protons along chains of hydrogen-bonded water molecules adsorbed on the surface of the globular protein lysozyme. The hydration dependence of the dielectric relaxation time is fitted by a modified Vogel-Fulcher-Tamman equation, in which the variable temperature has been replaced with hydration. We find that the relaxation time diverges at a singular hydration that coincides with the critical water content required to trigger lysozyme enzymatic activity. This surprising correlation suggests a direct coupling between protein function and glasslike behavior, with possible implications for the turnover number of the enzyme.

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Proteins, as individual molecules, possess the complexity necessary to exhibit cooperative dynamics comparable with those of the simpler glass-forming liquids and polymer systems [1,2]. A wide range of time scales characterizes the dynamics of a protein macromolecule, the dynamical spectrum ranging from rapid librations and vibrations to slow motions [3]. However, there is a need for experimental information on these motions and their relationship to protein stability, folding, and function, which are topics of great current interest, both fundamental and practical [4]. In analogy with glass-forming liquids, the relaxation process governing large scale motions is nonexponential in time and its temperature dependence does not follow the Arrhenius law [5]. These features are usually taken as the canonical signatures of relaxation in glassy systems [1], and are strongly influenced by the presence of water molecules around the protein, as shown by experiments [4,6–9] and computer simulations as well [10,11]. In this context, the role played by hydration water in determining the glass character of a protein is not fully clarified [12,13], and it is fair to say that we are still far from a quantitative treatment of the role of motions in functions, and no generally applicable theory exists [14]. 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, safely neglecting macromolecular motion, such as rotation and translation which obviously occur in protein solutions. In particular, the lysozyme-water system has been studied by several experimental techniques [15] and for a review see Ref. [16]. Of particular interest is the presence, at room temperature, of a percolative transition of the protein-water conductivity at a critical hydration level $h_c = 0.16$ g water/g dry protein (corresponding to about 130 water molecules per single protein molecule) coinciding with the onset of enzymatic activity. The percolation model is a general statistical physics approach, applicable to a wide range of phenomena where spatially random events and topological disorders are of intrinsic importance [17]. The most striking feature of a percolation process is the presence of a sharp transition, a collective effect where long-range connectivity among the elements of the system suddenly appears at a critical concentration of such elements. The conductivity percolative transition found for the lysozyme-water system is due to proton displacements along hydrogen-bonded water molecules adsorbed on the protein surface, with ionizable groups as sources of migrating protons [18,19]. Recently, the dynamics of these migrating protons over the lysozyme surface has been investigated by means of conventional dielectric technique over a limited frequency and temperature range [20,21]. Data were collected over the frequency window 100 Hz–1 MHz with temperature ranging from 265 to 290 K, and obtained results indicated interesting analogies with the dielectric behavior of fragile proton glasses, the electric counterparts of magnetic spin glasses [20,21]. In particular, the three canonical features of a glassy system [1] such as non-Arrhenius temperature dependence of the dielectric relaxation time, nonexponential relaxation processes, along with nonergodic behavior below a transition temperature were observed. To study a possible functional link between the glassiness of the subsystem represented by protons migrating along hydrogen-bonded water molecules on the lysozyme surface, and lysozyme enzymatic activity, we have investigated the hydration and temperature dependences of the dynamics of protons on the lysozyme surface. These migrating charges appear to be a natural choice, as far as catalytic activity is concerned, given their previously underlined role in the percolative process at room temperature. Since catalytic activity is a slow process, characterized by turnover numbers in the 10^{-1}–10^{3} s^{-1} range [13], we performed broadband dielectric spectroscopy to observe dielectric relaxation of migrating protons in the frequency range 10^{-3}–10^{6} Hz over temperatures from 210 to 310 K. Broadband dielectric spectroscopy is a powerful experimental method to investigate the dynamical behavior of a sample through the analysis of its frequency dependent dielectric

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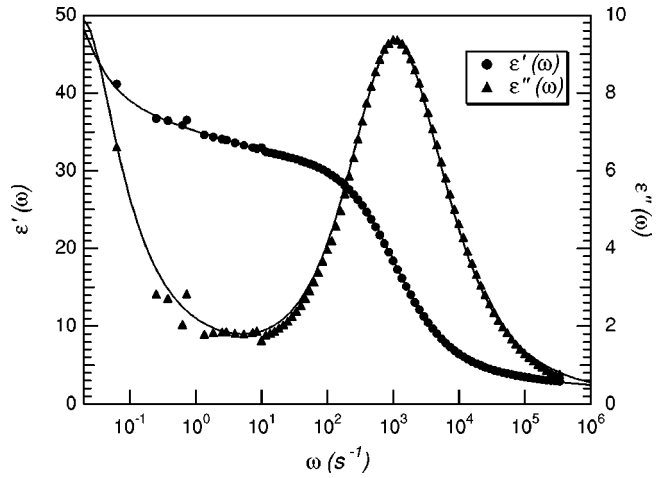

