TRENDS IN HIGH PRESSURE BIOSCIENCE AND BIOTECHNOLOGY

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PREFACE

The papers in this book comprise the proceedings of the First International Conference on High Pressure Bioscience and Biotechnology (HPBB-2000), organized by R. Hayashi, C. Balny, J.C. Cheftel, K. Heremans, S. Kaneshina, C. Kanno, C. Kato, D. Knorr, S. Kunugi, H. Ludwig, A. Suzuki, J. Frank, K. Gekko, H. Iwahashi, J. Jonas, N. Klyachko, P. Masson, A. Noguchi, M. Osumi, J.L. Silva and K. Yamamoto, which was held during Nov. 26 to 30, 2000 in Kyoto, Japan.

A number of conferences devoted to various aspects of high pressure in the field of biological science have been organized and industrial applications of high pressure techniques in food processing has been developed in Japan over the past ten years: Domestic symposia, organized by the Japanese High Pressure Group, on biologically related fields have been held each year since 1988; The European High Pressure Research Group (EHPRG) has a long tradition in bringing together European scientists from various fields; The concept of having joint Japanese and European meetings on High Pressure Bioscience was elaborated by a small group of scientists and the first meeting was held in France (1992) and subsequent meetings have been held in Japan (1995), Belgium (1996) and Germany (1998). During the development of this area of science and technology, it became necessary to organize a formal international association, because of the world wide interest and the establishment of such a group was proposed at the last joint meeting in Heidelberg, Germany. HPBB-2000 in Kyoto represented the first conference sponsored by the Association and it was a great pleasure to welcome about 200 participants from more than 20 countries in Asia, Australia, Europe, North and South America, in addition to large delegations from France and Germany.

Interest in high pressure has become widespread in the field of bioscience including food sciences, pharmacy and medical fields. Some high pressure techniques are used in the production of industrial products. Moreover, high pressure is a valuable tool for the study of natural macromolecules including biomembranes which are composed, primarily, of lipid and protein. Many intermediate processes in the pressure-induced protein unfolding have been discovered, as a result. This book covers the entire range of current high pressure bioscience and its possible applications.

We wish to thank the authors for their prompt submission of manuscripts, and all participants of the Conference for creating a cordial atmosphere and for then frank and open-minded discussion.

We hope that this publication will stimulate further developments in basic and applied research as well as a new area of scientific-industrial cooperation.

September 2001, Kyoto

Editor
Rikimaru Hayashi

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CONTENTS

Trends in High Pressure Bioscience and Biotechnology
R. Hayashi (editor)

High pressure bioscience and biotechnology: A century and a decade perspective

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In the last decade of the past century we have noticed an explosion in activities in the field of high pressure bioscience and biotechnology. It is a fascinating history of new experimental approaches, new concepts and new applications that has his roots in many branches of the history of scientists, science and technology.

1. INTRODUCTION

It all started a century ago with the innovative technological developments of Bridgman which allowed him to reach pressures far beyond those available at that time and his famous "observation of possible biological interest" on the white of an egg. But people were more interested in the "hot ice" that he made! During the time that Bridgman continued his physical studies, Basset and Macheboeuf laid the foundation for a long tradition of biological high pressure studies in France. Suzuki is at the origin of the "Kyoto Hot Spot". The number of laboratories in Kyoto in the field of high pressure bioscience is indeed remarkable.

During the last decade we have noticed interesting applications of high pressure technologies of the food industry in Japan, followed by the USA. Europe issued Novel Foods Regulations because, as seen in other issues, it is more hesitant to start new adventures.

This First International Conference on HP Bioscience & Biotechnology (HPBB-2000) is the outcome of a long process that started in Japan from the Japanese High Pressure Group in the Biologically Related Fields, a group that was and still is the brainchild of Prof. R. Hayashi. As we gather in this beautiful Kyoto during the last few weeks of the present century, it is appropriate to express our thanks to Prof. R. Hayashi and his team of staff and students under the silent guidance of Miss Setsuko Yasui. They will take care of us in this beautiful part of the world. When walking through Kyoto, I always keep in mind the advertisement that I discovered in the subway: "If you do not love your own garden, you cannot appreciate the beauty of the garden of your neighbour". Indeed, Kyoto is the not only the historical and cultural center of Japan, it is also an intellectual and spiritual center of the world.

2. A CENTURY PERSPECTIVE: THE FASCINATING HISTORY

If we extend the scope of our perspective over more than a century, it becomes clear that the development of the concept of pressure gradually developed into the simple idea of a force on a surface.

Whereas the contributions of Archimedes of Syracuse (c287-212 BC), Blaise Pascal (1623-1662) and Evangelista Toricelli (1608-1647) are well known, it was Simon Stevin van Brugghe (1548-1620) who stated and explained the "hydrostatic paradox", the fact that pressure exerted by a fluid is determined by the vertical height and not by the quantity of the fluid [1]. It was Toricelli, one of the last students of Galilei who observed that the "The height of the mercury column varied from day to day". It was Pascal who extended the analysis of Stevin and supplemented the work of all his predecessors by combining the statics of liquid and gases. His famous experiment on the Puy de Dôme showed that the mercury column was held up by the pressure of the air and not by the abhorrence of nature for vacuum as has been assumed since the time of Aristotle. Further details can be found in the monograph of Dijksterhuis [2].

