The solution structure of bovine pancreatic trypsin inhibitor at high pressure

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Abstract

The solution structure of bovine pancreatic trypsin inhibitor (BPTI) at a pressure of 2 kbar is presented. The structure was calculated as a change from an energy-minimized low-pressure structure, using ¹H chemical shifts as restraints. The structure has changed by 0.24 Å RMS, and has almost unchanged volume. The largest changes as a result of pressure are in the loop 10–16, which contains the active site of BPTI, and residues 38–42, which are adjacent to buried water molecules. Hydrogen bonds are compressed by 0.029 ± 0.117 Å, with the longer hydrogen bonds, including those to internal buried water molecules, being compressed more. The hydrophobic core is also compressed, largely from reduction of packing defects. The parts of the structure that have the greatest change are close to buried water molecules, thus highlighting the importance of water molecules as the nucleation sites for volume fluctuation of proteins in native conditions.

Keywords: Pressure; protein compression; chemical shifts; NMR; buried water; active site

Supplemental material: See www.proteinscience.org.

In order to gain an increased understanding of the dynamics of proteins, and the forces governing their structure, it is useful to have experimental measurements on how protein structures change with pressure. Pressure is an important environmental variable, reaching values in excess of 1 kbar (100 MPa) in deep sea trenches (Yayanos 1995; Heremans and Smeller 1998). It is also a variable whose effect can be simulated readily, and which is valuable for understanding the thermodynamic parameters that govern protein stability, because pressure affects the internal energy of proteins directly by its effect on partial molar volume.

Despite the importance of pressure, it has proved experimentally difficult to obtain protein structures at high pressure. Crystallography of proteins at high pressure requires

specialist cells, and suffers from absorption of the x-rays by the cell. In addition, crystals tend to dissolve or crack at high pressure. Consequently, there are only two crystal structures of proteins at elevated pressure: lysozyme and myoglobin, both at 2 kbar (Kundrot and Richards 1987; Urayama et al. 2002). It is also possible to collect NMR spectra at high pressure (Akasaka and Yamada 2001; Jonas 2002). There are two methods by which this has been achieved. In the first, a specialized high-pressure probe assembly is used. It has the advantage that very high pressures can be achieved, but suffers from the important disadvantage that the detection coil generally lacks the resolution, pulse programming flexibility, and solvent suppression that would permit high-resolution spectroscopy in water. In the second method, a thick-walled cell is connected to an on-line pressure source, and inserted into a normal commercial probehead. This method has the disadvantage that the sample volume is limited and consequently the sensitivity is low, but it has the important advantage that the spectra are of comparable quality to those obtained using conventional NMR tubes. We have developed this technique, and have

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used it to acquire two-dimensional spectra of proteins, including bovine pancreatic trypsin inhibitor (BPTI; Li et al. 1998, 1999). The spectra showed linear and reversible changes in chemical shift up to 2 kbar, demonstrating that the protein undergoes a reversible pressure-dependent conformational change.

We recently showed that changes in ^1H chemical shifts can be used to calculate changes in protein structure, provided that the structural changes are small and incremental (Iwdate et al. 2001; Refaee et al. 2003). This method is therefore ideally suited to calculation of protein structures under pressure. Chemical shifts are governed by the structure of the protein. We showed that, although the calculated values of chemical shifts are not sufficiently accurate to permit us to calculate or refine a protein structure using ^1H chemical shifts as direct restraints (Williamson et al. 1995), the changes in the shifts caused by a small change in the structure are much more accurate, and do permit a reliable calculation of the change in structure. We therefore use *changes* in shift to calculate *changes* in structure. This means that, starting from a structure whose chemical shifts are known *and match the experimental shifts*, we can use the change in chemical shifts on application of pressure to calculate the change in structure. The above condition is an important one. It is highly unlikely that any starting structure will give calculated shifts that exactly match the experimental shifts. Therefore, we developed a method that allows us to start from a high-quality structure, and we use the experimentally derived changes in shift with pressure as restraints to calculate the change in structure with pressure. The method was recently used to calculate the change in structure of lysozyme with pressure (Refaee et al. 2003). Here we present the results obtained from applying the method to BPTI, and show how the structure changes with pressure.

