Protein Compressibility, Dynamics, and Pressure

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ABSTRACT The relationship between the elastic and dynamic properties of native globular proteins is considered on the basis of a wide set of reported experimental data. The formation of a small cavity, capable of accommodating water, in the protein interior is associated with the elastic deformation, whose contribution to the free energy considerably exceeds the heat motion energy. Mechanically, the protein molecule is a highly nonlinear system. This means that its compressibility sharply decreases upon compression. The mechanical nonlinearity results in the following consequences related to the intramolecular dynamics of proteins: 1) The sign of the electrostriction effect in the protein matrix is opposite that observed in liquids—this is an additional indication that protein behaves like a solid particle. 2) The diffusion of an ion from the solvent to the interior of a protein should depend on pressure nonmonotonically: at low pressure diffusion is suppressed, while at high pressure it is enhanced. Such behavior is expected to display itself in any dynamic process depending on ion diffusion. Qualitative and quantitative expectations ensuing from the mechanical properties are concordant with the available experimental data on hydrogen exchange in native proteins at ambient and high pressure.

INTRODUCTION

The dynamics of protein molecules is described in terms of a multidimensional energy hypersurface with multiple states and substates (Frauenfelder et al., 1990; Leeson and Wiersma, 1995), as the molecule consists of a large number of atoms rather flexibly linked. However, because of the large number of degrees of freedom in such systems, many of their general dynamic properties can be successfully described in terms of bulk quantities—such as packing density, compressibility, or other coefficients of elasticity, etc.—which are typically applied to macroscopic systems. The protein molecule is a nearly macroscopic system; the validity of such an assumption has been widely demonstrated in the literature (Blumenfeld, 1974; Shakhnovich and Finkelstein, 1989).

The elastic properties of proteins are directly related to the mechanical part of the free energy and to the frequencies and amplitudes of intramolecular motions (Jacobson, 1947; Cooper, 1976; Morozov and Morozova, 1986, 1990, 1993; Kharakoz, 1990; Kobayashi et al., 1997). The elastic strain of proteins can serve as a reservoir of energy in the intermediate steps of a catalytic pathway (Chernavskii et al., 1967; Blumenfeld, 1974). Creation of internal cavities associated with a deformation of the protein interior is involved in a number of intramolecular dynamic processes, including hydrogen exchange (Richards, 1979; Woodward et al., 1982; Abaturov et al., 1983), the ion-mediated quenching of fluorescence (Calhoun et al., 1983; Shlyapnikova et al., 1986), and aromatic ring flips (Wagner, 1982). Therefore, these phenomena must depend on protein rigidity.

Not only the elasticity coefficients themselves but also their dependence on compression—the mechanical nonlinearity—is an important feature determining protein dynamics. The pressure dependence of protein compressibility has become a matter of increasing interest in the last decade. A number of experimental works and computer simulations on this subject have appeared to date (Sarvazyan, 1991; Sarvazyan and Chalikian, 1989; Chalikian, 1989; Suzdalev et al., 1991; Panchenko and Shaitan, 1992; Paci and Marchi, 1996; Paci and Velikson, 1997). However, the dynamic consequences of mechanical nonlinearity have not been considered so far.

As seen from this overview, the elasticity-dynamics relationship is an old matter of interest to protein scientists. A large body of experimental data has been accumulated, and reasonable qualitative ideas have been suggested. Nonetheless, this relationship remains poorly investigated quantitatively. Furthermore, even on the qualitative level, some elasticity-related aspects of dynamics are still not understood.

Many of the dynamic features of proteins follow from the fact that, by its elastic and other physical properties, the native protein globule resembles a solid particle (reviewed by Morozov and Morozova, 1990, 1993). In this paper, the available experimental data on the intrinsic compressibility and mechanical nonlinearity of protein molecules and their relation to intramolecular dynamics are critically reviewed. Only one type of nonlinearity is of concern here—the dependence of the compressibility and related elasticity coefficients on bulk compression. The following particular aspects are discussed: 1) the energy of elastic deformation induced by creating an internal cavity and its dependence on local packing defects; 2) the unusual sign of the electrostriction effect in the protein matrix, which is considered an additional indication of "solid-likeness" of the protein molecule; 3) the volumetric effects of transfer of small ions

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from the bulk water phase into the protein matrix and the nonmonotonic pressure dependence of the transfer, and 4) hydrogen exchange in proteins at ambient and elevated pressure as compared with the expectations from the elastic properties.

A general remark on terminology should be made. By "elasticity" I mean the ability of a system to undergo a reversible and gradual change in its shape or density. Elastic deformation proceeds through two more or less distinguishable steps: upon applied stress, a system undergoes an instantaneous change of interatomic distances followed by a structural relaxation prolonged in time. This point must be stressed here because the term "elastic" is often used in a narrow sense of the word, to denote only instantaneous changes. Although the deformation proceeds through many elementary steps on the level of molecular structure, it is elastic until remains reversible on the time scale considered. For example, rubber is a highly elastic material, although its stretching evidently proceeds through a multitude of molecular events.

NONLINEAR COMPRESSIBILITY OF PROTEINS

Theoretical background

The isothermal compressibility coefficient, β, characterizes the ability of a system to undergo a change in bulk volume, V, or density, ρ, upon pressure, P. By definition,

$$\beta \equiv -(\partial \ln V/\partial P)_T \equiv (\partial \ln \rho/\partial P)_T, \quad (1)$$

where T is the temperature. Other elasticity constants, Young's modulus, M_Y, and the shear modulus, M_S, are related to compressibility by standard equations valid for isotropic materials:

$$M_Y = \beta^{-1} \times 3(1 - 2p) \quad (2)$$

and

$$M_S = \beta^{-1} \times (3/2)(1 - 2p)/(1 + p), \quad (3)$$

where p is Poisson's ratio (Poisson's ratio in an isotropic material is the ratio of the relative decrease of a sample in a transverse dimension to a relative increase in the longitudinal dimension along which a deforming force is applied).

A characteristic feature of condensed matter is a highly nonlinear response to mechanical disturbance. This means, in particular, that the increment of density is not proportional to applied pressure. Over a wide range of temperature and pressure, the nonlinearity follows the Moelwyn-Hughes' isotherm (Moelwyn-Hughes, 1961), which is presented below in two equivalent forms:

$$(\partial \ln \beta/\partial \ln V)_T \equiv -(\partial \ln \beta/\partial \ln \rho)_T = \mu, \quad (4a)$$

or

$$(\partial \beta^{-1}/\partial P)_T = \mu. \quad (4b)$$

Here μ is Moelwyn-Hughes' nonlinearity index, a dimensionless parameter that is virtually invariant with changes in pressure and temperature. (In physical and medical acoustics, another parameter is widely used to characterize the nonlinear properties (Sarvazyan, 1991): B/A, the ratio of the second to the first virial coefficient in the power series $P = P_0 + A(\rho - \rho_0)/\rho_0 + (B/2)(\rho - \rho_0)^2/\rho_0^2 + \ldots$ It can easily be shown that $\mu = B/A + 1$.) The nonlinearity index is directly related to the anharmonicity of intermolecular interactions. This is clearly seen when the interaction is approximated by the Mie potential: $\varphi = ar^{-n} - br^{-m}$ (where r is the distance between the centers of particles). In this case $\mu = (m + n + 6)/3$ (Moelwyn-Hughes, 1961). Therefore, the value μ is equal to 8 for a Lennard-Johns system (when $m = 6$ and $n = 12$) and equal to 1 for a harmonic system (when $m = -1$ and $n = -2$). Experimental values for organic materials, whether liquid or solid, range between 6 and 11 (see Table 1).

Assuming p = const., one finds similar relations between the nonlinearity index and other elasticity constants:

$$\partial M_Y/\partial P = \partial M_S/\partial P = \partial \beta^{-1}/\partial P = \mu \quad (5)$$

(hereinafter, the index T is omitted when isothermal conditions are meant).

The so-called Grüneisen constant, γ, is also widely used to characterize anharmonicity in solids (Landau and Lifshitz, 1995), including organic polymers (Perepechko, 1977). Within the framework of Debye's theory of solids, this constant is defined as

$$\gamma = -\partial \ln \omega/\partial \ln V, \quad (6)$$

where ω corresponds to the frequencies of phonons in the solid (the Grüneisen constant, thus defined, may differ considerably from that calculated by means of a commonly used relation of thermodynamic coefficients; see discussion below). A relation between the two characteristics of anharmonicity for an isotropic solid is given by the expression

$$\mu = 2\gamma + 1/3, \quad (7)$$

derived in Appendix 1.