The development of high pressure bioscience is not documented in a single volume. Several books contain chapters that discuss some topics related to bioscience. Bridgman himself always kept a lively interest in the field as may be judged from the last chapter in his book "The physics of high pressure"[3] and in the many reviews that are collected in his "Collected experimental papers"[4]. The most extensive source on the older literature is probably to be found in "The kinetic basis of molecular biology" by Johnson et al. [5] or the newer edition "The theory of rate processes in biology and medicine" [6]. Books that concentrate only on pressure effects are: "High pressure effects on cellular processes" by Zimmerman [7] and "The effects of pressure on organisms" a symposium volume edited by Sleigh and Macdonald [8].

Over a period of about a century the emphasis in our understanding of the effect of pressure on organisms shifted from the question of how organisms can survive under those conditions to the recent discovery that some organisms actually like these extreme conditions. This contrast can be illustrated from two quotations from the end of the 19th century and the end of the 20th century. In 1884 Regnard wrote: "Le physiologiste n'a donc plus (qu') à rechercher si, sous ces pressions énormes de plus de 600 atmosphères que supporte le fond de l'Océan, la vie existe; il doit maintenant essayer de déterminer dans quelles conditions elle peut se produire". This phrase was written with the reporting of the fascinating observations made during the "Talisman" Atlantic Deep-Sea Expedition in 1883 [9].

The recent volume on Extremophiles by Horikoshi and Grant [10] shows how well life is adapted to the extreme conditions of temperature, pressure, cold, salinity, pH, etc. An important point that is becoming clear is that under such extreme conditions one encounters almost exclusively prokaryotes, many of which belong to the archaea, an evolutionary line of microorganisms quite distinct from the other prokaryotes. The book of Gross, "Life on the edge" nicely illustrates the biotechnological developments that have resulted from the research in that field [11].

My personal view, which is strongly biased because of my interest in the behavior of molecules, is that the story really started with the observations of Bridgman. In 1914 P.W. Bridgman – he obtained the Nobel Prize for Physics in 1946 - reported "a fact of possible biological interest": "If the white of an egg is subjected to hydrostatic pressure at room temperature, it becomes coagulated, presenting an appearance much like that of a hard boiled egg". Being interested in phase transitions of compounds as a function of pressure and temperature, he notes: "The effect of temperature, which is not large, seems to be such that the ease of coagulation increases at low temperatures, contrary to what one might expect" [12].

It may be noted that nobody was interested in this observation for immediate exploitation. To the surprise of Bridgman, there was some interest from the pressmedia in the "hot ice" that he made! The complete story can be found in Bridgman's biography [13]. With a strategy developed to search for new pressure-induced transitions, Bridgman observed new phases of water among them the so-called "hot ice". He reports: "It is well known that under ordinary conditions water is abnormal in many respects. The effect of high pressure is to wipe out this abnormality...the modification of ice stable at high pressures giving indications of being the last form, corresponding to the completely normal liquid" [14].

Bridgman continued his studies on the physical properties of liquids and solids. Every new discovery stimulated him to develop new pressure equipment to reach higher pressures. These new technical developments were of great help to other scientists who, at about the same time, tried to develop procedures to sterilise milk with high hydrostatic pressure. It is of particular interest to read the reports of Hite [15] were the constant concern for technical problems with the pressure equipment hindered progress in the field. As may be judged from his reviews, Bridgman took an interest in the biological effects of pressure. The lifelong interest of Bridgman in the challenge for the making of diamonds is also well documented [16].

In France, Basset and Macheboeuf reported their results on the effect of high pressure on the inactivation of enzymes, viruses, antigens and antibodies, some of them being quite pressure stable, microorganisms, tissues and cells. In the USA, Johnson and coworkers at Princeton, studied various biosystems at low pressures in order to get insight in the physicochemical mechanisms [5, 6].

In Japan, the research concentrated on the denaturation of proteins. Here we see the first report on the pressure and temperature effects on ovalbumin and hemoglobin with the data reported as a phase diagram [17]. At low temperatures and high pressures, negative activation energies are reported and interpreted as the pressure-induced penetration of water into the protein as the first step in the denaturation process. High pressure computer simulations support this proposal [18]. The developments since then have been reviewed elsewhere [19]. It is now possible to make a unified phenomenological description of the cold, heat and pressure denaturation of proteins.

3. A CENTURY PERSPECTIVE: NEW CONCEPTS

This conference takes place almost exactly a century after Max Planck held his famous lecture, to be precise on December 14, 1900, on the interpretation of the black-body radiation. This lecture was considered as the birthday of Quantum Physics. It was the time of the development of chemical thermodynamics especially by Josiah Willard Gibbs. But since his papers were rather mathematical, he was understood by only a few. The contributions of van't Hoff, Helmholtz and Planck were more accessible for chemists. In 1884 van't Hoff derived an equation for the effect of temperature on a chemical equilibrium ($\delta \ln K/\delta T = \Delta H/RT^2$) based on the Clapeyron-Clausius equation (1834). Incidentally, he noticed that the heat absorbed or released is not the driving force for a chemical equilibrium as proposed by Bertholet. A few months later, Le Chatelier, stated the *Le Chatelier principle* based on the equation proposed by van't Hoff. Van't Hoff also derived an equation for the effect of temperature on the reaction

rate, but the equation became known as the Arrhenius (1889) equation ($\delta \ln k/\delta T = Ea/RT^2$). More details on the development of chemical thermodynamics can be found in Laidler [20].

