Protein unfolding, amyloid fibril formation and configurational energy landscapes under high pressure conditions

Filip Meersman,*[a] Christopher M. Dobson[b] and Karel Heremans[a]

Received 22nd June 2006
First published as an Advance Article on the web 21st July 2006
DOI: 10.1039/b517761h

High hydrostatic pressure induces conformational changes in proteins ranging from compression of the molecules to loss of native structure. In this *tutorial review* we describe how the interplay between the volume change and the compressibility leads to pressure-induced unfolding of proteins and dissociation of amyloid fibrils. We also discuss the effect of pressure on protein folding and free energy landscapes. From a molecular viewpoint, pressure effects can be rationalised in terms of packing and hydration of proteins.

Introduction

Crystalline solids and molecular liquids undergo structural transformations, thereby forming different polymorphs, in response to pressure and temperature.[1] The pressures required to achieve such transformations are often in the range of tens of gigapascals (GPa). The application of pressure and temperature can also induce structural changes in biological molecules. However, as studies on biological systems are performed in aqueous media the maximum pressure reached in these experiments is dictated by the phase behaviour of water, *i.e.* by the pressure range where water remains liquid. Applied *hydrostatic* pressures therefore never exceed 1.0–1.5 GPa. We note that $p\Delta V$ has the dimensions of energy, and that for a typical protein unfolding process accompanied by a volume change of -30 ml mol^{-1} (and $p_{1/2} \approx 500$ MPa), the total energy input in the system is only of the order of 15 kJ mol^{-1}. Thus, in contrast to the phase transitions occurring in molecular systems, covalent bond angles and bond lengths can be considered to remain unchanged in the pressure range that is relevant to biological systems. Consequently, pressure enables one to tune the non-covalent interactions, and in particular the hydrophobic effect and hydrogen bonding, that are responsible for the stabilisation of biological molecules.

In the present review we consider the effects of high hydrostatic pressures on proteins, and refer the reader interested in lipids and nucleic acids to recent reviews elsewhere.[2,3] High hydrostatic pressures are required to cause changes in protein conformation, resulting in unfolding and loss of function, but even relatively low pressures can cause an elastic response of the protein. Such an elastic effect is associated with the compression of a protein molecule as a result of changes in hydration, in non-bonded interatomic distances and in the size of cavities that arise from the

[a]*Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200F, B-3001, Leuven, Belgium. E-mail: filip.meersman@chem.kuleuven.be; Fax: 0032-16-327990; Tel: 0032-16-327355*
[b]*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, UK CB2 1EW*

Filip Meersman

Filip Meersman received his PhD in Biochemistry from the Katholieke Universiteit Leuven in 2002, working with Karel Heremans on infrared spectroscopic investigations of protein unfolding and aggregation, with particular emphasis on the effects of high hydrostatic pressure. He then went on to join the group of Professor Christopher Dobson at the University of Cambridge as a Marie Curie Intra-European Fellow, where he studied the stability, structure and formation mechanism of amyloid fibrils. He is currently a fellow of the Research Fund Flanders at the Katholieke Universiteit Leuven, where he pursues his research interests in the pressure–temperature phase behaviour of proteins, protein assemblies and water-soluble polymers.

Christopher M. Dobson

Christopher M. Dobson received his doctorate from the University of Oxford in 1976, having worked on the application of NMR spectroscopy to define the structures and dynamics of proteins in solution. After a short period as a Research Fellow he moved to Harvard University as an Assistant Professor of Chemistry. In 1980 he returned to the University of Oxford first as a Lecturer and later as Professor and Director of the Oxford Centre for Molecular Sciences. His research interests increasingly focused on defining the mechanism of protein folding and more recently on understanding the consequences of misfolding particularly in terms of its relationship to human disease. In 2001 he moved to the University of Cambridge as John Humphrey Plummer Professor of Chemical and Structural Biology.

imperfect packing of the amino acid side chains. As we discuss below, protein compressibility is an interesting parameter as it is associated with changes in protein dynamics. An explanation of the effects of pressure at the molecular level will also, however, contribute to our understanding of the fascinating phenomenon of life under extreme conditions. Many creatures have been found to thrive in deep-seas where they can be exposed to hydrostatic pressures of up to 100 MPa and at both high and low temperatures. Their survival under those conditions depends on the piezophilic adaptation of their constituent molecules.

We discuss first the mechanism of pressure-induced protein unfolding and its consequences. Novel microscopic information about this mechanism has been obtained from computer simulations, which suggest that the contribution of the solvent to the energetic and volumetric changes associated with pressure-induced unfolding is the dominant factor. Secondly, we focus on pressure and protein aggregation. In the final section we discuss the influence of pressure on protein folding and the free energy landscape in general. But before that we shall introduce some key thermodynamic and statistical mechanical concepts.

Free energy derivatives: volume and compressibility

For a reversible, two-state folding/unfolding process (N (Native) ↔ U (Unfolded)), the pressure and temperature dependence of ΔG, the difference in Gibbs free energy between U and N, is given by

$$\mathrm{d}(\Delta G) = -\Delta S\ \mathrm{d}T + \Delta V\ \mathrm{d}p \tag{1}$$

where ΔS is the change in entropy, ΔV is the volume change, and p and T represent the pressure and temperature, respectively. From this equation it is clear that, at constant temperature, the first derivative of ΔG with respect to pressure is given by the volume change ΔV. This observation reveals at once a major advantage of pressure as a perturbation tool over temperature (at constant pressure), which will affect the kinetic energy as well as the volume of the system. Indeed, pressure effects on molecular systems are governed simply by the principle of Le Châtelier, which states that a pressure increase will shift a given equilibrium to the side that occupies the smallest volume.

Integration of eqn (1), assuming that both ΔS and ΔV are temperature and pressure dependent, gives the following expression:

$$\Delta G(p,T) = \Delta G^o - \Delta S^o(T-T_o) + \Delta C_p[(T-T_o) - T\ln(T/T_o)] + \Delta V^o(p-p_o) - \frac{\Delta\beta}{2}(p-p_o)^2 + \Delta\alpha(T-T_o)(p-p_o) \tag{2}$$

where ΔG°, ΔV° and ΔS° refer to the reference conditions, taken to be p_o (0.1 MPa) and T_o (298 K), and the second order terms $\Delta\alpha$, $\Delta\beta$ and ΔC_p are proportional to the differences in thermal expansion factor, compressibility factor and heat capacity between the unfolded and the native state of the protein, respectively. These parameters are assumed to be independent of pressure and temperature, and are defined as follows:

$$\begin{aligned} \Delta\alpha &= (\partial\Delta V/\partial T)_p = -(\partial\Delta S/\partial p)_T \\ \Delta\beta &= -(\partial\Delta V/\partial p)_T\ \Delta Cp = T(\partial\Delta S/\partial T)_p \end{aligned} \tag{3}$$

