Ultrasonic Study of the Compressibility of Globular Proteins

蛋白質壓縮性之超聲研究

by

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Abstract

Ultrasonic technique has been employed to study the adiabatic compressibility of different myoglobin derivatives (aquomet, fluoromet and azidomet myoglobin) at neutral pH, and aquomet myoglobin as a function of pH in 1 - 10 MHz frequency range at 20 °C. No difference was observed in the adiabatic compressibility of the various myoglobin derivatives. This indicates that the binding of different ligands to myoglobin does not lead to a difference in the structural flexibility of the protein. The finding is consistent with the result of the hydrogen exchange rates experiment and supports the idea that the two kinds of experimental techniques, the adiabatic compressibility and hydrogen exchange, are useful in the study of the protein dynamics. The adiabatic compressibility of myoglobin decreases by $5 \times 10^{-12}$ ($\text{cm}^2/\text{dyne}$) upon acid-induced denaturation. This trend is in contrast with the results of the isothermal compressibility measurements. The discrepancy may be explained by assuming that there exist low frequency ($< 1 \text{ MHz}$) conformational fluctuations or transitions in the denatured protein.
Chapter 1

Introduction

In recent years, there is considerable interest in the study of fast dynamic processes in protein molecules. The techniques employed usually involve the study of relaxation behaviour after a perturbation is applied to the system. Some examples of the perturbation techniques which have been extensively used are: the classical temperature jump method, the pressure jump method and the method of ultrasonic absorption. For reactions with relaxation times shorter than a few microseconds, difficulties arise with the slower T and P jump experiments and the ultrasonic techniques are thus preferable.

In an ultrasonic experiment, the perturbation applied is a continuous sinusoidal pressure wave in the MHz frequency range. In a protein solution, this wave causes alternating compressions and rarefactions. Since the period is short compared with the time required for the thermal equilibrium of the protein solution, the process is reversible and adiabatic. By measuring the decrease in the amplitude of the pressure wave after traversing the sample, the ultrasonic absorption of the protein solution can be determined. On the other hand, from the measurement on the change in the velocity of the sample as a function of protein concentration, the adiabatic compressibility, \( \beta_{ad} \), of the protein solution can also be deduced.
Since the early 1960's, it is known that protein solutions exhibit frequency dependent ultrasonic absorption in the MHz frequency range. The ultrasonic absorption consists of two contributions: pH-dependent and pH-independent. The pH-dependent component exhibits peak at pH 3 and 11.5, which have been attributed to proton-transfer reactions of protein side groups. However, the origin of the pH-independent absorption, which is present in all proteins, is still a matter of controversy. In a previous work, we have studied the excess ultrasonic absorption in several globular proteins. In the native state, the absorption of various proteins were found to be proportional to their compressibilities, which leads us to suggest that a large part of this absorption (i.e. pH-independent) is related to the conformational fluctuation of protein molecules.

Compared to absorption measurements, the study on ultrasonic velocity in a protein solution (i.e. adiabatic compressibility) has been relatively scarce. This may be partly due to technical difficulties, as very precise measurements of velocity have to be made. Compressibility is a volume property of the protein system. As the system is under pressure perturbation, the individual species, such as protein molecules, hydration water, solvent molecules, etc. undergo volume changes. Thus, for a composite system, the measured compressibility corresponds to that of the entire
system. If we can separate or estimate the contribution due to the other components from that of protein molecules, the compressibility of protein molecules can then be obtained. Since the compressibility can be related to the volume fluctuation, $\delta V_{\text{rms}}^6$, so a compressibility experiment measures essentially the volume fluctuation associated with various processes taking place in the protein system. These processes can be conformational fluctuation or chemical process at the protein surface such as ionization and solvation of side chains.

Some work on adiabatic compressibilities of protein solutions have been reported\(^7\)\(^-\)\(^9\). The reported values for the protein compressibility are generally around $10^{-12}$ cm\(^2\)/dyne, which is only 10 to 20 percent of that of water and approaches the value of soft metals. The low internal protein compressibility reflects a very compact, solid-like interior. On the other hand, experimental techniques such as relaxation and fluorescence quenching indicate that there are large conformational fluctuations inside the proteins, which suggests a relatively fluid protein interior. To compromise such apparently conflicting pictures, the existence of "dynamic solid domains" inside the proteins has been proposed by Gavish et al.\(^7\).

The compressibility of the native protein changes upon denaturation. In 1970, Brandts et al.\(^{10}\) found that upon denaturation, the isothermal compressibility (obtained by
measuring the volume change of the system as a function of applied static pressure) of Ribonuclease A is increased. This increase has been explained by the high local concentration of nonpolar groups of denatured protein. However, from measurement on the adiabatic compressibility, $\beta_{\text{ad.}}$, of metmyoglobin upon denaturation\textsuperscript{7}, the $\beta_{\text{ad.}}$ follows an opposite trend, i.e. it decrease upon denaturation. The reason on the difference observed in the two types of experiment is not known.

In another experiment, Eden et al.\textsuperscript{11} found that upon oxidation of cytochrome c, the apparent adiabatic compressibility of the protein increased significantly. It was suggested that the difference in compressibility reflects an increase in volume fluctuations of the oxidized state. All these experiments thus show that measurement on the compressibility of proteins is a sensitive means which can yield valuable information on the static as well as dynamic structure of the protein molecules.

In this work, we have measured the adiabatic compressibility of a well studied protein, myoglobin, under different conditions. The objective of the experiment is two fold:

1) **Ligand Effect.** The work of Eden et al.\textsuperscript{11} on cytochrome c has shown that the dynamic structure of a protein can be significantly altered upon a small perturbation, namely, changing the oxidation state of the iron atom. Thus, it would be interesting to know if the
binding of a different ligand in myoglobin would lead to similar effects. Existing information\textsuperscript{12,13} indicates that the nature of the ligand affects strongly the conformational stability of myoglobin molecules (reduced cytochrome c is also known to be much more stable than the oxidized protein\textsuperscript{14,15}), thus it is not unreasonable to expect that a difference in the conformational flexibility might exist for different myoglobin derivatives.

(2) Effect of Denaturation. As indicated above, there is at present a disagreement on how the protein compressibility changes upon denaturation as obtained by the two types of compressibility (isothermal and adiabatic) measurements. The only adiabatic measurement is on denaturation of myoglobin in the alkaline pH by Gavish et al.\textsuperscript{7}. In such a pH range, the measurement is complicated by the existence of relaxation contributions due to proton-transfer reactions of protein side groups. On the other hand, acid-induced denaturation of myoglobin occurs at a relatively high pH\textsuperscript{5,16} (pH \(\sim 4\)) where little proton-transfer relaxation contributions are present. Thus, we have determined in detail the compressibility of myoglobin at different stages of acid-induced denaturation as a model to study how the compressibility of protein changes upon unfolding. The results can provide useful information on the structure of a denatured protein.
[2.1] Adiabatic compressibility in a composite system

The adiabatic compressibility is defined as

$$\beta_{\text{ad.}} = -\frac{1}{V} \left( \frac{\partial V}{\partial P} \right)_S$$  \hspace{1cm} (2-1)

where $V$ is the volume, $P$ is the pressure and the subscript, $S$ (entropy), denotes the process is reversible and adiabatic.