Results

High-pressure structures

Starting from the crystal structure 5pti, the structure of BPTI was refined iteratively in a full XPLOR force field. The structure altered over the first few iterations, reaching a stable conformation (i.e., one that had no systematic structural changes on further iteration) after iteration 6 (data not shown). In order to check the reproducibility and stability of the calculations, all subsequent structure calculations were carried out in parallel on three refined structures, these being the structures from iterations 8, 13, and 19. These structures were chosen essentially at random from the iterations 6 through 20, and are respectively 1.344, 1.335, and 1.329 Å RMS different from the starting crystal structure (calculated using backbone heavy atoms). They are 1.335 ± 0.008 Å different from each other. The three structures are there-

fore essentially identical, and are at least as similar to the starting crystal structure as different crystal structures of the same protein are to each other. We have therefore taken them to be equally good samples of the conformational ensemble produced by low-temperature unrestrained molecular dynamics. The chemical shifts of each of these 'starting structures' were then calculated within XPLOR.

To generate reference low-pressure structures, calculated in the closest possible way to the high-pressure structures, the calculated chemical shifts of the starting structures were used as restraints for low-temperature molecular dynamics simulations. Families of 50 low-pressure structures were calculated, and were similar to the starting structure (e.g., family 8 differed by 0.211 ± 0.125 Å from the starting structure). Each family of 50 structures was averaged and energy-minimized.

To generate the high-pressure structures, the 159 measured experimental changes in chemical shift on going from 30 bar to 2 kbar were added to the calculated shifts of the starting structures, and used as restraints for molecular dynamics simulations, followed by averaging and energy minimization in an identical manner. Thus, although the chemical shift restraints used to generate this structure are not directly experimental, the difference in shifts is experimental. Therefore the difference between the high-pressure structure calculated in this way and the low-pressure reference structure is also based on direct experimental measurements. These high-pressure structures were similar to but significantly different from the starting structure (e.g., family 8 differed by 0.235 ± 0.016 Å from the starting structure). More importantly, the high-pressure structures differed from their low-pressure reference structures by small but meaningful amounts (0.235, 0.228, and 0.247 Å respectively for families 8, 13, and 19). All of the structures satisfied the chemical shift restraints within experimental error (e.g., differences of $[-0.15 \pm 4] \times 10^{-3}$ ppm and $[-1 \pm 7] \times 10^{-3}$ ppm for the low-pressure and high-pressure structures of family 8, respectively). Low- and high-pressure coordinates have been deposited with the Research Collaboratory for Structural Bioinformatics Protein Data Bank (Berman et al. 2000; accession codes 1oa5 and 1oa6, respectively).

Comparison of low- and high-pressure structures

The most obvious global measure of structural change with pressure is molecular size, as measured for example by radius of gyration, moments of inertia, surface area, or volume. These measures all suggest that the overall structure is in fact slightly expanded (Table 1), with an increase in volume of 0.17% (a compressibility of $-0.9/\text{Mbar}$). A negative compressibility is surprising, if not completely unknown (Gekko and Hasegawa 1986). In order to understand this result, it is necessary to examine the structural change in more detail. First, we note that the compression is far

Table 1. Structural change calculated for BPTI between 30 and 2000 bar

Measure	30 bar			2 kbar			% change		
	Family 8	Family 13	Family 19	Family 8	Family 13	Family 19	Family 8	Family 13	Family 19
R_g (Å) ^a	10.87	10.89	10.89	10.94	10.94	10.94	0.64	0.46	0.46
I_{xx} (Å) ^b	19055	18956	18965	18949	18945	18858	-0.56	-0.05	-0.56
I_{yy} (Å) ^b	28699	28818	28664	28989	29071	29023	1.01	0.88	1.26
I_{zz} (Å) ^b	64609	64806	65014	65646	65646	65891	1.61	1.3	1.35
Area (Å ²) ^c	4066	4135	4112	4128	4152	4119	1.52	0.41	0.17
Vol (Å ³) ^d	11930	11950	11940	11950	11970	11960	0.17	0.17	0.17
Cavity vol (0 Å probe) ^{d,e}	485.2	500.7	483.6	454.8	370.5	421.4	-6.3	-26.0	-12.9
Cavity vol (1.2 Å probe) ^e	336.1	293.3	407.2	293.7	223.5	269.3	-12.6	-23.8	-33.9

Values are shown for each family separately.

