BRIEF COMMUNICATION

THERMAL-STIMULATED PRESSURE AND CURRENT STUDIES OF BOUND WATER IN LYSOZYME

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ABSTRACT The state of bound water in crystalized lysozyme was studied by four techniques: electret thermal depolarization currents, thermal-stimulated pressure, isothermal polarization decay, and thermogravimetry. Hydration levels ranged from 0 to 40 mg water/g protein. Desorption of bound water dipoles was found to be the main process responsible for electrical depolarization. Two different binding sites for water were identified with long relaxation times at room temperature (order 10^2 s) and activation energies of 0.34 ± 0.02 eV and 0.55 ± 0.04 eV.

INTRODUCTION

The interaction of water with biological molecules plays an important role for determining their structure and biological activity (1, 2). This role, however, is still not understood. In the past, several techniques such as dielectric relaxation, nuclear magnetic resonance, (NMR), and others (3–5) have been used to study the problem. More recently it has been shown that bound water in proteins and other biopolymers may store large amounts of electrical polarization via the electret state (6, 7). The importance of water in relation to electrical properties in proteins and particularly in enzymes has been discussed recently (8, 9). The electric properties can be investigated by thermal-stimulated depolarization (TSD). Its advantage for studying bound water is that: (*a*) it can detect processes with very long relaxation times (10^{-1}–10^3 s) previously inaccessible by other techniques (10, 11), and (*b*) it is highly sensitive, able to detect effects due to bound water at hydration levels as low as 10 mg water/g of protein. In this communication we report observations on bound water in lysozyme using four different techniques: TSD; thermal-stimulated pressure (TSP); isothermal polarization decay (IPD); and thermogravimetric analysis (TGA). Through the analysis of

This work was part of a Ma.Sci. Thesis presented by S.C. to the University of São Paulo at São Carlos (June, 1977).

our experimental results, further understanding of the interaction of water with lysozyme was obtained, such as activation energies and relaxation times for the depolarization and water desorption and number of binding sites for water. We have also shown that the desorption of oriented bound water dipoles is the main mechanism for electrical depolarization. Lysozyme was chosen because previous dielectric experiments have shown it to be fairly resistant to thermal denaturation (4).

METHODS

Samples were pressed pellets of crystalized lysozyme (Sigma Chemical Co., St. Louis, Mo., chicken egg-white lysozyme, three times recrystalized, dialyzed, and lyophilized). Dimensions were 1 mm in thickness and 1 cm^2 in area. For the electrical measurements metal electrodes were applied to the sample, placed in a metal cryostat described elsewhere (12, 13). Temperature of the sample could be varied and recorded continuously with thermocouples from −200°C to 90°C. Hydration levels ranged from 0 to 40 mg water/g of protein and were measured by weighing.

TSD

Polarization fields of 5 kV/cm were used to induce the electret state, with polarization times of about 5 min at room temperature (RT). After the sample was cooled to liquid nitrogen temperature in the vacuum cryostat, the field was switched off and the sample was warmed at a constant rate (0.10°K/s). The depolarization current was measured with a Keithley 602C electrometer as a function of temperature (Keithley Instruments, Inc., Cleveland, Ohio).

TSP

During warm-up of the sample, desorption occurs. This was monitored by the water vapor pressure in a fixed volume as a function of temperature. We have introduced TSP as a new technique to study structural bound water in biopolymers.

The hydrated sample was cooled to liquid nitrogen temperature, the container was initially evacuated, and the temperature was increased at a linear rate. The pressure was continually monitored and recorded as a function of temperature. A second curve was obtained with the dry sample to correct for any changes in baseline. The maximum pressures attained for our experimental conditions are low enough so that the ideal gas approximation could be used to calculate the number of molecules desorbed.

For the TSP experiments walls of the sample container were heated so that no water would be adsorbed on them. Experimental details are given elsewhere (12).

IPD

The depolarization decay of the electret at a fixed temperature as a function of time was measured in short-circuit condition after charging. As shown by B. Gross (14), this technique is useful to complement TSD and gives information on different relaxation process.

TGA

A thermogravimetric micro balance was used (Rigaku-Denki, Japan) to measure changes in weight of sample during warm-up as a function of temperature.

RESULTS AND DISCUSSION

Results from TSD and TSP are shown in Figs. 1 and 2 as a function of hydration, h (continuous lines). The data clearly indicate that the desorption process (TSP) and

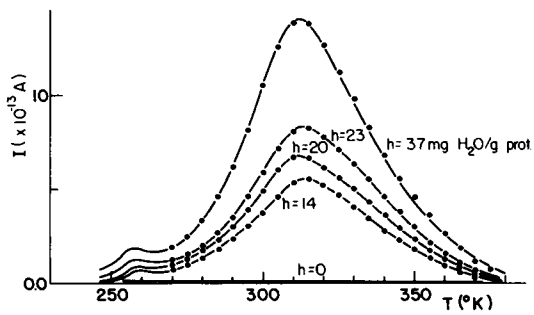

FIGURE 1

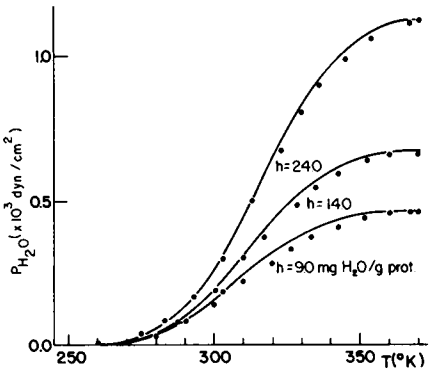

FIGURE 2

FIGURE 1 TSD curves for lysozyme (smooth curve) for several hydrations (field 5 kV/cm). Observe absence of effect for dehydrated sample. Circles are calculated points from Eq. 2 for two relaxation sites. Small peak at low temperature is due to ice formation on electrodes.
FIGURE 2 TSP curves for lysozyme (smooth curves) for several hydrations. Circles are points calculated by the integral (Eq. 1) of TSD curves.