We note that eqn (2) originates from a Taylor expansion of $\Delta G(p,T)$, with a cut-off after the second order terms. The meaning and measurement of the thermal expansion, the compressibility and the heat capacity have been discussed elsewhere.[4,5] Here we emphasise the importance of the compressibility in pressure-induced phenomena. At constant temperature eqn (2) can be rewritten as:

$$\Delta G(p) = \Delta G^o + \Delta V^o(p-p_o) - \frac{\Delta\beta}{2}(p-p_o)^2 \tag{4}$$

The last term on the right hand side of this equation reflects the pressure dependence of the volume change ΔV, which, at high pressures, can no longer be predicted from ΔV° alone. The compressibility factor $\Delta\beta$ is related to the isothermal compressibility β_T ($\beta_T = -V^{-1}\ (\partial V/\partial p)_T$), which is the second derivative of the free energy with respect to pressure, by $\Delta\beta = V\ \Delta\beta_T$. The isothermal compressibility of a system is of particular interest because its difference between the native and unfolded states reflects the pressure dependence of ΔV, and therefore influences the response of the protein to pressure. In addition, there exists a statistical mechanical relationship between the isothermal compressibility and the volume fluctuations of the system:[6]

$$<\delta V^2> = k_B\ T\ V\ \beta_T \tag{5}$$

where k_B is the Boltzmann constant, T is the absolute temperature, and V is the intrinsic volume of the system. In the case of a protein the system volume has to be related to the partial molar volume of the protein. Hence the isothermal compressibility not only provides insight into the effect of pressure on protein structure, but also into the dynamics of the native protein in terms of volume fluctuations. It should, however, be emphasised that protein volume fluctuations and protein flexibility are not the same. Moreover, it has been

Karel Heremans

Karel Heremans studied chemistry at the Katholieke Universiteit Leuven. In 1965 he obtained his PhD under the supervision of Paul Putzeys with a thesis on the molecular light scattering of proteins. During a postdoctoral period he studied protein–protein interactions with light scattering under high pressure. He worked on the effect of pressure on phase transitions in lipids, the activity of lipid-bound enzymes, nucleic acids, ribosome and microtubule assembly and on reactions of proteins in general. At present his research concentrates on the stability of biopolymers as a function of pressure and temperature, and its correlation with protein aggregation. He was twice chairman of the European High Pressure Research Group.

pointed out that eqn (5) is only valid for a system with a constant number of particles, such as the native state of a protein.[7] In contrast, highly hydrated states, such as molten globules and unfolded states, are characterised by a constant exchange of internal and bulk water. Thus the fluctuations in the number of internal water molecules have to be taken into account.

Typical values of β_T for native proteins are in the order of 0.25 GPa^{-1} at 25 °C.[4] For comparison, the compressibility of solids, including molecular crystals, soft and hard polymers, is in the range of 0.065 to 0.40 GPa^{-1} and the compressibilities of water, benzene and hexane are 0.45, 0.96 and 1.65 GPa^{-1}, respectively. On the basis of these values one can conclude that the protein interior is well-packed and more solid-like than liquid-like. This conclusion is also consistent with the packing density of proteins, with the proportion of space occupied by the component atoms being about 75%, which is just above even the upper limit for the packing of simple organic molecules (74%), and substantially greater than that of a glass (65%).[8]

The protein volume paradox

One of the early questions that arose in efforts to understand protein folding was related to the nature of the forces that drive a polypeptide chain to adopt a collapsed, globular conformation. The dominant force was suggested to be the hydrophobic effect, which results in a clustering of non-polar residues to minimise their interaction with solvent water. The hydrophobic effect has been modelled by the transfer of non-polar compounds, such as pentane, from non-aqueous to aqueous media. This reaction is highly disfavoured and is associated with a large increase in heat capacity, a characteristic that is typically observed during the thermal unfolding of proteins. Moreover, there is a close resemblance between the temperature dependence of protein folding and the temperature dependence of the free energy for the transfer of non-polar compounds from water to non-polar media. Thus the liquid hydrocarbon model has been quite successful in explaining the energetic properties of thermal unfolding.

Based on studies of the transfer of small hydrophobic compounds to water, the volume change upon unfolding of proteins is predicted to be negative and very large in absolute value. Moreover, it is also predicted that the volume change for this transfer will become positive with increasing pressure. In contrast, at 0.1 MPa the sign of ΔV can be positive or negative, and may depend on the nature of the observed transition, *e.g.*, native-to-molten globule or native-to-unfolded.[4] However, at high pressure protein unfolding is invariably accompanied by small and negative volume changes, typically in the order of −10 to −100 ml mol^{-1}.[9] This contradiction is the so-called *protein volume paradox* and was already recognized by Kauzmann who stated that "the liquid hydrocarbon model fails almost completely when one attempts to extend it to the effects of pressure on protein denaturation".[10]

What is the molecular interpretation of the volume change? The partial molar volume of a protein in solution, V_i, is defined as the change in volume of the solution as a small amount of solute is added divided by the total number of moles of added solute keeping the amount of the other components constant. For an ideal solution, this would be the difference between the solution volume and the original solvent volume. However, due to hydration effects dissolution of a protein will also affect the solvent volume. Therefore, V_i can be expressed as the sum of an intrinsic term and a hydration term:

$$V_i = V_{atom} + V_{cavities} + \Delta V_{hydration} \quad (6)$$

where V_{atom} is the sum of the van der Waals volumes of the constituent atoms, $V_{cavities}$ is the volume of the cavities that originate from imperfect packing in the native conformation, and $\Delta V_{hydration}$ is the volume change resulting from the interaction of the protein with the solvent.[6] Upon protein unfolding the van der Waals volumes will not change, so the volume change accompanying the unfolding can be written as:

$$\Delta V = \Delta V_{cavities} + \Delta\Delta V_{hydration} \quad (7)$$

Evidence for the role of cavities comes from mutagenesis experiments, where the creation of new cavities as a result of amino acid mutations results in larger negative volume changes upon unfolding compared to the native protein.[11] Contributions to $\Delta\Delta V_{hydration}$ arise mainly from hydration of hydrophobic groups and from the hydration of cavities previously devoid of water. Clearly, the hydration and cavity terms are difficult to separate. In principle, the largest contribution to $\Delta\Delta V_{hydration}$ would arise from the exposure to or removal from water of charged groups due to electrostriction: the formation of an ion in solution results in a strong attraction of the dipoles of nearby water molecules by the Coulombic field of the ion. In general, however, most charged side chains are solvent exposed in the native state, and hence this term is relatively small.