^a Radius of gyration.

^b Moments of inertia in three principal directions (smallest first).

^c Measured by the program AREAIMOL.

^d Measured by the program VOIDOO (Kleywegt and Jones 1994). The protein was rotated through random orientations and the calculation repeated at least 10 times to get reliable and consistent values. By using a primary grid spacing of 0.4 Å and shrinking the grid by a factor of 0.93 per refinement step, coefficients of variation for volume calculations between different orientations could be reduced below 15%, even for cavity calculations.

^e Cavity volumes measured by VOIDOO, using a range of rotations as above. Cavities were measured using a probe of radius 0 Å, to give the total cavity volume including packing defects; and also using a probe of radius 1.2 Å, to give the volume of all cavities large enough to contain a water molecule. There are a large number of packing cavities, but they do not add up to a very large volume.

from isotropic, because the changes in the three principal moments of inertia are very different (Table 1). The smallest moment of inertia has decreased, whereas the other two have increased, implying that the structure has become 'longer and thinner'. Second, we note that there is a large compression of cavities in the protein, considering both small cavities (packing defects) and large, potentially water-filled cavities (Table 1). This is confirmed by the radial distribution functions, shown in Figure 1, which also depicts the difference in radial distribution between high- and low-pressure structures (Fig. 1B). The latter shows that the distribution of interatomic distances for bonded contacts remains fairly constant [the height of the peaks at 1.1 Å (representing bonds to H) and 2.2 Å (representing heavy atom-heavy atom bonds) are very similar for the high and low-pressure structures], but that each peak of nonbonded contacts in Figure 1A tends to give rise to a +/− difference peak in Figure 1B, with the positive difference at shorter distance, showing that nonbonded contacts tend to move to shorter distances.

To compare the structures at low and high pressure, we used a difference distance matrix. This is obtained by calculating the distances between all Cα atoms in each structure separately, and subtracting one matrix from the other. The difference shows which parts of the structure have moved closer to or further away from other parts of the structure, and is less biased as a structure comparison tool than superimposition, because the result from superimposition depends on which atoms are chosen for the calculation. The results are shown in Figure 2, which constitutes a powerful demonstration that the changes calculated on the basis of chemical shifts are consistent among the three families calculated, and thus that the changes calculated do not de-

pend on the starting structure but are a real reflection of the chemical shift restraints.

The difference distance matrices show that residue 15 has moved closer to the rest of the structure, whereas the other main change is that residues 12, 26, 38, and 50 have moved further away. Compression is therefore by no means uniform across the structure, with some parts even expanding (as expected from the overall small increase in volume). Interestingly, residues 12, 15, 26, and 38 are all in loops, suggesting that most of the structural change is located in the loops, and that regular secondary structure is affected to a lesser extent. As another way of visualizing structural change, the low- and high-pressure structures were superimposed using backbone atoms in regular secondary structure. The RMS distance between backbone heavy atoms of corresponding residues in the two structures is shown in Figure 3 (for family 19; the other two families have very similar values). The residues with an RMS value greater than 0.35 Å are shown as black regions in Figure 4. This allows us to summarize the main structural changes as follows: The loops at K26 and C38 rotate out and away from the protein (this being the origin of the changes in moment of inertia described above); the loop at K15 folds into the protein, and the structure is distorted in the long loops shown at the top left side of Figure 4.