Experimental data

The protein interior is not uniformly compressed under the action of pressure; there is a wide distribution of rigid and soft elements in the protein structure (Kundrot and Richards, 1987; Kobayashi et al., 1997; Li et al., 1998). Here, however, only the bulk compressibility of the molecule as a whole is considered. To date, a number of experimental approaches to the estimations of the intrinsic compressibility of proteins have been employed. The results are summarized in Table 1.

TABLE 1 The compressibility coefficient (β, Mbar^{-1}) and mechanical nonlinearity index ($\mu = \partial\beta^{-1}/\partial P$) of a native protein molecule, compared to macroscopic organic materials under normal conditions

Substance and method	β	Reference*	μ	Reference*
Native protein molecules				
Acoustic measurements in protein solutions	10–25†	1	12, if $\beta = 25$	2
NMR spectroscopy of pancreatic trypsin inhibitor in solution	15‡	3		
Electrostriction of cytochrome *c* in solution			12, if $\beta = 25$	4
Dynamic Young's modulus of protein crystals	10–20§	5		
Pressure dependence of Rayleigh scattering of Mössbauer radiation in a wet powder of human serum albumin			7, if $\beta = 25$	6
Pressure dependence of the unit-cell volume in lysozyme crystals	10–20¶	7	(0–10)‖	8
Molecular dynamics simulation in superoxide dismutase	20 ± 4**	9	10 ± 1**	9
Computation of normal modes in TIM-barrel protein	16	10		
Organic materials††				
Liquids	22–130	11	6–11	11
Solids, including molecular crystals, hard polymers, and soft polymers	6.5–40	12	7–11	13
Only hard polymers	12–20	13	≈7‡‡	14
Ideal reference systems§§				
A Lennard-Johns system ($m = 6$, $n = 12$)			8	
An ideal harmonic system ($m = -1$, $n = -2$)			1	
Ideal gas			1	
A hypothetical system in which β is independent of compression			0	

*The references to the literature sources are specified as follows. 1: Jacobson (1950); Sarvazyan and Kharakoz (1977); Gekko and Noguchi (1979); Gavish et al. (1983); Kharakoz and Sarvazyan (1993); Chalikian et al. (1996); Priev et al. (1996). 2: Sarvazyan and Chalikian (1989); Chalikian (1989). 3: Li et al. (1998). 4: Kharakoz and Mkhitaryan (1986). 5: Morozov and Morozova (1993). 6: Suzdalev et al. (1991); Panchenko and Shaitan (1992). 7: Kundrot and Richards (1987); Katrusiak and Dauter (1996). 8: Katrusiak and Dauter (1996). 9: Paci and Marchi (1996); Paci and Velikson (1997). 10: Kobayashi et al. (1997); 11: Moelwyn-Hughes (1961); Kaporovskii et al. (1985). 12: Vinogradov (1991); Kaporovskii et al. (1985). 13: Vinogradov (1991). 14: Perepechko (1977).

†The range of values reflects mainly the differences in the methods employed to evaluate the protein hydration contribution to the measured compressibility of solution.

‡Calculated from a pressure-induced shortening of hydrogen bond length; pressures 0–2 kbar.

§Assuming the Poisson ratio to be within 0.33–0.41, as in glassy polymers (Perepechko, 1977).

¶Extrapolated to ambient pressure by means of Moelwyn-Hughes' isotherm, Eq. 4b.

‖Reported data contain a large uncertainty.

**The volume compressions, determined in the original works by means of the Voronoi polyhedra method, were approximated here by Moelwyn-Hughes' isotherm, Eq. 4b.

††Obtained by means of the mechanical or acoustic measurements.

‡‡In addition, the data on the Grüneisen constant of these polymers at the temperature 240 K have been reported (Perepechko, 1977), from which the range μ = 1.5–11 is obtained by means of Eq. 7. However, the reported Grüneisen constants have been determined by means of Eq. 9, which often gives substantially underestimated values for the Grüneisen constant because of structural relaxation in polymers (see text, under A Comment on the Definition of the Grüneisen Constant . . .).

§§As follows from the physical meaning of μ (see text, under Theoretical Background).

Most of these values were derived from ultrasonic measurements in aqueous solutions, at frequencies on the order of 10^7 MHz (Jacobson, 1950; Sarvazyan and Kharakoz, 1977; Gekko and Noguchi, 1979; Gavish et al., 1983; Kharakoz and Sarvazyan, 1993; Chalikian et al., 1996; Priev et al., 1996). This method allows measurement of the partial compressibility of proteins, the negative pressure derivative of partial volume. To a first approximation, the partial compressibility is composed of an intrinsic term and a contribution from the hydrated surface. Therefore, evaluation of the intrinsic compressibility essentially depends on the approach used to evaluate the hydration contribution (Kharakoz and Sarvazyan, 1993; Chalikian et al., 1996; Paci and Velikson, 1997). Different approaches give intrinsic

compressibility values ranging from 10 to 25 $Mbar^{-1}$. The difference between the isothermal and the acoustically determined pseudoadiabatic compressibility of the protein interior is negligible (Kharakoz and Sarvazyan, 1993) and is ignored here.

An independent estimate of the compressibility can be obtained from the dynamic (measured at frequencies of tens of kHz) Young's modulus of the protein molecule, $M_Y = 0.05$ Mbar. This value has been determined (Morozov and Morozova, 1993) from data on the elasticity of protein crystals. Assuming Poisson's ratio to be within $p = 0.33$–0.41 (the range characteristic of glassy polymers; Perepechko, 1977), one obtains from Eq. 2 that $\beta = 10$–20 $Mbar^{-1}$.

High-pressure NMR spectroscopy makes it possible to characterize quantitatively small displacements of atoms in proteins (Akasaka et al., 1997; Li et al., 1998; Inoue et al., 1998). It has been reported that the length of hydrogen bonds in pancreatic trypsin inhibitor under elevated pressure (up to 2 kbar) reduces linearly with an average coefficient of -5×10^{-6} bar^{-1} (Li et al., 1998). In terms of volume compression, this corresponds to $\beta = 3 \times (5 \times 10^{-6}) = 15$ $Mbar^{-1}$. Note that this value reflects the compressibility of relatively rigid hydrogen-bonded elements and, hence, should be regarded as a lower bound for the intrinsic compressibility. The accuracy of the method did not allow the authors to detect the nonlinearity in the pressure range explored.

The compressibility of lysozyme crystals of two different crystallographic groups was studied by means of x-ray diffraction under high pressure (Kundrot and Richards, 1987; Katrusiak and Dauter, 1996). A pressure-induced contraction of the unit-cell volume was monitored. The contraction corresponded to a bulk compressibility of 10 $Mbar^{-1}$, in the work by Kundrot and Richards, and to one on the order of 20 $Mbar^{-1}$ in the work by Katrusiak and Dauter (the latter value was calculated here by an extrapolation of the reported data to ambient pressure). The protein crystal is a porous system filled with water, which freely diffuses into and out of the crystal. Hydrostatic pressure is thus almost uniformly distributed within the pores, and the constituent protein molecules undergo a bulk compression. In this case, if the applied pressure does not cause an essential repacking in the crystal structure, the relative contraction of the unit cell is equivalent to that of the constituent molecules. Therefore, the above values ($\beta = 10$–20 $Mbar^{-1}$) can be considered a close approximation of intrinsic protein compressibility. A lower x-ray-based estimate, 5 $Mbar^{-1}$, was obtained by Kundrot and Richards when they calculated the contraction of the protein as a change in its molecular volume, determined by the so-called rolling-ball method. This low value is excluded from our consideration because the "rolling-ball" method probably systematically underestimates protein contraction, as reported recently (Paci and Velikson, 1997).