The cavity and hydration terms are generally considered to be negative contributions to ΔV. The small magnitude of ΔV from experimental measurements, however, suggests that there is also a positive contribution to ΔV that, at least in part, compensates for the negative contributions discussed above. The origin of this contribution is still the subject of debate, as is the sign of the net hydration term $\Delta\Delta V_{hydration}$.[9] There may be no need for a compensating factor, however, if it turns out that, in terms of volumetric properties, hydrocarbons are a poor model for amino acids.[8] Using other compounds such as ketones and amides, that can form hydrogen bonds, the volume changes upon transfer to water are found to be less negative than in the case of methane, and more predictive of the properties of real proteins. Moreover, attributable to the tight packing of their interior regions, protein molecules are far less compressible than liquid hydrocarbons.

Mechanistic aspects of pressure-induced protein unfolding

The water penetration model

Pioneering observations of the pressure effects on the behaviour of proteins were made by Bridgman and Suzuki.[6]

Both found that, contrary to expectation, the rates of the pressure-induced unfolding increase as the temperature is reduced, implying that the process is characterised by a negative activation enthalpy. Such negative activation energies have also been observed in the urea-induced unfolding of proteins. To explain his observations Suzuki proposed the following mechanism:

$$P + nH_2O \Leftrightarrow P(H_2O)_n \rightarrow P_D \quad (8)$$

where P is the native protein, $P(H_2O)_n$ is the hydrated protein and P_D the unfolded protein. Thus, he suggested that pressure results in the penetration of water molecules into the protein interior in a strongly exothermic step that, thereby, results in unfolding. Evidence in support of this model has been obtained from the results of more recent experiments. Weber and co-workers showed that lysozyme remains globular at high pressure, although its hydrodynamic volume has increased by 60–80% and fluorescence probes have undergone a blue shift, indicative of an increased polarity of their environment.[12] In an elegant study Lesch *et al.* determined the distance dependence of chromophore–solvent interactions in cytochrome c and found that, as a lower estimate, the solvent has to be within ~4.5Å of the chromophore in order to cause a blue shift in the fluorescence spectrum.[13] This distance is much smaller than the radius of cytochrome c, suggesting that water indeed has to penetrate the protein to explain the blue shift. Others have used scattering techniques to determine the radius of gyration of proteins under pressure.[14,15] For example, the R_g of staphylococcal nuclease increased from 16.3Å at 0.1 MPa to 34.7Å at 310 MPa, an expansion that is comparable to the increase in R_g resulting from urea-induced unfolding ($R_g \approx 33$Å at 8 M urea), but is still much less than in the case of heat-induced unfolding ($R_g \approx 65$Å).[15] The latter value approximates the value expected for a random coil. The data also indicated that the protein remained globular at 310 MPa, even though an increase in the R_g by a factor of two corresponds to an eight-fold increase in volume. Further characterisation of the unfolded state indicates a persistence of at least some native secondary structure at high pressure.[14–17] Thus the pressure-unfolded state can be considered to be a swollen, hydrated globular structure with a partially unfolded conformation, as illustrated for bovine pancreatic trypsin inhibitor (Fig. 1). We note, however, that the pressure-unfolded state is distinct from the molten globular state, which is typically characterised by an increase in R_g by a factor of 1.1 to 1.4, indicating that this state is almost as compact as the native state. The persistence of secondary structure is interesting as it indicates that the penetration of water molecules into the protein does not cause further unfolding through, for example, competition of protein–protein hydrogen bonds for protein–water hydrogen bonds. It is therefore tempting to speculate that only those parts of the protein molecule that are adjacent to cavities are sensitive to high hydrostatic pressure. In this respect it is important to realise that the effect of pressure in the molecule is not uniform and that different areas of a protein can have a different local compressibility (see below for the role of the compressibility).[18] In fact, a recent NMR study on bovine pancreatic trypsin inhibitor under subdenaturing pressure conditions has indicated that the parts of the protein that undergo the largest changes in response to pressure are those that are close to buried water molecules.[19]

In recent years computer simulations, for example, using pairs of methane molecules in water as a simple model for the hydrophobic effect, have provided further microscopic details of the pressure-unfolding mechanism that support the model proposed from experiments.[20–23] Fig. 2 shows the variation of the potential of mean force for the association of methane as a function of pressure. It can be seen that a pair of methane molecules in contact with each other ($r \approx 0.39$ nm) is destabilised relative to a pair of molecules separated by solvent ($r \approx 0.79$ nm) as the pressure increases, implying a weakening of the hydrophobic interaction. The latter can be rationalised by the supposition that, as pressure increases, the average number of water molecules surrounding another water molecule increases and the average binding energy of the water molecules decreases.[24] Thus, as a result of a reduction in the tetrahedral symmetry of the hydrogen bond network the relative cost of inserting water molecules into an unfavourable non-polar environment is also reduced. Using a water-soluble polymer as a model system, the increased level of hydration of

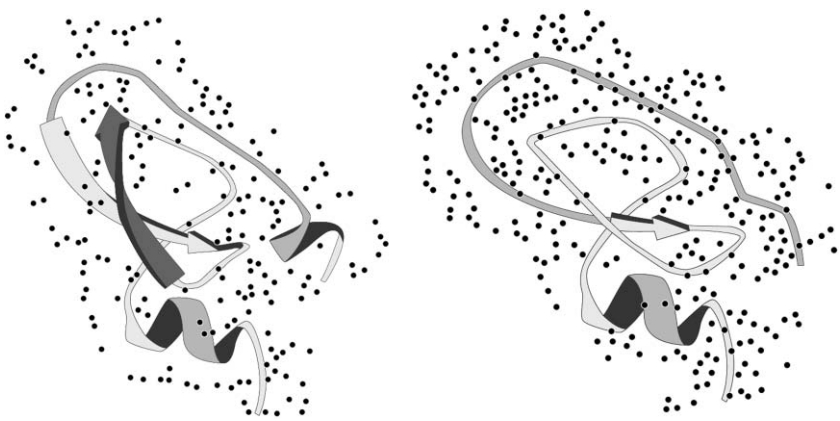

Fig. 1 Ribbon representation of BPTI and those solvent oxygen atoms that are less than 3.5Å apart from the main chain at 0.1 MPa (left) and 1.5 GPa (right). Note the persistence of certain elements of secondary structure and the increased number of solvent molecules in the interior of the protein. (Redrawn after ref. 51).