Backbone dihedral angles within BPTI changed by $4.6 \pm 4.9^\circ$ for ϕ , and $5.2 \pm 4.6^\circ$ for ψ (averaging over the three families). Side chain χ_1 angles had a greater variability, changing by $-0.2 \pm 7.2^\circ$, indicating that the side chains are more flexible under pressure than the backbone. This is not surprising, and was seen, for example, in the lysozyme crystal structure at high pressure (Kundrot and Richards 1987).

Figure 1. Radial distribution functions for low- and high-pressure BPTI structures. The family 8 results are shown as an illustration. Distances were calculated between all pairs of atoms (including hydrogens) and placed into 0.1 Å bins. The distributions were weighted by dividing the number in each bin by the square of the radius. (A) The functions for the low-pressure structure (solid line) and the high-pressure structure (dashed line). (B) The difference (high – low), such that a positive peak at any distance means that there are more atom pairs at this distance in the high-pressure structure than there are in the low-pressure structure.

The direction of movement of atoms within the structure was analyzed by superimposing the structures on their centers of mass (using all backbone heavy atoms). For each pair of corresponding atoms, the angle θ was calculated between the interatomic vector and the vector to the center of mass of the protein. For uniform compression (movement directly towards the center of mass), all atoms would have a θ of 0° . For random motion, atoms would have a flat normalized angular distribution. As shown in Figure 5, the frequency of different angles falls off approximately linearly with angle, indicating a general tendency for atoms to move towards the center of the protein. Similar distributions were reported previously (Kundrot and Richards 1987; Refaee et al. 2003). The similarity of the curves for the three families again demonstrates that the nature of the structural change calculated is independent of the exact starting structure.

The locations of hydrogen bonds were calculated (Kabsch and Sander 1983). A comparison of hydrogen bond

lengths in the low- and high-pressure structures (Fig. 6) shows that there is a slight tendency for hydrogen bonds to become shorter with pressure, but with quite a wide distribution of changes: Hydrogen bonds shortened overall by 0.029 ± 0.117 Å. Figure 6 also shows that longer hydrogen

Figure 2. Difference distance matrices, for (A) family 8, (B) family 13, and (C) family 19. The contours are plotted at contour levels of ± 0.6 , ± 0.5 , ± 0.4 Å, and indicate in red parts of the structure where distances are shorter and in blue parts where the distances are longer. The regular secondary structure elements of the structure are indicated.

Figure 3. RMS distance between corresponding backbone atoms in low- and high-pressure structures for family 19 by residue, after superposition of backbone heavy atoms in regular secondary structures. The line at 0.35 Å indicates the cutoff used for Figure 4.

bonds become preferentially shorter, particularly hydrogen bonds to buried water molecules. Figure 7 shows the locations of changes in hydrogen bond length on the structure.

Discussion

Validation of methodology

Our results demonstrate that there is virtually no overall volume change of BPTI with pressure, with some parts becoming more compressed and other less compressed. Overall, the structure becomes more elongated, with almost all of the structural distortions happening at loops. Because the method used is new (having been used only once previously, to calculate the structure of lysozyme under pressure; Refaee et al. 2003), it is appropriate to begin by examining the methodology, and ask whether it is sound, and whether the results are reasonable, particularly because here the overall volume increases with pressure.

We start by noting that chemical shift restraints are satisfied well, implying that the method meets its own targets. It is instructive to examine more closely the origins of the chemical shift changes. The larger chemical shift changes are to amide protons, which are generally the most sensitive ^1H signals to structural change. The largest change was of +0.198 ppm (i.e., a downfield shift) to HN of Cys55. This change is caused by a shortening of the hydrogen bond from Cys55 HN to Cys51 CO from 2.17 Å (using family 8 as an example) to 2.03 Å, simultaneous with a straightening of the H-N $\cdots$ O angle from 125° to 135°. The largest change to a non-amide proton is of -0.168 ppm to the H α of Cys51. The chemical shift value of this proton is dominated by a ring-current shift from Phe45, and the change in shift is caused

by a change in distance to the ring plane from 2.84 to 2.66 Å (Fig. 8). Thus, the local structural changes agree with what would be expected from the shift changes. Changes in the solvent hydrogen bonding network will also affect protein shifts, implying that some of the restraints to external amide protons in particular may be inaccurate. However, the size of the effect is difficult to estimate. Our guess is that the effect will be small, because solvent hydrogen bonds fluctuate much more than intramolecular bonds do (as seen, e.g., from analysis of amide proton temperature dependence; Baxter and Williamson 1997).

