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Review

# Protein structure and dynamics at high pressure[1]

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**Abstract**

The effect of pressure on the structure and dynamics of proteins is discussed in the framework of the pressure-temperature stability phase diagram. The elastic (reversible) properties, thermal expansion, compressibility and heat capacity, are correlated with the entropy, volume, and the coupling between entropy and volume fluctuations respectively. The experimental approaches that can be used to measure these quantities are reviewed. The plastic (conformational) changes reflect the changes in these properties in the cold, pressure and heat denaturation. 

*Keywords:* High pressure; Biospectroscopy; Phase diagram; Compressibility; Thermal expansion; Heat capacity

## Contents



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[1] Dedicated to the memory of Gregorio Weber (1916–1997), a pioneer in high pressure biophysics.

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## 1. Introduction

Proteins are unique biological macromolecules attracting active interest from various disciplines. For the physicist it is the structure which has the characteristics of order as well as of disorder; the chemist may be attracted by the unique properties that show up in the catalytic activity of enzymes and in the conversion of chemical into mechanical energy in muscle; biologists tend to put the emphasis on the functional role of proteins in various biological processes [1].

Although there are several good reasons for performing pressure experiments on proteins as compared to the usual approach which is to change the temperature, there is one reason which even favors pressure over temperature: namely that one can separate the effects of density and temperature. Whereas this review emphasizes on pressure effects, the relation to the effects of cold and heat will be discussed as well.

The pioneering work in high pressure protein research is that of Bridgman who observed that a pressure of several kbar will give egg white an outlook similar but not identical to that of a cooked egg [2]. Nowadays it is well known that proteins in solution are marginally stable under conditions of high temperature and pressure [3]. By contrast there is the observation that certain bacteria live under extreme temperature conditions and it is well known that bacteria can survive in the deepest parts of the ocean (ca. 1 kbar[2]). A few kbar is ordinarily sufficient to denature most of the dissolved proteins.

Besides the fact that high pressure denatures proteins, it solidifies lipids and destabilizes biomembranes. As a consequence, inactivation of microorganisms results. On the other hand. the observation that pressure treatment does not cause changes in the taste or the flavor of food materials is of special interest in view of possible applications in the food industry. Pressure treatment of certain food materials may thus be used as a possible alternative for temperature treatment. Besides the possibility of forming new textures, the conservation of natural flavor and other ingredients add to the potential of the technique. Future developments will decide whether bridgmanization (also called pascalization) will be a contribution as vital to biotechnology as the process of pasteurization is now. Besides the possible applications in food science, there is some perspective in the development of immunodesorption [4] and in vaccine development and virus sterilization [5]. A number of books and reviews have been published and should be consulted for more details [6–9].

If the conditions for equilibrium or isokineticity are plotted versus temperature and pressure, a phase or stability diagram is obtained with an elliptical shape [10–12]. One of the practical consequences is the stabilization against heat denaturation by low pressures. This has been observed in several proteins and enzymes [13–15]. As indicated, this also applies to the effect of pressure on the heat gelation of starch [16]. Of special interest is the observation that the inactivation kinetics of microorganisms shows diagrams similar to those of proteins [17–19]. This applies also to the cold inactivation of microorganisms [18].

The phase diagram on the pressure-temperature plane for the stability of proteins can be divided into two regions: at ambient pressure and temperature, when the protein is in its native state, effects of pressure and temperature result in elastic (reversible) changes [20]. These effects are discussed in Section 2. At high pressure and high or low temperature, changes are plastic or conformational (in most cases irreversible). These effects are discussed in Section 3.

## 2. Elastic properties

The elastic effect preserves the secondary structure. Changes in the bond length, the hydration and the cavities that result from the imperfect packing in the protein interior may be expected. If the temperature or the pressure is high enough, cooperative changes in the secondary structure may result, an effect known as plastic or conformational effect.

The partial molar volume, $V_i$, of a solute molecule or ion is defined as the volume change of the solution by the addition of a small amount of the solute over

[2] Units used for pressure: 1 kbar = 100 MPa; 10 kbar = 1 GPa; and for compressibility: 100 $Mbar^{-1} = GPa^{-1}$.

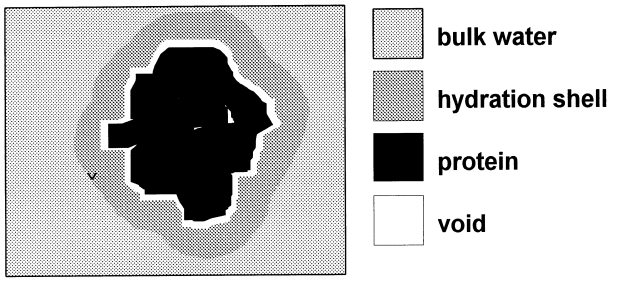


Fig. 1. Schematic representation of the volumes associated with the dissolved protein.

the number of moles of added solute keeping everything else constant.

$$V_i = \left(\frac{\partial V}{\partial n_i}\right)_{n_j,p,T}. \quad (1)$$

Here $V$ is the volume of the solution and $n_i$ is the number of moles of the solute added [21]. It is not equal to the volume of the molecule or the ions since it includes also the interaction with the solvent. For a protein $V_i$ can be written as the sum of three terms [22]

$$V_i = V_{\text{atom}} + V_{\text{cavities}} + \Delta V_{\text{hydration}} \quad (2)$$

where $V_{\text{atom}}$ and $V_{\text{cavities}}$ are the volumes of the atoms and the cavities respectively and $\Delta V_{\text{hydration}}$ is the volume change of the solution resulting from the interactions of the protein molecule with the solvent. Fig. 1 shows the different volumes associated with the protein solution. Care should be taken if quantities derived from the volume (such as compressibility) are interpreted. The results may depend on the sensitivity range of the method. Global measurements such as ultrasonics detect the whole molar volume, while some local probes may feel only the change of the protein interior volume. It should also be pointed out that notions of volume and surface are not easily defined in the molecular dynamical calculations. A critical discussion of this topic can be found in the paper of Paci and Velikson [23].