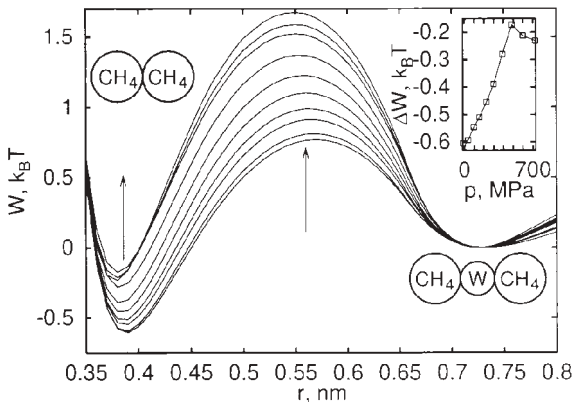

Fig. 2 The potential of mean force for the association of a pair of methane molecules as a function of pressure (−16 to 725 MPa) using the information theory. Arrows indicate the direction in which the pressure increases. The inset shows the pressure dependence of the free energy difference between the distance where the methane molecules are in van der Waals contact ($r \approx 0.39$ nm) and separated by water molecules ($r \approx 0.73$ nm). (Reproduced with permission from *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 1552. Copyright 1998 National Academy of Sciences, USA[20]).

both hydrophobic and polar moieties under pressure could also be demonstrated experimentally.[25] However, one should keep in mind that the above description of water under pressure is based on simulations of bulk water.[24] It is not clear to what extent hydration water will follow the same pattern of behaviour, assuming that the water molecules that insert into the protein are mainly water molecules that are involved in hydrating the protein at ambient pressure. In addition to influencing the structure of water by reducing its tetrahedral framework, pressure is also a necessary requirement for keeping water within the protein. It is well known from, for example, hydrogen exchange experiments that water molecules can penetrate into and escape from the protein interior on picosecond to millisecond timescales. It was found in a recent molecular dynamics simulation that water molecules inserted between a hydrophobic pair of amino acids only remained there at high pressure, whereas at 0.1 MPa the original hydrophobic contact was restored within the simulation time.[15]

The enthalpy and entropy contributions to the pressure-induced water penetration process have been calculated by Ghosh *et al.*[22] At low pressure the methane–methane contact pair is stabilised entropically, and neither the entropy nor the enthalpy are significantly affected by pressure. As the pressure increases the entropy contribution becomes slightly unfavourable, whereas the enthalpy term does not change. At this point a transition state is formed that is characterised by a solvent-excluded zone between the two methane molecules. A further pressure increase eventually results in an unfavourable entropy contribution that is offset by a negative enthalpy term, which arises almost completely from changes in solvent–solvent interactions rather than from methane–solvent interactions. A previous Monte Carlo study did not attempt to separate these two interactions, but did indicate that solvent–solvent and solvent–solute interactions are more important than solute–solute interactions.[23] A recent study, using a combination of X-ray crystallography and molecular dynamics simulations, has shown that a significant part of the favourable free energy for transferring a water molecule into a cavity is provided by hydrogen bond formation involving the water molecules that are inserted into the cavity, as well as by van der Waals interactions of these molecules with the surrounding medium.[26]

At this point a number of comments are in order. First, methane is clearly not an ideal model, at least in terms of size, for hydrophobic amino acid residues such as isoleucine and valine. Therefore Ghosh *et al.* also investigated the effect of the size of the hydrophobic solute on its response to pressure and observed qualitatively similar behaviour to that found for methane as the size of the solute increases, but the pressure effect is amplified.[21] Second, it is known that the properties of the hydration layer surrounding aromatic molecules are different from those of the hydration layer surrounding aliphatic molecules.[27] Consequently, the influence of pressure on aromatic–aromatic interactions may be different from the pressure effects on aliphatic interactions. Indeed, a recent simulation shows that aromatic pairs respond differently to an increase in urea concentration, which is not unlike an increase in pressure, compared to aliphatic pairs.[28]

Mechanistic implications of the pressure-dependence of the volume change

If we recall eqn (4) and define $\Delta\Delta G = \Delta G(p) - \Delta G_0$, then the pressure effect on the free energy can be discussed in terms of volume and compressibility, keeping in mind that higher order terms in the Taylor expansion have been ignored. Levy and co-workers have performed a Monte Carlo simulation in order to determine the contribution of these two terms to $\Delta\Delta G$, the free energy change for the association of two methane molecules.[23] Their findings, shown in Fig. 3, indicate that at low to moderate pressures the (negative) volume term dominates, which will cause association of the methane molecules. However, at higher pressures (>500 MPa) the compressibility contribution becomes dominant, resulting in the dissociation of the methane pair, *i.e.* of the hydrophobic

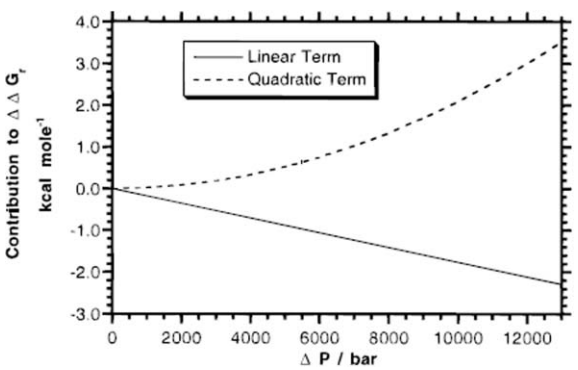

Fig. 3 The contributions of the change in partial molar volume and the change in isothermal compressibility, which are the linear and quadratic terms, to the change in the free energy of association $\Delta\Delta(G_r)$ as a function of pressure (1 bar = 0.1 MPa). (Reproduced with permission from *J. Phys. Chem. B*, 1997, **101**, 2054. Copyright 1997 Am. Chem. Soc.[23]).

contact. This suggests that in the case of proteins unfolding at moderate pressures is associated with a positive volume change, which then changes sign as the pressure is further increased if the compressibility change is positive. It also implies that the pressure-unfolded protein, including its hydration layer, has a higher compressibility than the native state, a conclusion completely consistent with experimental data.[4,29]

At atmospheric pressure most conformational transitions induced by chemical denaturants, pH and heat, except for those involving the formation of a molten globule from the native state, are invariably characterised by a negative $\Delta\beta$, meaning that the compressibility of the native state is larger than that of the unfolded state.[4] This begs the question as to why one observes the opposite behaviour under pressure. Computer simulations again prove to be valuable in addressing this issue, as they can separate the contributions from the protein from those of hydration and bulk water. It is generally assumed that the hydration layer of a native protein has a lower compressibility than the bulk solvent as most of the amino acid residues that are solvated in the native state are either polar or charged;[4,7] the polar residues form strong hydrogen bonds with water, whereas the charged groups are tightly solvated because of the electrostriction effect. As the pressure increases, however, more and more hydrophobic residues will become hydrated. Both experiments and simulations of model systems indicate that the hydration layer surrounding hydrophobic residues is more compressible than that around polar and ionic residues. Interestingly, experiments also predict that $\Delta\beta$ is positive for aromatic residues and indeed, simulations on methane and the protein staphylococcus nuclease indicate that the increased compressibility at high pressure mainly arises from the hydration of hydrophobic groups.[30,31] Importantly, the compressibility of the hydration water further increases as pressure is increased, whereas the compressibility of the bulk solvent decreases.