We also note that the changes observed here with pressure match those seen by others. Gekko and Hasegawa (1986) measured changes in compressibility of a range of proteins using ultrasound measurements, and noted that proteins with higher β -sheet content and with a higher density of disulfide bridges are less compressible. Of the 25 proteins they studied, a trypsin inhibitor was one of the least compressible. A few proteins even had negative compressibility, although it is important to remember that their results include a contribution from the compression of the hydration layer, which is generally negative. There are two crystal structures of proteins at high pressure (Kundrot and Richards 1987; Urayama et al. 2002). The changes in torsion angle and in hydrogen bond length seen in the present study are similar in magnitude to those seen in the crystal studies, and in our previous chemical shift-based studies (Iwadata et al. 2001; Refaee et al. 2003). They also match spectroscopic results (Shimizu et al. 1988; Heremans and Smeller 1998).

Figure 4. Ribbon representation of BPTI (drawn using GRASP; Nicholls et al. 1991). Residues colored black are those for which the RMS distance between corresponding residues in low- and high-pressure structures is greater than 0.35 Å (Fig. 3). Named residues are those that show large changes in their relative distances to other parts of the protein (Fig. 2). The four buried water molecules are also indicated.

Figure 5. Direction of movement of heavy atoms in BPTI with pressure. An angle of 0 corresponds to movement towards the center of mass. Random motion would give a horizontal distribution at $n = 1$. (Solid line) Family 8; (dashed line) family 13; (dotted line) family 19.

Our results also agree with these studies in a number of other important respects, showing for example that secondary structures are little affected by pressure, with a major site of compression being cavities and packing defects (Urayama et al. 2002).

It is noteworthy, and at first sight perplexing, that the protein overall shows a small expansion on application of pressure. The explanation lies in the fact that the protein is dissolved in water, and the fundamental thermodynamic requirement is for the system as a whole to compress. It must therefore be the case that the hydration layer has compressed more than the protein has expanded: We anticipate that the largest changes will be in the vicinity of the loops that move on application of pressure.

Finally, we note that probably the greatest objection to the method is that chemical shifts are very strongly dependent on the exact local geometry, and therefore have not been notably successful as structure refinement restraints. One could thus reasonably argue that the structural changes resulting from imposition of the chemical shift restraints are likely to be strongly dependent on the exact starting structure. However, the results shown here (e.g., Figs. 2, 5) demonstrate that very similar structural changes result from all three starting structures used. We are therefore confident that the structural changes seen here are real, and in particular that BPTI does indeed slightly expand overall with an increase in pressure, this expansion occurring despite very significant compression of internal cavities (Table 1).

Structural changes

There is no clear difference in compressibility of hydrogen bonds in different secondary structures (Fig. 6), although

there is evidence to suggest that hydrogen bonds to buried water molecules are more compressible than other hydrogen bonds. This is in agreement with our previous conclusions based only on chemical shift changes, that hydrogen bonds to water shorten more than intra-protein hydrogen bonds (Li et al. 1998). Longer hydrogen bonds are compressed more than shorter hydrogen bonds. This is again in agreement with our results on lysozyme (Refaee et al. 2003), and is most likely due to the longer hydrogen bonds having a greater intrinsic compressibility.

Analysis of the locations of hydrogen bond changes (Fig. 7) shows no obvious pattern. There is however some suggestion that hydrogen bonds on the periphery (e.g., the hydrogen bond at the top of the long β -sheet, and the hydrogen bonds on the outside face of the C-terminal helix) have the least tendency to compress, whereas those towards the center of the protein are most compressed.