From inspection of the data collected in Table 1, one finds that the upper estimate for proteins, 25 $Mbar^{-1}$, lies where the ranges of compressibility variations for solid polymers and low-compressible liquids overlap (see Table 1). Therefore, compressibility hardly allows one to characterize the state of the native protein molecule. The Young's and shear moduli, which are more sensitive to the physical state of matter, are more informative. They differ by several orders of magnitude between the hard and soft polymers and drop to zero when solids melt. It has been shown (Morozov and Morozova, 1990, 1993) that the dynamic Young's modulus of protein molecules, $M_Y = 0.05$ Mbar, is as high as those of glassy polymers and molecular crystals.

The Moelwyn-Hughes' nonlinearity index of proteins has never been estimated. There are a few experimental studies presenting data from which this can be done.

The partial specific compressibility of a number of proteins in solution has been studied in the range of pressures up to 1 kbar (Sarvazyan and Chalikian, 1989; Chalikian, 1989). From the data obtained, an average value of the pressure derivative of the intrinsic specific compressibility, $\partial k/\partial P$, has been determined (by definition, $k \equiv \beta v$, where v is the intrinsic specific volume of the protein). To do this, the hydrational contribution to the measured partial quantities was eliminated by means of two independent approaches: "regression" and "additivity" (for the formulation of the approaches see Kharakoz and Sarvazyan, 1993). These approaches yielded the same result: $\partial k/\partial P = -(5.5 \pm 1) \times 10^3$ $Mbar^{-2}$ cm^3 g^{-1}. Differentiation of the definition $k \equiv \beta v$ yields $\mu \equiv \partial(1/\beta)/\partial P = -(1/\beta^2 v)(\partial k/\partial P) - 1$. With good accuracy, $v = 0.7$ cm^3/g (Klapper, 1971; Richards, 1977; Pavlov and Fedorov, 1982). Therefore, the nonlinearity index can be calculated from a numerical expression,

$$\beta^2(\mu + 1) = (7.5 \pm 1.5) \times 10^3 \text{ Mbar}^{-2}, \qquad (8a)$$

given that the β value is known (see below for the results).

The nonlinearity index can also be approximately estimated from the redox-transition-induced changes in the intrinsic volume and compressibility of cytochrome *c* (Kharakoz and Mkhitaryan, 1986). Two independent methods employed in the cited work lead to similar results for the intrinsic properties: an increase in specific volume, Δv, of 0.0004–0.0012 cm^3/g is associated with an increase in specific compressibility, Δk, of 0.23–0.26 $Mbar^{-1}$ cm^3 g^{-1}. Thus, taking into account the definition $k \equiv \beta v$, and assuming that electrostatically induced compression follows isotherm 4, one obtains

$$\beta\mu = \Delta k/\Delta v = 300 \text{ Mbar}^{-1}. \qquad (8b)$$

(The latter estimate is valid within an order of magnitude, as the volumetric changes have been measured with a large uncertainty.)

The third possibility for evaluating the nonlinearity index is provided by the data on Rayleigh scattering of Mössbauer

radiation measured in a sample of human serum albumin under varying hydrostatic pressure (Suzdalev et al., 1991). In the framework of Debye's model of solids, the pressure dependence of the scattering is determined by the product of the compressibility coefficient and the Grüneisen constant. It follows from the experimental data reported that $\gamma\beta = (75 \pm 10)$ Mbar^{-1}. Then, according to Eq. 7,

$$\beta(\mu - 1/3) = (150 \pm 20) \text{ Mbar}^{-1}. \quad (8c)$$

In principle, the nonlinearity index could be directly determined by means of x-ray crystallography under high pressure. Unfortunately, in the only work (Katrusiak and Dauter, 1996) where the x-ray diffraction was measured over a wide range of pressures, up to 10 kbar, the accuracy of the data proved to be insufficient for this purpose. Likewise, the high-pressure NMR measurements (Li et al., 1998) are still not precise enough to detect the nonlinearity. Therefore, at present we can rely only on the data summarized in Eqs. 8a–c.

Most likely pair of β and μ

A large uncertainty in intrinsic compressibility results in an equally large uncertainty in the estimation of the nonlinearity index (see Eqs. 8a–c). Fig. 1 shows the lines of admissible values of the pairs μ and β, drawn according to Eqs. 8a–c. The lines converge in a range close to the maximum feasible value of the compressibility coefficient, 25 Mbar^{-1}.

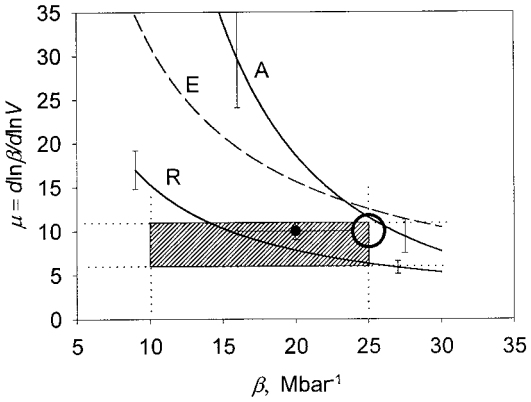

FIGURE 1 The lines of admissible values for the pairs μ and β, according to Eqs. 8a–c. Curve *A* corresponds to the data on acoustic measurements (Eq. 8a). Curve *E* corresponds to the data on electrostriction effect in cytochrome *c* (Eq. 8b) (this curve is drawn with a dashed line to stress the fact that the electrostriction effect has been measured with low accuracy; see text). Curve *R* corresponds to the data on Rayleigh scattering of Mössbauer radiation (Eq. 8c). The shaded area is restricted, along the abscissa, by the range of experimentally observed values of the intrinsic compressibility of proteins, and, along the ordinate, by the range of nonlinearity indexes of the organic materials, including liquids, molecular crystals, and polymers (Table 1). The filled circle shows the results of a computer simulation of molecular dynamics (Table 1). The open circle represents the most likely pair of values β and μ, the point where the data of all methods converge.

Therefore, the combined analysis of μ and β allows both an estimate for the extent of nonlinearity and a refinement of the value of intrinsic compressibility. For further calculations, we assume the values $\beta_0 = 25$ Mbar^{-1} and $\mu = 10$ (index 0 refers to normal pressure) are the most consistent with each other for the different experimental approaches (see Fig. 1).

However, I would like to warn the reader away from considering the obtained estimates as final. In all of the experimental methods used, the primary data are interpreted under simplifying assumptions. Not all of the factors contributing to the measured quantities are sufficiently investigated so far. In particular, a possible role of water as a plastifier is not yet clear. However, there are indirect indications that water might play a role other than just the hydration of the protein surface. For instance, water can diffuse into and out of native protein molecules (Kornblatt and Hoa, 1990; Kornblatt et al., 1998), thus contributing to the relaxation part of compressibility. It has been found recently in studies of proteins in the mixtures of water with polyols and carbohydrates (Priev et al., 1996; Almagor et al., 1998) that the intrinsic compressibility of proteins may decrease by more than 30% when the activity of water is reduced. Similarly, the mechanical compliance of protein crystals sharply decreases upon the drying of the crystal (Morozov and Morozova, 1993) or replacement water with other solvents (Pozharski 1998). Therefore, the obtained values of the intrinsic compressibility and nonlinearity index are expected to be refined in future. Especially promising are the methods of x-ray diffraction and NMR spectroscopy under high pressure, combined with theoretical approaches, as such methods provide specific information on particular elements of protein structure.

Comparison with theoretical works

Among the works on molecular dynamics simulation of proteins under high pressure, we refer to the most recent one (Paci and Marchi, 1996; Paci and Velikson, 1997), in which the simulation was performed at pressures varied between 1 bar and 10 kbar. The authors have shown that evaluation of the intrinsic compressibility essentially depends on the definition of the intrinsic volume. They reported that the most reasonable results on compression are obtained when the intrinsic volume is determined by means of Voronoi polyhedra tessellation of the protein structure, while the use of the excluded volume determined by means of the so-called rolling-ball method brings about a greatly underestimated compression. It follows from their data, treated with Eq. 4, that $\beta_0 = 20$ Mbar^{-1} and $\mu = 10$. These results are close to our estimate from experimental data.

Computation of normal modes (Yamato et al., 1993; Kobayashi et al., 1997) has shown that the local compressions (analyzed by means of Delauney tetrahedra tessellation) vary within one order of magnitude, the compressibil-

ity of the whole molecule being 15–17 $Mbar^{-1}$. This result is also within the range of experimental data.

