

# High Pressure Effects on Protein Structure and Function

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**ABSTRACT** Many biochemists would regard pressure as a physical parameter mainly of theoretical interest and of rather limited value in experimental biochemistry. The goal of this overview is to show that pressure is a powerful tool for the study of proteins and modulation of enzymatic activity. © 1996 Wiley-Liss, Inc.

**Key words:** hydrostatic pressure, secondary, tertiary, and quaternary structure, folding, molten globule, protein-nucleic acid interactions

## INTRODUCTION

Since Bridgman's pioneering work in 1914,<sup>1</sup> showing that a pressure of 7 kbar is able to denature proteins of egg white in a similar but not identical way as temperature, pressure has been long disregarded by biochemists. The reason was the absence of general ideas on what pressure can add to the understanding of the behavior of proteins. In addition, the lack of basic concepts about pressure effects on biochemical reactions and structure of biopolymers did not stimulate activities in this field. Marine biology and deep-sea diving physiology were the only exceptions. The oceans cover nearly 70% of the earth's surface and have an average depth of 3.8 km and an average pressure of 380 bar at the bottom (1 atm = 1 kg·cm<sup>-2</sup> ≈ 1 bar ≈ 0.1 MPa). Clearly, pressure plays an important role in the distribution of life in the world's oceans.<sup>2</sup>

Recent decades have, however, witnessed a growing interest on the part of researchers in introducing pressure as a variable acting on biosystems. One of the reasons is the possibility of applying pressure in specific biotechnological areas,<sup>3,4</sup> mainly for food processing. On the other hand, it also becomes clear that, along with such parameters as temperature and solvent conditions, pressure can be used for more detailed thermodynamic and kinetic description of bioprocesses and biosystems and regulation of their behavior.<sup>5</sup> For example, in protein denaturation studies, high hydrostatic pressure provides unique information on unfolding mechanisms.<sup>6,7</sup> Due to technical progress in the last decade, many

instrumental problems that hampered high-pressure research have been solved, and today almost all the methods used routinely at atmospheric pressure can be adapted to high-pressure studies (see Table I for a brief survey of the most useful methods).

Here we discuss recent selected examples of applying pressure in biochemical research with a special emphasis on proteins as the most intensely studied objects.

## BASIC CONCEPTS

Pressure effects are governed by Le Chatelier's principle, which states that at equilibrium a system tends to minimize the effect of any external factor by which it is perturbed. Consequently, an increase in pressure favors reduction of the volume of a system.

For an elementary equilibrium process  $A \leftrightarrow B$ , the following general expression holds:

$$\Delta G = -RT \ln K = \Delta E + p \Delta V - T \Delta S \quad (1)$$

where  $\Delta G$ ,  $\Delta E$ ,  $\Delta V$ , and  $\Delta S$  are the changes in free energy, internal energy, volume, and entropy;  $K$  is the equilibrium constant governing the process,  $T$  the temperature,  $p$  the pressure, and  $R$  the gas constant. An argument in favor of the use of pressure as a physical variable instead of temperature, which may change both the internal energy and the volume of the system, is that pressure only affects the volume of the system under study. The volume change, which is the difference between the volumes of the final ( $V_B$ ) and initial ( $V_A$ ) states, is given by the expression:

$$\Delta V = V_B - V_A = (\partial \Delta G / \partial p)_T = -RT \cdot (\partial \ln K / \partial p)_T. \quad (2)$$

For the rate constant  $k$  of an elementary process  $A \rightarrow B$ , analogous expressions can be derived between  $k$  and the activation parameters, with  $\Delta V^\ddagger$ , the activation volume, being the volume difference between the activated ( $V^\ddagger$ ) and ground ( $V_A$ ) states.

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**TABLE I. Major Methods in High-Pressure Studies of Protein Structure and Enzyme Kinetics**

Protein structure or function	Major methods*	Upper limit of applied pressures (kbar)
Primary		No effect of pressure
Secondary	Vibrational spectroscopy <sup>8</sup>	20
Tertiary	NMR spectroscopy <sup>7</sup>	5
	X-ray analysis <sup>9</sup>	1
	UV-vis and fluorescence spectroscopy <sup>6</sup>	10
	Molecular dynamic simulation <sup>10</sup>	
Quaternary	Electrophoresis <sup>11</sup>	5
	Ultracentrifugation <sup>12</sup>	1
	Fluorescence spectroscopy <sup>6</sup>	10
	NMR spectroscopy <sup>7</sup>	5
Ligand binding	Flash photolysis <sup>13</sup>	2
	Affinity electrophoresis <sup>11</sup>	2
Enzyme activity	Different spectroscopies <sup>6</sup>	10
	Stopped flow <sup>14</sup>	2
	Rapid sampling <sup>15</sup>	2

\*A single reference to each method was chosen, which describes the application of the method under high-pressure conditions in the most comprehensive manner.

The values of  $\Delta V$  or  $\Delta V^\ddagger$  are obtained by plotting  $\ln K$  or  $\ln k$ , respectively, versus  $p$ . Non-linear plots indicate that  $\Delta V$  or  $\Delta V^\ddagger$  are pressure dependent and that the reactants or their activated states have different compressibilities.

For a process with  $\Delta V$  or  $\Delta V^\ddagger$  of  $16 \text{ ml}\cdot\text{mol}^{-1}$ , a change in pressure of 1 kbar corresponds to an approximately twofold change in the equilibrium constant or reaction rate, respectively. On the other hand, typical values of  $\Delta V$  or  $\Delta V^\ddagger$  in biochemical processes are within the range of  $+50$  to  $-50 \text{ ml}\cdot\text{mol}^{-1}$ . The experimental consequence is that the pressure range to be scanned for determining reaction and activation volumes is from 1 bar to several kbar.