It is noteworthy that molten globule formation is also accompanied by a positive $\Delta\beta$, given its conformational similarity with the pressure-unfolded state.[4,7] Internal water has been found to be less compressible than the bulk solvent,[7] presumably because the hydration layer corresponds to the iceberg model for which a decreased compressibility is predicted.[30] Recent evidence, however, indicates that the hydration layer actually has a higher density than bulk solvent.[31,32] Even in cases where the density is assumed to be larger than that of the bulk solvent some argue that this result would decrease the compressibility of the system because of the reduced free volume at high density.[4] Here, one should consider the possibility of a compensation effect between a possible pressure-induced shortening of the hydrogen bonds[19] and a reduction of free volume on the one hand, and the constraints imposed by the directional nature of the hydrogen bond on the other hand. To complicate matters further the topography of the protein surface can also affect the nature of the hydration layer.[31]

Part of the difficulty in interpreting experimental data arises from the fact that one cannot separate experimentally intrinsic protein contributions from those of the hydration layer. One therefore has to rely on model systems and thermodynamic additivity assumptions. The latter have been suggested to break down when applied to biochemical systems, and indeed, recent work on the compressibility of proteins has indicated that, when the coupling between the solvent and protein fluctuations is strong, the additivity rule is no longer applicable.[33] Moreover, as pointed out in the previous section, most assumptions are based on model systems that may not be representative of the behaviour of amino acids in a well-packed protein interior.[8]

In closing this section we come back to the *protein volume paradox*. Given the fact that pressure-induced unfolding corresponds to the penetration of water into the protein core rather than to the exposure of the core residues to the solvent, as is usually the case in heat-induced unfolding, any estimation of ΔV on the basis of a random coil-like unfolded state will overestimate the hydrophobic hydration. Moreover, although the compressibility change $\Delta\beta$ is often assumed to be zero, Prehoda *et al.* showed that $\Delta\beta$ is significantly different from this value in the case of ribonuclease A and found that $\Delta V = -21$ ml mol^{-1} compared with -59 ml mol^{-1} when $\Delta\beta$ was assumed to be zero.[34] $\Delta\beta$, however, was found to be quite small in the case of staphylococcal nuclease.[35]

Probing the structure and formation of amyloid fibrils

Over the past few years it has become increasingly recognised that most natural proteins not only are able to fold into a functional, three dimensional conformation, *i.e.* the native state, but can also adopt other stable and metastable conformations. The latter process is referred to as misfolding and often involves protein aggregation. Although protein aggregation was originally considered simply as an undesired side effect of the experimental conditions used to study protein behaviour *in vitro* it is now the subject of intense investigation, since a number of highly debilitating and increasingly common human disorders, such as Alzheimer's and variant Creutzfeldt-Jakob disease, are associated with the formation of well-ordered protein aggregates *in vivo*, usually called amyloid fibrils. About 20 or so proteins are known to be involved in particular amyloid diseases, but the hypothesis has been put forward that under the proper conditions many, perhaps in principle all, proteins can be induced to form amyloid fibrils *in vitro*.[36] This finding has greatly enlarged the number of available model systems for the investigation of the structure and mechanism of formation of amyloid fibrils.

Probing the stability of amyloid fibrils towards high hydrostatic pressure can reveal information on their packing as well as the intermolecular interactions that are involved in their formation. This approach has been discussed in detail in a recent review[37] and we shall limit ourselves here to a brief description of the emerging trends. Pressures in the range of 200–300 MPa are found to be capable of dissociating early aggregates, which can have a spherical or a fibrillar morphology. As time progresses the fibrils become less and less pressure sensitive, suggesting that the fibrillar species undergo a maturation process. From the increased pressure stability one can infer that the maturation process involves a structural reorganisation resulting in an improved packing and

an enhanced hydrogen bonding. Kinetic and thermodynamic factors will also affect the observed stability. In a number of cases amyloid fibrils have been found to be dissociated by pressure although it is not clear whether such a phenomenon only occurs prior to the formation of the mature fibril.

An advantage of high pressure over, for instance, chemical denaturants, in dissociating early aggregates on the amyloid formation pathway is that it can shift more readily the reaction completely towards the monomer. Consequently, one can determine volume changes associated with the early steps of amyloid formation, which in turn can provide mechanistic information. However, extracting this information may not always be straightforward because the aggregates involved are often heterogeneous. Another observation of interest is that proteins, when submitted to high pressure, may form amyloid fibrils when the pressure is returned to ambient under conditions under which the native protein is normally stable, or amyloid fibrils may form on a shorter timescale than would be the case in absence of a compression–decompression cycle. This suggests that a pressure treatment may induce the formation of alternative conformations of the polypeptide that have a high aggregation propensity. Such a process may be potentially important in the case of globular proteins, as the first step in the amyloid formation process involves a partial unfolding of the protein,[38] and the pressure-unfolded state may well resemble such a partially unfolded state. In some cases, moderate pressures do not appear to affect the properties of the partially unfolded states, but rather slow down their aggregation rates (see below). Hence high pressure may be a useful perturbation method that enables one to characterise a protein conformation that is a precursor on the pathway to amyloid fibrils. When combined with the determination of $\Delta\alpha$, $\Delta\beta$, ΔC_p and ΔV, one can, within the thermodynamic framework described in the first section, relate conformational transitions to changes in the degree of hydration of a protein and in the fluctuations of enthalpy and volume.[39]

Pressure effects on protein energy landscapes

Energy landscapes reflect cooperative structural relaxation processes, from protein folding to glass transitions, and describe the energy of interaction between atoms or molecules as their relative positions are rearranged in order to obtain the ground state. When dealing with proteins, one should consider free energy landscapes rather than potential energy landscapes, as the conformational entropy of the polypeptide chain can play a major role in the relative stability of the different states. The process of protein folding involves a change in free energy when moving from the unfolded ensemble to folded (native) ensemble. However, due to the dynamical behaviour of proteins one can also explore changes in volume and energy within a single ensemble, *e.g.*, the native state, and depict this in terms of a free energy landscape. Thus an apparent single well (a local energy minimum) on the overall folding landscape contains many other local minima.[40] In what follows we shall address the influence of pressure on these two aspects of free energy landscapes.

Protein folding free energy landscapes

In general, the pressure dependence of a reaction rate k is given by:

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\#}}{RT} \tag{9}$$

where R is the ideal gas constant and $\Delta V^{\#}$ is the activation volume. Any reaction that is accompanied by a positive $\Delta V^{\#}$, *i.e.* if the transition state has a larger volume than the product, will be decelerated and *vice versa*. Pressure is a useful variable to investigate reaction mechanisms as studies of model systems have shown that pressure effects often determine the mechanism, whereas temperature mainly changes the frequency of the motions.

