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Pressure effects on weak interactions in biological systems

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Abstract
To understand the principles of pressure effects on biochemical processes, it is helpful to consider the effects of changing volume at molecular levels. Non-covalent interactions constitute the main target for the modulation of biomolecular characteristics through pressure, mainly because they stabilize nearly all the biochemical compounds. On the other hand, the two parameters pressure and temperature must be considered in close relationship since their effects on equilibrium or kinetics—where the role of weak interactions is predominant—are antagonistic in molecular terms. An interpretation of reaction and activation volumes is usually given in terms of intrinsic and solvent contributions. Most covalent bonds participating in the protein primary structure are pressure insensitive, at least up to 1000–1500 MPa. 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. These structural aspects will be discussed through significant model systems and biochemical examples.

1. Introduction: the importance of weak interactions in biochemistry

Native structure of biological compounds, i.e. the conformation that displays biological activity, is the result of a delicate balance between stabilizing and destabilizing interactions (strong or weak) within the polypeptide chains for proteins and with the solvent. Native biocompounds are stable in a narrow physical–chemical zone depending both on the nature of the biomaterials itself and on the environmental conditions (such as other proteins, nucleic acids, membranes, lipids, solutes, solvents, salts, pH, temperature). Weak interactions are, in biochemistry, one of the keys to understanding pressure effects on biomaterials. The main reason is because weak interactions are strongly involved in the structure of biomolecules and in the functions of many bioprocesses. The structures of nearly all the biocomponents are stabilized by such

interactions. In addition to these numerous forces, the role of solvation is important and so, consequently, is the relationship between the biocompounds studied and their environments, relationships driven by weak interactions [1].

For example, native globular proteins are mainly constituted of amino acids, some of them buried inside the structure with a low solvent accessibility. In contrast, other amino acids could be at the surface of the protein. The latter are solvated and they play an important role in the intermolecular interactions.

Proteins are tightly packed. They have a compact structure with ample intra-globular voids. The voids are small cavities subjected to dynamic fluctuations which can govern conformational dynamics, one source of protein activity. Both voids and weak interactions allow protein fluctuations which can be large, such as rotation of side chains and small protein inducing subconformations. A protein in solution is in a dynamic and thermodynamic equilibrium of various conformers. The multiple conformations depend also on the nature of the interactions involved. To understand these molecular fluctuations, one way involves perturbing them. This is why it is important to carry out both pressure and temperature experiments on these systems to separate thermal and volume effects.

However, weak interactions are not typical for just biosystems. They are also involved in understanding the pressure effects on many inorganic or bioinorganic reactions: synthesis, solvent exchange, ligand substitution, addition, elimination, electron transfer and radiation induced reactions [2–4].

2. Pressure and temperature parameters

The effects of pressure and temperature on the equilibrium 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. Ordering by pressure is offset by an increase in temperature. A clear demonstration of this pressure and temperature antagonistic behaviour was given more than 30 years ago by Hawley [5] showing the classical elliptic phase diagram of protein denaturation. In addition to the understanding of the fundamental thermodynamic concepts, these diagrams are useful for selecting biotechnological treatments for modulating the structure and stability of proteins [6].

3. The volume change; a useful tool for a 'quantitative' evaluation of pressure effects

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. An increase in pressure favours reduction of the volume of a system. The volume change is the difference between the volumes of the final and the initial states according various expressions substantially analysed in recent reviews [1, 6, 7]. Analogous expressions can be given for the rate constant of an elementary reaction process $A \rightarrow B$ where the activation volume is the difference between the activated and the ground states, according to the classical transition state theory of Eyring, assuming that between the two states A and B, there is a labile complex named the transition state. Pressure shifts equilibrium and accelerates processes for which the transition state has a smaller volume than the ground state. Therefore, pressure favours processes that are accompanied by negative volume changes. Unfortunately, in contrast to the case for simple chemical reactions, it is very difficult to give a precise physical meaning to the pressure effects for most biochemical reactions. However, the pressure parameter, like the temperature one, is used to affect enzyme selectivity and reaction rates [8, 9]. From a practical point of view, the volume changes of activation volumes are obtained by plotting the logarithm of the equilibrium con-

stant or the logarithm of the rate constant as a function of pressure. Generally, plots are linear in the pressure ranges explored for the biological processes studied, i.e. less than 1000 MPa.