Thermal expansion is defined as the relative change of the partial molar volume with temperature:

$$\alpha = \frac{1}{V_i}\left(\frac{\partial V_i}{\partial T}\right)_p \quad (3)$$

and the isothermal compressibility as the relative change of the volume with pressure:

$$\beta = -\frac{1}{V_i}\left(\frac{\partial V_i}{\partial p}\right)_T. \quad (4)$$

Table 1 gives the thermal expansion, the compressibility and the heat capacity for a number of small molecules, a polymer and for proteins.

As the volumes of the atoms may be considered, as a first approximation, to be temperature- and pressure-independent, it follows that both the thermal expansion and the compressibility are composed of two main terms, the cavity and the hydration terms. An estimate of the contribution of each factor is not easy to evaluate and relies on assumptions which are not easy to check experimentally. However, the compressibility of amino acids is negative because of changes in the solute-solvent interaction (i.e. the amino acid solution is less compressible than the pure solvent). It follows then that the contribution of cavities compensates this effect so that the compressibility of the protein in solution becomes positive. A more quantitative estimate is possible when one makes assumptions about the compressibility of the hydrational water [24].

Table 1
Thermal expansion, isothermal compressibility and heat capacity of water, benzene, hexane and nylon 6

| | Thermal expansion $10^{-6}$/K | Compressibility 1/Mbar | Heat capacity (Cp) kJ/kg K |
|---|---|---|---|
| Water | 210 | 45.8 | 4.17 |
| Benzene | 1220 | 96 | 1.7 |
| Hexane | 1380 | 166 | 2.26 |
| Nylon 6 | 80–100 | 15.2 | 1.6 (Cv) |
| Amino acids | 1000–1550 | −0.28–−0.62 | 0.78–2 |
| Proteins | 40–110 | 2–15 | 0.32–0.36 |

The quantities for amino acids and proteins are for dilute solutions in water.

An estimate of the compressibility of the cavities should be possible with positron annihilation lifetime spectroscopy. It has proved to be a useful tool for determining the size of cavities and pores in materials. The lifetime is sensitive to the size of the cavity in which it is localized. A number of empirical relations allows the correlation between the distribution of the lifetime and the free volume [25]. With this technique an increase of 0.6 $A^3$/K has been obtained for lysozyme. Data on the pressure effect on the lifetime are only available for polymers. For an epoxy polymer, the free volume reduces exponentially with pressure with a reduction to half of the free volume at about 2 kbar [26]. The compressibility of the cavities is ca. 250 $Mbar^{-1}$ (estimated from Fig. 6. in [27]), much higher than the ones characteristic for proteins. These results suggest that there may be a considerable contribution of the reduction in cavity size to the compressibility of a protein.

### 2.1. Compressibility and volume fluctuations

The compressibility is a thermodynamic quantity of interest not only from a static but also a dynamic point of view. Its relevance to the biological function of a protein can be understood through the statistical mechanical relation between the isothermal compressibility $\beta_T$, and volume fluctuations [28,29]:

$$\langle V-\langle V\rangle\rangle^2 = \beta_T k T_B V \quad (5)$$

where the expression between brackets indicates an average for the isothermal-isobaric ensemble. Because of the small size of the protein, the volume fluctuations are relatively large, they are of the order of the volume changes for the denaturation [28]. Kharakoz and Bychkova [30] pointed out that the above consideration is valid strictly only for a system consisting of a constant number of particles. If one considers an unfolded molecule, where the conformational fluctuations change the surface and therefore the size of the hydration shell, the fluctuation of the number of water molecules in the hydration shell must be taken into consideration. In the case of native, folded protein the fluctuation in the number of particles can be ignored. It should also be stressed that the compressibility also includes the fluctuations between different conformational substates as discussed by Frauenfelder et al. [31]. As pointed out by Richards, the expansion and contraction of the cavities is the only way to generate these volume fluctuations [32].

#### 2.1.1. Ultrasonic velocimetry

Most data on the compressibility of proteins have been obtained from ultrasonic velocimetry. The velocity of ultrasound ($u$) is related to the adiabatic compressibility, $\beta_S$, and the density, $\rho$, via the Laplace equation:

$$\beta_S = 1/\rho u^2. \quad (6)$$

The isothermal compressibility, $\beta_T$, can be obtained from the following relation:

$$\beta_T = \beta_S + \alpha^2 T/\rho C_p. \quad (7)$$

In this expression $\alpha$ is the thermal expansion, $\rho$ the density and $C_p$ the heat capacity. Sarvazyan [33] has drawn attention to the potentialities of ultrasonic velocimetry as a method to obtain information on all molecular aspects related to compressibility. Recently, the methodology has been extended to the measurement of the compressibility of proteins as a function of pressure [34].

The compressibility of amino acids in aqueous solution is negative. The compressibility of proteins in the native state is positive. The negative compressibility of the unfolded state is usually interpreted as the consequence of the reduced compressibility of the hydration shell [35]. However, the disappearance of the cavities would also result in a decrease of the compressibility.

#### 2.1.2. Luminescence spectroscopy

Marden et al. [36] studied the pressure-induced unfolding of several heme proteins from the fluorescence emission of the protein tryptophans (Trp). They suggested a method to obtain the compressibility of the protein prior to unfolding from the fluorescence intensity changes with pressure. The decrease in fluorescence yield with increasing pressure suggests an increase in energy transfer due to the protein compression. Ignoring the effect of pressure on the refractive index it can be shown on the basis of the Förster theory that the fractional change in fluorescence intensity is proportional to the difference in compressibility of the protein and the solvent. The observed decrease in intensity suggests that the

protein is more compressible than the water. This implies that the observed compressibility is much larger than that observed with ultrasound techniques. However, the fluorescence measurements refer to the Trp-heme distance whereas the sound velocity technique measures an average protein value.

The phosphorescence emission of Trp residues in proteins is a sensitive monitor of the local protein structure. The phosphorescence lifetime is an indicator of structural flexibility. The method has been used by Cioni and Strambini [37] to investigate pressure effects on monomeric as well as multimeric proteins. At low pressures ($<1$ kbar), the elastic effects predominate suggesting a tightening of the pressure on the protein. At higher pressures, a loosening effect is observed which is attributed to pressure-induced hydration of the protein. This seems to be consistent with the role of internal cavities and hydration of the polypeptide that contribute to the compressibility.