### Pressure and Temperature

The effects of pressure and temperature on equilibria or kinetics are antagonistic in molecular terms: as follows from the principle of microscopic ordering, an increase in pressure at constant temperature leads to an ordering of molecules or a decrease in the entropy of the system. A helpful practical rule is that ordering by pressure is offset by an increase in temperature. For a number of biochemical processes, like phase transitions in membranes, the numerical value of the coefficient for this temperature-pressure compensation is in the order of  $20^\circ\text{C}\cdot\text{kbar}^{-1}$  at pressures lower than 1 kbar.<sup>16</sup>

### Molecular Interpretation of Pressure Effects

An interpretation of reaction and activation volumes is usually given in terms of intrinsic and solvent contributions. Intrinsic contributions may occur as a consequence of changes in free volume due to the packing density and/or the formation or breaking of covalent bonds. In any real process, com-

pensation effects may occur that make a simple interpretation of a complex event, such as protein denaturation, a speculative undertaking.

The formation of *covalent bonds* has a  $\Delta V^\ddagger$  of  $\approx -10 \text{ ml}\cdot\text{mol}^{-1}$ , whereas  $\Delta V$  values for the exchanges in bonds or bond angle changes are nearly zero.<sup>17</sup> As a consequence, most covalent bonds participating in the protein primary structure are pressure insensitive, at least up to pressure values of 10–15 kbar. Volume changes that arise from changes in packing density are difficult to quantitate but are considered to be small. Thus, in the absence of covalent bond formation or breaking, the largest contributions are expected to arise from hydration changes that accompany non-covalent interactions. An estimate of the contribution of non-covalent interactions to the observed volume changes in proteins is based on model systems.

### Electrostatic interactions

When an ion is formed in solution, the nearby water dipoles are highly compressed by the coulombic field of the ion; this phenomenon, known as electrostriction, is accompanied by a decrease in volume. For the solvation of singly charged ions in water,  $\Delta V$  values are of the order of  $-10 \text{ ml}\cdot\text{mol}^{-1}$ , and this compression is stronger in non-polar liquids.<sup>17</sup> The dissociation of a neutral molecule into two ions induces a contraction of about  $-20 \text{ ml}\cdot\text{mol}^{-1}$ ; this is the ionization volume of pure water. Since the electrostriction is proportional to the square of the ionic charge, the volume change is much larger for the second ionization of di- and tribasic acids.<sup>17</sup> From these model data, it can be concluded that electrostatic interactions in biomolecules become much weaker at elevated pressures. A typical example is the pressure-induced reversible inactivation of chy-

motrypsin due to the dissociation of the salt bridge in the active site region.<sup>18</sup> The fact that the dissociation constants of weak acids vary with pressure also has important consequences for the analysis of pressure effects on pH-dependent processes. Closely related is the problem of maintaining a constant pH in high-pressure experiments since buffers, such as phosphate, have high ionization volumes. Fortunately, certain protonated bases, such as imidazole-HCl and tris-HCl, have nearly zero ionization volumes and are used to keep the pH constant over a large pressure range.<sup>5</sup>

### Hydrogen bonds

Study of model systems shows that hydrogen bonds are stabilized by high pressures.<sup>17</sup> This results from the smaller inter-atomic distances in the hydrogen-bonded atoms. The stabilizing effect of pressure on hydrogen bonding is indicated by the pressure dependence of the infrared spectra of the  $\alpha$ -helix in myoglobin<sup>19</sup> and from a comparison of the effect on the intermolecular interactions in hydrogen-bonded versus non-hydrogen-bonded amides.<sup>20</sup> However, a very small if not negligible  $\Delta V$  value is observed for processes in which there is an exchange between the existing hydrogen bonds.<sup>17</sup>

### Hydrophobic interactions

It is generally accepted that formation of hydrophobic contacts proceeds with positive  $\Delta V$  values and is therefore disfavored by pressure. Depending on the process selected to mimic formation of hydrophobic interactions (transfer of methane from non-polar solvent into water, dimerization of carboxylic acids, or micelle formation by surfactants), the increase in volume varies from  $+1 - +2 \text{ ml}\cdot\text{mol}^{-1}$  up to  $+10 - +20 \text{ ml}\cdot\text{mol}^{-1}/\text{CH}_2$  group.<sup>21,22</sup> Additional experiments with model systems are necessary for more precise evaluation of pressure effects on hydrophobic contacts. On the other hand, stacking interactions between aromatic rings show negative volume changes and are stabilized by pressure.<sup>17</sup>

At present it is far from clear to what extent these non-covalent forces contribute to protein stability. Each of them has been in the focus for some time and the results from pressure experiments have been interpreted according to the point of view that dominated at that time. It is obvious, however, that pressure effects are especially pronounced on processes in which the hydration state of the system components changes significantly.

## PRESSURE EFFECTS ON PROTEIN STRUCTURE

Protein volume (tens of  $\text{l}\cdot\text{mol}^{-1}$ ) in solution is the sum of three main constituents: the volume of individual atoms, the void volume of internal cavities due to the imperfect packing of amino acid residues,

and a contribution due to solvation of peptide bonds and amino acid side chains. The second term contributes positively and the third term contributes negatively to the total volume, and they tend to cancel each other.<sup>5</sup> Protein compressibility is largely determined by the compression of the internal cavities, which depends on the internal packing. Application of high hydrostatic pressure induces either local or global changes in protein structure and finally may lead to denaturation. Whereas pressures of 1–2 kbar are sufficient to cause dissociation of oligomeric proteins and of multiprotein complexes,<sup>6</sup> small monomeric proteins are usually denatured in the pressure interval from 4 to 8 kbar.<sup>16</sup>

### Local Effects of Pressure on Protein Structure

An image of local pressure-induced changes is obtained most clearly from x-ray structure analysis. In their pioneering work, Kundrot and Richards<sup>9</sup> found that different regions in the lysozyme molecule are compressible to different extents. At 1 kbar the root mean square shift for protein atoms is  $0.2 \text{ \AA}$  with a few atoms moving more than  $1 \text{ \AA}$ . Domain 2 (residues 40–88) is almost pressure insensitive while domain 1 (residues 1–39 and 89–129) and the inter-domain region are substantially compressible. Deformation of  $\beta$ -sheet regions is smaller than that of  $\alpha$ -helices, and this conclusion is consistent with some earlier indirect observations. The main-chain angles of helices and sheets are least affected by pressure, followed by the remaining main chains, side-chains of core amino acids, and side-chains of external amino acids of the lysozyme molecule. Pressure-induced changes in the hydration shell of lysozyme were not as pronounced, probably because of uncertainties in the coordinates of water molecules at  $2 \text{ \AA}$  resolution.