For a composite system, the total volume of the solution is given by

$$V_T = \sum_i N_i v_i = V_T \sum_i \frac{N_i}{V_T} v_i = V_T \sum_i v_i$$  \hspace{1cm} (2-2)

where $N_i/V_T$ is the molar concentration and $v_i$ is the fractional volume of the $i$th species. Thus, the adiabatic compressibility is given by:

$$\beta_{\text{ad.}} = \frac{-1}{V_T} \left( \frac{\partial V_T}{\partial P} \right)_S$$

$$= -\frac{1}{V_T} \sum_i \left( N_i \left( \frac{\partial v_i}{\partial P} \right)_S + v_i \left( \frac{\partial N_i}{\partial P} \right)_S \right)$$

$$= \sum_i \left( v_i \beta_i \right) - \sum_i \left( \frac{V_i}{V_T} \frac{\partial N_i}{\partial P} \right)_S$$

$$= \beta_s + \beta_r$$  \hspace{1cm} (2-3)

where $\beta_i$ is the intrinsic adiabatic compressibility of the
ith species. The first term of Eq. (2-3), $\beta_s$, reflects that
the total compressibility of a solution is the volume average of
the compressibility of the components. The second term,
$\beta_r$, is characteristic of the system under consideration and
depends on chemical relaxation processes present.

Aqueous protein solution is a composite system. Because
of the relaxational contribution such as that of titration of
the side chains of the protein molecules, the adiabatic
compressibility in a protein system consists of two parts:
(1) Nonrelaxational and (2) Relaxational.

[2.1.1] Nonrelaxational contribution

Physically, the nonrelaxational contribution term, $\beta_s$,
represents the process with the relaxation rate $1/\tau_i$ much
greater than the frequency, $\omega$, in the experiment (i.e. $\sim$
MHz). For the protein solution, they include the volume
average of the compressibility of the protein $\beta_p$, the pure
solvent $\beta_o$, and the solvent of hydration $\beta_h$, thus

$$\beta_s = v_p \beta_p + v_h \beta_h + (1 - v_p - v_h) \beta_o \quad (2-4)$$

where $v_p$ and $v_h$ are the corresponding volume fractions of the
protein and the solvent of hydration respectively and

$$v_p = \gamma w_p \quad (2-5)$$

where $\gamma$ is the partial specific volume of protein, $w_p$ is the
weight fraction of protein. Let $k = v_h / v_p$, then Eq. (2-4)
becomes:

\[ \beta_s = v_p \beta_f + v_p k \beta_h + (1 - v_p - v_p k) \beta_o \]
\[ = \beta_o + v_p (\beta_f - (1 + k) \beta_o + k \beta_h) \]  \hspace{1cm} (2-6)

If the change in \( \beta_s \) due to the addition of a volume fraction \( v_p \) of the protein is attributed entirely to the protein (i.e. neglect solvation effect), then an apparent value for the protein compressibility is obtained.

\[ \beta_s = (1 - v_p) \beta_o + v_p \beta_p^a \]  \hspace{1cm} (2-7)

The apparent compressibility of protein is related to the intrinsic one by

\[ \beta_p^a = \beta_p - k(\beta_o - \beta_h) \]  \hspace{1cm} (2-8)

[2.1.2] Relaxational Contribution

The second term of Eq. (2-3), \( \beta_r \), represents the relaxational contribution due to the change of chemical reaction under pressure perturbation. To evaluate \( \beta_r \), we note that it consists of real and imaginary parts\(^{17} \):

\[ \beta_r = \sum \beta_r^i = \sum \beta_r^i \Re - j \sum \beta_r^i \Im \]
\[ = \sum \frac{\beta_{ch}}{1 + \omega^2 \tau_i^2} - j \sum \frac{\beta_{ch}}{1 + \omega^2 \tau_i^2} \]  \hspace{1cm} (2-9)
where $\tau_i$ is the characteristic relaxation time of the $i$th process, $\beta_i^{ch}$ is the chemical contribution to the compressibility, and is given by:

$$\beta_i^{ch} = \frac{(\Delta V_i^0 - \frac{\alpha_p}{\rho C_p} \Delta H_i^0)^2}{RT \prod_i} \quad (2-10)$$

where $R$ is the gas constant, $T$ is the absolute temperature, $\alpha_p$ is the thermal expansivity of the solution, $\rho$ is the density of the solution, $C_p$ is the specific heat, $\Delta V_i^0$ and $\Delta H_i^0$ are the change of standard state volume and enthalpy for the reaction $i$, and $\prod_i$ is a concentration function which depends on the stoichiometric coefficient and equilibrium concentration of the reactants.

The component of the imaginary part, $\beta_i^{im}$, can be related to the corresponding excess or relaxational absorption per wavelength by

$$(\Delta \chi_i)_{ch}^m = \pi \frac{\rho u^2}{\Lambda^2} \frac{\beta_i^{ch}}{\omega^2 \tau_i^2} \quad (2-11)$$

Eq. (2-11) is commonly expressed in another form,

$$\frac{\Delta \chi_i^{ch}}{f^2} = \frac{(\Delta \chi_i)_{ch}}{uf}$$

$$= \frac{2\pi^2 \rho u \tau_i \beta_i^{ch}}{1 + \omega^2 \tau_i^2}$$
Experimental determination of the adiabatic compressibility

The propagation of a longitudinal wave in a particular direction, e.g. \( x \), through a homogeneous medium of uniform density, \( \rho \), obeys the following equation \(^{18}\)

\[
\frac{u^2}{\frac{\partial^2 \phi}{\partial x^2}} = \frac{\partial^2 \phi}{\partial t^2}
\]

\( (2-13) \)

where \( \phi \) is the velocity potential and \( u \) is the velocity of sound in the medium which is given by

\[
u^2 = \left( \frac{\partial P}{\partial \rho} \right)_S
\]

\( (2-14) \)

where \( P \) is the pressure and the subscript, \( S \) (entropy), indicates the process is reversible and adiabatic. Multiplying both sides of Eq. (2-14) by \( \rho \), the expression becomes

\[
\rho u^2 = \rho \left( \frac{\partial P}{\partial \rho} \right)_S
\]
so that
\[ \frac{1}{\rho u^2} = \frac{1}{\rho} \left( \frac{\partial \rho}{\partial p} \right)_S \]  \hspace{1cm} (2-15)

Since \( \rho = m/V \), where \( m \) is the mass and \( V \) is the volume, we thus have
\[ \frac{1}{\rho} \left( \frac{\partial \rho}{\partial p} \right)_S = - \frac{1}{V} \left( \frac{\partial V}{\partial p} \right)_S \]  \hspace{1cm} (2-16)