FIG. 1. Dielectric spectra. Angular frequency (ω) dependence of the real [$\epsilon'(\omega)$] and imaginary [$\epsilon''(\omega)$] components of the combined response of electrode polarization, interfacial dispersion, and hydrated powders of lysozyme. The powdered samples were prepared according to standard procedures [32] and equilibrated to *p*H7. The complex dataset shown in this figure has been obtained at $T=249.8$ K with a sample water content $h=0.28$ g water/g dry weight. The solid lines through the data are the result of a complex function fit procedure that takes into account the complex admittances of polarized electrodes, interfacial dispersion, and sample [23]. Details of the fitting procedure are given in Ref. [23].

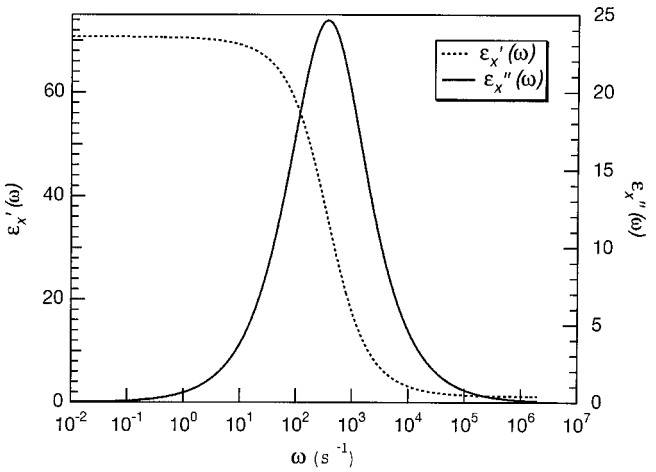

FIG. 2. Calculated angular frequency (ω) dependence of the real [$\epsilon_x'(\omega)$] and imaginary [$\epsilon_x''(\omega)$] components of the permittivity of a sample of hydrated powders of lysozyme. Sample water content is $h=0.28$ g water/g dry weight, sample temperature is $T=249.8$ K. The frequency response of sample relaxation [see Eq. (2)] contains fitted parameters whose values are $\epsilon_\infty=1.15\pm0.02$, $\epsilon_s=70.75\pm0.04$, $\tau=(2.77\pm0.08)\times10^{-3}$ s, $\alpha=0.78\pm0.01$, and $\beta=1.000\pm0.001$.

response. This technique is based on the measure of a complex quantity (i.e., admittance or impedance) as a function of angular frequency ω of a sample sandwiched between two electrodes. The measured admittance $Y_m(\omega)$ is directly related to the complex permittivity $\epsilon_m^*(\omega)=\epsilon_m'(\omega)-J\epsilon_m''(\omega)$ given that

$$\epsilon_m^*(\omega)=\frac{h}{J\omega\epsilon_o S}Y_m(\omega), \tag{1}$$

where $J=\sqrt{-1}$, ϵ_o is the permittivity of free space, and S and h are, respectively, the sample surface and thickness. If solid protonic conductors are investigated, the electrodes are usually ''blocking,'' namely they are able to eliminate free exchange between electronic and protonic charge carriers at the sample–electrode interface. Although required, the use of insulated electrodes results in large electrode polarization effects [22], and the correction for it is one of the major requisites in obtaining meaningful measurements on conductive samples [23]. The measured permittivity for a sample of powdered lysozyme (water content $h=0.28$ g/g and temperature $T=249.8$ K) sandwiched between two metallic electrodes and insulated from them by thin Mylar films is shown in Fig. 1. Electrode polarization is clearly visible in both real and imaginary components, as shown by the low-frequency tails of the permittivity data in Fig. 1. It is possible in principle to extract true sample permittivity from the measured frequency response by performing a complex function fit procedure that takes into account the fractal nature of electrode polarization [24], interfacial dispersion (also known as Maxwell-Wagner effect) [25], along with the frequency dependence of sample relaxation itself. This procedure has been recently tested with a reference sample (i.e., hexagonal ice obtained by cooling down water in the sample cell), yielding results in very good agreement with published data [23]. Solid lines in Fig. 1 represent the result of such a complex function fit procedure, and the good quality of the fit indicates that reliable true sample permittivity can be obtained. The more general expression for the frequency dependence of sample permittivity $\epsilon_x^*(\omega)$ can be written as