However, pressure-induced changes in the properties of water surrounding proteins have been visualized by other methods. For example, a molecular dynamics simulation<sup>10</sup> has shown that significant changes occur in the protein hydration shell at 10 kbar. Although the surface accessibility of protein is lowered by 7% under this pressure, the hydration energy is 4% or  $75 \text{ kcal}\cdot\text{mol}^{-1}$  higher than at 1 bar, and more than two-thirds of this energy increase is attributed to hydration changes in charged groups. As a consequence, at high pressure the solvation shell becomes more ordered. Moreover, within a distance of  $3.5 \text{ \AA}$ , the number of water molecules increases from 0.83 to 1.29/protein atom and the number of protein to water hydrogen bonds increases from 73 to 80. Proteins usually denature at 10 kbar where the simulation was done. The absence of unfolding is most probably due to the short simulation time (100 ps); however, the tendency revealed by these simulations, i.e., an increase in protein-water interactions, may be a characteristic feature of pressure-induced denaturation as well.

### Pressure-Induced Denaturation

Temperature-induced protein denaturation is often irreversible because of covalent bond breaking and/or aggregation of unfolded protein. An advantage of high pressure in inducing protein denaturation is that these effects are not expected to occur.<sup>16</sup> Also, temperature variations lead to changes in both the volume and the thermal energy of protein and it is difficult to separate these effects. By contrast, at constant temperature under high pressures the internal energy of the system is nearly independent of pressure, and internal interactions are affected solely by the changes in the volumes of the protein molecule and water structure.<sup>6</sup> Often denaturation by pressure can be described with a simple two-state thermodynamic formalism suggesting a transition between two states of a protein. However, there are recent indications<sup>7</sup> for the existence of pressure-induced predenaturation transitions, which in turn point to step-wise processes. In some respect, pressure denaturation studies can be also more straightforward, in comparison with denaturation induced by guanidinium chloride or urea at atmospheric pressure. A serious problem in the interpretation of chemical denaturation is caused by the fact that the thermodynamic parameters are also influenced by the binding of the denaturant molecules to multiple sites on a protein and this binding changes the chemical potential of the protein in an unpredictable way.

The fact that denaturation is induced by pressure means that the volume occupied by the compactly folded native conformation is larger than that of the unfolded one. Indeed, protein unfolding is characterized by a negative molar volume of denaturation:  $\Delta V_d$  is typically of the order of minus tens-hundreds of  $\text{ml}\cdot\text{mol}^{-1}$ ,<sup>6</sup> less than 2% of the volume of the protein molecule. This result may seem paradoxical but it should be realized that the net value of  $\Delta V_d$  includes the effects of formation and disruption of non-covalent bonds, changes in protein hydration and in conformation. The size of the protein hydration shell increases by attraction of new water molecules by the newly surface-exposed amino acid residues but this increase is more than compensated by the negative contribution to  $\Delta V_d$  from the disruption of electrostatic and hydrophobic interactions and disappearance of voids in the protein not accessible to solvent molecules.

The pressure-unfolded states of proteins have been studied by a wealth of experimental methods, which are summarized in Table I.

### Secondary structure

Due to actual technical problems in adapting circular dichroism spectroscopy to high pressures, emphasis in the secondary structure studies has shifted towards the use of Fourier transform infrared

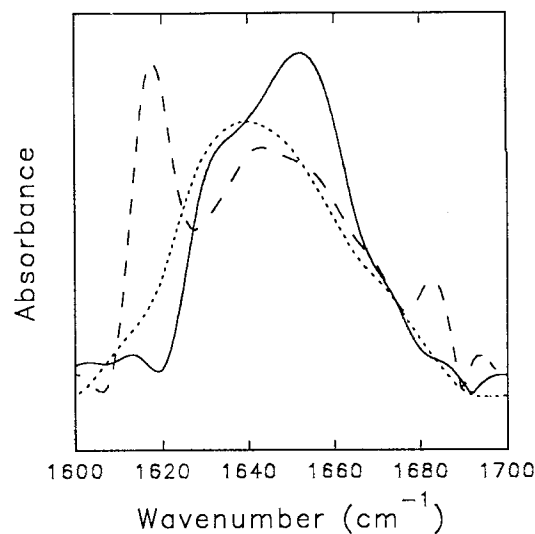


Fig. 1. Deconvolved infrared spectrum of lipoyxygenase in the amide I' region. Spectrum at ambient conditions (solid line), temperature-denatured protein (dashed line), and pressure-denatured protein (dotted line). (K. Heremans and J. Frank, unpublished observations.)

(FTIR) spectroscopy.<sup>8</sup> With the existing computational methods, one can derive detailed pressure-induced changes in protein secondary structure from characteristic shifts in the band frequencies in FTIR spectra. These data are complementary to those obtained by other techniques such as fluorescence spectroscopy and nuclear magnetic resonance (NMR). High-solution concentrations of proteins used in infrared spectroscopy studies often lead to irreversible inactivation. However, in the few instances when high- and low-concentration techniques (e.g., fluorescence spectroscopy) have been applied to the same protein, almost identical denaturation pressures have been found, clearly illustrating that the techniques explore different aspects of the same phenomenon.