Freiberg et al. [38] measured the pressure effects on the absorption and fluorescence emission spectra of photosynthetic light-harvesting pigment-protein complexes from the purple bacterium *Rhodospirillum rubrum* at room temperature and at 77 K. The pressure-induced frequency shifts of the pigments were interpreted with the Laird and Skinner theory [39] in terms of the compressibility of the protein matrix. It was found that compressibilities of the proteins surrounding the different pigments is different thus revealing the local specificity of the elastic properties of the proteins. A compressibility of $25\pm5$ Mbar$^{-1}$ is obtained for the bacteriochlorophyll surroundings and $10\pm2$ Mbar$^{-1}$ for the surrounding of the spirilloxanthin pigment.

A more sophisticated luminescence method, the so called spectral hole burning, was applied to measure protein compressibility [40,41] in the low pressure region. In this site selective spectroscopical technique a laser with very narrow bandwidth ($10^8$ Hz) is used to burn a spectral hole into the inhomogeneously broadened absorption band. The hole arises because the laser light alters photochemically or photophysically a significant population of those molecules with transition frequencies corresponding to the laser (burning) frequency.

One can obtain the compressibility of a protein matrix surrounding the chromophore from the pressure-induced frequency shifts. This is based on the microscopic model of Laird and Skinner [39] which takes into account the long range induced dipole-induced dipole interactions between the protein matrix and the chromophore.

$$\Delta\nu = 2(\nu_b - \nu_{vac})\beta\Delta p \quad (8)$$

where $\Delta\nu$ is the shift of the hole frequency caused by the $\Delta p$ pressure change, $\nu_b$ is the frequency where the hole was burnt and $\nu_{vac}$ is the frequency of the electronic transition of the isolated chromophore. Because of the high spectral resolution effects of pressures as low as 1–2 MPa can be measured.

The compressibility values determined at 4 K for horseradish peroxidase are in the range of the compressibilities obtained for proteins at room temperature. Binding of an aromatic hydrogen donor substrate to the free base mesoporphyrin-substituted horseradish peroxidase was found to change the protein dynamics. This is reflected in the drastic increase of the compressibility [42]. Although this technique is restricted to chromoproteins and very low temperature conditions (ca. 4 K), it is, in theory, possible to extend the method to the aromatic chromophores of the side chains of the proteins. A detailed review of the hole burning measurements can be found elsewhere in this issue [43].

It has to be mentioned that all of the above luminescence methods feel the compressibility of the interior of the protein molecule, which is different from that determined by ultrasonic measurements. By luminescence one measures the changes in the distances of the atoms of the protein. These distances change mainly because of the compression of the voids, while the ultrasonic measurements detect the effect of hydration as well.

### 2.1.3. UV/visible spectroscopy

Jung and coworkers have used the Laird and Skinner theory to calculate the local compressibility of the heme pocket of cytochrome P450-CO complexed with substrate analogues, by following the pressure-induced red shift and the broadening of the Soret band at room temperature [44]. The pressure-induced red shift is assumed to result from the interaction between the heme group and the active-site water molecules and/or polar amino acid residues near the heme. In contrast to the observed pressure-induced broadening of the Soret band for cytochrome

P450 complexes, a band narrowing is observed for the myoglobin-CO complex. As for the luminescence measurements, this approach gives local compressibilities of the protein interior.

*2.1.4. Vibrational spectroscopy*

Information about the protein structure can be obtained from the so called amide vibrations [45]. The most characteristic vibrational band is the amide I band. 85% of the energy of this vibration comes from C=O stretching. Since the oxygen atom is hydrogen-bonded to the other part of the protein chain in a folded protein this is a conformation-sensitive band. Its frequency is in the range of 1600–1700 $cm^{-1}$ depending on the actual secondary structure. The usual effect of pressure on the vibrational bands is a blue shift (higher frequency), but hydrogen-bonded systems are exceptions, showing a red shift [46,47]. This is valid for the proteins as well. The increasing frequency with increasing pressure demonstrates the strengthening of the hydrogen bond, which decreases the electron density of the C=O bond. Except for a few high pressure Raman studies [48,49] most of the vibrational spectroscopy was done with FTIR (Fourier transform infrared) spectroscopy. The infrared spectroscopical experiments must be done in $D_2O$ because of the overlapping of the amide I band with the strong water vibration at 1640 $cm^{-1}$. The use of $D_2O$ involves the possibility of H/D exchange. While this effect becomes significant at the conformational changes, where new amino acids will be exposed to the solvent, it can also be used to probe the dynamics of proteins by estimating the internal accessibility of the polypeptide chain (see Section 3).

Several proteins were investigated by Heremans et al. [50,51]. The decreasing amide I frequency was found to be a general feature in the elastic pressure regime (typically below ca. 5 kbar). An example of sperm whale and horse myoglobins is given in Fig. 2. The maximum position of the amide I′ band (where the prime indicates that the solvent is $D_2O$) has a negative slope in the elastic region according to the above detailed picture. One has to mention that the band maximum as such can be a misleading parameter if the protein has several domains with different secondary structure, because the amide I band is then composed of several overlapping components, and

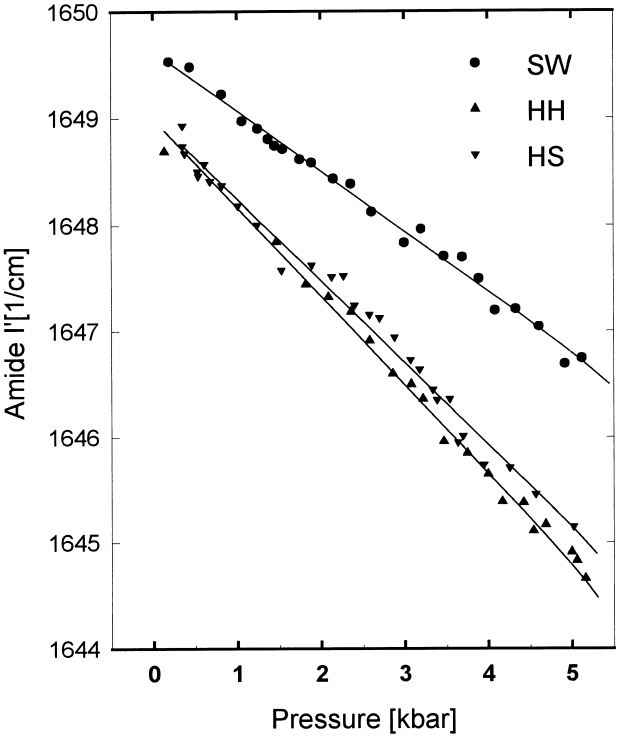


Fig. 2. Amide I′ band maximum versus pressure for sperm whale (SW), horse heart (HH) and horse sceletal muscle (HS) myoglobins.

the relative intensity changes can also be observed as frequency shifts of the overall band. A detailed analysis can be made with resolution enhancement and band fitting [52–54], but to perform it properly very good quality spectra are needed.

