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Adiabatic compressibility of globular proteins

(sound velocity / protein structure / volume fluctuations)

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ABSTRACT The adiabatic compressibility of several globular proteins has been measured by using an ultrasonic technique in the frequency range 0.5 to 10 MHz. The contributions to the measured compressibility from the protein matrix and from surface processes involving ionization of side chains and solvation effects are discussed. The internal protein compressibility is very low, indicating the existence of "dynamic domains" which are tentatively assigned to secondary structure elements.

During the past few years, considerable interest in the problem of fast conformational fluctuations in protein systems has developed (1–3). Many techniques have been used to study fluctuations in biomolecules. Generally they involve the study of relaxational behavior after a perturbation is applied to the system (4). The external perturbation can take the form of a step or a continuous sinusoidal function. We have studied the behavior of protein systems under continuous-wave pressure perturbation. A pressure wave in a fluid causes alternating compressions and rarefactions. Because the period is short compared with the time required for thermal equilibrium of the solution, the process is reversible and adiabatic. By using pressure waves, one can extract information about mechanical and thermal properties of the system. The frequency dependence of these properties arises from relaxational processes, yielding valuable information on the time constants of the different mechanisms involved.

The theory of pressure wave propagation in solutions can be found in standard texts (for instance, see ref. 5). The adiabatic compressibility is defined by \( \beta_{ad} = -1/V (\partial V/\partial P) \), where \( V \) is the volume of the sample and \( P \) is the pressure. \( \beta_{ad} \) can be obtained by a measurement of the density \( \rho \) and sound velocity \( c \). \( \beta_{ad} \) = \( 1/\rho c^2 \). For a solution containing protein, the protein adiabatic compressibility may be determined by measuring the density and sound velocity in the solution as a function of protein concentration.

Adiabatic compressibilities of protein solutions have been reported (6–11). Quite recently this method has been applied to the study of the compressibility of cytochrome c in the oxidized and reduced forms (12). The reported values for apparent protein compressibility are generally low, on the order of 10–20% of the compressibility of water which is a relatively incompressible liquid. This low value is generally explained by the observation that the constituent amino acids have a negative apparent compressibility. The apparent positive value observed for globular proteins then reflects a very compressible protein interior (8).

In order to explain the increase in compressibility upon denaturation, which seems to contradict the hypothesis of a high compressibility of the protein interior, a very high local concentration of nonpolar groups is assumed for a denatured protein. Sarvayyan and Hemmes (6) discussed the possible contribution of relaxations to the compressibility on the basis of sound absorption data. However, no direct measurements of compressibility of a protein as a function of the frequency have been available.

METHODS

Sound Velocity Measurements. The sound velocity in the solution is measured with an acoustic resonator similar to that described by Eggers and Funk (13). It consists of two quartz transducers 2.5 cm in diameter whose fundamental frequencies are 10 MHz; they are placed facing each other at a distance of about 1 cm. The acoustic resonator is tuned by using a spectrum analyzer. A standing wave is produced in the tank if the length of the resonator is a multiple of half a wavelength of the sound wave: \( L = n\lambda/2 \). The corresponding resonance frequency is \( f_{res} = n c/2L \), \( c \) being the velocity of sound. If \( n \) and \( L \) are known, measurement of \( f_{res} \) gives \( c \). The accuracy in the determination of \( f_{res} \) depends on the Q of the resonator. The Q is a very complex function. At frequencies <1.5 MHz it is determined mainly by the mechanical construction of the tank; at higher frequencies it depends on the sound absorption in the solution (13, 14).

Use of the acoustic resonator allows the measurement of the sound absorption coefficient. However, in the experiments described in this work, only sound velocity was measured.

A typical resonance peak is shown for distilled water in Fig. 1. For a 1% protein solution the position of the peak is shifted by a factor of 2–3 \( \times 10^{-3} \), and the width of the peak increases by a factor of \( \approx 3 \) over pure water. The exact value of the resonance frequency is measured by using an "autoresonator" technique (Fig. 2). The electronic system will be described elsewhere.

The resonance frequency was measured with an accuracy of 1.0 Hz over the entire range. The stability of the resonance depends primarily on temperature. In practice, two identical tanks were used and only differences in sound velocity were measured. The system was accurately thermostated. During a measurement the absolute temperature variations were \( <0.03 \) K, and the differential temperature changes between the two identical resonators were \( <10^{-3} \) K. With all factors taken into account, the accuracy in a relative sound velocity measurement was \( \approx 3 \times 10^{-6} \).

Density Measurements. A vibrating densimeter similar to that described by Kraty et al. (15) was used. In our system there are two identical vibrating capillaries to compensate for temperature changes. The overall accuracy in the determination of the relative density of the solution was approximately \( 5 \times 10^{-6} \). Before each run, an absolute calibration was performed with distilled water and a salt solution of known density.