Figure 1 shows an example of the infrared spectrum in the amide I' region of the enzyme lipoyxygenase, corresponding to the vibration of the C=O bond of the amide group. This vibration is sensitive to the conformation of the polypeptide. The spectrum, whose resolution is enhanced by Fourier self-deconvolution, shows large differences between the temperature- and the pressure-denatured proteins. The bands at around 1620 and 1680  $\text{cm}^{-1}$  in the spectrum of the temperature-denatured protein are typical for the intermolecular hydrogen bonds that are formed due to aggregation. However, these bands are absent in the pressure-denatured protein.

### Tertiary structure

Major advances in describing structural changes in proteins that accompany pressure-induced dena-

uration have been obtained by combining high-resolution NMR techniques with high pressure.<sup>7</sup>

By studying proton resonances of His 15, Leu 17, Trp 28, Cys 64, and Trp 108, volume changes in different regions of the lysozyme molecule were followed in response to reversible unfolding at high pressure.<sup>23</sup> From the differences in  $\Delta V$  values for these residues, it was concluded that unfolding of the  $\beta$ -sheet region in lysozyme is less pronounced than for  $\alpha$ -helices, which is in agreement with the differences in compressibility between these regions that can be concluded from X-ray data.<sup>9</sup> When the substrate analogue tri-N-acetylglucosamine is bound to lysozyme, volume changes derived from the resonance of Trp 108 upon pressure denaturation are nearly 2 times as negative as the value for the free enzyme, whereas  $\Delta V$  values for the other four residues studied remain practically unchanged. A possible explanation is that the substrate occupies about half of the active site, thus producing a large free volume in the vicinity of Trp 108. Another important conclusion is that structural changes upon pressure denaturation of lysozyme are distinct from temperature denaturation of this protein and are smaller in scale.<sup>23</sup> This suggests that pressure-denatured protein is infiltrated by water. The weakest non-covalent interactions between amino acid residues (like London dispersion forces) that support the protein tertiary structure are first destabilized at high pressure and then replaced by protein-water interactions, which have a smaller volume.<sup>6,24</sup> However, secondary structure remains essentially unaltered because the replacement of intra-protein hydrogen bonds by protein-water hydrogen bonds on their putative destruction would be accompanied by rather small changes in volume and energy. In these structural features, as proposed recently,<sup>6</sup> pressure-denatured proteins resemble molten globules. In these states, although proteins retain most of their secondary structure, their hydrodynamic radii are 10–20% greater than that of the native conformations.<sup>25</sup> In addition, in the molten globule state, patches of hydrophobic residues become solvent exposed, thus increasing binding of hydrophobic probes such as ANS (8-anilino-1-naphthalene-sulphonate), and promoting aggregation. For this reason, interest in studies of pressure-denatured proteins could increase<sup>26–29</sup> since the importance of the molten globule state as an intermediate in the folding process has been recognized.<sup>25</sup>

Similar conclusions about the structure of pressure-denatured proteins were obtained from <sup>1</sup>H NMR spectra of cold-denatured ribonuclease A at high pressure.<sup>7</sup> By applying pressures of several kbar, the freezing point of water was decreased down to  $-25^{\circ}\text{C}$ . This approach may be less damaging to proteins than other procedures used to shift the temperature of denaturation experiments to the subzero region, e.g., the application of cryosolvents

(like methanol), emulsions of protein solutions in a nonpolar solvent, or supercooled aqueous solutions. Experimenting at subzero temperatures under high pressure also avoids addition of chemical denaturants, like guanidinium chloride or urea in protein denaturation studies. For example, denaturation of cowpea mosaic virus particles is obtained in the presence of 5 M urea at room temperature. When temperature is decreased to  $-15^{\circ}\text{C}$  under a pressure of 2.5 kbar, the virus denatures in 1 M urea, and under higher pressure at  $-20^{\circ}\text{C}$ , the denaturation occurs in the absence of urea.<sup>30</sup>

Recent pressure-jump relaxation studies of the refolding of staphylococcal nuclease constitutes one of the first examples of direct measurements of volume changes accompanying protein refolding.<sup>31</sup> The volume of the protein-solvent system in the transition state was found to exceed that of either the folded or unfolded states by several tens of  $\text{ml}\cdot\text{mol}^{-1}$ . These  $\Delta V^{\ddagger}$  values were interpreted to be associated with the rate-determining step of nuclease folding, i.e., hydrophobic collapse of the polypeptide chain with concomitant desolvation, leading to a molten globule-like, loosely packed protein structure in the transition state.

Important information on the structure of proteins in the molten globule state can be obtained by using ultrasound velocimetry.<sup>32</sup> With this method the compactness of the protein molecule in different conformational states can be evaluated by measuring the compressibility of the molecule. The results obtained for cytochromes<sup>33</sup> and ribonuclease<sup>34</sup> indicate that the extent of "softening" of the protein molecule in the molten globule state is small in comparison to the variation in compressibility observed for different native proteins, and this state is much more compact than thermally and chemically denatured proteins.