The derivation of the equations describing the effect of pressure on the equilibrium was then a trivial matter except for the fact that it is much more difficult to verify experimentally. In 1887 Planck proposed the equilibrium equation ($\delta \ln K/\delta p = - \Delta V/RT$), followed by the kinetic equation ($\delta \ln k/\delta p = - Va/RT$) proposed by van 't Hoff (1901) and Evans and Polanyi (1935). This last equation is often found in high pressure articles or books as it gives for the first time a molecular interpretation of the activation volume in terms of intrinsic volume effects and solvent effects.

The combined effects of pressure and temperature on physical equilibria was first discussed by Bridgman in 1915. The application of these equations was used in the early seventies by Brandts and Hawley. The treatment makes use of the change of the Gibbs free energy change as a function of pressure and temperature:

$$d(\Delta G) = (\Delta V)\, dp - (\Delta S)dT$$

With the relation between ΔG and the equilibrium constant K, an expression is obtained, assuming that ΔV and ΔS are pressure and temperature dependent, that is used to analyze thermodynamic as well as kinetic data for the denaturation of proteins, the inactivation of enzymes and microorganisms, and many other biochemical and microbiological phenomena. The most salient feature of the equation is that it described the cold, the heat as well as the pressure denaturation of proteins.

A molecular interpretation of kinetic and thermodynamic data is usually based on data obtained from low molecular weight model systems. This is done in terms of intrinsic volume and solvent effects as first proposed by Evans and Polanyi. But the separation of these effects can only be based on molecular models. These models, again, start from some basic assumptions about the pressure and temperature behavior of matter. Occasionally one can hear very lively discussions among chemists at conferences up to the point that a lonely physicist in the audience asks the question "How real are these activation volumes?". The audience starts laughing, but during the coffee break, the reaction was different: People realize that there is thermodynamics and kinetics on the one hand and the molecular reality on the other hand and that it is not at all obvious how to bring them together even in the post-Boltzmann era.

For proteins and other biopolymers there is the additional factor of uncertainty: the packing of the atoms which is not always perfect. In order to take this effect into account Kauzmann (1959) proposed to consider the volume of a protein in solution as being composed of three contributions: The atoms, the cavities and the hydration. It is clear that pressure as well as temperature effects give rise to changes in the cavities as well as the hydration. Independent data on the cavities and the hydration are hard to get and most investigators are forced to make assumptions on the contributions of these factors. One possible technique that could be used to probe the role of cavities in proteins is positron annihilation lifetime spectroscopy. It is a powerful technique for determining the size of cavities in synthetic polymers.

4. A DECADE PERSPECTIVE: NEW APPLICATIONS

Somewhat a decade ago, the use of high pressure found new applications and industrial realizations, following the suggestions made by Hayashi and coworkers. Although Hite in the USA, Macheboeuf and coworkers in France, had studied the possible applications of high pressure for the treatment of milk and vaccines respectively, it was in Japan that the first products were put on the market. This was followed by an explosion of new papers exploring various possibilities mostly in food science but also in medical and pharmaceutical applications. The challenge for the industry was to develop new high pressure machines that could handle the vast amount of material that is usually treated in food preservation technologies.

This was not an easy job, both technically and from a commercial point of view. The developments made in Japan are recorded in the volumes (in Japanese with one page abstracts in English) edited by R. Hayashi under the title: High Pressure Bioscience Conferences in Japan [21]. A total of eight volumes has been published up to now.

The first conference on High Pressure Bioscience & Biotechnology in Europe was organized by C. Balny in 1992 in Montpellier (France). The publication of the proceedings proved to be a commercial success [22]. Other conferences were held, both in Japan and Europe, the proceedings of which are available [23]. There is also the long NATO Advanced Study Institute tradition, the proceedings of the most recent one contains a number of chapters on bioscience [24]. Planned high pressure conferences can always be found on the website of the European High Pressure Research Group [25].

The European Community supported the first research project on the application of high pressure in food science in 1992 under the direction of D. Knorr (TU Berlin). Other projects followed on dairy products under the direction of B.E. Brooker (IFR, Reading) and on the Kinetic aspects of high pressure treatment of food components, directed by M. Hendrickx (K.U. Leuven).

5. THE FUTURE: THE NEVER-ENDING HISTORY

Should we look for more molecular details? Or should we try another approach, e.g. the "polymer science" approach? Should we pay more attention to the dynamics of the glassy state, to water dynamics? Or should we look for a fresh instrument? "A fresh instrument serves the same purpose as foreign travel; it shows things in unusual combinations". This quotation by A.N. Whitehead dates from 1925 but is still valid today!

From molecules to macromolecules, from macromolecules to genes, from genes to cells and from cells to organisms. These are the levels for the conceptual interpretation of our experiments. When we try to relate these levels, we must be careful in our reasoning. Or should we make more errors in order to stimulate progress in our knowledge? We certainly must open the doors and windows of our mind and look for "unusual combinations"! Not only in theory, but also in practice. Many of our established industrial processes are not very well understood at the molecular level. In this repect there is still a lot to be learned from Mother Nature! We look forward seeing all of you in Dortmund for HPBB-2002 reporting on your new findings!