The theoretically obtained compressibilities are lower than our estimate of the most likely value. This is probably due to limitations in the theoretical approaches. Simulation of molecular dynamics is limited by very short times and cannot monitor slow conformational movement. The method of normal modes enables one to study only the harmonic components of the motions.

A comment on the definition of the Grüneisen constant and experimental data on Rayleigh scattering of Mössbauer radiation

The Grüneisen constant is related to the anharmonicity of systems, indicating how fast the frequency of molecular motions changes upon compression (see Eqs. 6 and 7). The first attempt to estimate this property for the protein interior was undertaken by Morozov and Morozova (1990). They obtained a very low value, $\gamma = 0.6$. However, the nonlinearity index estimated above, $\mu = 10$, shows a high anharmonicity of protein. When expressed in terms of the Grüneisen constant, it is $\gamma = (\mu - 1/3)/2 \approx 5$. Such a high anharmonicity is known for some polymers (e.g., for nylons) (Perepechko, 1977). Why such a great difference between our estimate and that obtained by Morozov and Morozova?

These workers calculated γ by means of the original and commonly used definition, which is also known as Grüneisen's law:

$$\gamma = \alpha/\rho c\beta, \tag{9}$$

where α is the coefficient of thermal expansion at constant pressure and c is the specific heat capacity at constant volume. Defined as such, the constant generally cannot be considered a characteristic of the anharmonicity of a system, unless Debye's model of the solid state is applicable to the system under consideration. In real polymers at normal temperature, there is a substantial contribution of structural relaxation to the thermodynamic coefficients measured. It is important to note that relaxation processes always contribute positively to c and β, while their contribution to α may be both positive and negative. (This statement can be clarified by a qualitative consideration of a polymer having a number of equilibrium substates that differ from each other in their partial volume and enthalpy. When a pressure is applied, first the substates themselves are compressed according to their partial compressibilities—this causes the instantaneous part of compression. Then, according to thermodynamic principles, the system relaxes in such a way that the equilibrium shifts toward the denser substates, resulting in additional compression. Therefore, the structure relaxation always contributes positively to the compressibility. Similarly, if the temperature is increased, the equilibrium shifts toward the substates with higher enthalpy, resulting in additional heat absorption. Therefore, the structure relaxation can only increase the heat capacity of the system. At the same time, a higher enthalpy of a substate does not necessarily correspond to a higher volume. Therefore, a temperature-induced shift of the equilibrium between the substates can result in expansion and contraction of total volume. This qualitative consideration is valid for structural relaxation of any kind.) Therefore, there is a high probability for γ to be underestimated when calculated with Eq. 9. Indeed, it is known that, when applied to polymers, Eq. 9 often yields substantially lower values of Grüneisen constant in comparison to those obtained from directly measured pressure dependence of compressibility or sound velocity (Perepechko, 1977). One can also refer to a clear example illustrating the above statement. Formally, Eq. 9 can be applied to liquid water at temperatures ranging within 0–4°C. This is the range of the density anomaly of water, where $\alpha < 0$, and thus, one obtains a negative value of γ. It is well known the negative sign of α, which is due to the highly pronounced relaxation of water structure, and it is obvious that the negative sign of γ is physically meaningless if γ is regarded to be a characteristic of anharmonicity. As for proteins, an indirect indication that Morozov and Morozova used an underestimated value of the thermal expansion coefficient ($\alpha = 1.4 \times 10^{-4}\ K^{-1}$) comes from a recent volumetric work by Chalikian et al. (1996), who have studied 15 globular proteins and found that the average intrinsic thermal expandability of proteins is twice as great, $\alpha = 3 \times 10^{-4}\ K^{-1}$.

The underestimated anharmonicity of protein molecules caused a misinterpretation of the data on the pressure dependence of intramolecular motions, studied by means of Rayleigh scattering of Mössbauer radiation (Suzdalev et al., 1991; Panchenko and Shaitan, 1992). When analyzed in terms of the solid state, the observed pressure dependence yielded the value $\gamma\beta = 75\ Mbar^{-1}$. This value was an order of magnitude greater than the authors expected for a solid with $\gamma = 0.6$. This observation seemed to be inconsistent with the solid-state model of native proteins. However, the experimental data presented above (under Experimental Data) reveal that, quite the contrary, the value $\gamma\beta = 75\ Mbar^{-1}$ is even lower than expected from independent data on the nonlinear protein compressibility (compare the *curves* in Fig. 1). Therefore, the pronounced pressure dependence of Rayleigh scattering does not contradict the solid-state interpretation but reflects the highly nonlinear compressibility of native proteins.

COMPRESSIBILITY AND THE ENERGY OF EXPANSION OF A CAVITY

Energy of elastic deformation

Formation of a cavity in a solid is accompanied by a deformation in the surrounding medium. To calculate the

energy of deformation let us assume it is purely elastic. Ignoring the nonelastic component results in an overestimation of the energy. Therefore, we will obtain a maximum estimate.

For elastic deformation, Frenkel (1946), in his study of defects in solids, used an approximate formula for the reversible work, W, against elastic forces applied to an expanded cavity: $W = 8\,\pi N_A M_S \times r_1(r_2 - r_1)^2$, where M_S is the shear modulus of the solid medium, and r_1 and r_2 are the initial and final radii of the cavity, respectively. Avogadro's number, N_A, appears to express the work per mole of cavities, for further convenience. To apply this formula to the cavities of molecular dimension, one should take into consideration the following point. To the extent that intermolecular interactions are considered to represent the central forces, it can be reasonably assumed that the elastic force is applied to a surface drawn through the centers of the atoms contacting the cavity. This means, in the case of a cavity capable of accommodating a hard sphere of radius r, that the elastic force is applied to a sphere of radius $r + \delta$, where δ is the average radius of the surrounding atoms. Therefore, the term $(r + \delta)$ should be written in place of r. Also taking into account Eq. 3, one writes

$$W = 8\pi N_A M_S \times (r_1 + \delta)(r_2 - r_1)^2 = A\beta^{-1} \times (r_1 + \delta)(r_2 - r_1)^2, \quad (10)$$

where $A = 12\pi N_A(1 - 2p)/(1 + p)$.

This equation has been derived under simplifying assumptions, in particular, that the solid material is isotropic, the relative deformation is small ($\Delta r/r \ll 1$), and the deformation is purely elastic. Moreover, the proximity of the cavity to the protein surface was not taken into account. Therefore, Eq. 10 can give us only an idea of the magnitudes of deformation energy in proteins. A more accurate consideration will be presented elsewhere.

There is a wide size distribution of cavities in the static structure of proteins (Rashin et al., 1986; Hubbard et al., 1994). They vary from $r_1 = 0.05$ nm, in close-packed regions, to $r_1 \geq 0.17$ nm, for large packing defects. For the final size of an expanded cavity, r_2, we adopt the value 0.17 nm, which is equal to the effective van der Waals radius of water. This value is larger than the commonly used one, 0.14 nm; the reasons for the difference were discussed earlier (Kharakoz, 1992). The water radius is used because one purpose of this paper is to analyze the permeation of water or its ions into the protein interior (see below). Let us assume that $M_S = 0.015$ Mbar (this corresponds to $\beta = 25$ Mbar^{-1} and a Poisson ratio of 1/3). Then Eq. 10 gives the energy ranging from $W = 65$ kJ/mol, in the close-packed regions ($r_1 = 0.05$ nm), to $W = 0$, in the highly defective ones ($r_1 \geq r_2$), with an average $W \approx 30$ kJ/mol. The variation in the energy reflects the size distribution of preexisting cavities (cf. Fig. 2).

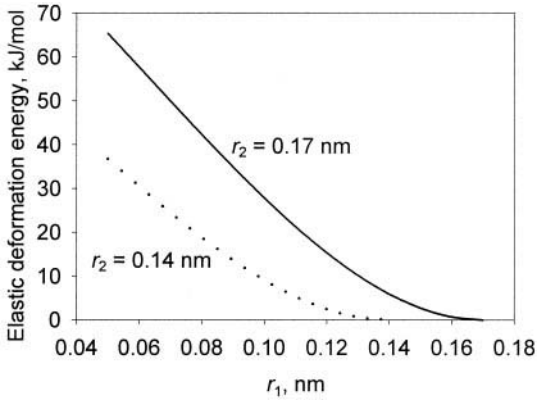

FIGURE 2 The energy of elastic deformation in the protein interior caused by the expansion of the internal cavity from an initial radius r_1 to the radius $r_2 = 0.17$ nm. The latter corresponds to an approximate van der Waals radius of water and OH^- ion. For comparison, a curve is shown for $r_2 = 0.14$ nm that is commonly used to represent water (*dotted line*). Calculated with Eq. 10.