As shown in Figure 4, the region of the protein that is deformed most under pressure (undergoing expansion as well as compression) is in the vicinity of the active site (the bond between residues 15 and 16). It is tempting to speculate that this reflects the fact that the active site of protease inhibitors (and of BPTI in particular) is held rigidly in a conformation that matches the protease active site (Hubbard et al. 1991). Thus, it may be that this region is more strained than the rest of the protein, and therefore more easily forced to adopt different conformations. In this context, it is of interest to note that we recently studied in detail the regions of BPTI that can access alternative conformations under normal solution conditions (Baxter et al. 1998). These regions were found to be similar to those observed here to

Figure 6. Hydrogen bond lengths in low- and high-pressure structures. Hydrogen bonds were identified using the method of Kabsch and Sander (1983). (●) Helical structure; (▲) sheet structure; +, loop; (■) hydrogen bond to buried water molecule. The line of best fit is indicated by a solid line, and the $y = x$ diagonal by a dashed line.

Figure 7. Changes in hydrogen bond length with pressure, showing locations on the structure of BPTI. Hydrogen bonds are indicated by thick colored lines, color-coded by the change in distance with pressure: dark blue, shorter by more than 0.1 Å; cyan, shorter by less than 0.1 Å; yellow, longer by more than 0.1 Å; green, longer by more than 0.1 Å. Figure produced using MOLSCRIPT (Kraulis 1991).

show the greatest movement (cf. Fig. 4), namely residues 13 and 15 near the active site, and 35 and 41–43 close to the buried water molecules. More studies are required to tell whether this observation can be generalized to other proteins, but it should be pointed out that in our study of lysozyme (Refaee et al. 2003) we also observed some of the largest structural changes close to the active site.

One of the most striking results is the finding that the largest changes in the structure tend to be close to buried

Figure 8. Relative locations of Cys51 and Phe45 in low- and high-pressure structures. The two structures were superimposed on the ring atoms of Phe45.

water molecules (Fig. 4). The same conclusion was reached in our study on the compression of lysozyme under pressure, and agrees with our observation in a range of proteins that nonlinear pressure-dependent changes in chemical shift are correlated with the density of cavities large enough to contain buried waters (Akasaka and Li 2001). We note that there is a linear relationship between compressibility and volume fluctuations (Cooper 1976). This implies that volume fluctuations are greatest close to buried water molecules even under normal conditions, and highlights the importance of buried waters for affecting the amount by which a protein can undergo local structural fluctuations ('breathe'). It is conventional wisdom that the burying of water molecules inside proteins produces a more ordered, lower entropy state (Dunitz 1994). However, it has more recently been suggested that the burial of water molecules need not reduce entropy and can even increase it (Denisov et al. 1997), and a computational analysis subsequently suggested that binding of a buried water to BPTI makes the protein more flexible (Fischer and Verma 1999). Our results support these conclusions, in that they identify hydrated cavities as sources of conformational plasticity. At least in BPTI (and also in lysozyme; Refaee et al. 2003), buried water molecules are found in regions of the protein that undergo the greatest structural changes with pressure. Volume changes are necessary for such diverse functions as ligand binding, channel opening, amide proton exchange, and pressure denaturation, and our results implicate buried water molecules as important nucleation sites for such events.

Materials and methods

Changes in the ^1H chemical shifts of BPTI on going from 30 bar to 2 kbar were taken from Li et al. (1998, 1999), which used the on-line pressure cell method on a 750-MHz Bruker DMX spectrometer, collecting homonuclear 2D spectra using conventional parameters and processing. All chemical shift changes in BPTI were reversible and linear with pressure. The low-pressure shifts were acquired at 30 bar rather than 1 bar for experimental simplicity, but do not differ measurably from the shifts at 1 bar.