4. The nature of the weak interactions involved in biosystems, and the pressure effects on them

Biological systems are very diversified and, in this review, we mainly focus on proteins in solution, a large subject in itself. These macromolecules are composed of polypeptide chains in close interactions. It is possible to distinguish two main classes of interactions, the strong one and the weak one. Both are involved in protein stabilization.

Covalent bonds are strong interactions as defined in chemistry. Their formation and/or breaking are responsible for changes in both free volume and activation volumes which control reaction pathways. For many other cases, such breaking may occur and yield protein denaturation. The ΔV values for exchanges in covalent bonds are nearly zero. As a consequence, most covalent bonds participating in the protein primary structure are pressure insensitive at last up to pressure values of 1000–1500 MPa. Volume changes that arise from changes in packing density are considered to be small.

In contrast, electrostatic interactions, hydrogen bonds and hydrophobic interactions are pressure sensitive weak interactions, and their contribution to the observed ΔV is based on model systems [10].

4.1. Electrostatic interactions

When an ion is formed in solution, the nearby water dipoles are highly compressed by the Coulombic field of the ion. This is electrostriction accompanied by a decrease in volume. From model data, it was concluded that electrostatic interactions in biomolecules are weaker at elevated pressure. A typical example is the pressure induced reversible denaturation of chymotrypsin due to the dissociation of a salt bridge in the active site region. Another example is given by the dissociation constants of weak acids which vary with pressure. Phosphate buffer has high ionization volumes in contrast with the protonated bases such as imidazole-HCl or tris-HCL which have nearly zero ionization volumes [11]. For the solvation of singly charged ions in water, the ΔV values are about -10 ml mol^{-1} and the dissociation of a neutral molecule into two ions induces a contraction of about -20 ml mol^{-1}.

4.2. Hydrogen bonds

These are stabilized by high pressure and a typical example is given by the pressure dependence of the infrared spectra of the α-helix in myoglobin. A very small ΔV value is observed for processes in which there is an exchange between the existing hydrogen bonds [12].

4.3. Hydrophobic interactions

The hydrophobic interactions are the most important weak interactions for protein stabilization. The formation of hydrophobic contacts proceeds with positive ΔV values disfavoured by pressure. The increase in volume for the formation of hydrophobic interactions varies from +1 to +20 ml mol^{-1} depending on the system studied. Stacking interactions between aromatic rings show negative volumes changes and are stabilized by pressure [13].

5. General pressure effects on biosystems [1, 7, 14, 15]

Pressure is able to affect the protein structure, i.e. its architecture. There are four molecular levels of structure. The primary structure is simply the sequence of amino acids stabilized by

covalent connections. The secondary structure relates to the steric relationship of amino acid residues that are close to one another in the linear sequence. Some of these steric relationships are of a regular kind: a periodic structure such as the α helix or the β pleated sheet. The tertiary structure relates to the steric relationship of amino acid residues that are far apart in the linear sequence. The dividing line between secondary and tertiary structure is arbitrary. The quaternary structure relates to proteins which contain more than one polypeptide chain. Thus, the term 'quaternary structure' refers to the way in which the chains are packed together. Each polypeptide chain is called a subunit.

We will examined the pressure effects on these structures.

The protein quaternary structure, maintained by hydrophobic (weak) interactions, is the most pressure sensitive. Moderate pressures ($<$150 MPa) were found to favour the dissociation of oligomeric proteins, a phenomenon always accompanied with negative and sometimes very large volume changes. Dissociation of lactic dehydrogenase is accompanied with a negative volume variation of 500 ml mol^{-1}. Dissociation can be followed by subunit aggregation or by precipitation. Generally, oligomer dissociation occurs at pressure $<$150–200 MPa, lower than those at which unfolding of monomers is observed. Pressure above 150–200 MPa induces protein unfolding and reassociation of subunits from dissociated oligomers.

To be affected by pressure, the tertiary structure must be subjected to higher pressure than the quaternary structure. Changes are observed beyond 200 MPa. However, reversible unfolding of proteins can occur at higher pressure (400–800 MPa) showing that the volume and the compressibility changes during denaturation are not completely dominated by hydrophobic effects. Denaturation affecting the tertiary structure is a complex process sometimes involving intermediate states (or forms) such as the molten globule state, leading to multiple denatured forms.

The secondary structure changes take place at a very high pressure, above 300–700 MPa, leading to non-reversible protein denaturation. This depends on the compression rate and on the extent of the secondary structure rearrangements.

The primary structures, maintained by covalent (i.e. strong) interactions, are nor pressure sensitive at ordinary temperatures.