Therefore, the free energy of deformation can exceed the thermal motion energy (2.5 kJ/mol at normal temperature) by one order of magnitude. Hence the elasticity of proteins should play an essential role in any processes associated with the transfer of small particles in the interior. This conclusion agrees with the experimental data on the kinetics of hydrogen exchange in proteins (discussed below). It is worth comparing our estimate with independent data on cavity formation in proteins and model systems.

Other data on the energy of cavity formation

The scaled particle theory allows one to calculate the free energy of cavities in nonpolar liquids. It follows from the theory that the energy is 15 kJ/mol for a cavity capable of accommodating a particle with a radius equal to that of water (Pierotti, 1963).

Molecular dynamics simulation in liquids (Kocher et al., 1996) gives similar results. For a cavity capable of accommodating a hard sphere of radius 0.16 nm, the energetic cost is 15 kJ/mol, in the case of hexane, and 23 kJ/mol, in the case of water. As far as liquids are considered, these values represent mainly the energy of "surface tension" on the boundary of the cavity because there is no contribution from shear deformation in liquids. Therefore, the values can serve as a measure of "surface" contribution in the case of cavities in protein.

In the same work (Kocher et al., 1996), molecular dynamics simulation has been applied to globular proteins. It has been found that, in the protein interior, the average free energy of a cavity of radius 0.16 nm amounts to 40 kJ/mol (extrapolated from the reported data). To a first approximation, this value consists of two parts: the "surface" component and the component resulting from elastic deformation.

Given that "surface tension" contributes 15–23 kJ/mol, as just discussed, one finds that the deformation gives a substantial contribution of ~20 kJ/mol. The work cited presents averaged values throughout the globule. Therefore, one should compare them with the average of our elasticity-based estimate, 30 kJ/mol, to find that the results are comparable.

Studies of the thermodynamic stability of mutant proteins provide additional means of characterizing the energy of cavities. It has been reported that replacing an internal nonpolar side group with a smaller one results in the destabilization of proteins (Kellis et al., 1988; Karpusas et al., 1989; Daopin et al., 1991). When the size of the deleted group is equivalent to the size of methane, the destabilization effects can amount to 20 kJ/mol. A quantitative interpretation of the data is aggravated by at least two factors. First, an equilibrium cavity, resulting from deletion, may have a smaller size than that of the deleted group (due to a surface tension effect shrinking the cavity). Second, one should take into consideration that the interaction of the deleted group with the environment is different in the native and denatured states of the protein. Nevertheless, it is noteworthy that the energetics of the defects in mutants is close to that of elastic deformation.

MECHANICAL NONLINEARITY AND THE SIGN OF ELECTROSTRICTION IN PROTEIN

Theoretical background

The specific volume of liquid dielectrics decreases under the action of an electrostatic field. This electrostriction takes place near ions and charged groups in liquid solutions. However, in solids, the electrostriction may be of the opposite sign (Landau and Lifshitz, 1982). The sign and magnitude of the volume change depend on the sign and magnitude of the pressure derivative of the dielectric constant, ε, according to the thermodynamic relation (Desnoyers et al., 1965)

$$(-1/V)(\partial V/\partial E)_{g,T} = (E/4\pi)(\partial\varepsilon/\partial P)_{E,T}. \qquad (11)$$

Here E is the electrostatic field strength; g is the chemical potential of the medium. The macroscopic polarizability of liquids is, to a first approximation, proportional to the density of dipoles, whether the atomic polarizability or the orientation of permanent dipoles is the dominating mechanism of polarization. In this case, the higher the pressure and density, the higher the dielectric constant: $(\partial\varepsilon/\partial P)_{E,T} > 0$, and, correspondingly, $(\partial V/\partial E)_{g,T} < 0$. In nonpolar solids, derivatives of the same sign are observed, because the polarization in this case is determined by the density of the induced atomic dipoles.

Unlike the above-mentioned systems, in polar solid dielectrics, where the reorientation of permanent dipoles contributes to polarization, the polarizability depends not only on the density itself, but also on the elastic forces impeding the reorientation. The higher the compliance of the solid medium, the greater the reorientation and polarizability, all else being equal. Therefore, the sign and magnitude of $(\partial\varepsilon/\partial P)_{E,T}$ are determined by two counteracting factors. On the one hand, an increase of density itself results in an increase in ε. On the other hand, because of the mechanical nonlinearity, compression leads to a sharp decrease in compliance, thus reducing the reorientation and the dielectric constant. The resulting change in ε is determined by the following approximate relation (derived in Appendix 3):

$$(\partial\varepsilon/\partial P)_{E,T} = \beta(\varepsilon - 1)\left(1 - \mu\frac{\varepsilon - \varepsilon_a - 1}{\varepsilon - 1}\right), \qquad (12)$$

where ε_a is the contribution of atomic polarizability to the dielectric constant. The sign of $\partial\varepsilon/\partial P$ is determined by the fraction of ε_a in the dielectric constant and by the nonlinearity index, μ. In protein-like organic materials, the contribution of atomic polarizability is normally 1.1 and never exceeds 1.6. This follows from the dielectric constants measured at optical frequencies (Krishtalik et al., 1997), implying that $\varepsilon_a = \varepsilon - 1$. For a limiting estimate, let us assume $\mu = 6$ (the lowest possible value for organic materials; see Table 1). Then, as seen from Eqs. 12 and 11, the volume will expand under the action of an electric field if $\varepsilon > 2.8$. In a protein interior having $\varepsilon \approx 4$ (Pethig, 1979) this condition certainly holds.

Therefore, if the protein globule is a solid with orientational polarizability, then its volume and compressibility should increase under the action of an embedded electric charge.

Experimental data

The only experimental result known so far that could provide information on the sign of electrostriction in proteins was obtained in a study of the redox transition in cytochrome *c* (Kharakoz and Mkhitaryan, 1986). In this protein, the heme iron is embedded deep in the interior. Upon oxidation, the electric charge of the iron is increased from +2 to +3. This causes an increase in intrinsic molar volume and compressibility of 10 ± 6 cm^3/mol and 3 ± 1 $kbar^{-1}$ cm^3 mol^{-1}, respectively. Although the accuracy of these values is not high, one can certainly conclude that the sign of the electrostriction effect is opposite that in liquids and is concordant with the expectation for a solid dielectric with orientational polarizability. This is an additional indication that the protein molecule behaves like a solid particle.

ELECTROSTRICTION AND THE VOLUME EFFECT OF ION TRANSFER INTO PROTEIN

The pressure dependence of the transfer of an ion from solvent to the protein interior is determined by the molar

volume and compressibility changes upon transfer. Let us estimate the expected changes for the OH^- ion, taking into account the foregoing consideration of electrostriction. The results will be used below, in an analysis of hydrogen exchange in proteins—a process in which the diffusion of OH^- plays a key role.

The changes in molar volume (ΔV) and compressibility ($\Delta K = -\Delta\Delta V/\Delta P$) upon transfer are determined by the following factors.

1. The OH^- ion leaves the bulk water phase. This results in a decrease in the volume of the system by 1.4 cm^3/mol and an increase in compressibility of 5.2 $kbar^{-1}$ cm^3 mol^{-1}, according to the partial volume and compressibility of this ion in water (Millero, 1972; Mathieson and Conway, 1974).

2. A cavity suitable to accommodate OH^- is formed inside the globule. The intrinsic volume of the ion is ~10 cm^3/mol, according to Bondi (1964). Because the average packing density of the protein interior is ~0.7 (Klapper, 1971; Richards, 1977), the ion occupies a space of 10/0.7 = 14 cm^3/mol. There are preexisting cavities of different sizes in the interior, including those capable of accommodating this particle without a mechanical strain. Therefore, the volume effect of cavity formation varies from 0 for loosely packed regions to 14 cm^3/mol for close-packed ones. This estimation implies (Frenkel, 1946) that the deformation of the medium around a cavity occurs without a change in its density (see Appendix 3 for a comment on this assumption). The volume change of the whole macromolecule is thus equal to the volume change of the cavity, $\Delta V = 0$–14 cm^3/mol. As the density of the protein material is unchanged and the cavity finally formed retains a constant size (the size of the inserted ion), the total compressibility does not change either, $\Delta K = 0$ at this step.