Eq. (2-1), (2-15) and (2-16) give
\[ \frac{1}{\rho u^2} = - \frac{1}{V} \left( \frac{\partial V}{\partial p} \right)_S = \beta_{ad}. \]  \hspace{1cm} (2-17)

where by definition \( \beta_{ad}. \) is the adiabatic compressibility of the medium. Thus, experimentally, by measuring the velocity and density of the medium, the \( \beta_{ad}. \) can be determined. However, it is worth noting that only the real part of the \( \beta_{ad}. \) is measured in the experiment, i.e.
\[ \beta_{ad.} = \beta_{ad.}^{re} = \beta_S^{re} + \beta_k^{re} \]  \hspace{1cm} (2-18)

The reason is given as follows: By considering a plane wave propagating in a dispersive medium, the propagating vector \( \mathbf{k} \) consists of real and imaginary parts and can be written as
\[ \mathbf{k} = k^{re} - i\alpha \]  \hspace{1cm} (2-19)

where \( k^{re} \) and \( \alpha \) are real numbers.
The wave will attenuate with the distance of travel, \( x \), and is given by \( e^{-\left(\alpha + jk^{\text{re}}\right)x} \). In our resonance setup, the wave is propagating between two reflecting surfaces with separation \( L \), and the pressure at a distance \( x \) from one of the reflecting surface is

\[
P(x) = P_0 \left[ e^{-\left(\alpha + jk^{\text{re}}\right)x} + e^{-\left(\alpha + jk^{\text{re}}\right)(2L+x)} + e^{-\left(\alpha + jk^{\text{re}}\right)(4L+x)} + e^{-\left(\alpha + jk^{\text{re}}\right)(2L-x)} + e^{-\left(\alpha + jk^{\text{re}}\right)(4L-x)} + \ldots \right] \quad (2-20)
\]

where \( P_0 \) is the initial amplitude of the wave and we have assumed that the reflection coefficients of all reflecting surfaces are equal to one and there is no diffraction loss.

Summing the series, one obtains

\[
P(x) = P_0 \frac{e^{-\left(\alpha + jk^{\text{re}}\right)x} + e^{-\left(\alpha + jk^{\text{re}}\right)(2L-x)}}{1 - e^{-\left(\alpha + jk^{\text{re}}\right)2L}} \quad (2-21)
\]

and

\[
P(0) = P_0 \frac{1 + e^{-\left(\alpha + jk^{\text{re}}\right)2L}}{1 - e^{-\left(\alpha + jk^{\text{re}}\right)2L}} \quad (2-22)
\]

The maximum of \( P(0) \) occurs at the resonance condition

\[
2k^{\text{re}}L = 2n\pi
\]

i.e. \( k^{\text{re}} = \frac{n\pi}{L} \) \quad (2-23)
where \( n \) is a positive integer. Denoted \( u^{\text{re}} \) to be the velocity of sound in the medium due to the contribution only by the real part of the propagating vector, i.e. \( k^{\text{re}} \), we have

\[
 k^{\text{re}} = \omega / u^{\text{re}}
\]  
(2-24)

From Eq. (2-24), with \( \omega = 2\pi f \) where \( f \) is the resonance frequency, which is the quantity determined in the experiment, Eq. (2-23) can be expressed as

\[
f = \frac{n u^{\text{re}}}{2L}
\]  
(2-25)

The relationship between \( u^{\text{re}} \) (i.e. what has been measured) and \( \beta_{\text{ad}} \) (i.e. what is going to be determined) can be deduced by considering a damped plane wave

\[
\phi = \phi_0 e^{-\lambda x} e^{j(\omega t - k^{\text{re}} x)}
\]  
(2-26)

In this case, the quantity \( u^2 \) in Eq. (2-13) is a complex number and for a single relaxation process, from Eq. (2-3), (2-9) and (2-17), it is given by

\[
u^2 = 1/\left\{ \beta_S + \frac{\beta_{\text{ch}}}{1+\omega^2c^2} - j\frac{\beta_{\text{ch}}}{1+\omega^2c^2} \right\}
\]  
(2-27)

Substituted Eq. (2-26) into Eq. (2-13), the expression becomes

\[
u^2 (\lambda + jk^{\text{re}})^2 = -\omega^2
\]
\[ \frac{1}{u^2} = - \frac{(\alpha + jkr_{\text{re}})^2}{\omega^2} \]  \hspace{1cm} (2-28)

Combining Eq. (2-27) and (2-28) gives

\[ \omega^2 \phi \left( \beta_S + \frac{\beta_{\text{ch}}}{1 + \omega^2 \tau^2} - j \frac{\omega \tau \beta_{\text{ch}}}{1 + \omega^2 \tau^2} \right) \]

\[ = (kr_{\text{re}})^2 - \alpha^2 - j2\alpha kr_{\text{re}} \]  \hspace{1cm} (2-29)

Eq. (2-29) can be easily solved and gives

\[ (kr_{\text{re}})^2 = \frac{\omega^2 \phi (\beta_S + kr_{\text{re}})}{2} \left[ 1 + \frac{\omega^2 \tau^2 (kr_{\text{re}})^2}{\left( \beta_S + kr_{\text{re}} \right)^2} \right] \]  \hspace{1cm} (2-30)

where \( kr_{\text{re}} = \frac{\beta_{\text{ch}}}{1 + \omega^2 \tau^2} \) and has been defined in Eq. (2-8).

The negative solution is neglected since \( kr_{\text{re}} \) is real. For a medium with small absorption, \( \beta_S \gg \beta_{\text{ch}}, (kr_{\text{re}})^2 \) is equal to:

\[ (kr_{\text{re}})^2 = \frac{\omega^2 \phi (\beta_S + kr_{\text{re}})}{2} \left( 1 + 1 \right) \]

\[ = \omega^2 \phi \left( \beta_S + kr_{\text{re}} \right) \]  \hspace{1cm} (2-31)

By combining Eq. (2-24) and (2-31), it gives

\[ 14 \]
Thus, the measurement of $u^r e$ only gives the real part of $\beta_{\text{ad}}$ (i.e. $\beta_{\text{ad}}^r$). Finally, with Eq. (2-9) and (2-18), the adiabatic compressibility can be expressed as

$$\beta_{\text{ad.}} = \beta_S^r + \sum_i \frac{\beta_i^{ch}}{1 + \omega^2 \tau_i^2}$$

(2-33)

where $\beta_i^{ch}$ has been given in Eq. (2-10) with the expression

$$\beta_i^{ch} = \frac{1}{RT} \left[ \Delta v_i^0 - \frac{\chi_p}{\beta_{C_p}^0} \Delta H_i^0 \right]^2$$

[2.3] Volume fluctuation and the adiabatic compressibility

The isothermal compressibility $\beta_T$ is related to the volume fluctuation, $\delta V_{\text{rms}}$, of the system by

$$(\delta V_{\text{rms}})^2 = K T V \beta_T^2$$

(2-34)

where $K$ is the Boltzmann constant, $V$ is the volume of the protein. Thus, to find the corresponding volume fluctuation, we need to relate the isothermal compressibility with the adiabatic one by:
where $C_v$ is the specific heat capacity at constant volume.