Elastic effects can also be followed using the ring vibration of the tyrosine side chain. Here the usual blue shift is observed. To correlate the *dv/dp* values with the compressibility is not as straightforward as in the fluorescence and visible/UV spectroscopy. The main difference is that the short range forces play a more important role than in the electronic transitions. The vibrational frequency is proportional to the second derivative of the potential with respect to the normal coordinate. The simplest calculation for the frequency shifts deals with a diatomic oscillator for which the vibrational frequency and equilibrium bond distance undergo slight changes. To the first order there is a simple relation between these changes [46]:

$$\frac{\Delta\nu}{\nu_0} = -a\frac{\Delta r}{r_e} \tag{9}$$

where the factor $a$ depends on the leading terms in the series expansion of the potentials. There are the intrinsic potential of the oscillator and the interaction potential to be taken into account.

The pressure effect depends on how the vibration affects the volume of the molecule. Gardiner et al. [55] presented an example of this effect in the case of toluene, where the $d\nu/dp$ value is small for the vibration not affecting the volume, while the breathing vibrations, show higher $d\nu/dp$. This means that the Laird and Skinner theory is not applicable here. The connection between the compressibility and $d\nu/dp$ would have to include the nature of the vibrational mode. Even if one could calculate the coefficient giving the connection between the $d\nu/dp$ of the amide I′ band and the compressibility, this compressibility would be different from the overall one, and would mainly reflect the compressibility along the C=O bond length, because the majority of the vibrational energy comes from the C=O stretching.

Fourier self-deconvolution was applied for resolution enhancement to separate the components of the amide I band characteristic for the different secondary structure by Heremans et al. [50,51]. The $d\nu/dp$ for the component bands of α, β and unordered structure were measured in case of several proteins. These $d\nu/dp$ values are typically in the range of −0.2 to −0.5 $cm^{-1}$/kbar for the α and β structure. The band corresponding to the unordered structure shows a positive (0.4 $cm^{-1}$/kbar) slope.

Pressure-induced vibrational wavenumber shifts in solids or polymers have been characterized by the volume-independent Grüneisen parameter [56].

$$\gamma_i = -\frac{d\ln\nu_i}{d\ln V} = -\frac{V d\nu_i}{\nu_i dV}. \quad (10)$$

If this parameter is assumed to be the same for all vibrations, one can obtain a bulk thermodynamic definition expressing the relation between the volume, the thermal expansion, the compressibility and the heat capacity at constant volume:

$$\gamma = \frac{V\ \alpha}{C_V \beta_T}. \quad (11)$$

The bulk Grüneisen parameter is found to be ca. 4 for polymers from the effect of pressure on the velocity of sound [57]. The data suggest that for the heat capacity only the interchain contribution should be taken into account. With this assumption, an order of magnitude calculation with the data available in Table 1 shows that the bulk Grüneisen parameter for proteins is of the same order of magnitude as that of polymers. This suggests that the thermal expansion and the compressibility of proteins reflect primarily the domains between the secondary structures. These movements are reflected in the low frequency part of the vibrational spectrum. Unfortunately, no experimental data are available on the effect of pressure on these vibrations.

### 2.1.5. Nuclear magnetic resonance

The pressure-induced changes in the overall folded structure of lysozyme was studied with NMR at 750 MHz [58]. The chemical shifts of 26 protons were followed up to 2 kbar. The main result is a compaction of the hydrophobic core consisting of bulky side chains. By contrast, it was found that the compaction is restricted to the α helical region in the crystal structure (see Section 2). The pressure effect on the structural dynamics of bovine pancreatic trypsin inhibitor was recently estimated by the same research group from the chemical shifts of the individual hydrogen bonds of the peptide bonds [59]. From the linear dependence of the chemical shifts on pressure, a pressure-independent compressibility was assumed up to 2 kbar. This is a rather surprising result. But given the rather small pressure range, the change in compressibility might be under the limit of experimental detection.

### 2.1.6. X-ray crystallography

The compressibility of lysozyme up to 1 kbar has been obtained from X-ray diffraction by Kundrot and Richards [60]. As expected the compression is non-uniformly distributed. The domain of the α helices is found to be more compressible (5.7 $Mbar^{-1}$) than the other domain which is essentially β structure. It should be noted that this compressibility does not include the hydration water.

### 2.1.7. Normal mode calculations

The compressibility of deoxymyoglobin has been estimated from normal mode analysis calculations up to 100 MPa by Yamato et al. [61]. In general, the helices are rigid but the interhelix regions are soft. The intrahelix rigidity is consistent with the

findings of Kundrot and Richards for lysozyme [60]. Interestingly, the large cavities in the hydrophobic clusters do not make these clusters very compressible. Their relative compressibility depends on the size of the cavities, the larger cavities showing the largest compressibility. In agreement with NMR work of Morishima and Hara [62] the distal cavity is also found to be the most compressible.

### 2.2. Thermal expansion and volume-entropy fluctuations

Like the compressibility and heat capacity, the thermal expansion can also be related to the fluctuations of the system; however, this relation is not as widely known as the others mentioned above. The thermal expansion is proportional to the cross-correlation of the volume and entropy fluctuations [29]:

$$\langle SV-\langle S\rangle\langle V\rangle\rangle = k_b TV\alpha. \quad (12)$$

This is in accordance with the intuitive picture, that the thermal expansivity characterizes some kind of coupling between the thermal ($T$, $S$) and the mechanical ($p$, $V$) parameters.