3. The volume and compressibility of the protein matrix increase because of the electrostriction effect of the inserted ion. To estimate these contributions, one can refer to the above-discussed experimental data on the redox transition in cytochrome *c* (Kharakoz and Mkhitaryan, 1986). Recall that, during the oxidation, the electric charge of the heme iron increased from +2 to +3, which resulted an increase in volume of 10 cm^3/mol and an increase in compressibility of 3 $kbar^{-1}$ cm^3 mol^{-1}. To exploit these data, one should take into account that the electrostriction effect is proportional to the electric charge squared (one can make sure of it by integrating Eq. 11). Hence the effects of the charge alteration from +2 to +3 should exceed the effects of a monovalent ion by a factor of $(3^2 - 2^2)/1^2 = 5$. Therefore, for a monovalent ion, the volumetric effects are $\Delta V = 2$ cm^3/mol and $\Delta K = 0.6$ $kbar^{-1}$ cm^3 mol^{-1}.

Summing the contributions listed gives the following result for the overall changes in the system: $\Delta V = 1$–15 cm^3/mol and $\Delta K = 6$ $kbar^{-1}$ cm^3 mol^{-1}. The major factors determining these changes are 1) the release of a portion of water that was contracted around the hydroxyl ion when it was in the bulk water phase and 2) an increase in intrinsic volume and compressibility of protein caused by the ion inserted into the protein matrix. The latter factor results from the protein nonlinearity.

The expectations inferred from this calculation are as follows. Under normal conditions, when the volume effect of transfer is positive, elevated pressure will suppress ion transfer. However, the increased compressibility means, according to definition, that the volume effect decreases with increasing pressure. Therefore, the sign of the volume effect may reverse at a certain pressure. Then a further increase in pressure will promote the transfer instead of suppressing it. These expectations are supported by the data on the hydrogen exchange discussed below.

HYDROGEN EXCHANGE IN NATIVE PROTEINS

Introducing remarks

The basic features of hydrogen exchange between the solvent and the buried atomic groups in native proteins are described here according to reviews (Woodward et al., 1982; Abaturov et al., 1983; Wagner, 1982; Englander and Kallenbach, 1984; Englander et al., 1997). Only the features directly related to the matter under consideration are presented. We restrict our analysis to the exchange process occurring under conditions far from denaturation, to avoid the necessity of considering an influence of unfolding on the exchange rate. Formally, the process passes through two steps:

$$\mathbf{A} \leftrightarrow \mathbf{I} \leftrightarrow \mathbf{B}. \tag{13}$$

The first is the transition of a protein from the ground state **A** to an intermediate one **I** in which a given internal atomic group becomes competent for the second, chemical step—the exchange reaction itself. For simplicity, the first step is called hereinafter initiation. During the initiation, an internal group comes in contact with the hydrogen carrier (a water molecule or its ions, which are the catalyzers of the process). The thermodynamics and kinetics of initiation are studied by comparing the exchange kinetics in native proteins with that in unfolded models, oligo- and polypeptides. It is implied that the chemical step does not differ in the systems compared. The ratio of the exchange rate in peptides to that in native proteins is called the protection factor, meaning that the tightly packed native structure protects the buried group from coming into contact with water. The following features are important for our analysis:

1. According to one of the exchange mechanisms, the step of initiation represents the insertion of a hydrogen carrier (water or its ions) inside the globule by a mechanism similar to diffusion in solids. Therefore, the elastic deformation of the matrix should play an essential role. An indirect indication that just the solid-state nature of native protein impedes the penetration by water has been provided by recent studies showing that, in the case of the molten-

globule state, water readily flows inside the protein (Kharakoz and Bychkova, 1997), and the protection factors in this state are very low (Englander et al., 1997).

2. At pH above 3.5, the hydrogen exchange is base-catalyzed—the OH^- ion is the dominating hydrogen carrier. Thus electrostatic interactions should also play an essential role.

3. For the majority of exchangeable groups, the initiation is a quasiequilibrium step, so that the **A** and **I** states are populated according to the difference in the standard free energies. Therefore, details of the pathway of the catalyst from protein surface to a target group may be ignored, while the local conditions in close proximity to the group (packing density, compressibility, electrostatics, etc.) are of primary importance.

Hydrogen exchange at ambient pressure

The standard free energy of initiation varies within the range of 0–65 kJ/mol (Woodward and Hilton, 1980). Surprisingly, this range, reflecting the differences in local conditions near exchanged groups, is the same as the above-discussed range of variation of the deformation energy—the variation determined by local packing in protein. Such good agreement may be coincidental, given the simplifications involved in our calculation. However, this strongly suggests that the variation in the deformation energy could be a major reason for the observed variations in the rates. One would therefore expect the rate to correlate with the protein compressibility and local packing. Although this possible correlation has been discussed before (Richards, 1979; Eden et al., 1982; Woodward et al., 1982; Abaturov et al., 1983; Nikitin et al., 1984; Kharakoz and Mkhitaryan, 1986; Kharakoz, 1990), no conclusive experimental evidence has been reported so far. Experimental data on the alterations in the compressibility and exchange rate are summarized in Table 2. In all cases but the redox transition in thioredoxin, the expected correlation is found: a smaller compressibility is associated with a slower rate. However, most of these data should be regarded with care for the following reasons. The compressibility changes in native proteins are very small unless a dramatic reorganization like denaturation occurs. Detection of such small changes requires special precautions against systematic errors. Reliable data have been reported so far in only two studies for only two processes: the binding of a trisaccharide to lysozyme (Nikitin et al., 1984) and the redox transition in cytochrome *c* (Kharakoz and Mkhitaryan, 1986). In these studies, the measurements were performed with a differential titration technique, when a change in protein state was induced and registered within the same sample (otherwise, a systematic error caused by the Donnan effect or specific interactions would have been observed; see comments by Kharakoz and Mkhitaryan, 1986). These two cases qualitatively confirm

TABLE 2 The changes in compressibility and hydrogen exchange rate observed in various processes*

Protein, process	Intrinsic compressibility of protein globule	Exchange rate	Correlation	
			Qualitative	Quantitative
Lysozyme, ligand binding (tri-*N*-acetylglucose)	Drops by 10–15%†‡	Retarded 5–10-fold§	Yes	Yes
Cytochrome *c*, ox-to-red transition ($Fe^{3+} \rightarrow Fe^{2+}$)	Drops by 2.5%†¶	Retarded 10–90-fold‖	Yes	No
	Drops 1.5-fold**††		Yes	
	(?)		(?)	
Myoglobin, ligand binding (water, azid, fluorine)	Unchanged**‡‡	Unchanged§§	Yes	
	(?)		(?)	
Thioredoxin, ox to red transition (SS → SH + SH)	Drops 2-fold**¶¶ (?)	Accelerated by 20%‖‖	No	
	(?)		(?)	

*Question marks indicate questionable data from the works where compressibility change was measured by comparing the separately prepared samples of two protein forms. This approach did not exclude a large systematic error resulting from different concentrations of components in the samples (see comments by Kharakoz and Mkhitaryan, 1986).

†Measured by means of a differential titration technique.

‡Nikitin et al. (1984); similar results have been obtained for the change in compressibility of the protein solution by Gekko and Yamagami (1998) and for the change in mechanical compliance of a crystalline lysozyme by Morozova and Morozov (1983).

§Tsuboi and Nakanishi (1979).

¶Kharakoz and Mkhitaryan (1986).

‖Ulmer and Kägi (1968); Patel and Canuel (1976).

**Determined by comparing the separately prepared samples of the two protein forms (a large systematic error is possible).

††Eden et al. (1982).

‡‡Leung et al. (1986).

§§Benson et al. (1973).

¶¶Kaminsky and Richards (1992a).

‖‖Kaminsky and Richards (1992b).

the correlation between the compressibility and exchange rate (Table 2).

Unfortunately, only one of the cases demonstrates the quantitative agreement with expectation—namely, the binding of the ligand to lysozyme. According to Eq. 10, the observed 10% reduction in compressibility upon binding should cause an increase in the energy of cavity formation of 6.5 kJ/mol. The experimentally observed alteration of the energy of initiation is close to this estimate: 4–6 kJ/mol, as follows from the 5–10-fold decrease in the exchange rate (Table 2).