From Eq. (2-8) and (2-35), the expression

$$\beta_T = \beta_p \frac{C_p}{C_v} = \beta_p + \left( \chi_p^2 \frac{T}{C_p} \right)$$

(2-35)

can be obtained.
Chapter 3
Experimental

[3.1] Sample Preparation

The protein samples including bovine serum albumin (BSA) and horse heart myoglobin (Mb) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and were used without further purification. The first part of the experiment concerns with the ligand effect. Aquomet, fluoromet, azidometmyoglobin derivatives were prepared by dissolving myoglobin into aqueous solution with respectively 0.1M of sodium chloride (NaCl), sodium fluoride (NaF) and sodium azide (NaN₃). The second part of experiment concerns with the effect of acid-induced denaturation. In this part of the experiment, myoglobin was dissolved into 0.1M of acetate buffer with pH ranging from 5 to 3.5. The pH value of the solution was measured by means of a pH meter to an accuracy of 0.05 pH unit.

In all experiments, the concentration of myoglobin were smaller than 1% by weight. The absolute concentration of myoglobin was determined by optical density measurement in the visible region using a Hitachi 220 double beam spectrophotometer.

All the protein solutions were out-gassed before being introduced into the ultrasonic cell. This is necessary because any air bubble trapped in the solution will scatter
the ultrasonic wave and cause artifacts in the measurement.

[3.2] **Ultrasonic wave velocity measurements**

[3.2.1] **Acoustic Resonator**

In the measurements of the sound velocity, an acoustic resonator (Fig. 3.1) similar to that described by Eggers et al. was used. Protein solutions were placed in between two parallel x-cut quartz transducers with a fundamental resonance frequency of 1 MHz and a diameter of 4 cm spaced about 1 cm apart. Ultrasonic wave was generated by the transmitting transducer driven by a Hewlett Packard model 3325A frequency synthesizer. The wave propagates between the two reflecting transducers and a resonant condition is set up when the frequency equals \( f = n u^r / 2L \) (see Eq. (2-25)). The electrical signal from the receiving transducer was amplified and then fed into a Fluke 8920A RMS voltmeter. The output of the voltmeter was monitored as a function of frequency on an oscilloscope and plotted using a Hewlett Packard 7045A x-y chart recorder (Fig. 3.2).

[3.2.2] **Calibration of the resonance cell**

The separation, \( L \), of the transducer is given by Eq. (2-25)

\[
\begin{align*}
  f &= n u^r / 2 L \\
  L &= n u^r / 2 f
\end{align*}
\]

First, the resonance frequency of distilled water in
Fig. (3.1) Cross section of the inner part of the "4cm" resonator cell with X-cut quartz transducer (Q₁, Q₂: diameter=4cm) mounted between silicone rubber o-rings S; A: alignment screw; C: clamp; E: ground contact plate; FH: filling hole; G: viton o-rings; I: insertion hole for the platinum resistance thermometer; LO: liquid outlet; T: teflon ring; V: evacuation hole.
Fig. (3.2) Schematic of the setup for measurement of sound velocity. R: resonator; F: frequency synthesizer; A: attenuator; W: wide band amplifier; V: AC RMS voltmeter; O: oscilloscope; P: X-Y plotter; T: temperature control unit (see Fig. (3.3)).
the resonance cell was measured. The sound velocity in distilled water at the experimental temperature (i.e. 20 °C) could be obtained from literature\textsuperscript{22}. From Eq. (3-1), the proportional constant

\begin{equation}
z = 2 \frac{L}{n} = \frac{u^\text{re}}{f}
\end{equation}

was determined. By keeping \( L \) fixed, protein solutions with weight fraction \( w_p < 1\% \) was introduced into the resonance cell. The change in the resonance frequency was so small that the integer \( n \) in Eq. (3-2) was unchanged. Thus, once the proportional constant, \( z \), was determined using distilled water, the sound velocity in the protein solution could be directly obtained from the resonance frequency measurement (i.e. by Eq. (3-2)).

\[3.2.3\] Temperature Control unit

The sound velocity measurement is strongly affected by small changes in temperature. To maintain a constant temperature, the acoustic resonantor was placed inside a double-wall stainless steel tank in which water was circulating between the walls. An Haake F3-C circulator was used to maintain the circulating water at 20 °C to within ± 0.02 °C. To minimize the effect due to the variation of the room temperature, the outside of the tank was also thermally insulated by a thick layer of polystyrene foams. Due to the large heat capacity of the resonator, the short term (a few minutes) temperature fluctuation of the resonant cell was found to be about 0.002 °C.
The temperature was measured by an Omega 50 $\Omega$ miniature platinum resistor which was inserted into the body of the resonator (refer to Fig. (3.1)). The resistance of the platinum resistor was monitored by a Leeds & Northrup model 8069-B high precision mueller temperature bridge (Fig. (3.3)) and a Keithley model 150B microvoltmeter was used as null detector. Since the bridge could detect changes in resistance down to 0.0001 $\Omega$, temperature changes as small as $5 \times 10^{-4}$ °C can be detected.

(3.2.4) Experimental procedures and performance of the system

(A) Protein solution at temperature slightly above 20 °C was introduced in the ultrasonic cell. By setting the temperature of the circulating water slightly below 20 °C, the temperature of the protein solution drifted very slowly across 20 °C. To ensure the protein solution in the cell was in good thermal equilibrium during the sound velocity measurement, the drift of the temperature at 20 °C was made to be less than $10^{-3}$ °C/min by fine tuning the temperature setting in the Haake water circulator. Usually, we measured three resonance frequencies at about 1.5MHz, 2.5MHz and 9.3MHz, corresponding to different values of $n$ as shown in Eq. (3-1). These measurements took about 2 minutes and the temperature drift during this period as recorded by the bridge is about $2 \times 10^{-3}$ °C.

To check the effect of the temperature drift in the
Fig. (3.3) Block diagram of the temperature control unit.

TC: temperature-control circulator;
IT: well insulated tubing; CW: circulating water;
IL: thermally insulated layer;
D: double-wall tank; R: resonator;
RS: resonator support;
PT: 3-lead platinum resistance thermometer;
B: Mueller temperature bridge; G: microvoltmeter.
protein solution on our result, we had performed the following experiments:

(a) With outgased distilled water introduced into the resonance cell, a series of ultrasonic resonance frequency measurements was taken with the sample temperature changing very slowly. The results were very reproducible and we found that a change in $2 \times 10^{-3}$ °C caused a frequency shift of about 6 Hz at 1.5 MHz (Fig. (3.4)).

(b) The outgased distilled water in the cell was replaced for many times. During each time the resonance frequency at 20 °C was measured. The greatest difference among the measurements was 8 Hz at 1.5 MHz and the mean deviation was only about 6 Hz.

