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Pressure-dependent changes in the structure of the melittin α-helix determined by NMR

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Abstract

A novel method is described, which uses changes in NMR chemical shifts to characterise the structural change in a protein with pressure. Melittin in methanol is a small α-helical protein, and its chemical shifts change linearly and reversibly with pressure between 1 and 2000 bar. An improved relationship between structure and HN shift has been calculated, and used to drive a molecular dynamics-based calculation of the change in structure. With pressure, the helix is compressed, with the H—O distance of the NH—O=C hydrogen bonds decreased by 0.021 ± 0.039 Å, leading to an overall compression along the entire helix of about 0.4 Å, corresponding to a static compressibility of 6×10^{-6} bar^{-1}. The backbone dihedral angles φ and ψ are altered by no more than ± 3° for most residues with a negative correlation coefficient of −0.85 between ϕ_i and ψ_{i-1}, indicating that the local conformation alters to maintain hydrogen bonds in good geometries. The method is shown to be capable of calculating structural change with high precision, and the results agree with structural changes determined using other methodologies.

Introduction

The effect of pressure on the structure of proteins is of interest to a wide range of disciplines ranging from physical chemistry to microbiology (Gross and Jaenicke, 1994; Heremans and Smeller, 1998). Pressure in the sea ranges up to 1200 bar (120 MPa), with many organisms able to grow at pressures in excess of 500 bar. Pressures of this magnitude affect macromolecular structure and function, with important consequences for the chemistry of life at these pressures. Pressure also has the effect of reversibly denaturing many proteins, and can therefore be used as a means of investigating folding processes. It has an advantage over many other methods for investigating protein structure in that thermodynamic variables can be readily associated with pressure changes, implying that thermodynamic arguments can be used straightforwardly. In particular, variation of pressure at constant temperature can be related directly to volume changes. However, to date it has not been possible to characterise structural changes with pressure to atomic detail, either because the techniques do not contain information at atomic level, or because the changes are small in comparison with the inherent error in the techniques. There is therefore a pressing need to develop new techniques that are capable of revealing structural details. Here we show how ^{1}H NMR spectroscopy can be used in this way.

^{1}H chemical shifts of proteins are highly sensitive to secondary and tertiary structure (Pardi et al., 1983; Szilágyi and Jardetzky, 1989; Wishart et al., 1991; Smith et al., 1996; Asakura et al., 1997; Li

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et al., 1998; Sitkoff and Case, 1998). However, the problem with using shifts as structural constraints has always been that calculation of chemical shifts from structure has been too inaccurate. We have previously developed methods for calculating chemical shifts for HC (Williamson et al., 1992; Williamson and Asakura, 1993) and HN (Asakura et al., 1995) in proteins that give agreement with experimental shifts with standard deviations of ca. 0.25 ppm and 0.5 ppm, respectively (using good models of the structures, namely high-resolution crystal structures). It was shown (Williamson et al., 1995) that for HC calculations in which the NMR solution chemical shifts were predicted based on the X-ray crystal structure, there is a good correlation between the resolution of the crystal structure and the accuracy of the predicted shifts. In other words, a significant part of the 0.25 ppm 'error' for a 1.6 Å structure derives from the fact that the crystal structure has small random errors. On extrapolation of this correlation to a nominal 0 Å resolution, there is still an error of ca. 0.12 ppm in the predicted shifts. This implies that for typical calculations, very roughly 0.12 ppm of the error derives from inaccuracies in the calculation method, while the rest comes from the fact that the real (time-averaged) solution structure is different from the crystal coordinates used to calculate the shifts. For typical 2.0 Å resolution structures, with an error of 0.3 ppm, this implies that ca. 60% of the error (i.e. the difference between calculated and observed shift) is due to structural differences between the structure used to calculate the shift and the true solution structure, with the remaining 40% coming from deficiencies in the calculation such as incorrect parametrisation and oversimplified models.

Although this inaccuracy in calculations of ^{1}H shifts is too large to allow them to be used readily for structure calculation, it does not preclude the use of *changes* in shift to follow *changes* in structure. Our justification is as follows. Chemical shifts can be calculated from structures and compared to the experimental shifts. We divide the calculated secondary shift (the difference between random coil and observed shifts) into two parts: the result of the calculation, and an error, which (following the argument above) derives both from inaccurate structures and from calculational errors:

$$\delta_{sec} = \delta_{calc} + E = \delta_{calc} + E_{struct} + E_{calc}$$

with E_{calc} roughly 40% of E. Following a small perturbation to the structure, e.g. an elastic change due to the application of pressure, there is a change in the observed secondary shift, which is given by

$$\Delta\delta_{sec} = \Delta\delta_{calc} + \Delta E_{struct} + \Delta E_{calc}$$