Different biophysical methods can be used in conjunction with pressure to study such pressure effects on protein structure (and function) [7]. Electrophoresis, ultracentrifugation, fluorescence spectroscopy and NMR spectroscopy are used to study pressure effects on the quaternary structure. For the studies of the pressure effects on the tertiary structure, the major methods used are NMR spectroscopy, x-ray analysis, UV/visible and fluorescence spectroscopy, and pressure jumps. As regards the secondary structure, only vibrational spectroscopy can give suitable information. The majority of these methods have been adapted to work up to 500–700 MPa, some of them being specially adapted to perform experiments up to several GPA such as vibrational spectroscopy (using diamond anvil cells).

For the studies on pressure effects on ligand binding and enzyme activity, dynamic methods have been adapted to high pressure experiments, such as flash photolysis, stopped flow and rapid sampling. For the two last methods, due to the technical high pressure adaptation difficulties, the working pressure is generally limited to 200–300 MPa.

6. The biological role of weak interactions

The exhaustive list of implications of weak interactions for biological functions is too long to quote in this paper. Among recent newly explored fields, important implications in medicine have been demonstrated. This includes for example the case of some diseases where protein conformations are involved. Neurodegenerative diseases such as Alzheimer's dementia,

Parkinson's disease and Huntington syndrome have become major concerns for public health as life expectancy increases. Another case relates to the devastating symptoms of transmissible spongiform encephalopathies, or prion diseases, for which, up now, no cure is available, but which are caused by an aberrant conformational change of a protein (i.e. the cellular prion protein) leading to its deposition in the form of amyloid fibrils [16]. For all these diseases, weak interactions are involved, and as we will see below, pressure could be a useful tool for understanding the mechanisms of such aberrations.

7. Pressure effects on 'biochemical' weak interactions: some significant examples

7.1. High pressure as a tool for studying reversible protein folding–unfolding

A typical example is given by the study of the monomeric 33 kDa protein isolated from spinach photosystem II particles which has been used to explore high pressure protein unfolding [12]. This work has been achieved in collaboration with K Ruan (Shanghai, China). The protein molecule consists of only one tryptophan residue and eight tyrosine residues which can be used to probe the protein conformation and structural changes under pressure using either fluorescence spectroscopy or fourth-derivative UV absorbance spectroscopy. An extrinsic fluorescent probe, ANS (8-anilino-naphthalino-sulfonic acid) was also used to measure pressure induced protein unfolding via hydrophobicity variations. Its fluorescence is enhanced when it is bound to solvent-exposed hydrophobic areas of proteins. The above probes detect only the changes in local regions. For instance, the tryptophan fluorescence study detects the conformation change around tryptophan residue; the ANS fluorescence study detects the hydrophobic core. Local changes represent the behaviour of the whole protein with some exceptions. As regards the latter, we have already observed the effect of pressure on trypsin, with the observed fluorescence variations being attributed to the difference in compressibility of the various regions of the protein [17].

Experiments as a function of pressure indicated that the protein shows a reversible two-state transition from atmospheric pressure to about 180 MPa. Different weak interactions are involved in the process. Salts (NaCl) significantly protect the protein from pressure induced unfolding. Energetic values of the process have been determined showing that the salt effect also modifies the standard free energy and the volume variation. The presence of salt greatly reduced ΔV. This may be explained by two hypothesis: firstly, the salt could shield the electrostatic interactions, resulting in a reduction of ΔV; secondly, the salt could play a role in osmotic stress which can internally dehydrate the protein, resulting in a reduction of ΔV.

For the same system, it has been found that sucrose and glycerol have profound effects on pressure induced unfolding [18]. The additives shift the equilibrium to the left, causing a decrease in the standard volume change ΔV, related to the additive concentrations. The decrease in ΔV varied with the additive. The theoretical shift of the half-unfolding pressure calculated from the net increase in free energy upon addition of additives was lower than that obtained from experimental measurements. This indicates that the free energy change caused by preferential hydration of the protein is not the only factor involved in the protein stabilization. The origin of the ΔV reduction could involve many factors. The compressibility must be taken into consideration. On the other hand, the contraction of the protein–solvent interface caused by the increase in chemical potential is accomplished by water release from the inside of the protein, influencing weak interactions and consequently increasing the core density. This is the problem of preferential hydration. At the end, the reduction in ΔV might be the result of osmotic stress which could be the result of the exclusion of the adducts from the protein core.