The effects of pressure on folding and unfolding rates have been investigated by pressure-jump and high pressure stopped flow experiments for a number of proteins and in most cases pressure is found to decrease the folding rate and to increase the unfolding rate.[14,41–44] Using an off-lattice minimalist model Hillson *et al.* demonstrated that, depending on the nature of the atomic interactions involved in the transition state, pressure may lower or increase the transition state energy.[45] Thus the folding rate can, in principle, increase or decrease with pressure, although only decreases in folding rates have been observed so far with an increase in pressure. The latter phenomenon is due to the fact that, as pressure increases, the reconfigurational diffusion coefficient, which characterises the average rate of local motion, will become smaller, and this effect in practice dominates any pressure-induced lowering of the transition state energy. Because the reconfigurational diffusion coefficient is a function of the fold of the native protein and the roughness of the energy landscape, one can conclude that the free energy landscape is rougher at pressures different from ambient. An important consequence of this conclusion is that metastable states may reside in their local minima for longer times, thereby enabling their characterisation. In this respect it is also of interest to note that the pressure-unfolded states of several proteins have been suggested to resemble intermediates in the folding process.[16,17] For example in the case of ribonuclease A, hydrogen–deuterium exchange protection factors and the secondary structure of the pressure-unfolded state are similar to those found for a previously characterised early folding intermediate,[17] suggesting that high pressure studies may provide important information on such species.

In order to go from the unfolded to the folded state the polypeptide chain has to cross a free energy barrier, which corresponds to the transition state. The properties of this transition state are rather elusive given its transient nature; structural information has been obtained mainly through mutational (ϕ-value) analysis, and computational methods.[46] The transition state has been found to be a rather heterogeneous ensemble of conformations, whose major, defining feature is an overall native-like topology. One important question concerns the role of water in the folding mechanism and whether the rate-limiting step involves desolvation?[47] This question can be addressed by determining the hydration properties of the transition state ensemble (TSE). Pressure

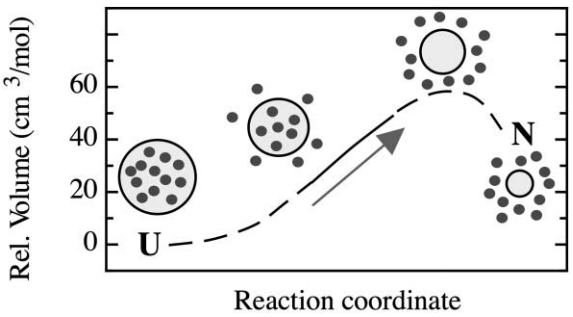

Fig. 4 Volume changes along the reaction coordinate of tendamistat folding. The arrow indicates the shift of the transition state ensemble upon addition of guanidinium chloride (from 0 M to 8 M). The circle represents the protein and the dots represent water molecules. (Reproduced with permission from *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 17. Copyright 2000 National Academy of Sciences, USA[42]).

studies can provide information on the TSE by measuring the activation volumes of folding (ΔV_f) and unfolding (ΔV_u). In the case of tendamistat, for instance, the respective activation volumes are +25 and −16 ml mol^{-1}, indicating that the TSE is closer to the native than to the unfolded state on the reaction coordinate and that it is still partially hydrated (Fig. 4).[42] Upon addition of guanidinium hydrochloride the volume of the TSE was found to increase, a finding that was interpreted as a shift of the TSE towards the native state due to further desolvation. However, Jacob *et al.* suggested that binding of the denaturant to the non-native state could be an alternative explanation for the observed volume increase.[41] Most measurements of activation volumes for folding suggest that the TSE is largely dehydrated, a finding which seems to differ from the conclusions of most computational studies and ϕ-value analyses.[44] However, it has been shown for staphylococcal nuclease that although the wild type protein has a highly dehydrated transition state, some of its mutants containing ionisable residues, have a hydrated TSE, *i.e.*, the absolute value of $\Delta V_f < \Delta V_u$.[44] Taken together these examples suggest that the degree of hydration of the TSE depends on the properties of the particular protein as well as on the experimental conditions. Moreover, a recent simulation study comparing implicit and explicit solvation models showed that although both models are qualitatively in agreement with each other, the explicit model does indicate that the TSE is more hydrated.[47] Part of the apparent contradiction mentioned above can therefore be related to the fact that most simulations deal with water implicitly and that ϕ-value analysis is also interpreted in terms of an implicit role of the solvent. As a consequence, high pressure methods may provide the best experimental approach to characterise the TSE in terms of hydration.

Protein dynamics: accessing conformational substates

Proteins are dynamic molecules, a characteristic which enables them to perform functions such as ligand or substrate binding and release. The fluctuations that underlie this dynamic behaviour cause the protein to adopt numerous conformations, which are commonly referred to as conformational substates.[40,48] Thus the native state of a protein is actually an ensemble of nearly isoenergetic substates, which may perform different functions. Experiments have shown that within these substates one can also identify statistical substates, which perform the same function, but with different rates. Two types of statistical substates, α and β, can be identified, which are coupled to dielectric (α) fluctuations of the bulk solvent and β-fluctuations in the hydration shell, respectively; these two substates are referred to as solvent-slaved and hydration shell-coupled states, respectively. Not all protein fluctuations, however, are coupled to fluctuations of the bulk solvent or of the hydration layer.

Pressure is a useful tool to explore the conformational substates in an energy landscape as it can shift the population from one substate to another on the basis of the volumetric properties of the respective substates. In addition, pressure can also change the reaction rate k with which a given substate perfoms its function, as its value depends on the activation volume $\Delta V^{\#}$ [eqn (9)], which may be different for different substates, as well as on the properties of the solvent (*e.g.* viscosity). This type of experiment can lead to new insights into the dynamics and reactions of proteins, such as the binding mechanism of carbon monoxide and oxygen to myoglobin.[40,48]

From free energy landscapes to pressure–temperature phase diagrams

Life on Earth can thrive in environments having a wide range of pressures and temperatures. In order to understand this ability on the molecular level it is necessary to consider pressure effects on proteins, and living systems in general, over a wide temperature range. A plot of transition midpoint for pressure unfolding *versus* temperature yields an elliptical phase diagram (Fig. 5), which, interestingly, is also found when plotting inactivation rates of microorganisms or the phase separation behaviour of water-soluble polymers.[5] On the basis of the contours of the phase diagram, with its re-entrant behaviour at low temperature, cold unfolding of proteins was predicted. It is worth noting that, at least at elevated pressures, cold and pressure unfolding are thermodynamically (Fig. 5) and mechanistically similar.[16] Pressure is often used in cold unfolding experiments because pressures of ~200 MPa reduce the freezing point of water by ~20 °C, thus enabling experiments at low temperature in the liquid state. The phase diagram can be described by eqn (2) and its features have been discussed in detail elsewhere.[5] Here we limit ourselves to a number of brief comments.