$$\epsilon_x^*(\omega)=\left[\epsilon_s+\frac{\epsilon_s-\epsilon_\infty}{[1+(J\omega\tau)^\alpha]^\beta}\right], \tag{2}$$

where ϵ_s is the limiting low frequency sample permittivity, ϵ_∞ is the limiting high frequency sample permittivity, and τ is the sample relaxation time. The two exponents α and β appearing in the relaxation term of Eq. (2) provide an empirical generalization of the ideal Debye relaxation characterized by a single relaxation time instead of a more realistic asymmetric distribution centered around the most probable τ. This generalization, usually referred to as Havriliak-Negami relaxation [26], better describes the frequency dependence of dielectric properties of a very wide class of samples [27]. Calculated real and imaginary components of sample permittivity are shown in Fig. 2. It should be noted that neither the amplitude, $\Delta\epsilon=\epsilon_s-\epsilon_\infty$, nor the position, τ^{-1}, of the sample relaxation could be drawn by looking at the measured frequency response shown in Fig. 1, as done in the past [20,21]. In particular, $\Delta\epsilon=70$ and $\tau^{-1}=361$ Hz should be compared with 30 and 1000 Hz, respectively, as resulting from the measured frequency response of the sample sandwiched between insulating electrodes (Fig. 1). In Figure 3 we show the relaxation time τ, obtained from the complex function fit and attributed to migrating protons, as a

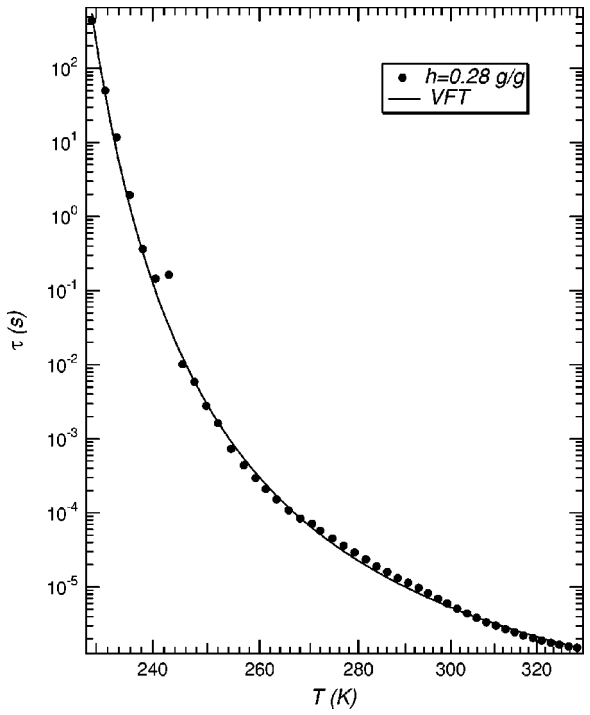

FIG. 3. Temperature dependence of the dielectric relaxation time $\tau(T)$ of migrating protons along water molecules hydrogen bonded to the lysozyme surface. Values of the relaxation time are obtained from fits of the measured frequency response due to electrode polarization, interfacial dispersion, and hydrated powders of lysozyme. Hydration level of the sample is $h=0.28$ g water/g dry weight. The solid line through the data represents the fit with a VFT equation. The inverse of the attempt frequency (τ_{o_T}) was found to be $3.71\pm0.28\times10^{-9}$ s, the amplitude of the potential barrier (B_T) was found to be 454 ± 58 K, and T_o was found to be 210.0 ± 2.3 K.