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Trends in High Pressure Bioscience and Biotechnology
R. Hayashi (editor)

Structural features and dynamics of protein unfolding

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The heat and pressure induced unfolding process was compared for 3 model proteins: Sso7d from the thermophilic archaebacterion Sulfolobus solfataricus, Ribonuclease A, and trypsin. The methods of choice were fluorescence and UV absorbance (4th derivative mode) under equilibrium conditions, unfolding kinetics after a sudden change of the equilibrium by a pressure jump, and by molecular dynamics simulation. Protein stability appears to depend strongly on hydrophobic interactions, such as stacking between aromatic residues and van der Waals interactions. The mechanism of stabilization appears to be protein specific, and it is yet too early to draw general conclusions. However, evidence for an energetic equivalency of unfolded states could be found. Furthermore, the dynamics of protein unfolding can be interpreted in terms of the so-called "new view". The p-jump technique appears to be promising for further kinetic investigations.

1. INTRODUCTION

Proteins are built up of typically many hundred amino acids. Each amino acid residue interacts with all others to a different degree by electrostatic and hydrophobic interactions or by hydrogen bonding. Furthermore, proteins interact strongly with their solvation shell (usually water), and sometimes also between each other. Their folding and unfolding processes (which may be induced by pressure, temperature or by a chemical denaturant) may therefore be expected to be complex: multiple pathways comprising many intermediates are theoretically possible. Indeed, some first studies with single molecules suggest stepwise folding/unfolding pathways [1]. Nevertheless, the most frequently observed re-sult is a two-state transition between a native and a denatured protein structure. Sometimes, an intermediate state (it is fashion to call it «molten globule») is detected.

The two- or three-state transition is generally interpreted as resulting from a high cooperativity of the various elementary folding/unfolding steps. This is of course in contradiction with the stepwise unfolding observed for single molecules.

For a better understanding of the principles underlying protein unfolding mechanisms, more experimental as well as theoretical work is needed. Our approach is a thorough comparison of temperature and pressure induced protein unfolding by fluorescence and 4th derivative UV absorbance spectroscopy, as well as by a kinetic analysis of pressure jump induced protein folding and unfolding reactions. Furthermore, we used molecular dynamics simulation in order to get an insight into the very first protein unfolding events.

We used 3 very different protein models. Sso7d is a very small (7 kDa) ribonuclease and DNA binding protein from the hyperthermophilic archaebac-terion Sulfolobus solfataricus, an organism which grows optimally at 89 °C in the acid hot springs of Southern Italy [2]. The protein, which has an SH3 like structure of orthogonally disposed β-sheets, in addition to a small external α-helix, is characterized by a small hydrophobic core and a positively charged envelop. Its binding to DNA has been shown to considerably increase the DNA melting temperature. For this study, we used wild-type Sso7d, as well as its F31A mutant [3].

Another model was Ribonuclease A from bovine pancreas. This model was chosen because it is one of the best known proteins in the field of protein unfolding. We used the wild-type protein, but also 14 mutant forms, each differing in a single point mutation of a chain folding initiation site. All muta-tions were conservative. They concerned the aliphatic chain of several selected hydrophobic amino acid residues [4].

The last model was trypsin, which is a relatively large globular protein. This protein was chosen because of its hysteresis like structural response as a function of pressure [5]. The 2 other models mentioned above, do not show such a phenomenon.

2. MATERIAL AND METHODS

Proteins

Bovine pancreatic ribonuclease A was from Sigma. It was further purified as described [6]. Mutant and wild type genes were expressed in Escherichia coli and isolated as reported previously, using an FPLC Mono S HR 5/5 cation-exchange column[4]. By this procedure the following RNase A variants were prepared: I106A/L/V, I107A/L/V, V108A/G, A109G, Y115W, V116A/G, and V118A/G.

Sso7d from the archaebacterium S. solfataricus was expressed in E. Coli and purified as described by Fusi et al. [2]. The F31A mutant form was obtained by site-directed mutagenesis as described [3].

Bovine pancreatic trypsin was purchased from Sigma. The fluorescent probe 8-anilinonaphthalene-1-sulfonate (ANS) was from Molecular Probes Co.

Methods

The UV spectra were recorded with a Cary 3E (Varian) spectrophotometer. The monochromator proceeded in steps of 0.1 nm with a data acquisition time of 0.3 s per step and a bandwidth of 1 nm. Each spectrum was the result of 5 accumulations. The 4th derivatives of the UV spectra were evaluated as described [7,8], using a mean derivation window of 2.6 nm, optimal for tyrosine. The resulting derivative spectra reflect the averaged polarities of the tyrosines. Fluorescence spectra were taken with an Aminco Bowman Series 2 luminescence spectrometer with excitation and emission slits of 4 nm.

Spectral (absorbance and fluorescence) measurements were performed using a thermostated high pressure cell which was placed into the sample compartment of the spectrometers. The high pressure cell was of Marval X12 steel, the windows of sapphire, and the sample cuvette (quartz) was closed by a Dura-Seal polyethylene stretch film. Pressure jumps were performed by opening a pneumatic valve between the cell containing the sample and another cell which had been pressurized differently. This procedure allowed an equilibration of pressure between the two cells within 5 ms. The equipment permitted to undertake positive and negative pressure jumps within the range of atmospheric pressure up to 700 MPa. Typically, pressure jumps of 50 or 100 MPa were done. The accompanying small change of temperature could be neglected.

For RNase A and Sso7d, the buffer was MES from Sigma. Experiments with trypsin were performed in 1 mM HCl at 1 °C [5].

3. RESULTS

Ribonuclease A

Under high pressure, as well as at high temperature, the UV absorbance spectrum of RNase A was blue-shifted. This spectral change appears very clearly in the 4th derivative mode, as shown in Figure 1. This blue-shift reflects the increase in polarity in the tyrosine environment due to hydration as the protein unfolds. The spectral changes are characterized by clear isosbestic points, suggesting that the protein unfolding is a two-state transition. The transition was found to be completely reversible. From the change of the signal amplitude as a function of temperature and pressure, the thermodynamic parameters ΔG, ΔH, ΔS, and ΔV were determined for each mutant.