The pressure–temperature dependence of the volume change ΔV is defined as:

$$\Delta V(p,T) = \Delta V^{\circ} + \Delta\alpha(T - T_o) - \Delta\beta(p - p_o) \qquad (10)$$

where the second term $\Delta\alpha(T - T_o)$ represents the temperature dependence of the volume. In fact, ΔV is found to have a strong temperature dependence, with ΔV becoming less negative as the temperature increases.[35] However, in the case of ribonuclease A the changes in ΔV with temperature have been shown to depend on experimental conditions.[49] In practice, the stability of a protein will depend strongly on

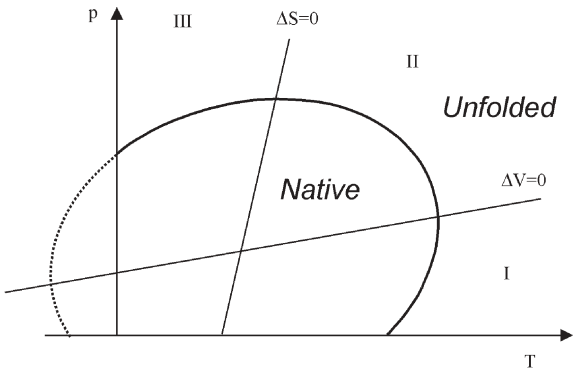

Fig. 5 A schematic representation of the pressure–temperature phase diagram of a protein. The boundary of the ellipse is given by $\Delta G = 0$. In zone I ΔV and ΔS are positive, in zone II ΔV is negative and ΔS is positive and in zone III both ΔV and ΔS are negative. Zone III is where one generally observes pressure unfolding at ambient temperature. Note the positive dp/dT at high temperature and low pressure. The region of the cold unfolding is indicated by a dotted line.

solution conditions such as pH, the presence of chemical denaturants or co-solutes. The addition of co-solutes or co-solvents can have a large effect on the volume change, and Scharnagl *et al.* have recently given a comprehensive thermodynamic description of the effect of co-solutes and co-solvents on the stability of the protein.[50]

The slope of the equilibrium line in the diagram (Fig. 5) is given by:

$$\frac{dT}{dp} = \frac{\Delta V^o - \Delta\beta(p-p_o) + \Delta\alpha(T-T_o)}{\Delta S^o - \Delta\alpha(p-p_o) + \Delta C_p((T-T_o)/T_o)} \qquad (11)$$

Note that, if $\Delta\beta$, $\Delta\alpha$ and ΔC_p, are zero this equation is reduced to the classical Clapeyron equation ($dT_m/dp = T_m \Delta V/\Delta H$), which describes the behaviour under pressure of, for instance, simple liquids, lipids and nucleic acids. This demonstrates clearly the importance of the second order terms in the elliptic nature of the phase diagrams of proteins. Higher order terms, describing the temperature and pressure dependence of $\Delta\beta$, $\Delta\alpha$ and ΔC_p, have been ignored in eqn (2), but for at least one protein, ribonuclease A, a pressure dependence of ΔC_p has been reported.[49] Inclusion of such higher order terms distorts the diagram, but does not change completely its overall appearance.

One of the interesting features of the phase diagram is that for most proteins dT_m/dp is positive at low pressures and high temperatures (Fig. 5), suggesting that pressure increases the stability of the protein towards thermal unfolding. As a result it is possible to refold a thermally unfolded protein (at 0.1 MPa), at temperatures just above the unfolding temperature, by increasing the pressure. The fact that in this low pressure-high temperature region the unfolding is associated with a positive volume change has been attributed to the difference in the thermal expansion of the folded and unfolded states.[35]

Conclusion and outlook

The application of high hydrostatic pressures is a useful tool for investigating the packing and hydration properties of proteins and protein assemblies. Although the thermodynamic basis of the effects of pressure on proteins is now well understood in terms of the volume change ΔV and the isothermal compressibility β_T, the molecular interpretation of these parameters requires further investigation. Further progress in this field is expected to come from a comparison of computer simulations with experiments, as the simulations seem to be able to predict the experimentally observed behaviour and can separate contributions arising from the protein, the hydration layer and the bulk solvent. Eventually one should be able to explain not only why $\Delta\beta$ is positive for pressure-unfolding, but also why it is negative for most other types of unfolding. Together with the determination of other volumetric properties, such as thermal expansion, one should obtain a detailed picture of the pressure- and heat-induced unfolding of proteins, and the origin of their mechanistic and thermodynamic differences. In addition, the pressure dependence of $\Delta\beta$ remains to be explored.

Computer simulations of model systems so far have focused on the effect of pressure on the hydrophobic effect, which is undoubtedly a major driving force in protein folding. On the other hand, in simulations of proteins one tends to focus on hydration, and hence on the role of hydrogen bonds. Obviously, the structure of the hydrogen bond network in the solvent and the hydrophobic effect are tightly intertwined. Nevertheless, both approaches reveal new aspects of different sides of the same coin. Furthermore, in order to obtain a complete molecular interpretation of pressure effects on aromatic–aromatic interactions in proteins they should also be investigated under pressure, as new data on the hydration of aromatic compounds and the effect of urea on their interactions suggest that their pressure behaviour may be significantly different from that of aliphatic–aliphatic contacts. From the viewpoint of understanding free energy landscapes, pressure seems to be a promising variable to contribute to an elucidation of the folding mechanism, and in particular the role of solvent water. It may also provide an alternative approach to populate and characterise otherwise fast relaxing or aggregating conformations. The effect of co-solutes and co-solvents on the pressure stability of proteins has been investigated. Such experiments may also provide new evidence for the role of water in the mechanism of protein unfolding and enhance our overall understanding of protein folding and unfolding not just *in vitro* but also in the complex milieu of the living cell.

Acknowledgements

We are grateful to Mr Johan Smeyers for producing Fig. 1 and Prof. Kiefhaber for providing us with Fig. 4. F.M. is a Postdoctoral Fellow of the Research Foundation—Flanders (FWO—Vlaanderen). The work of C.M.D. is supported in part by Programme Grants from the Wellcome Trust and Leverhulme Trust.

Notes and references

1 P. F. McMillan, *J. Mater. Chem.*, 2004, **14**, 1506.
2 R. Winter, *Biochim. Biophys. Acta*, 2002, **1595**, 160.
3 R. B. Macgregor, Jr., *Biochim. Biophys. Acta*, 2002, **1595**, 266.
4 T. V. Chalikian, *Annu. Rev. Biophys. Biomol. Struct.*, 2003, **32**, 207.