#### 2.2.1. Dilatometry

The thermal expansion has been obtained by Bull and Breese [63] from dilatometry. The experimental observations are discussed in terms of changes of the hydration only, water being released at higher temperature. A minimum is observed in the $dV/dT$ against temperature which is interpreted as a disappearance of water molecules into the interior of the protein. It is suggested that this is related to a predenaturational stage. At higher temperatures, a maximum in $dV/dT$ is observed which is related to the formation of an extensive interaction network between the denatured proteins.

#### 2.2.2. Densitometry

The thermal expansion has recently been obtained by Chalikian et al. [64] from the partial molar volumes. The interpretation focuses on the inapplicability of the small molecular weight model systems to the understanding of the behavior of proteins. It is suggested that mutual thermal motions of macromolecules and solvating waters involve modes that are absent in small molecules. More specifically, the hydration of non-polar groups on the surface of proteins is estimated to be different from the low molecular weight model systems. The same authors discussed this topic in more detail in a more recent paper in terms of the 'thermal volume' a volume that results from the thermally induced molecular vibrations. This volume is directly proportional to the isothermal compressibility [65].

#### 2.2.3. Ultrasonic velocimetry

In a seminal paper, Gekko and Hasegawa [24] have estimated the contribution of cavities and hydration to the temperature dependence of the adiabatic compressibility of proteins. Bovine serum albumin and lysozyme show an increase in adiabatic compressibility with increasing temperature. The authors attribute this mainly to a diminished hydration at higher temperature. At lower temperature the compressibility of both proteins becomes negative which is interpreted as a decreased contribution from the cavities.

The adiabatic compressibility of glycyldipeptides increases (becomes less negative) also with increasing temperatures [66]. This was interpreted as a decrease in electrostriction of the water surrounding the charged endgroups as the temperature is increased. Interestingly, the compressibility of dipeptides containing hydrophobic groups is less temperature-dependent.

In both cases it is possible to present an alternative interpretation: the solvent structure changes with temperature thus accounting for the changes in volume change of the hydration.

#### 2.2.4. X-ray crystallography

Frauenfelder and coworkers [20] determined the thermal expansion of metmyoglobin from X-ray data. This refers to the expansion of the space inside the molecular surface and does not include the hydration water. The *linear* thermal expansion coefficient is estimated to be $115\times10^{-6}$/K, a value more than twice that of liquid water ($70\times10^{-6}$/K) but less than that of benzene ($410\times10^{-6}$/K). It is concluded that the expansion is mainly due to the increase in volume of the numerous tiny packing defects in the protein, i.e. the subatomic free volumes between the atoms. The bulk of the overall effect is the separation between secondary structure units as the temperature

increases. In the same temperature range the thermal expansion of ribonuclease A is about 2–3-fold smaller [67].

### 2.3. Heat capacity and entropy fluctuations

In a thermodynamic system with constant T and p, the isobaric heat capacity can be regarded as the measure of the entropy fluctuations of the system [29]:

$$\langle S-\langle S\rangle\rangle^2 = k_B C_p. \quad (13)$$

The partial molar heat capacity has been considered to be composed of intrinsic and hydration contributions. The intrinsic component contains contributions from covalent and noncovalent interactions. It has been shown that about 85% of the total heat capacity of the native state of a protein in solution is given by the covalent structure [68]. Changes in the heat capacity upon unfolding are primarily due to changes in the hydration. A physical picture of entropy fluctuations means changing the conformation between ordered and less ordered structures. This can be achieved by hindered internal rotations, low frequency conformational fluctuations and high frequency bond stretching and bending modes.

## 3. Conformational (plastic) properties

When a protein unfolds or denatures, the thermal expansion, the compressibility and the heat capacity change. This follows from the observation that the reaction volume and enthalpy for the denaturation are temperature- and pressure-dependent. The first experimental evidence for such behavior came from the kinetic studies of Suzuki [10]. A complete thermodynamic description was given by Hawley [11] but the equations have been derived by Bridgman for the general case of temperature and pressure dependence of phase changes [69]. A recent example of such a stability study was published by Masson et al. [15]. The mathematical implications and assumptions that are usually made in the analysis of the data has been recently discussed by Smeller and Heremans [70]. Of particular interest is the cold denaturation of proteins that was predicted on the basis of the model describing the temperature dependence of the reaction enthalpy accounting for the large increase in heat capacity upon denaturation. This has been reviewed in detail by Privalov [71]. As we shall see, the effects of heat, cold and pressure on proteins are interconnected.

### 3.1. Phase diagram for the reversible/irreversible denaturation

#### 3.1.1. Thermodynamic theory

The elliptic phase diagram on the $p$-$T$ plane is characteristic for proteins. Mathematically this kind of shape originates from the fact that second order terms give a significant contribution to the $\Delta G$ ($\Delta G = G_{\text{denatured}}$-$G_{\text{native}}$). Physically these second order terms are proportional to $\Delta\beta$, $\Delta C_p$, $\Delta\alpha$, the changes in the compressibility, the heat capacity and the thermal expansion respectively between the denatured ($D$) and the native ($N$) state of the protein. $\Delta G$, the difference in free energy between $D$ and $N$, can be written in the form of [69,70]:

$$\Delta G = \Delta G_0 - \Delta S_0(T-T_0) - \frac{\Delta C_p}{2T_0}(T-T_0)^2 + \Delta V_0(p-p_0) + \frac{\Delta\hat{\beta}}{2}(p-p_0)^2 + \Delta\hat{\alpha}(p-p_0)(T-T_0) + \text{higher order terms} \quad (14)$$

where $\Delta\hat{\beta}$ in the compressibility factor difference ($\hat{\beta} = \beta V$) and $\Delta\hat{\alpha}$ the difference of the thermal expansion factor ($\hat{\alpha} = \alpha V$) of the denatured and native states of proteins.

The $\Delta G = 0$ curve is an ellipse on the $p$-$T$ plane and it describes the equilibrium border between the native and denatured state of the protein. This curve is known as the phase or stability diagram [10–18]. This is visualized in Fig. 3.