ΔE_{struct}, the change in the error of the calculation because of the use of an inaccurate starting structure, will be small provided *both* that the initial structure is close to the correct structure *and* that the function relating structure to chemical shift is smooth in the vicinity of the correct structure. This is true for ^{1}HN, which depends almost entirely on distance and angle to hydrogen bonded carbonyls (Asakura et al., 1995). Therefore we can to a first approximation ignore ΔE_{struct}, and approximate the change in shift with pressure as

$$\Delta\delta_{sec} \approx \Delta\delta_{calc} + \Delta E_{calc}$$

The range in E is roughly 40% of the range in δ_{calc} (e.g. for HN, $\pm$ 0.5 ppm in E compared to $\pm$ 1.3 ppm in δ_{calc}; Asakura et al., 1995). For a small perturbation to the structure, the size of ΔE_{calc} is therefore ca. 0.4 $\times$ 0.4 or 20% of the size of $\Delta\delta_{calc}$. In other words, if we use the change in shift of ^{1}HN to derive the change in structure in the vicinity of the amide, then we expect that there will be an associated structural error of ca. 20%. This is a large error, but small compared to that of any existing method (bearing in mind that the changes induced by pressure are of the order of 0.1 Å), and implies that NMR shifts are useful probes for structural change in proteins.

The above argument relies on the assumption that increased pressure does not introduce novel calculational errors, for example changes in susceptibilities or charge distribution within the peptide bond. There have been a wide range of spectroscopic studies (reviewed in Gross and Jaenicke, 1994) that suggest that such changes are minimal up to 2 kbar. The assumption is therefore justifiable.

We have previously described an on-line pressure cell (Yamada, 1974; Yamada et al., 1994; Li et al., 1998) that has been attached to a 17.6 Tesla (750 MHz) NMR spectrometer (Akasaka et al., 1997) and used to measure NMR spectra for a range of proteins in solution (Inoue et al., 1998; Akasaka et al., 1999; Li et al., 1999; Kalbitzer et al., 2000). Chemical shift changes are usually linear and reversible. For hydrogen bonds in BPTI, pressure shifts were used to estimate distances shortened by pressure for individual NH—O=C hydrogen bonds by using an empirical shift-distance correlation (Li et al., 1998). However, to date we have not attempted a quantitative elucidation of pressure-induced changes of the

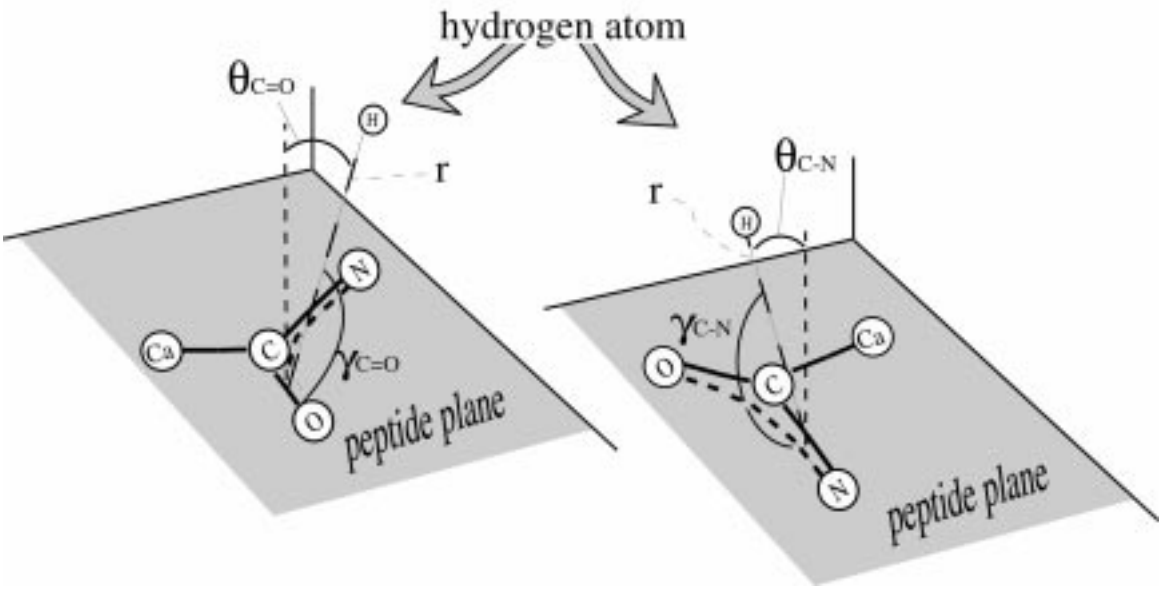

Figure 1. Definitions of the parameters used to calculate chemical shift effects arising from bond magnetic anisotropy.

entire three-dimensional structure of a protein. In this paper we present the results of such a study, using melittin, a toxic 26-residue polypeptide from bee venom (Hebermann, 1972) with primary sequence GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2, which has a simple helical structure in methanol (Bazzo et al., 1988; Pastore et al., 1989; Buckley et al., 1993). We show that the change in structure with pressure for a helical peptide can be calculated, with results that agree with expectations from other experiments.