Surprisingly, we obtained virtually identical free energy values for pressure and temperature induced unfolding. This fact is reflected in Figure 2: clearly, the ΔG_u values determined from pressure and temperature effects are strongly correlated. This indicates that the unfolded states of RNase A are degenerate: temperature and pressure induced denaturation lead to equivalent energy levels. This is surprising, as temperature and pressure are expected to induce different protein conformations [9].

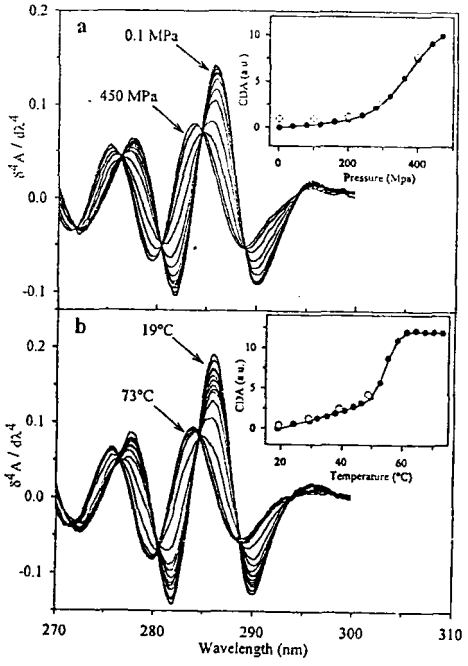

Figure 1. Effects of pressure and temperature on the 4th derivative spectrum of RNase A V116A (1 mg /ml, at pH 5). a) effect of pressure at 40 °C, b) effect of temperature at atmospheric pressure. The inset shows the temperature unfolding curve for this variant. The open circles show the folding reaction. The cumulative difference amplitude (CDA) corresponds to the total signal change. The solid line is the nonlinear least-squares fit of the data based on a two-state model.

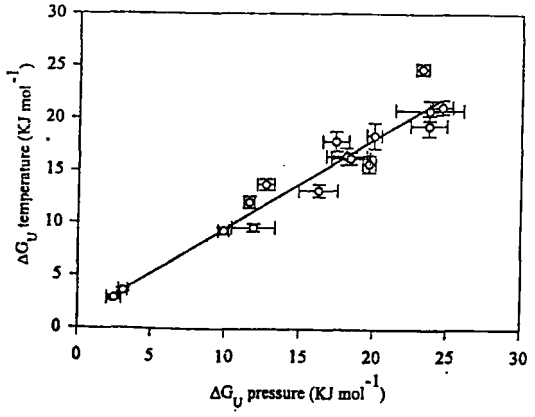

Figure 2. Correlation between the unfolding energies of the different RNase A variants determined from pressure and temperature denaturation curves at 40°C and 0.1 MPa. The standard errors are indicated.

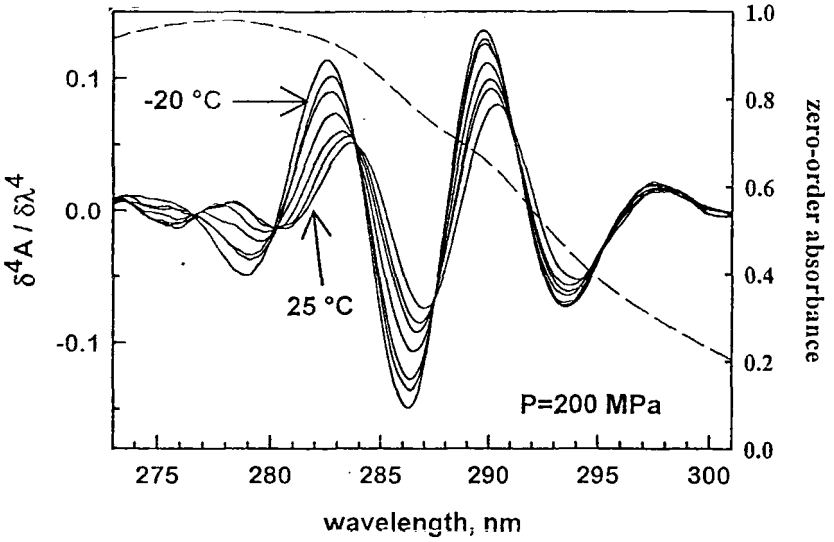

Figure 3. Fourth derivative UV spectra of Sso7d F31A as a function of temperature. Cold denaturation between +25 and –20 °C; phosphate buffer, 50 mM, pH 7.4.

Sso7d

The power of the 4th derivative method is immediately evident from Figure 3. Whereas the absolute UV spectrum was rather broad, a significantly better resolution was obtained in the 4th derivative mode. The Figure shows the result obtained by a cold denaturation experiment. That is, first pressure was raised to 200 MPa, and then temperature was decreased from +25 to –20 °C. Again, clear isosbestic points were obtained, indicating a two-state transition from the folded to the unfolded protein. The results obtained with heat, as well as with high pressure, yielded very similar results: each time a pronounced blue-shift of the tyrosine bands was observed, and the transitions were reversible. In order to obtain a further mechanistic insight, we investigated the kinetics of the unfolding / folding process. For that, we used both, experimental and theoretical appreoaches: pressure jumps, and molecular dynamics simulations.