An equation similar to the well known Clausius-Clapeyron equation can be obtained for the slope of the phase boundary curve:

$$\frac{\partial T}{\partial p} = \frac{\Delta V_0 + \Delta\hat{\beta}(p-p_0) + \Delta\hat{\alpha}(T-T_0)}{\Delta S_0 - \Delta\hat{\alpha}(p-p_0) + \Delta C_p\dfrac{T-T_0}{T_0}}. \quad (15)$$

This expression reduces to the classical Clausius-Clapeyron equation when the difference in compressibility, thermal expansion and heat capacity vanish as is

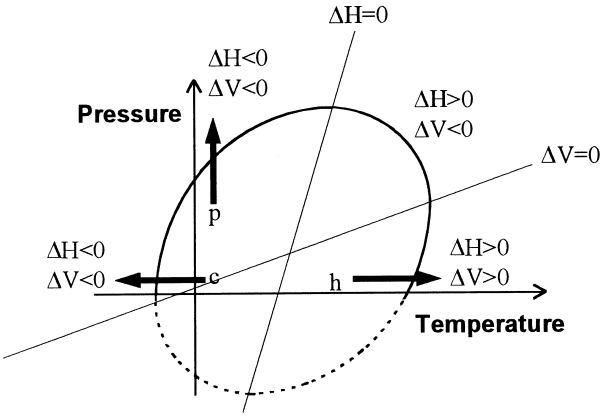


Fig. 3. Elliptic phase diagram of proteins. The protein is stable in its native state inside the ellipse. The arrows denoted by h, p, c show the three most common denaturation ways, i.e. heat, pressure and cold denaturation respectively.

observed for most phase transitions in lipids [72,73]. The shape of the phase diagram for proteins is of considerable interest since, as pointed out in Section 2, it contains information on the volume and entropy fluctuations and on the coupling between volume and entropy fluctuations [28].

### 3.1.2. Kinetic theory

Fig. 3 shows the three common possible ways to denature a protein. These are heat, pressure, and cold denaturation. According to the above thermodynamic description the denaturation could be a reversible process. However, the denatured protein in some circumstances tends to produce a gel stabilized by a network of intermolecular hydrogen bonding, which prevents the refolding to the native spatial structure. Since the equilibrium theory describes the denaturation by treating only two states, the irreversibility is not included in that model. The kinetic theory cannot distinguish between two denatured states, a metastable state where the protein is reversibly denatured ($D$) and an irreversibly inactivated ($I$) one [9]:

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} D \xrightarrow{k_3} I$$

We should keep in mind that this scheme is especially important in the case of experiments where the sample is subjected to high pressure, but where the amount of inactivation is measured after the pressure treatment on the depressurized sample [74]. There are a few special cases of interest regarding the rates of the three conformational transitions.

(i) When the rate constant $k_3 \ll k_1$, $k_2$ then there is an equilibrium between the $N$ and $D$ states (with the equilibrium constant, $K = D/N$), while the $D \rightarrow I$ process is slow. In this case the above described experiment reflects the kinetics of the $D \rightarrow I$ process with an apparent rate constant of $k_{obs}$:

$$k_{obs} = (k_1/k_2)k_3 = Kk_3. \quad (16)$$

(ii) In the opposite limit, ($k_1$, $k_2 \ll k_3$), all the reversibly denatured proteins will be irreversibly captured in an intermolecular network of the reversibly unfolded state. In this case the kinetic experiment reflects the first step of the two-step denaturation process.

## 3.2. Local probes

### 3.2.1. Fluorescence spectroscopy

Weber and Drickamer introduced the fluorescence emission of tryptophan and small molecules bound to proteins to follow the pressureiinduced unfolding of single-chain proteins [75]. It was observed that the tryptophan environment becomes more polar at high pressures and that the volume changes are invariably small ($< 1\%$) compared to the volume of the protein itself. Similarly, fluorescence polarization experiments suggest that, upon unfolding at high pressures, there is considerable penetration of water into the protein interior. Although the observed effects are in most cases reversible, Raman and infrared spectroscopy studies on the same proteins but at higher concentrations revealed irreversible effects from extensive aggregation of the proteins [48,76].

More recent work of the same group has concentrated on the effect of pressure on subunit interactions in multimeric enzymes and proteins [77]. It is invariably observed that pressure dissociates multisubunit proteins into monomers at low pressures ($< 3$ kbar). In many cases the effects are reversible. Of particular interest is the observation that the recovery of the enzyme activity lags behind the reassociation of the subunits. This suggests a 'conformational drift' of the isolated subunits. Dissociation of tetramers and large aggregate, such as viruses, reveals an apparent violation of the law of mass action. This can be understood in terms of deterministic

equilibria in contrast to the classical stochastic equilibria of dimers. The role of deterministic equilibria is even more evident in the case of assembly and disassembly pathway of viruses [5]. The overall conclusion from this work is that pressure affects non-covalent interactions in protein folding, assembly of proteins and viruses. Many additional effects can be explained by assuming that pressure induces a number of intermediate conformations between the folded and unfolded state of proteins. This demonstrates the high plasticity of proteins.

### 3.2.2. UV/visible spectroscopy

The first systematic studies on pressure- and temperature-induced protein unfolding were performed with UV spectroscopy on ribonuclease A [78], chymotrypsinogen [11] and with spectroscopy in the visible region for metmyoglobin [12]. This provided the first systematic thermodynamic description of the stability diagram of a protein as given in Section 3.

Lange and coworkers [79] developed fourth derivative UV spectroscopy as a tool to evaluate changes of the dielectric constant in the vicinity of the aromatic amino acids in proteins which undergo pressure induced structural changes. Thus they detected in ribonuclease A a pressure-induced intermediate that also occurs in the high temperature-induced unfolding [80].

Using the same approach, Mombelli et al. [81] studied the cold, heat and pressure denaturation of ribonuclease P2 and two mutants from the thermophilic archaebacterium *Sulfolobus solfataricus*. The extreme stability of this protein against pressure and temperature treatment is assumed to be due to a hydrophobic core containing three aromatic acid residues. A strong destabilization takes place when $Phe^{31}$ is replaced by $Ala^{31}$. The response of the protein was found to be different towards cold, heat and pressure. The authors suggest that the cold and pressure denaturation may result in intermediate states in which the hydrophobic core is preserved whereas the outer part of the protein undergoes unfolding. The infrared studies on this protein are discussed in Section 3.