We had compared the result in (a) with those of Eggers et al. for aqueous solutions at room temperature. According to Eggers, the drift of the resonance frequency $\Delta f_n(T)_{H_2O}$ of water is related to the temperature drift $\Delta T$ by:

$\Delta f_n(T)_{H_2O} = 1.8 \times 10^{-3} f_n \Delta T \tag{3.3}$

Setting $\Delta T = 2 \times 10^{-3}$ °C and $f_n = 1.5$ MHz in Eq. (3.3), we obtained $\Delta f_n = 5.4$ Hz, in good agreement with our result of 6 Hz.

(b) The system was also checked by a well-studied protein sample, i.e. bovine serum albumin (BSA). Different amount of
Fig. (3.4) Typical resonance frequency shift for distilled water at 1.5 MHz with a temperature difference of $2 \times 10^{-3}$ °C.
BSA was dissolved into distilled water to obtain protein solutions with weight fractions, $w_p$, varying from 0 to 1.6%. The weight fractions were also determined by optical density measurements at the ultraviolet region. With each protein weight fraction, the velocity of sound, $u$, of the solution was determined by the procedures given in sections [3.2.2] and [3.2.4(A)]. Plotting $u$ vs. $w_p$, a straight line with correlation coefficient better than 0.9998 (by least square fit method), was obtained (Fig. (3.5)). We had repeated the experiment twice and two more set of data were obtained. From these sets of data, the slope of the straight lines ($u$ vs. $w_p$) were found to be 255, 253 and 252 m/s respectively. Hence the greatest deviation between them was only about 1%, which indicated that our experiment was highly reproducible. However, our results were consistently greater than the value of 222 m/s as given by Gekko et al. The difference might probably be due to the difference in the preparation of the protein sample.

[3.3] Density measurements

The density measurement was based on the Archimede's principle. The weight of a small glass plummet was first determined by an electronic balance with accuracy good to 0.01 mg. The plummet was then immersed into the protein solution which was contained in a double-wall, thermally insulated test tube, and weight of the plummet was measured. From these two measurements, the density of the protein solution could be determined. We found that the accuracy of
Fig. (3.5) Sound velocity of bovine serum albumin solution at 20 °C as a function of the weight fraction protein, \( w_p \) (at 1.5 MHz).
the density measurement was highly dependent on the temperature fluctuation of the protein solution, and we recorded the temperature of the protein solution in each measurement using a thermometer read to 0.1 °C. All measurements were conducted at about 20 °C and then normalized to 20 °C using a calibrated measurement of the density of distilled water as a function of temperature at around 20 °C.

Density measurements of 0.1M NaCl solutions were carried out with this method. The values determined were ranging from 1.0022 to 1.0018 gcm⁻³. Comparing our result with the value of 1.0022 given by Millero et al.²⁴ with the measurement at 25 °C, we noted that although the fluctuation of the density data was not small, it was still acceptable in this study. The inherent limitations of the accuracy of the data were probably due to the temperature fluctuation of the sample and the viscosity of the solution.
Chapter 4

Results

[4.1] Aquometmyoglobin solution

[4.1.1] Sound velocity measurements

Fig. (4.1) shows a typical resonance peak for myoglobin in 0.1M NaCl at 1.5 MHz. Referring to the experimental section, the resonance cell is first calibrated by distilled water and the velocity of sound in the protein solution can then be determined from the variation of resonance frequency, \( f \), with protein concentration. By the measurement of the 3 dB bandwidth (i.e. half-power bandwidth (HPB)), \( \Delta f \), the absorption of the protein solution can also be deduced.

The sound velocity measurement is carried out at 20°C with different protein concentration (expressed as weight fraction, \( w_p \leq 0.0087 \)). The result is plotted as a function of \( w_p \) and a straight line is obtained, i.e.

\[
 u = u_0 + (du/dc) w_p
\]  

(4-1)

where \( u_0 \) (cm s\(^{-1}\)) is the velocity of sound in the solvent and \( du/dc \) is the slope. By least square fitting to the data, \( u_0 \) and \( du/dc \) are determined. The velocity of each protein concentration is recorded in three different frequencies, namely, 1.5 MHz, 2.5 MHz and 9.3 MHz (Table (4.1)). The result (Fig. (4.2a), (4.2b) and (4.2c)) shows no significant difference which reflects that within the experimental frequency range (i.e. 1 MHz to 10 MHz), the sound velocity
Fig. (4.1) Typical resonance peak for 0.2 % Mb in 0.1M NaCl.
Fig. (4.2) Sound velocity, $u$, of aquometmyoglobin solution (at 20 °C) as a function of the weight fraction protein, $w_p$, at various frequencies.
measurement of aquometmyoglobin in 0.1M NaCl does not depend on the frequency. The y-intercepts of the graphs give the velocity of sound in the solvent (i.e. 0.1 M NaCl), \( u_0 \). The relative velocity of sound, \( \Delta u \) (i.e. \( u_0 - u_w \), where \( u_w \) is the velocity of sound in the distilled water), is equal to 6.6 m/s which agrees very well with that of 6.4 m/s given by the literature.

4.1.2 Density measurements

As density, \( \rho \), of the protein solution is plotted against the concentration (i.e. \( w_p \)), a linear graph is obtained. The measurement is not very good and is chiefly affected by inherent limitations including viscosity and the temperature effects. On the whole, the measurement is reproducible and can be expressed as

\[
\rho (\text{gcm}^{-3}) = 1.0019 + 0.268w_p \tag{4-2}
\]

where the standard deviations are \( +0.0001 \) and \( +0.017 \) for the y-intercept, \( \rho_o \) (i.e. the density of the solvent) and the slope respectively (Fig. (4.3a)).

To determine the density of the protein, \( \rho_p \), the same data may be plotted as \([1-w_p]\rho_p^{-1} \text{ vs. } w_p(1-w_p)^{-1}\) and the slope of the straight line obtained gives \( 1/\rho_p \) (Fig. (4.3b)). The density of myoglobin, \( \rho_p \) thus determined is given by

\[
\rho_p (\text{gcm}^{-3}) = 1.36 \pm 0.03 \tag{4-3}
\]

which agrees with the values 1.37 and 1.35 reported by the
Fig. (4.3(a)) Density, $\rho$, of myoglobin solutions at 20 °C as a function of the weight fraction protein, $w_p$.

Fig. (4.3(b)) Plot of $[(1-w_p)\rho]^{-1}$ versus $w_p(1-w_p)^{-1}$. 
other workers\textsuperscript{7,8}.