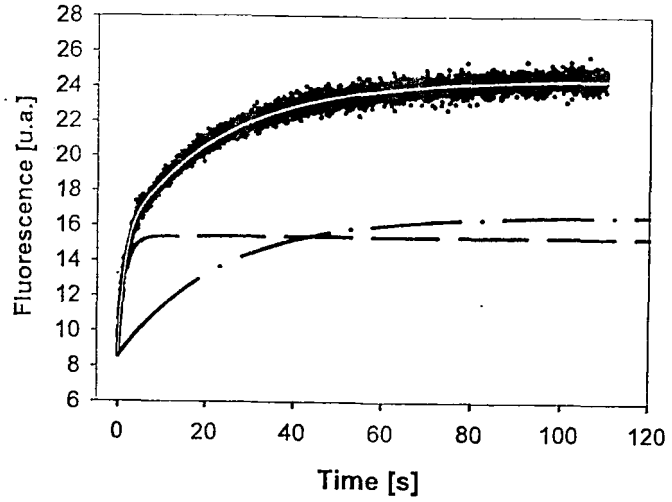

Figure 4. Kinetics of Sso7d F31A after a pressure jump from 300 to 350 MPa in the presence of ANS. The broken lines represent the fitted fast and slow phases.

- *Pressure jumps.* We used ANS to probe the exposition of hydrophobic domains in course of the protein unfolding. Indeed, when structured hydrophobic protein domains become accessible to ANS, the ANS fluorescence increases strongly (normally it is quenched by the polar water molecules). As shown in Figure 4, a positive pressure jump induced a strong enhancement of ANS fluorescence. The kinetics of this fluorescence increase reflect the protein unfolding kinetics. Clearly, 2 kinetic phases can be distinguished. This suggests, that protein unfolding of Sso7d occurs within at least two steps.

- *Molecular dynamics simulation.* We simulated the trajectory of the unfolding events after a sudden heating of the molecule to 400 K. The simulations suggested a complex unfolding mechanism within the first nanosecond. The most intriguing feature was that the small C-terminal α-helix which lies upon the surface of the protein, starts suddenly to unwind, and to swing around like a whip. After that, other parts of the protein outer layer began to change conformation. However, the very solid hydrophobic core in the center remained in its original configuration [10].

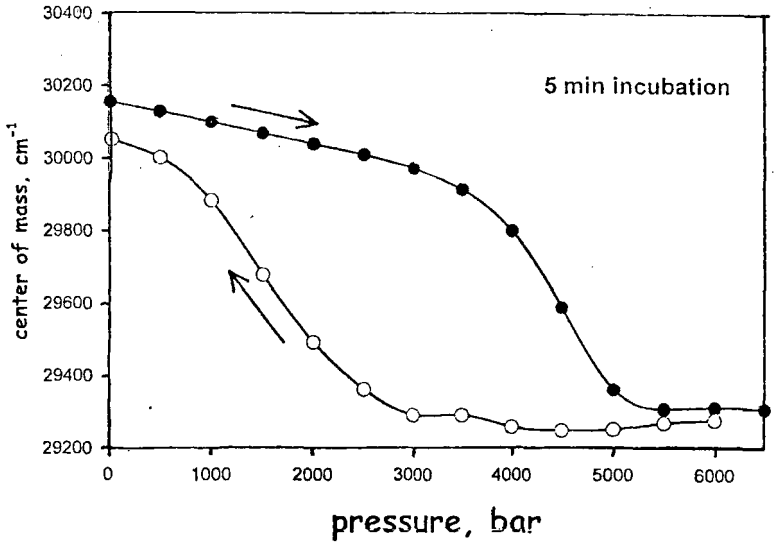

Figure 5. Pressure induced unfolding of trypsin at pH3 and 0 °C. Center of mass of the fluorescence spectra as a function of increasing and decreasing pressure.

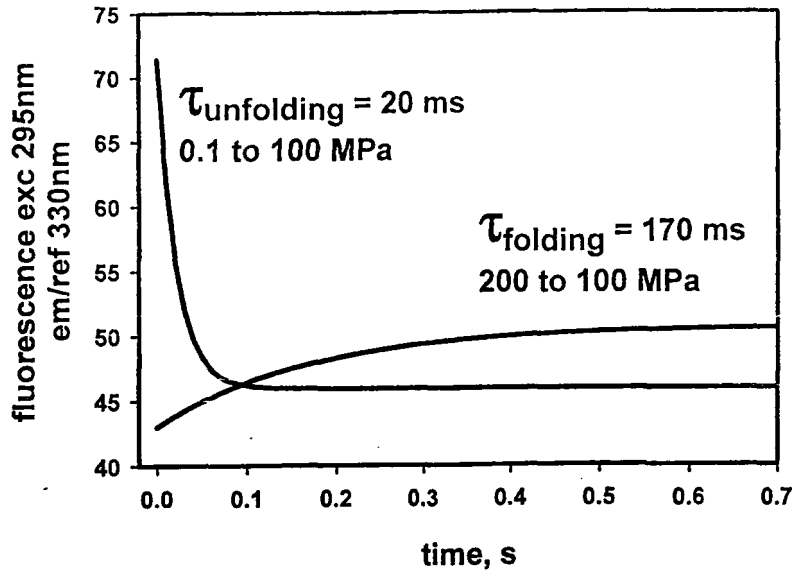

Figure 6. Kinetics of pressure induced folding and unfolding of trypsin. The experimental conditions were those of Figure 5. The final pressure was 100 MPa.