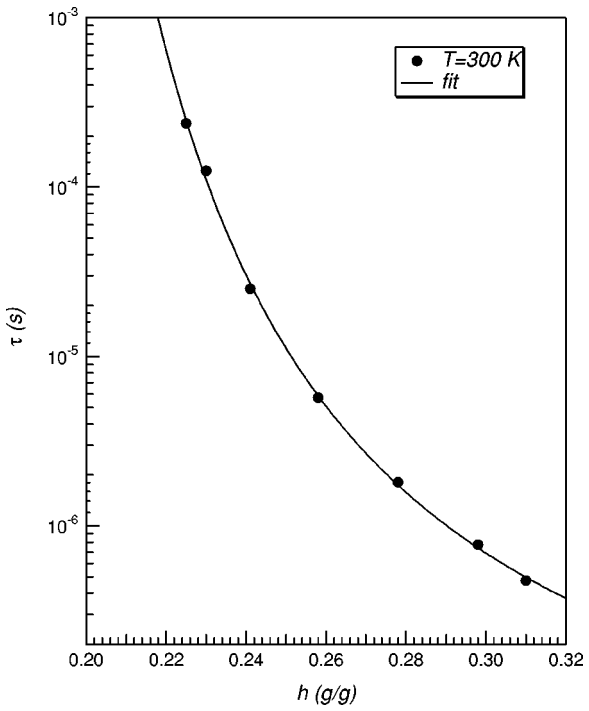

FIG. 4. Hydration dependence of the dielectric relaxation time $\tau(h)$ at constant temperature ($T=300$ K). The water content of each lysozyme sample was adjusted by exposing it over saturated salt solutions producing different water vapor pressures. The solid line through the data represents the fit with a modified VFT equation in which the independent variable temperature has been replaced with hydration. Fitted values of the parameters appearing in the fitting function were found to be equal to $5.7\pm0.6\times10^{-9}$ s, 0.65 ± 0.03 g water/g dry weight, and 0.16 ± 0.01 g water/g dry weight for the inverse of the attempt frequency (τ_{o_h}), the amplitude of the energy barrier (B_h), and the ''freezing'' hydration (h_o), respectively. Note that h_o coincides with the percolation threshold h_c triggering the onset of lysozyme catalytic activity at room temperature.

function of temperature at constant hydration $h=0.28$ g/g. The temperature dependence of the relaxation time is clearly non-Arrhenius [$\log\tau(T)\sim1/T$], and it can be successfully described by a Vogel-Fulcher-Tamman (VFT) relation

$$\tau(T)=\tau_{o_T}\exp\left(\frac{B_T}{T-T_o}\right), \tag{3}$$

as shown by the solid line, fitting the relaxation time data from 10^3 to 10^{-6} s, thus confirming and extending the overall conclusions reached by Refs. [20,21]. The three parameters, τ_{o_T},B_T, and T_o, appearing in the VFT equation are, respectively, the inverse of the attempt frequency (τ_{o_T}) of the proton transfer process across the energy barrier (B_T), and the so-called ideal glass transition temperature (T_o) indicating a divergence of the relaxation time τ. To study the hydration dependence of the migrating protons relaxation time, we performed the same kind of experiments described above with powdered samples of lysozyme at different water contents in the range $0.22\leqslant h\leqslant0.32$ g/g. It should be noted that at all water contents investigated, the protein is enzymatically active [15] with hydration levels larger than the percolation threshold $h_c=0.16$ g/g. The set of relaxation time values, obtained from the complex function fit applied to all samples, at constant temperature $T=300$ K is shown in Fig. 4. Reassuringly, the relaxation time moves to longer times with decreasing water contents, in agreement with published data [18]. The limited range of relaxation times shown in Fig. 4 is due to the fact that at hydration lower than the percolation threshold, i.e., 0.16 g/g, the dielectric relaxation time is simply unmeasurable [16], and above $h=0.33$ g/g instead of a hydrated protein powder one has a protein solution along with unwanted complications such as macromolecular motions. The limited range of relaxation times is thus linked to the limited hydration range; however, it should be noted that such a small range of feasible water contents yields a three-order of magnitude change of the relaxation time (Fig. 4), similar to the four-order of magnitude variation produced by the temperature going from 240 to 340 K (Fig. 3). At this stage we have reasonably assumed that hydration, instead of temperature, should be taken as the natural variable triggering catalytic processes as the number of water molecules available for binding to a single protein in intracellular solution can be varied by the presence of several natu-

ral solutes [28,29]. Therefore we have rewritten the VFT equation substituting the variable temperature (T) with the variable hydration (h), so that the hydration dependence of the migrating proton relaxation times can be expressed as

$$\tau(h) = \tau_{o_h} \exp\left(\frac{B_h}{h - h_o}\right). \quad (4)$$

The solid line fitting the data in Fig. 4 indeed represents the modified VFT relation. The possibility of such description of the measured relaxation time is clearly unexpected, and a number of issues should be discussed. First of all, the values of the inverse attempt frequency (τ_{o_T} and τ_{o_h}) are of the same order of magnitude (10^{-9} s), suggesting that the same dynamic process is responsible of the measured relaxation time, independently from the relation used to describe it. Moreover and most importantly, the fact that the singular hydration at which the relaxation time diverges is identical to the percolation threshold of the dc protonic conductivity of the same system at the same temperature is an unpredictable finding relating a well established property of the protein with its functional glassiness. This indicates that the dynamics of the migrating protons is blocked below a critical hydration threshold that coincides with the water content required to trigger lysozyme enzymatic activity. The divergence of $\tau(h)$ for h approaching h_c suggests the absence of long range connectivity between hydrogen bonded water molecules. At hydration below h_c, the dynamics of the system of charges over the protein surface becomes nonegodic, in analogy with the behavior of glasses below T_o. Proton transport along water chains is essential for the translocation of protons over large distances in proteins. However, this is a very fast process typically occurring in the picosecond time scale [30,31]: the glassiness of the migrating protons, resulting in a characteristic slowing down on the time scale, might have been envisaged to couple such a fast process with the much slower process governing enzymatic activity.