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where $N_i/V_T$ is the molar concentration and $v_i$ is the fractional volume of the $i$th species. The adiabatic compressibility is then

$$
\beta_{ad} = -\frac{1}{V_T} \left( \frac{\partial V_T}{\partial P} \right)_s = -\frac{1}{V_T} \sum_i N_i \left( \frac{\partial V_i}{\partial P} \right)_s + V_T \left( \frac{\partial N_i}{\partial P} \right)_s
$$

$$
= \sum_i \left( v_i \beta_i - \frac{v_i}{V_T} \frac{\partial V_i}{\partial P} \right)_s. \tag{1}
$$

$\beta_i$ is the intrinsic adiabatic compressibility of the $i$th species. The first term of Eq. 1 states that the total compressibility of a solution is the volume average of the compressibility of the components. The second term is characteristic of the system under consideration and depends on chemical relaxation processes. In particular, if one existing equilibrium is pressure dependent—i.e., $\Delta V \neq 0$ in the corresponding free energy term—it contributes to the solution adiabatic compressibility.

Using the theory of chemical relaxation, one can obtain expressions for isomerizations, proton transfer, solvation, coupled equilibrium reactions, etc.

The relaxational term is frequency dependent, the characteristic frequency being determined by the rate constant of the reaction. In general, the adiabatic compressibility takes the form

$$
\beta_{ad} = \sum_i \left[ v_i \beta_i + \frac{\Delta V_i^2}{RT} \Gamma_i \frac{1}{1 + \omega^2 \tau_i^2} \right]
$$

where $\Delta V_i$ is the volume change associated with a particular chemical reaction, $\Gamma_i$ is a factor characteristic of the chemical reaction, and $\tau_i$ is the relaxation time for the reaction (5). At neutral pH, ionization of side chains can be neglected and only solvation effects are considered (7, 17). The nonrelaxing part of Eq. 1 contains three unknown parameters: the intrinsic protein compressibility $\beta_p$, the compressibility of the bound water $\beta_{bw}$, and the fractional volume of the bound water with respect to the protein $k = v_i/v$. Here, $v$ is the fractional volume of protein.

$$
\beta_{sol} = \beta_p + v[\beta_p - \beta_p(k + 1) + k\beta_{bw}]. \tag{2}
$$

This equation is identical to equation 13 of ref. 12. Some observations on the nature of this equation are useful in order to clarify the meaning of the quantities we are considering. (a) Eq. 1 expresses the solution compressibility in terms of the intrinsic compressibility of the components. In Eqs. 1 and 2, all compressibilities are positive. (b) The coefficient that multiplies $v$ in Eq. 2 can be negative or positive. Generally the coefficient is negative for proteins and electrolytes. This coefficient has an absolute minimum when two of the solution components—i.e., protein and bound water—are incompressible. In this case $\beta_{sol} = \beta_p - v \beta_p (k + 1)$. This value never can be reached. (c) Small molecules, typically electrolytes, are incompressible. Eq. 2 then can be used to determine $k$, the amount of water electrostricted around the electrolyte. (d) If the change in the solution compressibility due to the addition of a volume $v$ of protein is attributed entirely to the protein, neglecting solvation effects (i.e., $k = 0$ in Eq. 2), then an apparent value for the protein compressibility is obtained. The relationship between the apparent and intrinsic values is

$$
\beta_{app} = \beta_p - k(\beta_{bw} - \beta_{sol}).
$$

If $k(\beta_{bw} - \beta_{sol}) > \beta_p$, then $\beta_{app}$ can be negative. A negative value of $\beta_{app}$ has no particular significance other than an indication of solvation contributions to the observed apparent compressibility.

The experimental results of $\beta_{sol} = \frac{1}{\rho_{sol}v_{sol}^2}$ as a function of

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**MATERIALS**

Solutions of sperm whale myoglobin (Sigma) in water were centrifuged and passed through a 1.2-μm Millipore filter. The pH was varied from 6.7 to 13. Salt-free lysozyme ( Worthington) was dissolved in 35 mM citrate/phosphate buffer and passed through a Sephadex G-75 column. The pH was varied from 3.5 to 5.9. Human hemoglobin was prepared by B. Alpert as described (16). The concentration of the solutions was determined by a dry weight method. The reproducibility was ±0.3 mg or 1% of the protein weight.