Experimental methods

Experimental

Melittin was obtained from Sigma and purified according to the literature (Battenburg et al., 1987). It was dissolved in methanol-$^2H_3$ (Euriso-Top) to a concentration of 5 mM. The apparent pH of the solutions measured with a glass electrode was 3.0 (uncorrected glass electrode reading).

High-pressure ^{1}H NMR measurements were performed on a Bruker DMX-750 spectrometer operating at a frequency of 750.13 MHz with deuterium field-frequency lock under varying hydrostatic pressure between 30 and 2000 bar at 25 °C by utilizing the on-line high pressure-cell NMR technique used in previous studies (Akasaka et al., 1997, 1999; Li et al., 1998, 1999; Inoue et al., 1998). The sample cell was placed in a Bruker 5 mm probe with inverse ^{1}H detection with x,y,z-field gradients. The high pressure cell was made of quartz with an inner diameter of about 1 mm, which was connected to a kerosene line. The pressure was maintained using a hand pump located remote from the 17.6 Tesla magnet (Japan Magnet Technology). The choice of 30 bar for low pressure instead of atmospheric pressure was purely for a technical reason, the spectrum at 30 bar being indistinguishable from that at 1 bar.

Two-dimensional spectra were obtained at various pressures to perform sequential assignments in the phase-sensitive mode with time-proportional phase incrementation (TPPI), using spectral widths of 8993 Hz in both dimensions, 64 transients, and 2K data points for 512 t_1 increments with a recycle delay of 1.7 s. DQF-COSY spectra were recorded with presaturation of water signals. The OH signals of methanol in TOCSY and NOESY spectra were suppressed using WATERGATE (Piotto et al., 1992). The TOCSY spectra were recorded with MLEV-17 using a spin-lock of 70 or 110 ms and a 2.5 ms trim pulse before and after mixing. NOESY spectra were collected with mixing times of 100 and 300 ms. Data were processed on an Indigo-2 computer with the Bruker UXNMR software. Apodization with a Lorentzian to Gaussian transformation or with a shifted squared sine-bell window function was applied in both dimensions, and data were zero-filled to 4K points in t_2 and to 2K points in t_1 prior to Fourier transformation. Fifth-order polyno-