A high-resolution crystal structure of BPTI was used as the initial structure, namely 5pti, which is a joint X-ray/neutron diffraction refinement, to a resolution of 1 Å (X-ray) and 1.8 Å (neutron; Wlodawer et al. 1984). Water molecules were removed, except for four molecules that are completely buried (with no solvent-exposed surface, as measured using the program AREAIMOL from the CCP4 routines; Bailey 1994). These were water molecules 59, 60, 61, and 62. Protons were removed and then re-added automatically by XPLOR (Brünger 1993), except for the four water molecules, for which the deuterium positions in the original structure were used. The structure was then refined using XPLOR in a force field that contained the usual nonbonded forces, including van der Waals forces (a Lennard-Jones potential out to 4 Å, decaying smoothly to zero at 5 Å) and electrostatic forces (using a $1/r$ -dependent dielectric constant, switched off between 4 and 5 Å). All masses were set at 100 throughout the calculation.

The refinement consisted of an initial energy minimization, followed by 6 ps (2000 steps of 3 fs) unrestrained Verlet molecular dynamics at a temperature of 200 K, and a final energy minimization step. The calculation was carried out 10 times using random Boltzmann distributions of starting velocities, and the resultant 10 structures were averaged and energy-minimized. This procedure caused a structural drift, in that the resultant structure had changed significantly (as assessed by a Student's *t*-test) from its starting point. The refinement was therefore repeated iteratively (i.e., the result from the previous iteration formed the starting point of the next iteration) a sufficient number of times that the structure no longer had a structural drift, after which a structure was chosen as the starting 'low-pressure' structure.

The chemical shifts of this low-pressure starting structure were calculated, and defined to be the starting (low-pressure) shifts. As noted in the introduction, these shifts are of course not identical to the experimental low-pressure shifts, although there is a good correlation (Williamson et al. 1995). The experimentally derived changes in chemical shift on going from 30 bar to 2 kbar were then added to the starting shifts, and formed the set of high-pressure shift restraints. A high-pressure structure was then calculated by re-refining the starting structure, in the same force field as used previously, but now with the addition of the high-pressure chemical shift restraints. Chemical shift restraints were used as presented in Kuszewski et al. (1995), which uses equations presented in Williamson and Asakura 1993 and Asakura et al. 1995. The original XPLOR program (a gift from Dr. G.M. Clore, NIDDKD, NIH, Bethesda, MD) was modified to omit chemical shift terms derived from electric field effects, because these are relatively small but fall off as r^{-2} (Williamson et al. 1992; Williamson and Asakura 1993), and so lead to problems of instability in the calculation, particularly for side chain protons. The modified program is available from M.P.W., together with typical scripts. In the method, the chemical shift energy is proportional to the squared difference between restrained shift and calculated shift. A strong chemical shift force constant of 4000 kcal/mole.ppm² was used. For pairs of resonances where no stereospecific assignment was available (e.g., β -methylene protons and valine methyls), the restraint consisted of the sum of the shift from both signals. The calculations were carried out using the same parameters as described above, except that the molecular dynamics simulation was run for a total of 1.25 ps. The initial part of the molecular dynamics calculation used short timesteps, to avoid having the program crash. Each calculation was carried out 50 times, using random starting velocities. The final structures were averaged and energy-minimized.

In order to be able to compare the high-pressure structure to the low-pressure structure, without introducing bias from the calculation method, a low-pressure calculation was carried out identically from the same starting structure, using the full set of low-pressure shifts as restraints.

The direction of movement of all backbone atoms was analyzed by superimposing the low- and high-pressure structures on their centers of mass (using all heavy atoms in regular secondary structures). For each atom, the direction of movement was calculated with respect to the vector from the unchanged position of the atom to the center of mass of the protein. Directions of motion (θ) were divided into 9° bins, and the frequencies were smoothed and normalized by multiplying by $180/2n\sin(\theta)$, where n is the number of atoms used.

Hydrogen bonds were identified using an in-house version of the Kabsch and Sander (1983) calculation. Volumes were calculated using VOIDOO (Kleywegt and Jones 1994), and surface area using the AREAIMOL routine in CCP4. Moments of inertia were calculated using XPLOR.

Electronic supplemental material

One page lists the high-pressure chemical shift restraints applied.

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