[4.1.3] **Adiabatic compressibility of the protein solution**

The adiabatic compressibility, $\beta_{\text{ad}}$, of the protein solution can be determined from the results in [4.1.1] and [4.1.2] with Eq. (2-17) (given in Chapter 2). Owing to the relatively large error in the density measurements, we take the density values at each protein weight fraction from the best fit straight line (i.e. Eq. (2-17)) in computing $\beta_{\text{ad}}$. When $\beta_{\text{ad}}$ is plotted against $w_p$, the graph obtained is a straight line in the form of

$$\beta_{\text{ad}} = \beta_0 + S w_p \quad (4-4)$$

where the y-intercept, $\beta_0$, represents the adiabatic compressibility of the solvent, and $S$ is the slope (i.e. change in compressibility of the solution per unit protein weight fraction, $w_p$). The results at different frequencies are shown in Fig. 4.4a, 4.4b and 4.4c with slope $-2.89 \pm 0.07$, $-2.88 \pm 0.07$ and $-2.90 \pm 0.07 (10^{-11} \text{ cm}^2/\text{dyne})$ respectively. The uncertainties indicated in the slope correspond to the calculated value by taking all the uncertainties (i.e. velocity, density) into account. As indicated in [4.1.1], there is essentially no frequency dependence on the velocity measurement and correspondingly, no difference in $\beta_{\text{ad}}$ as a function of $w_p$ in the three graphs is observed.
Fig. (4.4) Adiabatic compressibility, $\beta_{ad.}$ of aquometmyoglobin solution (at 20 °C) as a function of the weight fraction protein, $w_p$, at various frequencies.
Determination of the apparent adiabatic compressibility of the protein molecules

The result that there is no frequency dependence on the sound velocity measurements (see [4.1.1]) reflects that the relaxational contribution of the compressibility is negligible. (see discussion in Chapter 5). Thus, Eq. (2-3) can be rewritten as

\[ \beta_{\text{ad.}} = \beta_s \]  

(4-5)

From Eq. (2-7) and (4-5), the apparent adiabatic compressibility of the protein molecules, \( \beta_p^a \), is related to \( \beta_{\text{ad.}} \) (the adiabatic compressibility of the protein solution) by

\[ \beta_{\text{ad.}} = (1-v_p) \beta_o + v_p \beta_p^a = \beta_o + v_p (\beta_p^a - \beta_o) \]  

(4-6)

where \( v_p \) (the fractional volume of the protein in the solution) is given by

\[ v_p = \frac{v^p}{v^p + v^o} \]  

(4-7)

where \( v^p \) and \( v^o \) are the volumes of the protein and the solvent respectively. With \( V = \frac{\rho}{m} \), where \( m \) is the mass, Eq. (4-7) can be written as

\[ v_p = \frac{\rho_p}{\rho^p} \]  

(4-8)
Since the protein weight fraction, \( w_p \), in the experiment is small \((\sim 10^{-2})\), the approximation of \( \rho = \rho_o \), where \( \rho_o \) is the density of the solvent, will only introduce an error of \(\sim 0.3\%\) (refer to Eq. (4-2)). So Eq. (4-8) can be approximated by

\[
V_p = w_p \rho_o / \rho_p
\]

and thus Eq. (4-6) can be expressed as

\[
\beta_{ad.} = \beta_o + \frac{\rho_o (\beta_p^a - \beta_o)}{\rho_p} w_p
\]

By comparing Eq. (4-4) and (4-10), we have

\[
S = \frac{\rho_o (\beta_p^a - \beta_o)}{\rho_p}
\]

i.e.

\[
\beta_p^a = \beta_o + S \frac{\rho_p}{\rho_o}
\]

From our results, the \( \beta_p^a \) of aquometmyoglobin in 0.1M ionic concentration is determined to be \(5.67 \times 10^{-12}\) \((\text{cm}^2/\text{dyne})\), with the uncertainty of \(\pm 1.36 \times 10^{-12}\) \((\text{cm}^2/\text{dyne})\).

[4.2] Ligand effect

The compressibilities of two other myoglobin derivatives, fluoromet and azidomet are measured in the same way as that of aquomet as described above. Like aquometmyoglobin, the sound velocity measurements of the other two protein derivatives show no frequency dependence
(Table 4.1). In Fig. (4.5), the graphs of $u$ vs. $w_p$ of the protein for the three myoglobin derivatives are shown. The slope of the graphs show no significant difference. The difference in the y-intercepts is due to the fact that the sound velocities in different kinds of solvent media (0.1M NaCl, 0.1M NaF, 0.1M NaN$_3$) are not identical.$^{24}$

The measurement of density on the different protein derivatives also show no significant changes within the experimental uncertainty. In all cases, the values range from 1.34 to 1.38 (g/cm$^3$). So the value of $\rho_p$ (refer to Eq. (4-3)) is taken to be $1.36 \pm 0.03$ (g/cm$^3$) for the three derivatives.

The adiabatic compressibilities, $\beta_{ad}$, of the various protein derivatives versus $w_p$ have been plotted in Fig. (4.6) as in [4.1.3] with slope $-2.89 \pm 0.07, -2.85 \pm 0.07$ and $-2.87 \pm 0.07 (10^{-11} \text{ cm}^2/\text{dyne})$ for aquomet, fluoromet and azidometmyoglobin respectively. As expected from the results of $u$ and measurements, no significant difference on the slope can be observed, except that the y-intercepts of the curves is different which simply implies that the adiabatic compressibilities of the various kinds of solvent media are not the same.

From the above measurements, the $\beta_p^a$ of various myoglobin derivatives are determined by Eq. (4-11). The results are $5.67 \pm 1.36, 6.03 \pm 1.36$, and $6.06 \pm 1.36 (10^{-12} \text{ cm}^2/\text{dyne})$ for aquomet, fluoromet and azidometmyoglobin respectively.
### Table (4.1) \( \frac{du}{dc} \) (change of velocity per unit weight fraction protein) for the three myoglobin derivatives (at neutral pH) at various frequencies.

<table>
<thead>
<tr>
<th>Myoglobin derivatives</th>
<th>( f ) (MHz)</th>
<th>( \frac{du}{dc} \times 10^{-2} ) (cm sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquomet</td>
<td>1.5</td>
<td>279.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>279.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>281.8 ± 1.7</td>
</tr>
<tr>
<td>Fluoromet</td>
<td>1.5</td>
<td>275.4 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>275.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>278.3 ± 2.6</td>
</tr>
<tr>
<td>Azidomet</td>
<td>1.5</td>
<td>275.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>276.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>278.5 ± 1.8</td>
</tr>
</tbody>
</table>
Fig. (4.5) Sound velocity, $u$, of aquomet ($\phi$), fluoromet (●) and azidomet (▲) myoglobin solution (at 20 °C) as a function of weight fraction protein, $w_p$. 
Fig. (4.6) Adiabatic compressibility, $\beta_{ad}$, of aquomet (◊), fluoromet (●) and azidomet (▲) myoglobin solutions (at 20 °C) as a function of weight fraction protein, $w_p$. 
It is well known that the myoglobin exhibits an acid-induced denaturation at pH \( \approx 4 \) (the exact pH of the transition depends on the temperature and the ionic strength of the solution\(^5,\!^1\!^6\)). The measurement of the optical density of Mb (in 0.1M acetate buffer) versus pH (Fig. 4.7) shows that the denaturation for the sample starts at pH \( \approx 4.5 \) and is completed at pH \( \approx 3.8 \). To study the change in compressibility of myoglobin upon denaturation, we have thus conducted a detail study on the compressibility of protein solution in the pH range of 3.4 to 5.1 (0.1M acetate buffer).