5 F. Meersman, L. Smeller and K. Heremans, *Biochim. Biophys. Acta*, 2006, **1764**, 346.
6 K. Heremans and L. Smeller, *Biochim. Biophys. Acta*, 1998, **1386**, 353.
7 D. P. Kharakoz and V. E. Bychkova, *Biochemistry*, 1997, **36**, 1882.
8 F. M. Richards, *Ann. Rev. Biophys. Bioeng.*, 1977, **6**, 151.
9 C. A. Royer, *Biochim. Biophys. Acta*, 2002, **1595**, 201.
10 W. Kauzmann, *Nature*, 1987, **325**, 763.
11 J. Torrent, J. P. Connelly, M. G. Coll, M. Ribó, R. Lange and M. Vilanova, *Biochemistry*, 1999, **38**, 15952.
12 J. L. Silva and G. Weber, *Annu. Rev. Phys. Chem.*, 1993, **44**, 89.
13 H. Lesch, J. Schlichter, J. Friedrich and J. M. Vanderkooi, *Biophys. J.*, 2004, **86**, 467.
14 G. Panick, R. Malessa, R. Winter, G. Rapp, K. J. Frye and C. A. Royer, *J. Mol. Biol.*, 1998, **275**, 389.
15 A. Paliwal, D. Asthagiri, D. P. Bossev and M. E. Paulaitis, *Biophys. J.*, 2004, **87**, 3479.
16 F. Meersman, L. Smeller and K. Heremans, *Biophys. J.*, 2002, **82**, 2635.
17 J. Zhang, X. Peng, A. Jonas and J. Jonas, *Biochemistry*, 1995, **34**, 8631.
18 C. Schnell, M. Reif, C. Scharnagl and J. Friedrich, *Phys. Chem. Chem. Phys.*, 2005, **7**, 2217.
19 M. P. Williamson, K. Akasaka and M. Refaee, *Protein Sci.*, 2003, **12**, 1971.
20 G. Hummer, S. Garde, A. E. Garcia, M. E. Paulaitis and L. R. Pratt, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 1552.
21 T. Ghosh, A. E. Garcia and S. Garde, *J. Am. Chem. Soc.*, 2001, **123**, 10997.
22 T. Ghosh, A. E. Garcia and S. Garde, *J. Chem. Phys.*, 2002, **116**, 2480.
23 V. A. Payne, N. Matubayasi, L. R. Murphy and R. M. Levy, *J. Phys. Chem. B*, 1997, **101**, 2054.
24 F. Sciortino, A. Geiger and H. E. Stanley, *Nature*, 1991, **354**, 218.
25 F. Meersman, J. Wang, Y. Wu and K. Heremans, *Macromolecules*, 2005, **38**, 8923.
26 M. D. Collins, G. Hummer, M. L. Quillin, B. W. Matthews and S. M. Grunner, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 16668.
27 T. M. Raschke and M. Levitt, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 6777.
28 M.-E. Lee and N. F. A. van der Vegt, *J. Am. Chem. Soc.*, 2006, **128**, 4948.
29 M. W. Lassalle, H. Yamada and K. Akasaka, *J. Mol. Biol.*, 2000, **298**, 293.
30 N. Matubayasi and R. M. Levy, *J. Phys. Chem.*, 1996, **100**, 2681.
31 N. Smolin and R. Winter, *Biochim. Biophys. Acta*, 2006, **1764**, 522.
32 F. Merzel and J. C. Smith, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5378.
33 H. Pfeiffer and K. Heremans, *ChemPhysChem*, 2005, **6**, 697.
34 K. E. Prehoda, E. S. Mooberry and J. L. Markley, *Biochemistry*, 1998, **37**, 5785.
35 H. Seemann, R. Winter and C. A. Royer, *J. Mol. Biol.*, 2001, **307**, 1091.
36 C. M. Dobson, *Nature*, 2003, **426**, 884.
37 F. Meersman and C. M. Dobson, *Biochim. Biophys. Acta*, 2006, **1764**, 452.
38 M. Dumoulin, A. M. Last, A. Desmyter, K. Decanniere, D. Canet, G. Larsson, A. Spencer, D. B. Archer, J. Sasse, S. Muyldermans, L. Wyns, C. Redfield, A. Matagne, C. V. Robinson and C. M. Dobson, *Nature*, 2003, **424**, 783.
39 V. Smirnovas, R. Winter, T. Funck and W. Dzwolak, *J. Phys. Chem. B*, 2005, **109**, 19043.
40 P. W. Fenimore, H. Frauenfelder, B. H. McMahon and R. D. Young, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 14408.
41 M. H. Jacob, C. Saudan, G. Holtermann, A. Martin, D. Perl, A. E. Merbach and F. X. Schmid, *J. Mol. Biol.*, 2002, **318**, 837.
42 G. Pappenberger, C. Saudan, M. Becker, A. E. Merbach and T. Kiefhaber, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 17.
43 D. M. Korzhnev, I. Bezsonova, F. Evanics, N. Taulier, Z. Zhou, Y. Bai, T. V. Chalikian, R. S. Prosser and L. E. Kay, *J. Am. Chem. Soc.*, 2006, **128**, 5262.
44 L. Brun, D. G. Isom, P. Velu, B. Garcia-Moreno and C. A. Royer, *Biochemistry*, 2006, **45**, 3473.
45 N. Hillson, J. N. Onuchic and A. E. Garcia, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 14848.
46 M. Vendruscolo and C. M. Dobson, *Philos. Trans. R. Soc. London, Ser. A*, 2005, **363**, 433.
47 Y. M. Rhee, E. J. Sorin, G. Jayachandran, E. Lindahl and V. S. Pande, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 6456.
48 H. Frauenfelder, N. A. Alberding, A. Ansari, D. Braunstein, B. R. Cowen, M. K. Hong, I. E. T. Iben, J. B. Johnson, S. Luck, M. C. Marden, J. R. Mourant, P. Ormos, L. Reinisch, R. Scholl, A. Schulte, E. Shyamsunder, L. B. Sorensen, P. J. Steinbach, A. Xie, R. D. Young and K. T. Yue, *J. Phys. Chem.*, 1990, **94**, 1024.
49 T. Yamaguchi, H. Yamada and K. Akasaka, *J. Mol. Biol.*, 1995, **250**, 689.
50 C. Scharnagl, M. Reif and J. Friedrich, *Biochim. Biophys. Acta*, 2005, **1749**, 187.
51 B. Wroblowski, J. F. Diaz, K. Heremans and Y. Engelborghs, *Proteins*, 1996, **25**, 446.