However, in the case of redox transition in cytochrome *c*, there is no such quantitative agreement. Indeed, from the 2.5% reduction of compressibility one would expect a minor increase in the energy of cavity formation, of less than 1.6 kJ/mol, while the observed 10–90-fold retardation of exchange indicates a large increase in the energy of the **I** state, of 6–11 kJ/mol. Therefore, the alteration of the exchange rate is also determined by factors other than protein elasticity. One likely possibility is the electrostatic interaction between the negatively charged hydrogen carrier, OH^-, and the positive charge on the heme iron. The less positive the charge, the lower the probability of transfer of the anion inside. A simple electrostatic calculation (unpublished) shows that this factor can perfectly account for the observed large retardation of the exchange process.

Therefore, among the scanty set of available data, the most reliable experimental observations qualitatively support the expectation of a direct correlation between the compressibility and the exchange rate. In one of them, even quantitative agreement is found.

Recently, a set of literature data on the hydrogen exchange of individual amides in proteins has been statistically analyzed in our laboratory (Pozharski, 1998) by the use of a simple model. Both elastic and electrostatic contributions to the energy of OH^- transfer were considered explicitly in the model. The elastic energy was calculated by means of the approach described in this paper. A good agreement between calculation and experiment was found. If the elastic term in the model is ignored, the correlation worsens considerably. This is a direct indication that protein elasticity, indeed, constitutes an essential factor determining the ion transfer and exchange rate (to be published).

Hydrogen exchange at elevated pressure

Carter et al. (1978) studied the pressure dependence of the base-catalyzed hydrogen exchange in ribonuclease and lysozyme at room temperature. The exchange kinetics of bulk amides was monitored. It has been found that, with increasing pressure, the population of the **I** state decreases at first, and then, after an inversion point, it starts to increase. It has been found that the volume effect of initiation changes from $\Delta V = 3$ cm^3/mol at 1 bar to $\Delta V = -11$ cm^3/mol at 2.5 kbar. This means that the partial compressibility is increased upon initiation by $\Delta K = -\Delta\Delta V/\Delta P = 14/2.5 = 6$ kbar^{-1} cm^3 mol^{-1}.

Two possible qualitative explanations of this behavior were discussed by the authors. One was based on some qualitative ideas about water transfer into protein. Another one implied that, first, the protein suffers occasional unfolding-like destruction to allow the contact of water with buried groups. Second, the unfolding-like events become more probable at high pressure, which is reasonable (Kharakoz, 1997). On a qualitative level, both ideas provide a reasonable explanation of the data, and, hence, the two mechanisms remained undistinguished. However, a quantitative consideration based on the data presented here provides a criterion that allows one to discriminate between the mechanisms.

Indeed, comparison of the experimental numbers for ΔV and ΔK with our calculations of the volumetric effects of the ion transfer reveals that the experimental observations are in good agreement with the predictions ensuing from the diffusion mechanism (see Table 3), while, for the unfolding mechanism, one would expect substantially larger volumetric changes. The latter can be verified by analyzing the data on the hydrogen exchange rates in bovine pancreatic trypsin inhibitor (Wagner, 1982) observed at a high temperature, 60°C, which is close to the temperature of denaturation. A qualitatively similar, biphasic pressure dependence of the rates has been found in this case. The volume effect of initiation is a positive value at ambient pressure, $\Delta V = 20$ cm^3/mol, and decreases to the negative one at 2 kbar, $\Delta V = -14$ cm^3/mol. This means that the compressibility increases upon initiation by $\Delta K = 24$ kbar^{-1} cm^3 mol^{-1}. The values ΔV and ΔK have been calculated here by comparing the activation volumes reported by Wagner (1982) with those in the reference polypeptide chains, reported by Carter et al. (1978). The obtained magnitudes of ΔV and ΔK of initiation are too large to be rationalized in terms of a diffusion mechanism but are consistent with the initiation, in this case, corresponding to denaturation—namely, the native to molten-globule transition. Indeed, it is known that, for a transition of this kind, the partial volumetric effects vary within wide ranges: from -0.002 to 0.006 cm^3/g for volume and from -0.0022 to 0.0032 kbar^{-1} cm^3 g^{-1} for compressibility (reviewed by Kharakoz, 1997; see also a recent work by Chalikian et al., 1997). In molar units, these ranges correspond to -13 to 40 cm^3/mol and -14 to 21 kbar^{-1} cm^3 mol^{-1}, respectively, as calculated for the molecular mass of the protein under consideration. The volumetric effects of the hydrogen exchange initiation in Wagner's work are found to be in this range (cf. Table 3). This is not surprising, given that the experimental conditions in his work were close to denaturation (see also Tsuboi and Nakanishi, 1979).

Hitchens and Bryant (1998) have recently studied the hydrogen exchange of 44 individual amides in T4 lysozyme over a pressure range up to 2 kbar. They determined the

TABLE 3 Characteristics of the hydrogen exchange initiation (A ↔ I) in native proteins under conditions far from denaturation and close to denaturation: comparison of the expectations from the ion diffusion and from the protein melting mechanisms with experimental data*

	Far from denaturation (normal conditions)		Close to denaturation (a high temperature)	
Conditions: Quantity, units	Expectations from ion diffusion†	Experiment	Expectations from protein melting‡	Experiment
Standard free energy change (kJ/mol)	0 to 65§	0 to 65		
Volume change (at ambient pressure) (cm^3/mol)	1 to 15¶	−9 to 19¶ 3‖	−13 to 40	20**
Compressibility change ($kbar^{-1}$ cm^3 mol^{-1})	6	6‖	−14 to 21	24**

*See text for the details of calculations, the specification of proteins, and the references.
†The range of values reflects the size distribution of internal cavities existing in the static protein structure.
‡From experimental data on the native to molten-globule state transition of proteins; recalculated for a molecular mass of 6.5 kDa (pancreatic trypsin inhibitor).
§Only the deformational part of energy.
¶Individual residues.
‖Bulk residues.
**Individual residues averaged.

individual volume effects of A-to-I transition under the assumption of a linear dependence of the activation energy on pressure. The individual ΔV ranged from −15 to 13 cm^3/mol, with a mean value of −3.2 cm^3/mol. As the linear approximation was used, their results correspond to 1 kbar. The value agrees well with the value of −2.6 cm^3/mol obtained by Carter et al. (1978) for the same pressure. Analyzing individual amides, Hitchens and Bryant did not observe any clear nonlinearity in the pressure dependence of the individual activation energies. Unfortunately, they did not present in their paper a complete set of data for all 44 amides investigated. I suppose if the data were averaged, the authors would be able to detect the nonlinearity and, thus, to elucidate an average compressibility effect of the A-to-I transition. Let us assume that the compressibility effect is the same as that observed by Carter et al., $\Delta K = 6$ $kbar^{-1}$ cm^3 mol^{-1}. Then the diversity of ΔV reported should be corrected by 6 cm^3 mol^{-1} to obtain the range −9 to 19 cm^3/mol for ambient pressure. This is close to the above-discussed theoretical range for the effects of ion permeation to different sites of the protein interior, 1–15 cm^3 mol^{-1}. Therefore, the experimental study of the exchange of individual amides shows good agreement with the estimates based on the diffusion mechanism of exchange initiation.

Another study of hydrogen exchange at elevated pressure has recently been made by Fuentes and Wand (1998), who investigated individual amides of apocytochrome *b*562. They also observed a strong and nonmonotonic pressure dependence, indicating large volume effects, the effects characteristic of a denaturation-like process. However, apocytochrome *b*562 displays physical features commonly associated with the molten-globule state. Therefore, it is difficult to interpret these data, in terms of our approach, as valid for the native state.

The foregoing shows that quantitative volumetric analysis based on the experimental data on protein elasticity provides a new tool for discriminating between hydrogen exchange mechanisms.

CONCLUDING REMARKS

Since the very first studies of detailed crystallographic structure of proteins, it has become clear that the protein molecule resembles a solid body in which each atom "knows its place." Later on, investigations of bulk mechanical properties of protein globules as a whole have supported this idea by showing that, according to their average packing density (Klapper, 1971; Richards, 1977) and elasticity constants (Morozov and Morozova, 1990, 1993), native globules are as dense and hard as molecular crystals and glassy polymers. This fact implies a number of consequences that are important for understanding the dynamic features of proteins and that have been considered in the present work.