### Quaternary structure

Intersubunit regions of oligomeric proteins are rich in electrostatic and hydrophobic contacts, which stabilize quaternary structure; thus, it is not surprising that relatively moderate pressures (0.5–2 kbar) may induce dissociation of oligomeric proteins by weakening these interactions. Sometimes pressure-induced dissociation leads to formation of individual non-denatured subunits (as is, for example, the case with the  $\beta$ -subunits of tryptophan synthase at 1.5 kbar<sup>35</sup>), which is not easily accomplished when oligomeric proteins are dissociated by chemical denaturants or heat. More often this initial dissociation is followed by subsequent conformational changes in individual subunits. An example of such changes is presented by a recent study of the dissociation of a small dimeric Arc repressor protein using phase-sensitive two-dimensional correlated spectroscopy (COSY) and nuclear Overhauser effect enhancement spectroscopy (NOESY).<sup>36</sup> The inter-

subunit  $\beta$ -sheet in the native dimer is replaced by an intra-monomer  $\beta$ -sheet in the denatured state at a pressure higher than 2 kbar (Fig. 2), with formation of a denatured molten globule monomer. As evidenced by NMR, this intra-monomer  $\beta$ -sheet is absent in the repressor denatured by urea or heat. NMR spectra also displayed some initial pressure-induced small changes in the Arc repressor under conditions where the dimer is still intact. At 1 kbar, there are additional cross-peaks between Glu 9 and Trp 14 and between Glu 9 and Met 7 that disappear at 2 kbar. These changes in NMR spectra prior to dissociation point to the existence of a predissociated state (Fig. 2) that may correspond to an intermediate in the process of folding and subunit association of the Arc repressor.<sup>36</sup>

Often after rapid dissociation, structural adjustments in monomers proceed rather slowly, leading to a phenomenon known as "conformational drift."<sup>36</sup> The drift is observed as non-coincident plots of the degree of oligomer dissociation versus pressure in pressurization and decompression experiments. At high pressure, individual monomers may exist for a rather long time (hours and days under certain experimental conditions) in transient states that are often very sticky and readily form non-specific aggregates (see Fig. 3 for an example of such a study by electrophoresis). Conformational drift has also been observed during pressure denaturation of a monomeric phosphoglycerate kinase,<sup>38</sup> and this result may initiate studies on a better understanding of the molecular mechanism of this phenomenon.

One of us has found<sup>29</sup> that the tetrameric form of butyrylcholinesterase does not dissociate up to very high pressures, at least 3.5 kbar. This unexpected behavior suggests that the intersubunit area of this protein is mainly stabilized by either pressure-insensitive interactions, e.g., hydrogen bonds, or pressure-enhanced interactions such as aromatic-aromatic bonds. It follows from secondary structure predictions of the C-terminal peptide segment (exon 4) and site-directed mutagenesis experiments that eight aromatic rings within a ridge of the apolar side of a helical segment may face each other to form a zipper motif in the inter-subunit region connecting the segments from all the four subunits.

In conclusion, it is clear that hydrostatic pressure is a valuable additional tool for studying structural principles of organization of complex oligomeric assemblies. Among these we could mention myosin,<sup>39</sup> proteins of the chaperonine family,<sup>40</sup> and viruses<sup>30</sup>; we refer also to the comprehensive review of Silva and Weber<sup>6</sup> for other examples.

### PRESSURE EFFECTS ON ENZYME REACTIONS

General expressions in Eqs. 1 and 2 that govern pressure effects on reactions apply to an elementary act or a single step in a process.<sup>41</sup> The experimental

value of  $\Delta V^\ddagger$  for a multi-step enzyme reaction includes volume changes in the rate-determining step and also in the binding step and in the steps that precede this rate-determining step.<sup>42</sup> As a consequence, pressure dependencies of the rates of enzyme reactions often show curvature, maxima, minima, or breaks in plots of  $\ln k$  vs.  $p$ . By analogy to non-linear dependencies on temperature in plots of  $\ln v$  vs.  $T^{-1}$ , such non-linear dependencies may be explained by either a change in the rate-determining catalytic step, or a conformational change of the enzyme, or another more specific reason.<sup>5</sup> High-pressure studies of enzyme kinetics have appeared to be particularly informative in the two cases discussed below.

### Volume Profiles of Enzyme Reactions for Mechanistic Studies

Volume changes at different steps of a catalytic process may be complementary to the data of other methods for disclosing the mechanisms of enzyme reactions. Butz et al.<sup>43</sup> managed to determine volume changes at each step of fumarate conversion to L-malate by fumarase, as shown in Figure 4. Binding of substrates proceeds with a volume decrease in the transition state and an increase in the volume of the complexes EF and EM. These changes are explained by assuming a conformational transition enabling the substrate to enter the active site. In addition, the bound water molecules are released because correct substrate orientation in the active site involves ion pairing and the resulting increase in volume is reflected in  $\Delta V_{-1}^\ddagger$  and  $\Delta V_3^\ddagger$ . There is an additional rise in volume associated with fumarate hydration or malate dehydration ( $\Delta V_2^\ddagger$  and  $\Delta V_{-2}^\ddagger$ ), suggesting that the transition state has a large volume. It is only in the transition states that the volume reaches a maximum or a minimum (Fig. 4), while in the enzyme-substrate complexes the volume is similar to that in the free enzyme. Volume changes during enzyme turnover are relatively small compared with the total enzyme volume (about 0.3%).

### The Transition State and Effects of Solutes on $\Delta V^\ddagger$

Volume effects in enzyme catalysis originate from structural changes in the protein itself and in the hydration shell around the protein due to changes in exposure of protein functional groups.<sup>44</sup> Since both processes are highly sensitive to the medium composition, large changes in the values of  $\Delta V^\ddagger$  for enzyme reactions can be anticipated upon addition of solutes. Conversely, the sensitivity of  $\Delta V^\ddagger$  to a change in the medium composition, and to the lyotropic character of anions and cations, may indicate that the reaction involves large changes in enzyme conformation or hydration. A correlation between the effects of salts on the reaction rate and the ef-