The slope, \( \frac{du}{dc} \), of the protein solution (i.e. change of velocity per unit protein weight fraction) in Eq. (4-1) changes monotonically from 285 to 340 (cm/s) as the pH decreases from 4.5 to 3.8 (Fig. (4.8)). This result reflects that upon the acid-induced denaturation of myoglobin, the velocity of sound propagating in the denatured protein molecules is greater than that in the native one. All the measurements of sound velocities show no frequency dependence (Table 4.2) with different weight fractions, \( w_p \), of myoglobin solutions as in [4.1] and [4.2].

In the density measurement, on the whole, there is no appreciable changes for myoglobin in the different pH media. The result is

\[
\rho_d \, (\text{gcm}^{-3}) = 1.00034 + 0.267 \, w_p \quad (4-12)
\]
Fig. (4.7) Optical density ($\lambda=502$nm) of aquometmyoglobin solution ($9.6 \times 10^{-5}$ Molar) at 20°C as a function of pH.
Fig. (4.8) $\frac{du}{dc} 	imes 10^{-2}$ (cm sec$^{-1}$) (change of velocity per unit weight fraction protein) for aquometmyoglobin solution at 20 °C as a function of pH.
<table>
<thead>
<tr>
<th>pH</th>
<th>f (MHz)</th>
<th>$\frac{du}{dc} \times 10^{-2}$ (cm sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.10</td>
<td>1.5</td>
<td>227 ± 7</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>228 ± 7</td>
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<tr>
<td></td>
<td>9.3</td>
<td>220 ± 7</td>
</tr>
<tr>
<td>4.80</td>
<td>1.5</td>
<td>278 ± 7</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
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</tr>
<tr>
<td></td>
<td>9.3</td>
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<td>1.5</td>
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</tr>
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<tr>
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</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>9.3</td>
<td>304 ± 5</td>
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<tr>
<td>4.20</td>
<td>1.5</td>
<td>313 ± 5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>314 ± 5</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>313 ± 5</td>
</tr>
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<td>4.00</td>
<td>1.5</td>
<td>332 ± 5</td>
</tr>
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<td></td>
<td>2.5</td>
<td>334 ± 5</td>
</tr>
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<td></td>
<td>9.3</td>
<td>337 ± 5</td>
</tr>
<tr>
<td>3.75</td>
<td>1.5</td>
<td>342 ± 5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>342 ± 6</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>343 ± 5</td>
</tr>
<tr>
<td>3.40</td>
<td>1.5</td>
<td>344 ± 6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>345 ± 5</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>345 ± 5</td>
</tr>
</tbody>
</table>

Table (4.2) \( \frac{du}{dc} \) (change of velocity per unit weight fraction protein) for aquometmyoglobin solution (at 20 °C) as a function of pH at various frequencies.
with the standard deviations $\pm 0.0006$ and $\pm 0.012$ for the $p_0$ and the slope respectively. From Eq. (4-12), the density of the protein, $p'_p$, deduced is $1.36 \pm 0.03$ (g/cm$^{-3}$). The small change in the density of the protein upon denaturation is consistent with the results reported on the pressure-induced denaturation of chymotrypsinogen$^{25}$ and the alkaline denaturation of metmyoglobin$^7$.

For illustration, the adiabatic compressibilities as determined in [4.1.3] are plotted as a function of $w_p$ at pH 4.50 and 3.75 (Fig. (4.9)). A large difference between the slope of the two curves ($-2.92 \pm 0.06$ and $-3.29 \pm 0.06$ (10$^{-11}$ cm$^2$/dyne)) for the protein solution at the different pH is observed.

The apparent adiabatic compressibilities of the myoglobin molecules are plotted against pH in Fig. (4.10). In the figure, $p'_p$ decreases from $5.3 \times 10^{-12}$ (cm$^2$/dyne) at around pH 4.5 to $0.5 \times 10^{-12}$ (cm$^2$/dyne) at around pH 3.8. The mid-point of transition lies between pH 4.2 and 4.3, which is in agreement with the optical density measurement. This indicates that the compressibility of myoglobin decreases by about $5 \times 10^{-12}$ (cm$^2$/dyne) upon denaturation.
Fig. (4.9) Adiabatic compressibility, $\beta_{\text{ad.}}$, of aquometmyoglobin solution (at 20 °C) at pH 4.5 (▲) and 3.75 (●) as a function of weight fraction protein, $w_p$. 
Fig. (4.10) Apparent adiabatic compressibility of myoglobin, $\beta_p^a$, in 0.1M acetate buffer at 20 °C as a function of pH.
Chapter 5
Discussion

The apparent adiabatic compressibility of aquometmyoglobin $\tilde{\beta}_p$ at neutral pH determined in this experiment ($5.7 \times 10^{-12} \text{ cm}^2/\text{dyne}$) agrees very well with that of $6.0 \times 10^{-12} \text{ cm}^2/\text{dyne}$ measured by Gavish et al.\textsuperscript{7} but differs slightly from the value of $9.0 \times 10^{-12} \text{ cm}^2/\text{dyne}$ obtained by Gekko and Moguchi\textsuperscript{8}. $\tilde{\beta}_p$ is found to be unchanged in the frequency range of $1 - 10 \text{ MHz}$, indicating that relaxation contributions such as ionization and proton transfer reactions are negligibly small. Since large scale conformational changes which are present in extreme pH occur in a time scale of msec or slower, relaxation contribution of such reactions would not be observable in this frequency range covered in this experiment. Thus, the $\tilde{\beta}_p$ value should correspond to the intrinsic apparent compressibility of the protein system. As shown in the theoretical section (i.e. Eq. (2-8)),

$$
\tilde{\beta}_p = \beta_p - k(\beta_o - \beta_h)
$$

(5-1)

where $\beta_p$, $\beta_o$, and $\beta_h$ correspond respectively to the intrinsic adiabatic compressibility of the protein, the pure solvent and the solvent hydration, and $k$ is the fractional volume of the bound water with respect to the protein.