It has been shown that the native protein molecule is characterized by a high degree of mechanical nonlinearity. Because of this feature and the orientational mechanism of polarization, the protein molecule expands under the action of an electrostatic field. This behavior is in contrast to that of liquids and nonpolar solids.

A strong pressure dependence of intramolecular motions (indicated by Rayleigh scattering of Mössbauer radiation) and a nonmonotonic pressure dependence of the permeation of ions into the protein matrix and the related dynamic processes result from the nonlinearity of proteins. The set of experimental data on elastic properties of proteins discussed here allows a qualitative and quantitative explanation of the

other set of data—that of hydrogen exchange of buried atomic groups and its dependence on hydrostatic pressure. The data collected provide a new tool for discriminating between different mechanisms of the hydrogen exchange in native globular proteins.

The approach suggested here for an approximate calculation of the deformational energy of cavity formation could apply to studies of any processes dependent on the diffusion of particles into the protein matrix—for instance, in studies of the ion-mediated quenching of fluorescence, the transfer of ligands toward an internal site of binding, such as in myoglobin, etc. This simple calculation could serve as a preliminary step for an effective design of studies by other, more powerful and rigorous methods, such as computer simulation of molecular dynamics.

Some conclusions put forth in this paper require independent proof. For instance, the electrostriction effect has been considered here within a very simple approach. A more rigorous treatment of this phenomenon and more accurate experimental confirmation are desirable. However, a general conclusion from the data presented appears to be evident: the mechanisms of protein dynamics cannot be fully understood without considering the nonlinear compressibility of protein molecules.

APPENDIX 1: NONLINEARITY INDEX AND GRÜNEISEN CONSTANT

Within Debye's theory of solids (Landau and Lifshitz, 1995), the frequency of any phonon mode, ω, is related to the Grüneisen constant, γ, by the derivative

$$\gamma = -\partial \ln \omega / \partial \ln V = -\partial \ln \omega_D / \partial \ln V,$$

where ω_D is the characteristic Debye frequency, given by the relation

$$\omega_D = u(6\pi^2 N/V)^{1/3},$$

where N is the number of particles and u is an average of the transverse, u_t, and longitudinal, u_l, acoustic waves, defined as

$$3/u^3 = 2/u_t^3 + 1/u_l^3.$$

Then

$$\gamma = -\partial \ln u / \partial \ln V + 1/3. \quad (14)$$

The transverse and longitudinal sound velocities are determined by corresponding elasticity coefficients: $u_t^2 \approx M_S/\rho$ and $u_l^2 \approx \beta^{-1}/\rho$, where M_S is the shear modulus and β^{-1} is the bulk modulus of a solid. Assuming $M_S \approx \beta^{-1}$ (from Eq. 3 at p = const.), one writes $u^2 \approx \beta^{-1}/\rho$. Differentiating this proportionality, $\partial \ln u/\partial \ln V = -(\partial \ln \beta/\partial \ln V - 1)/2$, and taking into account the Moelwyn-Hughes isotherm, $\partial \ln \beta/\partial \ln V = \mu$, one rewrites Eq. 14 in the final form

$$\gamma = (\mu - 1/3)/2.$$

APPENDIX 2: ELECTROSTRICTION IN A SOLID WITH ORIENTATIONAL POLARIZABILITY

The macroscopic polarizability, χ, of a solid containing polar atoms consists of two parts, the atomic component caused by the displacement of electrons, χ_a, and the component resulting from the reorientation of permanent dipoles, χ_d:

$$\chi = \chi_a + \chi_d. \quad (15)$$

Upon compression, the polarizability increases because of the increased number of dipoles, whatever the permanent or induced atomic dipoles, in unit volume. At the same time, it decreases because of the sharply increased rigidity of the solid medium, impeding the permanent dipole reorientation. To a first approximation,

$$\chi_a \approx \rho \quad \text{and} \quad \chi_d \approx \rho\beta. \quad (16)$$

Compressibility is considered here to be reciprocal to the rigidity of the surrounding medium (this is valid under the assumption that the Poisson ratio does not change upon compression). Differentiation of Eq. 15 over pressure gives the following equation, expressed in terms of relative quantities:

$$\chi(\partial \ln \chi/\partial P)_{E,T} = \chi_a(\partial \ln \chi_a/\partial P)_{E,T} + \chi_d(\partial \ln \chi_d/\partial P)_{E,T}.$$

This equation, combined with the proportionalities in Eq. 16 and the definition $\beta \equiv (\partial \ln \rho/\partial P)_T$, yields

$$\begin{aligned}(\partial \ln \chi/\partial P)_{E,T} &= (1/\chi) \times (\chi_a\beta + \chi_d\beta + \chi_d\beta(\partial \ln \beta/\partial \ln \rho)) \\ &= \beta\left(1 + \frac{\chi - \chi_a}{\chi}\frac{\partial \ln \beta}{\partial \ln \rho}\right) \\ &= \beta\left(1 - \frac{\chi - \chi_a}{\chi}\frac{\partial \ln \beta}{\partial \ln V}\right).\end{aligned}$$

Taking into account that $(\varepsilon - 1) = \chi$ and $\mu = \partial \ln \beta/\partial \ln V$, one finally obtains

$$\begin{aligned}(\partial\varepsilon/\partial P)_{E,T} &= (\varepsilon - 1)(\partial \ln \chi/\partial P)_{E,T} \\ &= \beta(\varepsilon - 1)\left(1 - \mu\frac{\varepsilon - \varepsilon_a - 1}{\varepsilon - 1}\right),\end{aligned}$$

where ε_a $(= \chi_a)$ is the atomic polarization part of the dielectric constant.

APPENDIX 3: A COMMENT ON THE VOLUME EFFECT OF DEFORMATION

Frenkel (1946) determined that the density of an isotropic solid material does not change during the expansion of an internal cavity. Let us consider this issue more carefully. If the deformation upon the cavity expansion is purely elastic, and if the shear modulus is constant in the whole range of strain, then Eq. 10 describes the reversible work, W, of deformation. At constant temperature and pressure, the work is equal to the Gibbs free energy change. Then, according to thermodynamic definition, the volume effect of deformation is obtained by differentiation of W over pressure: $\Delta V = (\partial W/\partial P)_{T,\Delta r} = \mu\beta W$. Therefore, Frenkel's result, $\Delta V = 0$, holds true only in the case where $\mu = 0$ (meaning a hypothetical situation in which the compressibility coefficient does not change upon compression). For the formation of a large cavity in the highly nonlinear protein interior, considered above (under Compressibility and the Energy of Expansion of a Cavity, and Electrostriction and the Volume Effect of Ion Transfer into Protein), this equation would give an unreasonably large positive change of

volume. (While the compressibility would remain constant, as seen from the derivative $\Delta K \equiv -\partial\Delta V/\partial P = \mu\beta - \mu\beta = 0$.)

However, in reality, Hooke's law for shear deformation holds true only in a very limited range of strains—certainly less than 10% for native proteins (Gorelov and Morozov, 1987; Zenchenko and Morozov, 1995). Therefore, around a large cavity, the deformed area splits onto two regions. There is a distant one, in which the condition $\Delta r/r < 0.1$ is fulfilled (here r is the distance between the center of the cavity and any given point in the solid medium). For this region, the deformation follows Hooke's law. This "Hooke's" region encompasses a highly deformed layer in close proximity to the cavity—a layer that deforms in a quasiplastic way (this obvious qualitative consideration was supported by computer simulation work of Noguti and Go, 1989). The following features characterize the inner region: 1) Its shear modulus should abruptly decrease (although not to zero), and the layer should undergo a bulk compression resulting from the restoring elastic forces acting from the encompassing "Hooke's" region. 2) The energy expense of this compression should be less than would be required for the "Hooke's" shear deformation of the same layer. 3) The compression should compensate for the volume increase occurring in the "Hooke's" region; it can even reverse the sign of the overall volume effect.

This consideration should be regarded as a qualitative argument in favor of ignoring the volume effect of deformation in our treatment. A more accurate quantitative solution of the problem will be published elsewhere.

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