**RESULTS**

Compressibility is a volume property of the system. The total volume of the solution is given by

$$
V_T = \sum_i N_iV_i = V_T \sum_i v_i
$$

**Fig. 1.** Typical resonance peak for water at 1,209,400 Hz.

**Fig. 2.** Schematic of the sound velocity setup. T, acoustic resonator; A1, wideband amplifier, Tektronix type 1121; A2, filter amplifier, PAR type 113 preamplifier; A3, wideband driver amplifier; M1, high-frequency mixer, Minicircuits ZAD-1; M2, low-frequency mixer, Minicircuits ZAD-8. The frequency synthesizer is a Hewlett-Packard model 3325A.
v for myoglobin and lysozyme are reported in Fig. 3. Density and sound velocity in the solution are directly measurable quantities. The volume fraction v is the fractional weight of the protein times the ratio of the density of the protein to the density of the solution. From these data we can extract an upper limit for the intrinsic protein compressibility by estimating an upper limit for the contribution of the bound water to the total solution compressibility. The bound water is clearly nonhomogeneous. In a rough approximation we can distinguish three different kinds: (a) water bound to charged groups, (b) water bound to the amide backbone, and (c) water organized around water already bound and around other protein groups (18—20). Only bound water with different compressibility than bulk water needs to be distinguished from bulk water in Eq. 2. We assume that the electrostricted water bound to charged groups makes the dominant contribution. The number of charged groups varies from protein to protein and is a function of pH. For lysozyme at neutral pH we can estimate (18) an upper limit for the water associated with charged groups to be k = 0.10. This value is obtained by considering the total number of charged groups and assuming that 30 ml of water is associated with each mole of charged groups (21). The compressibility of the electrostricted water is probably very low (21—23). Neglecting the term $k\beta_{tw}$ in Eq. 2 and setting $k = 0.1$ will give an upper limit for the intrinsic protein compressibility $\beta_p$.

The values obtained for different proteins are listed in Table 1. The intrinsic protein compressibility is small but not negligible. An additional correction to the compressibility derives from the relaxation contribution due to proton transfer equilibrium at the ionizable side chains (6, 24—35). The correction deriving from this relaxation can be calculated from sound absorption measurements by using the expression $\Delta c_{rel}/c = \alpha k/(2\pi n\nu)$, where $\alpha$ is the ultrasonic absorption coefficient for the reaction, $\tau$ is the characteristic relaxation time, $2\pi n\nu$ is the frequency, and $\lambda$ is the wavelength of the sound. At neutral pH the correction is very small (5, 17). Furthermore, our measurements of compressibility as a function of frequency show no evidence for relaxation at neutral pH. Comparison of our experimental results with recent data on apparent compressibility of globular proteins (7, 8, 11) shows small discrepancies. Whereas the values of apparent compressibility for lysozyme and myoglobin are quite close to those reported by Sarvazyan and Hemmes (6), our value for hemoglobin is definitely smaller than the values reported in refs. 8 and 9. The errors attributed to compressibility values in Table 1 reflect the statistical distribution of our measurements for a given protein preparation. Slightly larger systematic errors have been observed when different protein preparations are used. The origin of this variation from preparation to preparation is unknown.

The changes in compressibility of myoglobin as a function of pH are particularly interesting. The apparent compressibility increases from the value at neutral pH up to about pH 12. Then a sharp decrease is observed. At 25°C, myoglobin undergoes reversible denaturation above pH 13 (36). Our measurements on compressibility are similar to the data on sound velocity reported by Sarvazyan and Hemmes (6). The increase in compressibility as the pH is raised can be understood in part by the neutralization of the basic protein side chains. However, an estimation of the amount of electrostricted water released and of the relaxational contribution at high pH shows that some other processes are taking place. The intrinsic protein compressibility $\beta_p$ also increases and then falls sharply after the denaturation is achieved. This result is in contrast with earliest observations on the isothermal compressibility of denatured proteins (37—39). It seems unlikely that this decrease in apparent compressibility can be explained solely by changes in solution upon denaturation. Instead, we believe that the decrease reflects a contribution from the internal protein structure.

![Graph](image)