Trypsin

At 0°C and pH3, the tryptophan fluorescence emission spectrum of trypsin was strongly red-shifted. This effect is best viewed by plotting the center of the spectral mass (csm) as a function of pressure (csm is the intensity weighted averaged wave-number of the spectrum [5]). As shown in Figure 5, again a two-state transition between the folded and the unfolded state appeared. However, a strong hysteresis effect was observed: for increasing and decreasing pressures, the values of $p_{1/2}$ were 450 and 150 MPa respectively. This argues for a more complex folding/unfolding mechanism, and we effectuated therefore a kinetic analysis.

Figure 6 shows the kinetics of the change in fluorescence intensity after a pressure jump. We compared positive and negative pressure jumps, the final pressure being 100 MPa in both cases. Now, from a thermodynamic point of view, it should not matter, whether the equilibrium is reached from a lower or from a higher pressure. However, our experiments showed that the relaxation times for the positive and the negative p-jump were significantly different (20 and 170 ms respectively). These experimental findings are therefore in contradiction with the classical thermodynamic theory.

4. DISCUSSION

In the light of several diseases, such as Creutzfeld-Jacob, Huntington, etc., which imply protein conformational changes, there is actually a real need for a better understanding of protein folding/unfolding mechanism. Indeed, classical thermodynamic theory does not seem to be appropriate to describe macromolecular conformational changes, and we have therefore to collect experimental information from as many proteins as possible. Here we compared the thermal and pressure stability, as well as the mechanism of unfolding of three very different proteins: RNase A as the classical folding model, Sso7d as the representative of proteins from thermophilic organisms, and trypsin, a mammalian protein of common properties.

Their common feature for structural stabilization is the importance of hydrophobic interactions [11, 12]. This appears very clearly for RNase A, where the mutants differing in the chain-length or the configuration of hydrophobic residues are considerably less stable. For Sso7d, it is the stacking of aromatic amino acids in the hydrophobic core, which determines the stability. And finally, for trypsin, the cold denaturation results point again in favor of stabilizing hydrophobic interactions.

As to the mechanism of the folding/unfolding processes, three results appear to be important:

a) In each case, spectral measurements at equilibrium can be interpreted by simple two-states transitions. This does not mean that the reactions occur without intermediates (for example, the molecular dynamics simulation with Sso7d suggests the occurrence of intermediates). However, it indicates that if there is an intermediate, it will be a kinetic and not a thermodynamic intermediate.

b) The results obtained with RNase A indicate that the energy levels of the unfolded states are equivalent. This is in opposition of our expectation of different structural effects of pressure and temperature. A way to overcome this problem is the interpretation by the so-called "new view" [13]. This theory describes the energetic landscape of a protein as a funnel. The most simple situation would be a smooth surface of the funnel. This would explain both the two-state transitions and the energetic equivalency of the unfolded states.

c) The real situation appears to be more complex: molecular dynamics simulations as well as experimental pressure jump kinetics indicate the presence of intermediates in the course of the unfolding process. Furthermore, as shown by the example of trypsin, the kinetics are path-dependent, i.e., the same equilibrium is obtained by different mechanisms depending on the initial conditions. This observation is in clear contradiction with classical thermodynamics. Interestingly, this does not appear to be an isolated observation: similar, path-dependent unfolding kinetics have been reported recently for the major heat shock protein of E. coli [14]. A possible interpretation of such "non-classical" kinetics can be given again by the concept of the 'new view'. Indeed, a tentative explanation would be that the funnel-shape energetic landscape of the protein folding / unfolding reaction is not smooth, but rugged.

5. CONCLUSION

Clearly, our understanding of protein folding/unfolding mechanisms is still at the beginning. Whereas much is known now about equilibrium conditions, relatively little information is yet available to explain the dynamics of these processes [15]. It appears therefore evident, that much more experimental kinetic data, as well as information from dynamics simulation studies are needed in order to understand the principles underlying the mechanism of protein folding and unfolding. Experimentally, pressure-jump kinetics may reveal as a very useful tool. By this method, irreversible side-reactions can be avoided. Furthermore, this method is suitable for both positive and negative jumps, and it allows also to conduct kinetic experiments in highly viscous media. The technical problems of this method are now resolved, and suitable instrumental set-ups for both fluorescence and absorbance measurements are available [15].

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A discussion of the physical basis for the pressure unfolding of proteins

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The application of pressures in the range below 8 kbar leads to the disruption of the native structures of most proteins in solution. This occurs because the volume of the system (protein + solvent) is smaller when the protein adopts an unfolded conformation. The underlying contributions to this decrease in volume are discussed. In addition for several protein systems, the kinetic basis for the destabilization has been determined. The results are analyzed in terms of the volumes of activation for the folding and unfolding reactions. These values in turn are interpreted in light of our limited understanding of the volume change. in terms of the position of the transition state along the reaction coordinate.