These data clearly show the potentiality for using high pressure as a tool to perturb weak interactions giving deep thermodynamic and structural information.

7.2. *Protein misfolding and neurodegenerative diseases*

As pointed out above, weak interactions are involved in protein misfolding and recent data from this laboratory have shown alternative prion structural changes revealed by high pressure [19]. The misfolded infectious isoform (PrP^{sc}) of the normal prion protein (PrP^{c}) has been proposed as the sole component responsible for both pathology and infectivity in transmissible spongiform encephalopathies [20]. Under this hypothesis, self-propagating conformational rearrangement to a β-sheet-rich structure leads to a β-amyloid fibrils. An understanding of the molecular basis of these structural changes where weak interactions are involved is required. Many experimental approaches, including the use of high temperature, chemical denaturants, salts etc, have shown that PrP can adopt completely different non-native conformational states. Despite many efforts, it has proved impossible to obtain *in vitro* formation of a PrP^{sc}-like state capable of causing disease. The experimental conditions used so far do not lead to the alternative folded form associated with infectivity.

High pressure has been used as an alternative for finding new pathways for prion structural changes [21]. Such a perturbation takes place by favouring the dissociation of electrostatic bonds and by favouring the solvation of hydrophobic residues. The pathway induced by high pressure may be different to that at high temperature. In some cases, the application of moderate pressures has been shown to protect unfolded proteins from aggregation and to assist disaggregation of protein aggregates. The reason for this structural effect of pressure can be understood as the hydration of buried hydrophobic residues.

Aggregation–disaggregation and protein changes of a recombinant mammalian PrP protein as a function of both pressure and temperature have shown that these parameters act in different ways on both PrP structural changes and aggregation. Different weak interactions have been affected, revealing several alternative prion structural changes. They differ from those that have been observed by more conventional methods (involving chemical compounds or temperature).

Evidence that pressure may provide us with a better understanding of the folding–unfolding pathways has been reported for another prion protein, the yeast prion protein Ure2 [22].

7.3. *Oligomeric dissociation and pressure aggregation*

The mechanisms of pressure dissociation are complex but it is a domain where weak interactions are deeply involved (see above). Pressure not only acts at the level of the interface between the subunits; protein conformational changes are also involved in the pressure denaturation processes of oligomeric proteins.

Subunits in oligomeric proteins and in supramolecular assemblies are mainly associated through hydrophobic bonds. Pressure acts as a destabilizer, inducing dissociation of oligomeric structures. This effect often occurs below 200 MPa and, after pressure release, the reverse transition may be shown and hysteresis phenomena are observed. A typical case is the hysteresis effects observed in the pressure induced dissociation of a protein β_2-dimer of tryptophan synthetase [23]. These time dependent conformational changes called ‘conformational drift’ are very often due to long-living metastable intermediate states [24].

Often, subunit dissociation is followed by subsequent conformational changes in individual subunits such as in the case of the pressure dissociation of a small dimeric Arc repressor protein. To investigate that, two high pressure NMR methods were used: phase sensitive two-dimensional correlated spectroscopy and nuclear Overhauser effect enhancement

spectroscopy [25]. Clearly, NMR changes in NMR spectra prior to dissociation point to the existence of a predissociated state that may correspond to an intermediate in the process of folding and subunit association of the Arc repressor. A conformational drift was then observed.

Aggregation of proteins is a problem affecting both industrial production of proteins and human health (cases of neurodegenerative diseases). Because pressure acts to disfavour the hydrophobic and electrostatic interactions that cause protein aggregation, studies conducted under pressure may allow protein aggregates to be formed reversibly. A growing literature suggests that pressure may be a useful tool for both understanding protein aggregation and reversing it in industrial applications [26].

7.4. Special cases

The literature reports several examples where there are no pressure induced oligomeric dissociations. This is the case for the sweet potato β-amylase which is an enzyme found in higher plants and composed of four identical subunits. Under high pressure, this tetramer does not dissociate, a phenomenon explained by the significant role of the pressure induced molecule hydration change.

Another example concerns the tetrameric form of butyrylcholinesterase which does not dissociate up to 350 MPa. This behaviour suggests that the inter-subunit area of this protein is mainly stabilized by either pressure insensitive interactions, i.e. hydrogen bonds, or pressure enhanced interactions such as aromatic–aromatic bonds [27].