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[1] J.L. Green, J. Fan, and C.A. Angell, J. Phys. Chem. **98**, 13780 (1994).
[2] H. Frauenfelder and P.G. Wolynes, Phys. Today 58 (February 1994).
[3] H. Frauenfelder, Nature (London) **338**, 623 (1989).
[4] G.U. Nienhaus, J.D. Mller, B.H. MacMahon, and H. Frauenfelder, Physica D **107**, 297 (1997).
[5] I.E.T. Iben *et al.*, Phys. Rev. Lett. **62**, 1916 (1989).
[6] G.P. Singh, F. Parak, S. Hunklinger, and K. Dransfeld, Phys. Rev. Lett. **47**, 685 (1981).
[7] W. Doster, A. Bachleitner, R. Dunau, M. Hiebl, and E. Lüscher, Biophys. J. **50**, 213 (1986).
[8] W. Doster, S. Cusack, and W. Petry, Nature (London) **337**, 754 (1989).
[9] R.B. Gregory, in *Protein-Solvent Interactions* edited by R.B. Gregory (Marcel Dekker, New York, 1995), p. 191.
[10] C. Arcangeli, A.R. Bizzarri, and S. Cannistraro, Chem. Phys. Lett. **291**, 7 (1998).
[11] A.R. Bizzarri, A. Paciaroni, and S. Cannistraro, Phys. Rev. E **62**, 3991 (2000).
[12] M. Diehl, W. Doster, W. Petry, and H. Schober, Biophys. J. **73**, 2726 (1997).
[13] R.M. Daniel *et al.*, Biophys. J. **77**, 2184 (1999).
[14] H. Frauenfelder, S.G. Sligar, and P.G. Wolynes, Science **254**, 1598 (1991).
[15] G. Careri, E. Gratton, P.H. Yang, and J.A. Rupley, Nature (London) **284**, 572 (1980).
[16] J.A. Rupley and G. Careri, Adv. Protein Chem. **41**, 37 (1991).
[17] D. Stauffer and A. Aharony, *Introduction to Percolation Theory* (Taylor and Francis, London, 1992).
[18] R. Pethig, Annu. Rev. Phys. Chem. **43**, 177 (1992).
[19] G. Careri, Prog. Biophys. Mol. Biol. **70**, 223 (1998).
[20] A. Levstik *et al.*, Phys. Rev. E **60**, 7604 (1999).
[21] G. Careri, G. Consolini, and F. Bruni, Solid State Ionics **125**, 257 (1999).
[22] Y. Feldman, R. Nigmatullin, E. Polygalov, and J. Texter, Phys. Rev. E **58**, 7561 (1998).
[23] F. Pizzitutti and F. Bruni, Rev. Sci. Instrum. **72**, 2502 (2001).
[24] S.H. Liu, Phys. Rev. Lett. **55**, 529 (1985).
[25] R. Pethig, *Dielectric and Electronic Properties of Biological Materials* (Wiley, Chirchester, 1979).
[26] A.K. Jonscher, *Dielectric Relaxation in Solids* (Chelsea Dielectric, London, 1983).
[27] *Impedance Spectroscopy*, edited by J.R. Macdonald (Wiley, New York, 1987).
[28] M.F. Colombo, D.C. Rau, and V.A. Parsegian, Science **256**, 655 (1992).
[29] D. Bulone, P.L. San Biagio, M.B. Palma-Vittorelli, and M.U. Palma, Science **259**, 1335 (1993).
[30] K. Drukker, S.W. de Leeuw, and S. Hammes-Schiffer, J. Chem. Phys. **108**, 6799 (1998).
[31] Z. Smedarchina, W. Slebrand, and A. Fernández-Ramos, J. Chem. Phys. **112**, 566 (2000).
[32] G. Careri, M. Geraci, A. Giansanti, and J.A. Rupley, Proc. Natl. Acad. Sci. U.S.A. **82**, 5342 (1985).