1. INTRODUCTION

Due to a decrease in system volume upon the unfolding of proteins in solution, the application of relatively low hydrostatic pressures leads to a decrease in the relative stability of the folded *vs.* unfolded state. This effect of pressure has been known since the 1940's, but continues to be perceived as counterintuitive, when it is not altogether ignored by the protein folding community. Despite this general lack of understanding and interest, the effects of pressure should be considered as fundamental (in the same right as the effects of temperature which are relatively well understood) to the study of protein stability and the specificity of the folded state. The characterization of the phase diagram of any material must be considered as the very least one should do to begin to comprehend its properties. To date, however, the phase diagrams of only a few protein systems have been reported. Below we discuss the various contributing factors to the pressure/temperature phase behavior of proteins. Secondly, in order to understand, and eventually modulate for a number of practical purposes any chemical reaction, the characterization of the kinetics of the reaction and the response of the kinetic parameters to changes in temperature and pressure must be undertaken. Interestingly, while a large number of studies have appeared describing the temperature behavior of protein folding and unfolding reaction kinetics, very few reports have appeared in which the pressure dependence of these properties has been investigated. We resume here the results from a few systems that we have studied and draw conclusions concerning the properties of the transition state of the reaction. We note that we consider here only systems in which the protein is in a simple equilibrium between a folded state and an unfolded ensemble. Pressure has also been used to characterize reaction intermediates, but that topic will not be addressed herein.

2. CONTRIBUTIONS TO THE VOLUME CHANGE OF UNFOLDING

Regardless of the atomic mechanisms underlying the decrease in system volume upon the unfolding of proteins, it is clear in general, that the effect arises from a change in the interaction of the protein with the solvent. Moreover, this decrease in volume upon unfolding is quite small in magnitude, ~-100 ml/mol, which for small single domain proteins corresponds to approximately 1% of the total protein volume. The task of identifying the underlying contributing factors remains relatively daunting. The volume of the solution is the sum of the volume of the atoms and covalent bonds, and the free volume. The covalent bonds of the protein do not change in length over this pressure range, and of course nor does the volume associated with the atoms of the solvent and the protein. Thus, we can consider that the volume change upon the unfolding of proteins arises due to changes in the free volume of the solution of protein and water. Changes in the free volume arise from changes in the non-covalent interactions between the atoms, and these changes occur due to the exposure of amino acid residues and the peptide backbone, that in the folded form are buried in the native structure, and inaccessible to solvent.

2.1 Electrostriction

Consider the disruption of an ion pair that is buried in the native structure and its subsequent exposure to the surrounding aqueous environment. Water molecules will interact more strongly (and thus at a shorter distance) with these exposed charged groups than with other water molecules, and a phenomenon termed electrostriction will occur. For a single ion pair such effects can be quite significant, resulting in a decrease in volume of nearly 20 ml/mol ion pair. This effect is responsible for the pressure dependence of the pKa of ionisable buffers, for example. The magnitude of the contribution of electrostriction to the total decrease in volume upon protein unfolding will depend upon the individual protein.

2.1 Exposure of polar moieties

The exposure of polar, uncharged moieties may also contribute to the decrease in volume, depending upon relation between the packing density of these groups in the protein interior, the specific volume of the bulk water and their specific volume in interaction with the new hydration layer. While the magnitude of this effect per mole exposed polar moiety, is surely much smaller than for an exposed disrupted ion pair, in general, the number of exposed polar moieties, including the backbone, is much larger than the number of exposed ion pairs. Hence, this contribution could indeed prove significant to the total decrease in volume.

2.3 Hydrophobic hydration

Proteins, in their native structure bury a significant proportion of their hydrophobic surface, which upon unfolding also becomes exposed to the solvent. Based on transfer studies of hydrophobic compounds from neat liquid to water (Table 1), it has been assumed by many that the transfer of hydrophobic groups from the interior of proteins to aqueous solution would lead to a very large decrease in volume. A few investigators have questioned this assumption (1-4). In fact, the density of neat liquids of hydrophobic compounds is much smaller than that of hydrophobic groups in proteins. In fact, proteins are very tightly packed. A methyl group in a protein interior has a specific volume which is approximately 5 times smaller than neat methane. Thus the large decrease in specific volume observed in transfer studies of hydrophobic compounds to water is largely due to their low density in the pure form. Interestingly, it has been noted in a transfer study of water into solutions of hydrophobic compounds that its specific volume increased (5) (Table 2). Thus, water

molecules hydrating hydrophobic moieties that are exposed upon unfolding may well lead to an overall increase, rather than a decrease in the system volume.

Table 1.
Specific volume of hydrocarbons in various solvents (ml/mol) (5).

Solvent	Methane	Ethanol
Per-fluoro-n-heptane	68.4	82.9
n-heptane	60.0	69.3
Carbon tetrachloride	51.7	66.0
Water	37.3	51.2

Table 2. Specific volume of water in various organic solvents (ml/mol) (6).

Solvent	Methane
1,2 dichloroethane	20.1 ± 1
benzene	22.1
1,1,1-trichloroethane	22.3
Carbon tetrachloride	31.6
Water	18.0

2.2 Loss of cavities and voids

As noted above, protein interiors are very well-packed. But as for all substances, according to scaled particle theory, there exist voids in the interior of proteins between the atoms. In addition, to the voids due to the spherical nature of the atoms, actual cavities exist in the protein structure, which are often small enough or inaccessible enough as to exclude solvent. Upon unfolding of the protein, the interaction of the exposed surface with the relatively small water molecules leads to a decrease in system volume akin to that which is achieved by adding small spheres to a box containing larger spheres. The smaller spheres can take up excluded volume in between the larger spheres. Moreover, the unfolding of the protein also eliminates the free volume due to actual cavities in the structure, and this space is occupies by water molecules. Thus, the elimination of packing defects and scaled particle free volume necessarily leads to a decrease in system volume upon unfolding. Actual cavities