The precise values of $k$ and $\beta_h$ are not known. From NMR data, $k$ is as large as $0.30^{26}$. However, according to the estimation of Gavish et al.\textsuperscript{7}, $k \sim 0.13$. If we assume, as Eden
et al.\textsuperscript{11}, that $\beta_h$ is about the same as that of ice ($18 \times 10^{-12}$ cm$^2$/dyne), $\beta_p$ will range between $9.2 \times 10^{-12}$ cm$^2$/dyne - $13.8 \times 10^{-12}$ cm$^2$/dyne for $k$ values of 0.13 - 0.30. These $\beta_p$ values are quite low ($\sim 25\%$ of that of water) comparing to other materials. However, they are much higher than those of small molecules, since in such cases, large covalent forces must be overcome to change their volume. For a large protein molecule occupying a definite volume in the solvent, the dominant contribution to its compressibility is the volume changes associated with low energy structural fluctuations of the entire molecule. The low compressibility observed for myoglobin and proteins in general\textsuperscript{7,8} reflects that their structures are quite compact dynamically. However, this seems to contradict the results of other experimental data (NMR, hydrogen exchange, x-ray temperature factor) which indicate that proteins can exhibit large amplitude fluctuations in the position of many atoms and thus suggest that the protein interior is relatively fluid-like. In view of the compressibility data, it is likely that the fluid-like regions only cover a part of the molecule and on the average the molecule has little flexibility. Along this line, Gavish et al.\textsuperscript{7} has proposed that the interior protein is made up of "solid-like domains" which correspond to regions of highly correlated motions.

The work of Eden et al.\textsuperscript{11} on cytochrome c reveals that a small perturbation (which corresponds to changing the oxidation state of the iron atom in the protein) can lead to
large differences in the compressibility of the protein
\( \beta_p(\text{red.}) = 8.5 \times 10^{-12} \text{ (cm}^2/\text{dyne}), \ \beta_p(\text{ox.}) = 14.2 \times 10^{-12} \text{ (cm}^2/\text{dyne}) \)\(^{11}\). It is thus interesting to see if such effects are common among proteins. In this work, we have studied the compressibilities of myoglobin when bound to different ligands. It is well known that different myoglobin derivatives exhibit differences in their structural and physical properties. In particular, among the three derivatives under examination, azidometmyoglobin is significantly more stable than the aquomet and fluoromet derivatives against denaturation by acid\(^{13}\) and by heat\(^{12}\). A question which naturally arises is whether there exists any correlation between the conformational stability and the compressibility for the various myoglobin derivatives. The results of this experiment show that the apparent compressibility of the various myoglobin derivatives are essentially the same. Since the hydration effects for the various derivatives are not expected to be very different, thus the intrinsic compressibility \( \beta_p \) of myoglobin is not altered by its state of ligation. Since \( \beta_p \) can be related to the volume fluctuation of the protein\(^6,11\), this implies that binding of different ligands to myoglobin does not lead to a difference in the flexibility of the molecule. This finding is consistent with that of Benson et al.\(^{27}\) who showed that different ligands have no discernible effect on the hydrogen exchange rates of myoglobin. Furthermore, the results support the idea of Eden et al.\(^{11}\) that an increase in compressibility should be accompanied by an increase in mean hydrogen
exchange rate. This hypothesis has been verified now for cytochrome c\textsuperscript{11} and for myoglobin. It will be useful to extend such studies to a larger number of systems to see if the results of the two techniques (hydrogen exchange and adiabatic compressibility) agree with each other. This correlation is essential in establishing the usefulness of the two different techniques in probing the dynamics of protein structures.

The change in the compressibility of proteins upon denaturation is particularly interesting as little work on this has been done. The method of denaturation of myoglobin by acid, adopted in this work, has the advantage that relaxation contributions to the measured adiabatic compressibility due to proton transfer reactions in the pH range of interest is small\textsuperscript{5}. This is evident by the fact that the compressibility is frequency independent in the range covered in the experiment. $\beta_p^a$ of metmyoglobin decreases monotonically from $5.3 \times 10^{-12}$ (cm\textsuperscript{2}/dyne) at around pH 4.5 to $0.5 \times 10^{-12}$ (cm\textsuperscript{2}/dyne) at around pH 3.8 with the extent of denaturation. This trend is consistent with that obtained by Gavish et al.\textsuperscript{7} on alkaline denaturation of myoglobin, except in that case the results were complicated by the presence of relaxation contributions and a more quantitative determination of the change in $\beta_p^a$ upon denaturation was not attempted. The decrease in $\beta_p^a$ for the denatured protein could be understood as follows. First, the protein unfolds upon denaturation, and such a nonstructured polypeptide
should be less compressible than a native protein (i.e. $\beta_p$ decreases upon denaturation). Second, the nonpolar groups buried in the protein interior are exposed to the solvent in the process of denaturation. This leads to an increase in hydrophobic or hydrogen bonded hydrations (i.e. $k$ for the denatured protein is larger), and thus a smaller $\beta^a_p$ for the denatured state (refer to Eq. (5-1)).

The present result on the change in adiabatic compressibility of a protein upon denaturation is in contrast with the results seen in the isothermal compressibility $\beta_T$ measurements$^{10,25,28}$. For myoglobin$^{28}$, $\beta_T$ of the denatured protein is smaller than that of the native one by only $3 \times 10^{-13}$ cm$^2$/dyne; and for both ribonuclease$^{10}$ and chymotrypsinogen$^{25}$, $\beta_T$ increase instead by about $1.5 \times 10^{-12}$ cm$^2$/dyne upon denaturation. This unexpected increase has been explained by proposing that there is a very high local concentration of nonpolar groups in the denatured protein$^{29,30}$. Such a proposal is not supported by the results of the adiabatic measurements.

Theoretically, the intrinsic $\beta_p$ and $\beta_T$ are related$^{19}$ by (see Eq. (2-35))

$$\beta_T = \beta_p + (\chi_p^2 T)/(\rho C_p)$$

This $\chi_p^2 T/\rho C_p$ term corrects for the change in volume with change in $T$ which is introduced in the adiabatic experiment, and is typically $3 \times 10^{-12}$ cm$^2$/dyne for native proteins$^{8}$. If
this term is much larger for the denatured protein, this may account for the discrepancy observed. Since $C_p$ of the protein is known to increase by about 50% upon denaturation, $\alpha_p$ for the denatured protein must be larger than that of the native one by as much as a factor of two in order to explain the observed difference between $\beta_T$ and $\beta_p$. We have not been able to find any data on $\alpha_p$ for denatured proteins, but it seems unlikely that $\alpha_p$ will be very different for the native and denatured proteins, considering the fact that the volume of the protein remains essentially the same after denaturation.

One possible explanation for the difference in the measured $\beta_T$ and $\beta_p$ is as follows. If there exists in the denatured protein large scale conformational fluctuations or transitions occurring in a time scale slower than 1 $\mu$sec, such processes will lead to an increase in $\beta_T$ for the denatured protein but will not give a contribution to $\beta_p$ which is conducted at a frequency of 1 MHz or higher. Thus, the observed discrepancy may be ascribed to low frequency relaxation contributions in the denatured protein. This explanation requires that there is some structure for the denatured protein which is normally assumed to resemble a random coil. Such structures could result from incomplete unfolding of the protein which is not uncommon for denaturation process in water, or from the various intermediates or incorrectly folded species in the refolding reaction. Further studies on the compressibilities...
of proteins will be required before the validity of this hypothesis can be established. Such work should lead to a better understanding of the dynamic structure of native as well as denatured proteins.
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