### 3.2.3. NMR spectroscopy

The contributions from high NMR spectroscopy for the behavior of proteins under pressure have been reviewed by Jonas and Jonas [82]. In the case of lysozyme it was possible to follow the unfolding from several amino acid residues located in different parts of the protein. As expected this reveals that different parts of the protein show slightly different pressure responses. The cold-induced unfolding of ribonuclease A was studied by Zhang et al. [83] with 1D and 2D $^1$H NMR. In these experiments advantage was taken of the fact that the freezing point of water is depressed by high pressure thus allowing experiments in the liquid state down to $-20$°C at 2 kbar. The experiments suggest that the cold- and pressure-denatured protein may contain partially folded structures that are visible as an intermediate in the heat denaturation.

These results are in contrast to recent NMR and circular dichroism work on a part of the lambda repressor, suggesting that the cold- and the heat-denatured states are thermodynamically and conformationally equivalent in the presence of 3 M urea [84]. It is assumed that the strong temperature dependence of the properties of the water and the way it interacts with the denatured protein is thought to be the primary mechanism of the denaturation. If the interpretation is correct, then it follows that similar conformational changes are expected for pressure-induced unfolding. However, this seems not to be the case as is evident from studies on two different proteins, ribonuclease A and ribonuclease P2, with different experimental approaches.

Hydrogen exchange kinetics of the pressure-assisted cold denaturation of ribonuclease [85] has revealed that this state differs markedly from the temperature and pressure denatured state. It is markedly different from a random coil due to patches of residual secondary structure. The situation is quite different for ubiquitin [86]. Here little secondary structure is found. Hydrogen exchange studied with IR is discussed in Section 3.

## 3.3. Global probes

### 3.3.1. Sound velocity

Tamura and Gekko [87] used precise density and sound velocity measurements to follow the thermal denaturation of ribonuclease A. The apparent molar volume decreased but the adiabatic compressibility increased. Similar observations have been re-

ported for chymotrypsinogen [33]. Compressibility has been proposed as a means to detect and characterize intermediate states in proteins [30,88,89]. More specifically, the compact intermediate state, sometimes called molten globule state, shows an increase in the compressibility compared to the native state.

The change in compressibility between the native and the pressure-induced state has been calculated from the stability diagram as discussed in Section 3. Recently it has been become possible to measure the adiabatic compressibility in situ under pressure [32,34,90]. For amino acids the compressibility increases with increasing pressure. The pressure induced intermediate state of cytochrome *c* at acid pH shows an increased compressibility to the native state. As pointed out by Kharakoz and Bychkova [30], the volume fluctuations for highly hydrated intermediate states cannot be calculated quantitatively without thermodynamic data on the transfer of water into the protein.

### 3.3.2. Vibrational spectroscopy

Raman and infrared spectroscopy allows one to obtain information on the secondary structure from the shape of the amide I band. The first Raman study on the effect of pressure on lysozyme revealed that the high pressure induced unfolding of the molecule is irreversible rather than reversible as observed with fluorescence techniques [48]. A subsequent infrared study on chymotrypsinogen gave similar results [76]. In both cases the high concentrations of protein used (ca. 1 mM), showed that the pressure-induced unfolding can lead to intermolecular interactions, thus giving rise to irreversibility. This phenomenon is well known for temperature-induced denaturation of proteins. The temperature-induced aggregation of proteins gives rise to specific bands in the amide I region of the infrared spectrum that are characteristic for intermolecular hydrogen-bonded structures [91,92]. Typical data for horse heart myoglobin are shown in Fig. 4. Such bands are, in general, not observed for the pressure-induced denaturation of proteins suggesting that the degree of unfolding is smaller than in the temperature-induced unfolding. A notable exception is the pressure-induced unfolding of chymotrypsin reversed micelles, where the bands typical for intermolecular hydrogen bonding develop after the pressure is released [93].

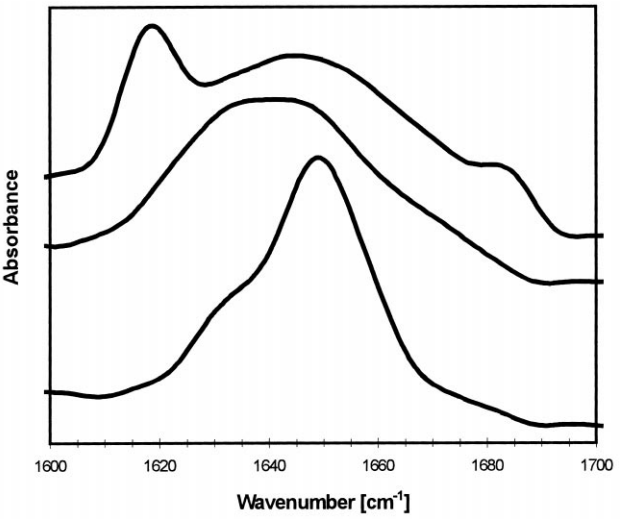


Fig. 4. Amide I′ band of the infrared spectra of pressure and temperature denatured myoglobin (from horse heart). Bottom curve: native protein at ambient pressure, room temperature; middle curve: pressure-denatured (p = 7.4 kbar) at room temperature; top curve: ambient pressure 80°C.

An interesting exception to the previous observation is ribonuclease A. Takeda et al. [94] studied the difference between temperature and pressure-induced denaturation at pH 7 and found no residual secondary structure above 5.5 kbar and 60°C. Both transitions are reversible although the pressure-induced transition shows some hysteresis. It is also suggested that the pressure-induced unfolding goes via an intermediate state. These results get support from the NMR work at pH 2 of Zhang et al. [83]. As discussed in Section 3, the cold and pressure denaturation of ribonuclease A suggest intermediates that are closer to the native state than the unfolded state which is observed in the heat denaturation. This also follows from small angle X-ray scattering experiments as discussed in Section 3. Work in progress on mutants of ribonuclease reveals interesting effects of site directed mutagenesis on the stability of the protein [95]. A Y115W substitution destabilizes the protein by about 1 kbar.

Infrared studies with the high pressure diamond anvil cell indicate a considerable stability for small proteins. Bovine pancreatic trypsin inhibitor (BPTI) shows pressure-induced changes that are reversible even after compression up to 14 kbar [49]. The

changes that take place above 10 kbar are reversible and can also be simulated with high pressure molecular dynamics (see Section 3). Ribonuclease P2 (see Section 3) shows no changes in its structure up to 14 kbar [96]. On the other hand, the site-directed mutation F31A in the hydrophobic core of the protein destabilizes the protein giving a reversible pressure-induced unfolding at 4 kbar.