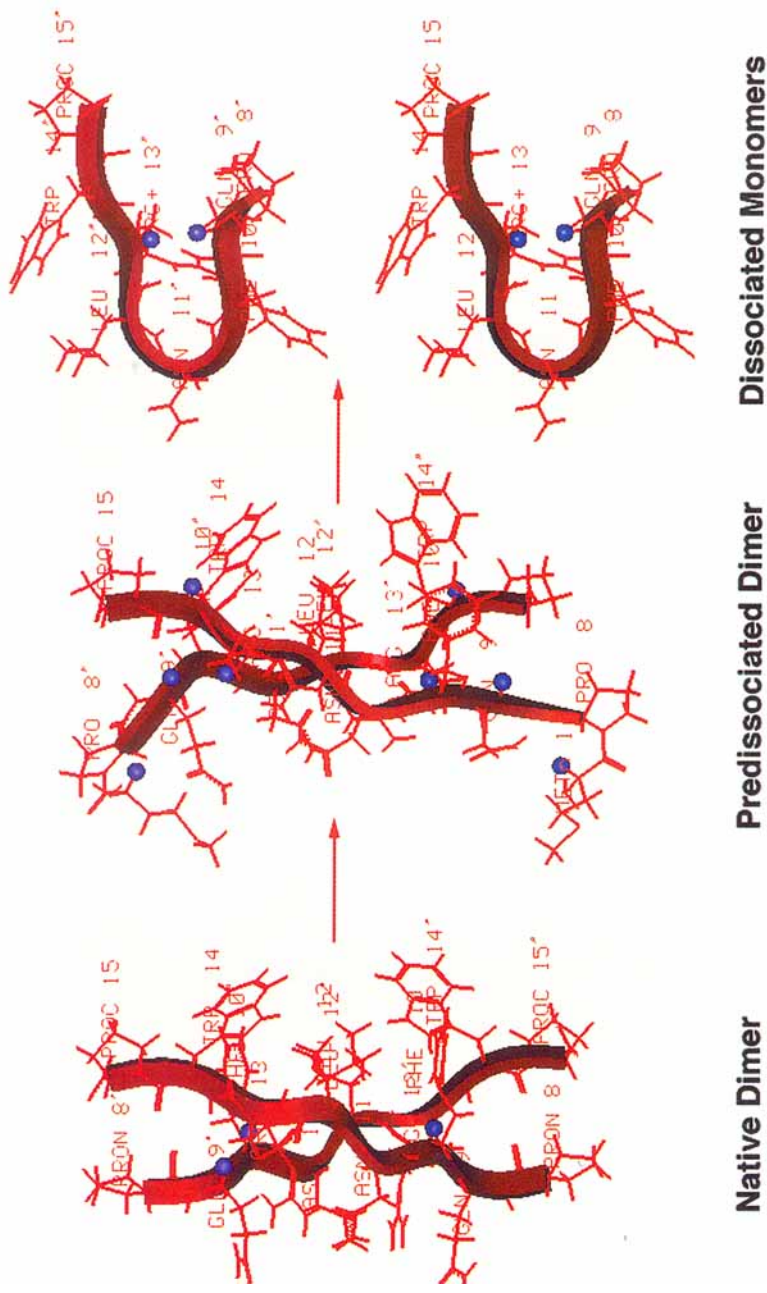


Fig. 2. Proposed  $\beta$ -sheet structure of the Arc repressor in the native state, the predisassociated state, and the dissociated molten globule state.<sup>23</sup> (Courtesy of J. Jonas, reproduced with permission from ref. 23. (Copyright 1994, American Chemical Society.)

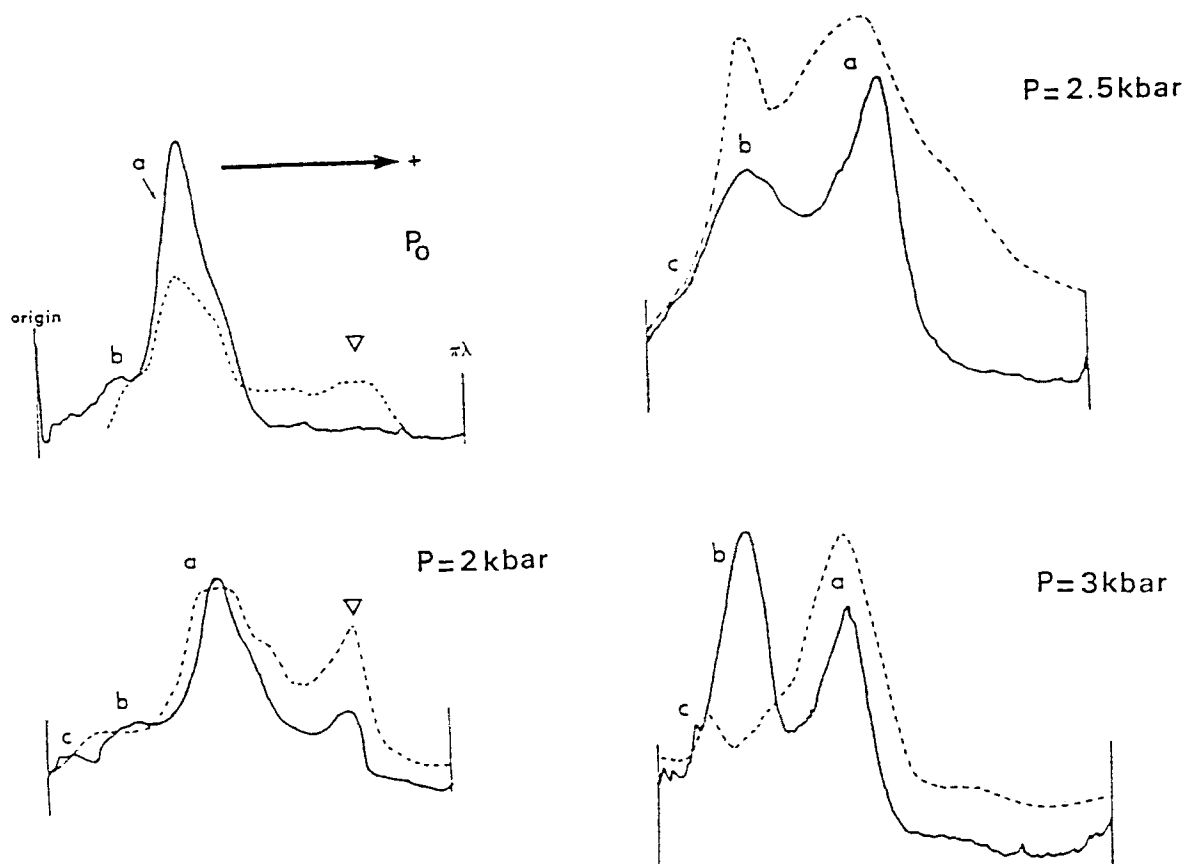
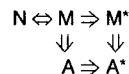