the electrical depolarization (TSD) occur in the same temperature range. TSD curves do not conform to first-order monomolecular kinetics usually observed for depolarization of rotating dipoles (15). However, a linear dependence between total polarization stored and field was found experimentally. The same was found for polarization as a function of hydration. This indicated the possibility of several independent relaxations of a dipolar nature. This was confirmed by our IPD experiments (Fig. 3) at RT, in which two relaxation processes are apparent, characterized by long relaxation times (10^2 s), indicating strong binding sites for water dipoles. With these experimental results, we proceed to interpret TSP and TSD spectra. The TSD could be interpreted in principle as due to two different mechanisms: rotation and thermal desorption of previously oriented water dipoles. From our data we concluded that the main mechanism for electrical depolarization is desorption. This can be seen by plot-

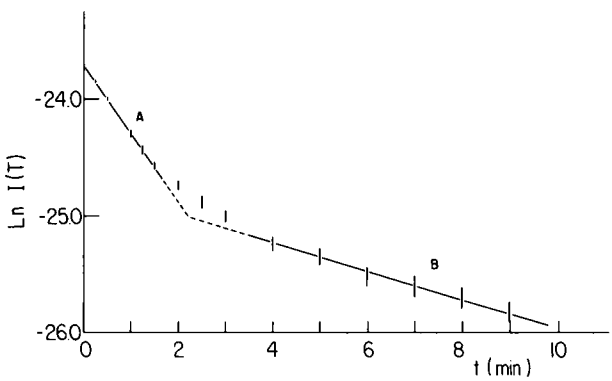

FIGURE 3 IPD curves for lysozyme, showing presence of two relaxation sites.

ting the integral from TSD curves $\int_{T_0}^{T} I(T')\,dT'$, where $I(T')$ is the depolarization current and T_0 is the initial temperature of the depolarization process) as a function of temperature T. The open circles in Fig. 2 indicate that practically all depolarization is due to the desorption. From Fig. 3, since two binding sites have been shown to be present, the total current in TSD can be written as a sum of two first-order monomolecular processes.

$$I_j(T) = \frac{Q_{oj}}{\tau_{oj}} \exp\left(-\frac{\Delta F_j}{kT} - \frac{1}{\tau_{oj} b}\int_{T_0}^{T} e^{-\Delta F_j/kT'}\,dT'\right), \tag{1}$$

$$I(T) = \sum_{j=1}^{2} I_j(T), \tag{2}$$

where Q_{oj} = stored charge, τ_{oj} = characteristic time, b = heating rate, ΔF_j = activation energy, and k = Boltzmann's constant.

Eq. 2 fits TSD experimental data very well (circles in Fig. 1). Relaxation times and activation energies obtained from TSD and TSP agree with values from a similar analysis of TGA data (not shown). The relaxation times also agree with those observed at RT by IPD.

CONCLUSIONS

(*a*) Water dipoles in lysozyme at low hydration levels are bound in two different sites characterized by relaxation times and activation energies. (*b*) Bound water dipoles are responsible for practically all the stored electrical depolarization in the electret state. (*c*) The electrical depolarization proceeds via the thermal desorption process, as indicated by the correlation between TSP and TSD curves. In view of the long relaxation times and values of the activation energies, it is suggested that water at these hydrations is directly bound to hydrophilic residues by two different degrees of hydrogen bonding. Finally, the linear dependence of stored polarization on electric field indicates that the mechanism of electrical polarization is rotational dipole orientation following a Langevin model. This is in contrast to preferential adsorption of oriented dipoles as found by Onsager, et al. (16) for amorphous ice. (*d*) The number of water dipoles as obtained from TSD equations agrees with the measured hydration as well as with the pressure data. (*e*) The stored polarization cannot be ascribed to Maxwell-Wagner polarization because it was found to depend linearly on hydration. Conductivity and dielectric constants are known *not* to vary linearly with hydration. Since interfacial polarization (Maxwell-Wagner polarization) should depend drastically on conductivity and dielectric constants, it can be ruled out here. The TSD cannot be due to thermoelectric effects since TSD is absent with nonpolarized samples, and also the depolarization current is irreversible during heating; that is, if at one point of the TSD the sample is cooled and then reheated (cleaning experiment), there is no current for the temperature interval where previous heating occurred. Also the TSD spectrum was found to be independent of the nature of metal electrode, ruling out electrode effects. In view of the linearity of the dependence of the stored polarization with field, space charge effects were considered to be absent.

As a closing comment we would like to add that the term "bound water" requires careful qualification and we suggest the following general description: (*a*) strongly bound water at low hydrations, that is below levels required to cover all hydrophilic sites, as in our present case. These have very long relaxation times and cannot be observed with usual AC dielectric methods. They do not appear in NMR spectra because of line broadening due to long correlation times. They may be observed by TSD or TSP and in favorable cases by different Fourier X-ray diffraction techniques; (*b*) first or subsequent water layers bound in a more complex way to the biopolymer and probably responsible for observed AC higher frequency dielectric absorption and NMR spectra; and (*c*) liquid water phase nearest to the biopolymer. Our description could also be phrased as a frozen gas bound water molecule, some degree of order or structuring in multilayers, and liquid phase.

We thank Drs. G. F. Leal Ferreira, B. Gross, and D. Channin for important discussions of our work.

This work was supported by FAPESP, Conselho Nacional de Pesquisas-National Science Foundation (Harvard-S. Carlos program), and FINEP.

Received for publication 12 July 1977 and in revised form 3 August 1977.

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