Hydrogen-deuterium exchange experiments give important information on the protein dynamics as shown by the dependence of the accessibility of the protein interior and the pressure and temperature dependence. Even small proteins such as BPTI and ribonuclease P2 show considerable differences in their exchange rates and accessibility under normal conditions. Whereas P2 exchanges all the hydrogen under normal conditions [96], all hydrogens in BPTI are exchanged after a pressure cycle of 5 kbar [51]. For larger proteins complete unfolding usually brings about a complete exchange of all hydrogens [76,97].

### 3.3.3. Light scattering

Changes in turbidity or light scattering have been used to study the effect of pressure on protein aggregation. Payens and Heremans [98] have studied the effect of temperature and pressure on the aggregation of β-casein. At room temperature the protein forms aggregates that are dissociated by high pressure. This is the expected behavior of protein aggregates under pressure. However, at 1.5 kbar, pressure induces a reversible aggregation which continues up to the highest pressure investigated (3 kbar). At 4°C, the protein is in the monomeric state at atmospheric pressure. Pressure has no effect under these conditions up to 2.5 kbar. At higher pressures reversible aggregation takes place similar to that observed at room temperature. It is suggested that pressure induces a conformation in the protein that, via a not understood mechanism, leads to pressure-induced aggregation. It is not excluded that the mechanism has resemblance with the pressure-induced stabilization of proteins against temperature denaturation.

An example of a pressure-temperature-induced irreversible process is the time-resolved turbidity study during the gelation of egg white by Kanaya et al. [99]. A phase diagram is found similar to that observed for protein denaturation, i.e. pressures below 1.5 kbar inhibit the temperature induced gelation process. Assuming a simple mechanism as proposed for the mechanism of protein denaturation (see Section 3), it is found that at low temperatures, the pressure-induced gelation process is slow compared to the denaturation process, whereas the reverse is true at high temperatures.

### 3.3.4. Small angle X-ray scattering

Small angle X-ray scattering (SAXS) is a technique that provides information about the overall shape and size of macromolecules. In this respect it gives complementary information on the unfolded state of a protein to approaches such as infrared and NMR spectroscopy. Kleppinger et al. [100] used SAXS and FTIR to follow the pressure-induced changes in ribonuclease A and concluded from Kratky plots and distance distribution functions that the protein remains compact up to 8 kbar although the radius of gyration increases slightly at 5 kbar were a pretransition is observed with infrared spectroscopy [48]. The main unfolding step takes place at 7 kbar. For lysozyme small changes in the radius of gyration have been reported to take place at about 4 kbar [101]. Winter and coworkers studied the pressure- and temperature-induced gel formation of β-lactoglobulin with FTIR and SAXS [102]. Under these conditions, 80°C/10 kbar, the radius of gyration increases dramatically. Since the protein is already highly associated under normal conditions, a quantitative analysis of the data is not a simple task.

## 3.4. Computer simulations

With the exception of the normal mode analysis study of the behavior of deoxymyoglobin under pressure discussed in Section 2, all high pressure computer simulations reported so far have been of the molecular dynamics (MD) type. The first high pressure MD simulations on BPTI were reported by Kitchen et al. [103]. No changes in the conformation were detected at 10 kbar, only the increased hydration of certain amino acids was observed. Subsequent MD simulations up to 20 kbar revealed changes in the secondary structure between 10 and 15 kbar [104]. These changes could be correlated with changes in the secondary structure observed with high pressure infrared studies [51]. Van Gunsteren and coworkers [105] studied the unfolding of lyso-

zyme. No net unfolding was observed at 10 kbar after 210 ps. However, fluorescence [75] as well as Raman [48] studies indicate that the protein unfolds at 5 kbar. Presumably, the origin of the absence of unfolding in the computer experiments might be kinetic. A central problem in MD calculations is the definition of the volume that gives the best agreements with experimental results. It was reported recently that the Voronoi volume and its related computed compressibility agrees best with the experimental intrinsic compressibility [23].

## 4. Outlook

What is the general picture that emerges from nearly a century of pressure studies on proteins? Although the prediction of the behavior of one particular protein is still far away, a number of simple physical factors that control the pressure behavior is becoming clear.

At low pressures ($<1$–3 kbar), one may assume that the compression of the cavities and an increased hydration is the main effect. This pressure regime can be studied by a number of experimental approaches. However, the connection between these approaches is not always clear. One of the most promising techniques is the compressibility as obtained from ultrasonic velocimetry. Gekko and coworkers have recently shown that changes in the compressibility can be found for protein mutants. Since volume fluctuations are proportional to the isothermal compressibility, changes in the compressibility reflect changes in the dynamic behavior of the mutant protein [106].

The role of the solvent in protein behavior is giving very useful insights into the dynamics. Studies by Priev et al. [107] have shown that glycerol decreases the compressibility of the protein interior. These studies suggest a role for water as a lubricant for the conformational flexibility of proteins.

Probing the difference between the pressure, heat and cold denaturation/unfolding is not easy in view of the possible side effects that results from the unfolding that make a simple analysis not straightforward. Heat denatured proteins are extremely prone to aggregation. This is especially clear from the infrared spectra where the formation of intermolecular hydrogen bonding gives rise to specific bands in the amide I region. As indicated, these specific structures may also occur, under certain conditions and with certain proteins, after compression. It remains to be investigated whether these effects are protein specific or not. It is also of particular interest that certain water soluble polymers show pressure-temperature phase diagrams similar to those of proteins [108].

The effect of various cosolvents on protein denaturation has been investigated by Timasheff [109]. Although the number of pressure studies are limited, it seems that the stabilizing effect against temperature denaturation are also found against pressure denaturation [48]. Kinetic studies under pressure of the folding of staphylococcal nuclease in the presence of xylose show that the sugar effect is primarily on the folding step suggesting that the transition state, a dry molten globule state, is close to the folded state [110].

Finally, one may ask the question whether the pressure-temperature behavior of proteins is unique among biomacromolecules. Hayashi and coworkers have shown that starch also forms a gel by the application of pressure [111]. This suggests that proteins and starch show a similar behavior with regard to temperature and pressure. This raises the question on the presumed role of hydrophobic interactions in the stability of proteins.

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