Fig. 3. Polyacrylamide gel electrophoresis of hydroxylamine oxidoreductase (HAO) in capillary gels. Densitometry patterns of the gels after electrophoresis at various pressures ( $P$ , in kbar;  $P_0$  is atmospheric pressure). Continuous curves are for gels stained for proteins; a, native enzyme (molecular weight of 150 kDa); b and c, aggregated forms of dissociated subunits. Dotted curves are for gels stained for enzyme activity;  $\nabla$  indicates the position of a fast migrating active form (dissociated subunit). The denaturation pathway of HAO up to 3 kbar may be depicted by the following scheme:



where N stands for the native enzyme, M the active subunit,  $M^*$  the inactive partially unfolded subunit, A active aggregates, and  $A^*$  inactive aggregates.<sup>37</sup> (Adapted with permission from ref. 37.)

fects on  $\Delta V^\ddagger$  was established for dehydrogenases<sup>45</sup>: the reaction was accelerated by salts that decrease the activation volume. As an explanation, it was suggested that the exposure to water of protein functional groups is changed during the conformational and hydrational events in catalysis. An important consequence from this correlation is the possibility of controlling the reaction rate by adding solutes; this effect can be predicted from the ion position in the Hofmeister series or by the direct salt effect on the free energy of transfer for protein groups from a nonpolar solvent to water.

An important role played by hydration has also been found for inactivation of enolase by high hydrostatic pressure.<sup>46</sup> In the transition state of dissociation, the previously buried interfaces of pressure-dissociated subunits of this dimeric protein become hydrated, and this hydration is favored by high pressures. On the other hand, addition of glycerol and

saccharides reduces protein hydration and stabilizes inter-subunit interactions.

### PROTEIN-NUCLEIC ACID INTERACTIONS

Interaction of proteins with nucleic acids has attracted much interest due to their importance in such in vivo key processes as transcription, replication, and restriction.<sup>47</sup> One of the best model systems for studying site-specific protein–DNA interactions is represented by restriction enzymes. The *EcoRI* restriction endonuclease is known to cleave the DNA sequence GAATTC between G and A on both strands. Under certain conditions, the enzyme specificity is relaxed, leading to a more efficient cleavage at other sites containing a noncanonical base pair (*EcoRI*\* or “star” activity). It has been proposed that discretely bound water molecules participate in the process of protein–DNA recognition and in the regulation of *EcoRI* specificity. In conjunction