**Fig. 3.** Solution compressibility relative to water as a function of volume fraction v. x. Lysozyme in 10 mM KP; at 22°C, pH 7.0; o, myoglobin in water at 25°C, pH 6.7.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Solvent</th>
<th>pH</th>
<th>Temp., °C</th>
<th>$1/\rho_i$, g/cm$^3$</th>
<th>$\beta_p/\rho_i$ vs. $\rho_i$</th>
<th>$k_{max}$ *</th>
<th>$(\beta_p/\rho_i)<em>{k</em>{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>50 mM KP</td>
<td>2.5—3.5</td>
<td>25</td>
<td>1.38 ± 0.01</td>
<td>0.152 ± 0.017</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>35 mM citrate/P</td>
<td>3.5—5.9</td>
<td>26.7</td>
<td>1.38</td>
<td>0.166 ± 0.013</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>100 mM KP</td>
<td>6.5—7.0</td>
<td>22</td>
<td>1.38</td>
<td>0.122 ± 0.027</td>
<td>0.10</td>
<td>0.22</td>
</tr>
<tr>
<td>metMb</td>
<td>H$_2$O</td>
<td>6.7</td>
<td>25</td>
<td>1.37</td>
<td>0.133 ± 0.017</td>
<td>0.13</td>
<td>0.26</td>
</tr>
<tr>
<td>metMb</td>
<td>H$_2$O</td>
<td>12.3</td>
<td>25</td>
<td>1.33</td>
<td>0.306 ± 0.030</td>
<td>0.05</td>
<td>0.36</td>
</tr>
<tr>
<td>metMb</td>
<td>H$_2$O</td>
<td>13.5</td>
<td>25</td>
<td>1.35</td>
<td>0.079 ± 0.030</td>
<td>0.04</td>
<td>0.12</td>
</tr>
<tr>
<td>Hb</td>
<td>(oxy &amp; deoxy)</td>
<td>100 mM KP</td>
<td>7.2</td>
<td>1.34</td>
<td>0.169 ± 0.030</td>
<td>0.09</td>
<td>0.26</td>
</tr>
<tr>
<td>apoMb</td>
<td>50 mM KP</td>
<td>7.2</td>
<td>22</td>
<td>1.37</td>
<td>0.052 ± 0.024</td>
<td>0.13</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Measurements at 9 MHz.
† $\rho_i$ is partial specific volume of protein.
‡ Apparent compressibility $\beta_p = 4.55 \times 10^{-12}$ m$^2$/N. Shown ± SD.
§ The maximal value of $k$ has been calculated by counting the number of charged groups per protein and assuming that 30 ml is the volume of electrostricted water per mole of charged groups.
¶ Upper limit for intrinsic compressibility.

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*Biophysics: Cavish et al.*

DISCUSSION

We have shown that the intrinsic protein compressibility is very low. Comparison with compressibilities of known substances can provide some information about the nature of the protein interior. Fig. 4 shows schematically the values of compressibility for associated and nonassociated liquids, polymers, glass, and metals (40). Compressibilities of globular proteins are lower than those of liquids and solid polymers but larger than those of metals and covalent solids. We believe that this fact reflects the particular nature of the protein interior: a covalently bonded structure along the polypeptide backbone and a weakly bonded structure in the perpendicular directions (41).

A large body of experimental data suggests that proteins exhibit relatively large conformational fluctuations on a time scale of 1 psec to 1 μsec (1, 3). Temperature x-ray factors (42, 43) indicate large amplitude fluctuations in the position of many atoms in some proteins. The observed range of amplitude points toward a relatively fluid protein interior. Experiments on oxygen quenching of the tryptophan fluorescence in many proteins show that diffusion of the gas through the protein is very rapid. The diffusion rate is less than that in liquid water only by a factor of 0.2 to 0.5 (44). This kind of internal dynamics is also shown by many other experimental techniques including NMR, hydrogen/deuterium exchange, fluorescence polarization, and dielectric constant (2).

If the local motions observed were independent, a large total volume fluctuation would be found. Our compressibility values indicate a very compact, solid-like interior. A compressibility experiment measures essentially the fluctuation in volume associated with dynamic processes taking place in the protein system (6, 12, 45). These dynamic processes can be conformational fluctuations or chemical processes at the protein surface. The latter are typically ionization and solvation of side chains, which have been extensively characterized (24–35). By using Eq. 1 it is possible to show that, at neutral pH, ionization effects are not very large. Volume changes associated with conformational transitions have been studied statically in high-pressure experiments (37–39, 46). Generally, conformational transitions are associated with volume changes on the order of 50–100 ml/mole. Under our experimental conditions, large conformational transitions are excluded, with the exception of myoglobin at high pH. Furthermore, these transitions occur at low frequency (0.1–100 Hz) and will not give a relaxation contribution at the frequency of our measurements. In order to resolve the apparent paradox of liquid-like high-amplitude motions such as those described above and the low intrinsic compressibility found, we assume that the interior of the protein is made up of solid-like domains. From a dynamic point of view, a domain is a region in the protein of highly correlated motions.

Protein consists of a covalently bonded polypeptide chain stabilized in a well-defined tertiary structure by weaker interactions (hydrophobic, hydrogen bonds, etc.). It is known that covalent bonds and angles cannot be easily compressed or bent by pressure (47). Some of the basic polypeptide structures, such as α-helix, and β-sheet, are expected to have a relatively low compressibility compared to hydrophobic regions. We tentatively associate dynamic domains with the protein secondary structure. The nonvanishing value of the protein compressibility arises from the existence of and interaction between dynamic domains. At present, no correlation between intrinsic protein compressibility and secondary structure have been proven. Studies of compressibility by using ultracentrifugation methods suggest that a protein with a high fraction of β structure such as IgG has the lowest compressibility (48). A denatured protein such as myoglobin at pH 13 has a very low value of compressibility, corresponding to the fact that a nonstructured polypeptide chain is less compressible than a native protein.

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