Pressure can induce structural modifications at the level of the dissociated monomers. This was found in the case of the oligomeric bacterial chaperones: tetradecameric and heptameric proteins. The pressure-dissociated monomers do not reassemble readily, indicating a pressure alteration of their native structure, a typical example of conformational drift [24, 28].

7.5. Dissociation and enzyme activity

Since hydrostatic pressure is viewed as a gentle and reversible perturber, several experiments have been reported studying the pressure induced dissociation of oligomeric enzymes with the goal of producing enzymatically active monomers. For many systems, the fundamental question is how to determine the location of the active site in an oligomeric enzyme structure, i.e. determining whether or not the monomers have catalytic activity. To explore such problems, pressure dissociation presents a great advantage in comparison with dissociation produced by chemical denaturants because, in addition to dissociation, the latter generally also induces monomer denaturation.

Typical experiments were carried out in this laboratory by Kornblatt (Montreal, Canada), in an effort to answer to the question: can monomers of yeast enolase have enzymatic activity [29]? All enolases are dimers with subunit molecular masses of 40–50 kDa. Using a combination of UV spectroscopy under pressure and stopped-flow kinetics under pressure, it has been demonstrated that the monomers of yeast enolase produced by hydrostatic pressure are inactive. The major change that occurs upon dissociation is in the average environment of tyrosine residues, which become less polar. There are a water-filled clefts between the subunits. In the dimer, this water is relatively rigid and therefore has a higher dipole than bulk water. Many of the subunit contacts involve loops where there are salt bridges and hydrogen bonds between monomers. There is a network of interactions that maintains a given conformation of the monomer affected by the pressure treatment.

Using similar approaches, inactivation of creatine kinase (a cytoplasmic dimeric enzyme involved in cellular energy metabolism) by high pressure was studied; the finding was that

inactivation precedes dimer dissociation [30]. The multistate transitions of creatine kinase caused both by pressure and guanidine denaturation are in a direct relationship with the existence of hydrogen bonds which maintain the dimeric structure of the enzyme.

Another example is provided by the pressure effects on apo wild-type glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* (a tetrameric enzyme) where denaturation precedes dissociation into subunits [31]. As expected, denaturation is accompanied by the loss of enzymatic activity. Due to the stabilizing effects of hydrogen bonds, the interfaces (which contain hydrogen bonds) are more stable than the subunits.

Recently, the process of pressure induced modification of horse liver alcohol dehydrogenase, a dimeric enzyme (HLADH), was studied [32]. Using tryptophan fluorescence measurements and activity kinetic data, results indicated that the pressure induced denaturation of HLADH was a process involving several transitions and that the observed transient states have characteristic properties of molten globules. Low pressure (<100 MPa) induced no important modification in the catalytic efficiency of the enzyme and slight conformational changes: a native state was assumed. Higher pressure (100–400 MPa) induced a strong decrease of HLADH catalytic efficiency and further conformational changes. At 400 MPa, a dimeric molten globule-like state was proposed. Further increase of pressure (400–600 MPa) seemed to induce the dissociation of the dimer, leading to a transition from the first dimeric molten globule state to a second monomeric molten globule. The existence of two independent structural domains in HLADH was assumed to explain this transition: these domains were supposed to have different stabilities against pressure induced denaturation where different weak interactions are involved. FTIR spectroscopy confirmed that the intermediate states have a low degree of unfolding and that no completely denatured form seemed to be reached, even up to 1000 MPa. Specific hydrophobic residues present at the subunit interface play a crucial role in these processes.

8. Conclusions

The role of weak interactions in biochemical processes is indeed complicated. The biochemist tries to apply what is learned from various model systems. For example the stabilizing effect of pressure on hydrogen bonding may be seen from a comparison of the effects on the intermolecular interactions in hydrogen-bonded and non-hydrogen-bonded liquid amides [33]. This model is an oversimplification of organized systems such as proteins. The fact that high pressure stabilizes hydrogen bonds has important consequences for the secondary structures of the proteins. This explains also the extreme stability of nucleic acids under pressure [34]. In fact, for many biosystems, all weak interactions are involved together and at the same time. Pressure is then a perturber displacing all the equilibriums which maintain the activity of biomolecules. This is the deep difference from the pressure action on chemical systems where, generally, not all of the weak interactions are involved all together, at the same time [35].

Weak interactions are substantially responsible for biocompound stability. The understanding of the pressure induced changes at the molecular level provides fundamental information that can be applied in practical and industrial applications, such as in food processing [36].

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