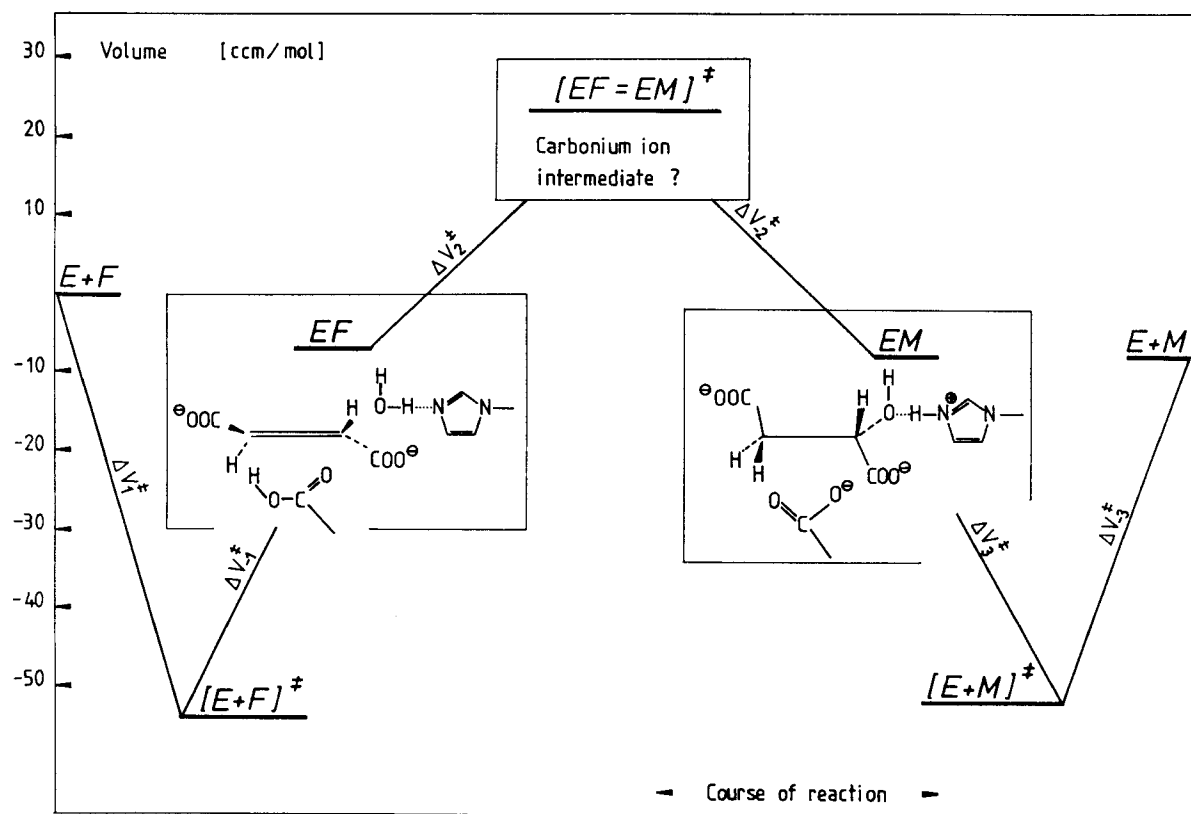


Fig. 4. Volume profile of the fumarase-catalyzed conversion of fumarate to L-malate at salt concentration of 0.3 M including a chemical mechanism at the active site. E, enzyme; F, fumarate; M, malate; EF and EM, enzyme-substrate complexes of fumarase with fumarate and malate;  $[E + F]^\ddagger$  and  $[E + M]^\ddagger$ , activation complexes of fumarase in binding of fumarate and malate;  $[EF =$

$EM]^\ddagger$ , activation complex for reaction;  $\Delta V_1^\ddagger$ ,  $\Delta V_{-1}^\ddagger$ ,  $\Delta V_2^\ddagger$ ,  $\Delta V_{-2}^\ddagger$ ,  $\Delta V_3^\ddagger$  and  $\Delta V_{-3}^\ddagger$ , activation volumes corresponding to different individual steps of the catalytic process.<sup>43</sup> (Courtesy of H. Ludwig, reproduced with permission from ref. 43. Copyright 1988 American Chemical Society.)

with calorimetry,<sup>48</sup> recognition mechanisms have been approached by hydrostatic pressure and osmotic pressure techniques to study hydration changes associated with *EcoRI*-DNA complex formation.<sup>49,50</sup>

Osmolytes (amino acids, sugars, and polyols), which are preferentially excluded from the protein surface, affect the binding of water to proteins, and their presence shifts the conformational equilibrium in macromolecules towards a state with the least amount of bound water.<sup>51</sup> A correlation between osmotic pressure, linked to osmolyte concentration, and binding affinity of a certain sequence of DNA to restrictase was predicted that may be interpreted as evidence for participation of bound water in this process. Indeed, in the *EcoRI*-DNA system, osmotic pressure, changed by the addition of different neutral solutes, increases enzyme-catalyzed cleavage at noncanonical "star" sites. The appearance of *EcoRI*\* "star" activity does not correlate satisfactorily with any solvent properties (dielectric constant, viscosity, etc.) other than osmotic pressure. The effect of hydrostatic pressure shifts the equilibria towards a more hydrated state with a smaller volume. As a

consequence, the effect of osmolytes is reversed by high hydrostatic pressure, restoring the natural specificity of *EcoRI* to its canonical site. The simplest molecular explanation of these effects is that during formation of the complex between the restriction enzyme and canonical site DNA, a portion of the water molecules is sequestered from the bulk solvent in order to hydrate the components. By contrast, these rearrangements in the components' hydration state are not so important when the enzyme is associated with the star sites.<sup>49</sup>

The approach described for the *EcoRI*-DNA system may provide a rather general method for studying water effects in mediating macromolecular interactions and may lead to a molecular understanding of solvation phenomenon in protein-nucleic acid recognition.<sup>50</sup> As suggested from high-pressure studies of another system, the Arc repressor-operator DNA complex, hydration effects also play a significant role in the ability of the protein to discriminate between specific and nonspecific sequences of DNA.<sup>52</sup>

Another important aspect investigated by high-pressure methods is the affinity of protein for nu-

cleic acids (measured at varying pressures and extrapolated back to atmospheric pressure) in the formation of specific, functionally significant complexes. Royer et al.<sup>53</sup> found that the *lac* repressor-operator complex, which plays an important role in the control of enzyme synthesis of the *lac* operon, is less stable than the tetrameric protein alone. On the other hand, Da Poian et al.<sup>27</sup> demonstrated that energy coupling between protein association and binding to RNA plays a crucial role in virus assembly. For cowpea mosaic virus, this stabilization effect of RNA is equal to 250 kJ per virus particle.

### CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In his discussion in 1987 on the applicability of hydrophobic liquid model to explain temperature and pressure effects on protein folding, Kauzmann<sup>54</sup> wrote: "Volume and enthalpy changes are equally fundamental properties of the unfolding process, and no model can be considered acceptable unless it accounts for the entire thermodynamic behaviour." He stated further: "Until more searching is done in the darkness of high-pressure studies, our understanding of the hydrophobic effect must be considered quite incomplete."

During the 8 past years impressive progress has been realized in this area. Adaptation to conditions under high pressure of methods such as X-ray structure analysis<sup>9</sup> and NMR<sup>7</sup> allowed the collection of high-resolution data from high-pressure experiments with proteins. This is illustrated by some of the recent results discussed in this overview. Concerning the structure-function and structure-stability relationship in proteins, the study of mutant proteins, obtained by site-directed mutagenesis, with high-pressure NMR and other spectroscopic approaches will certainly contribute increasingly to our understanding of protein structure and behavior.<sup>13,55</sup> There is a growing interest in studying volume changes in protein folding in relation to the molecular mechanism and the role of the molten globule in this process.<sup>6,31</sup> In combination with the effects of temperature and low-molecular-weight additives,<sup>56</sup> hydrostatic pressure will be more actively applied for studying mechanisms of enzymatic catalysis and modulation of activity and specificity of enzymes.<sup>4</sup> On the other hand, to facilitate interpretation of the results, a substantial effort should be made to eliminate the lack of model data describing the effects of pressure on the formation of hydrophobic interactions and hydrogen bonds. During the next few years it will become clear whether the growing optimism about the application of high pressure to studies in proteins will remain with us.

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