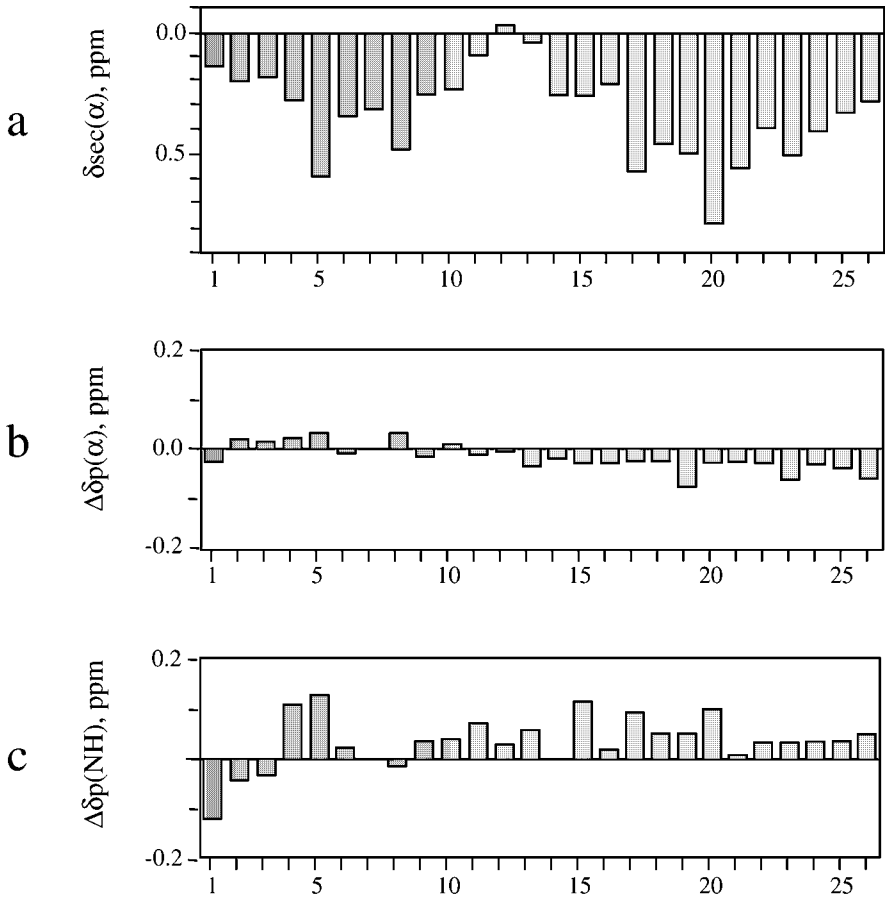

Figure 2. Pressure-induced shifts ($\Delta\delta p$) of individual α and amide protons of melittin in the α-helical state in methanol ($C^2H_3O^1H$) at 25 °C, plotted as histograms against the amino acid residue number. (a) Secondary shift for α protons, defined as shift in methanol minus shift in random coil (in water) at 1 bar; (b) pressure-induced shifts ($\Delta\delta p$), defined as the shift at 2 kbar minus the shift at 30 bar, for α protons; (c) the same for amide protons.

mial baseline correction was applied to all the spectra along the ω_2 axis.

Determination of bond magnetic anisotropy parameters of a peptide group for HN

The origins of conformation-dependent ^{1}H NMR chemical shifts in globular proteins include a number of contributions, namely local anisotropic magnetic field arising from anisotropic susceptibility of peptide groups and aromatic rings, effects from electric charges, effects from hydrogen bonding and effects from van der Waals interactions (Szilágyi and Jardetzky, 1989; Williamson et al., 1992; Sitkoff and Case, 1998). ^{1}H NMR chemical shifts of amide protons HN are strongly affected by relative positions of neighbouring peptide planes. In a simple alpha helix, the amide proton shifts will be determined primarily by the magnetic anisotropy in local peptide bonds. Therefore, we assume that for each amide proton the secondary shift δ_{sec} is given by $\sigma_{anisotropy} \times B_0$ where B_0 is the external magnetic field and $\sigma_{anisotropy}$ can be estimated from the following equation:

$$\sigma_{anisotropy} = \sum \frac{1}{3r^3} \cdot \left\{ \begin{array}{l} \left[\Delta\chi_1^{C=O} \cdot \left(3\cos^2 \phi_{C=O} - 1 \right) \right. \\ \left. + \Delta\chi_2^{C=O} \cdot \left(3\cos^2 \gamma_{C=O} - 1 \right) \right] \\ + \left[\Delta\chi_1^{C-N} \cdot \left(3\cos^2 \phi_{C-N} - 1 \right) \right. \\ \left. + \Delta\chi_2^{C-N} \cdot \left(3\cos^2 \gamma_{C-N} - 1 \right) \right] \end{array} \right\} \quad (1)$$

The summation indicates that Equation 1 takes into account the contributions from magnetic susceptibility anisotropy effects on HN from the C=O and C–N groups with distance r, and θ and γ angles as defined in Figure 1 (Williamson and Asakura, 1993; Asakura

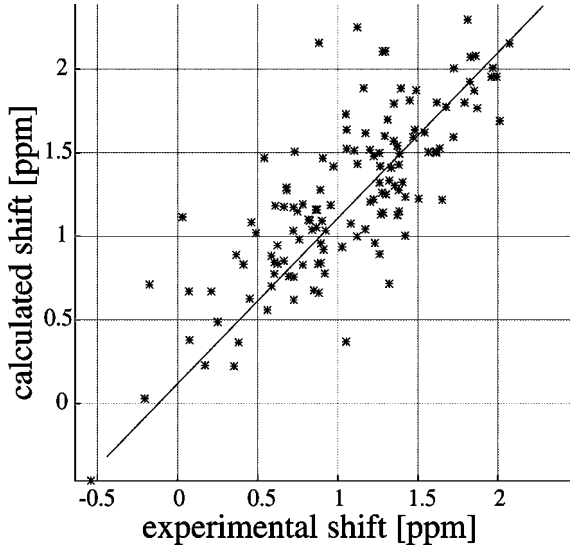

Figure 3. Comparison of calculated (δ_{calc}) and experimental secondary amide proton shifts, expressed as differences from the random coil values, for 159 amide protons from five globular proteins. Calculation was performed based on crystal structures (PDB codes: 193L, 1CHN, 1WEJ, 2MLT and 9ANT) in the Protein Data Bank, and the experimental shifts were obtained from literature (BMRB IDs: 1093, 3440, 1170, 245 and 1037). Positive and negative values indicate downfield and upfield shifts, respectively. The calculated and experimental shift values are correlated with a correlation coefficient of 0.78.

et al., 1995) from *all* the peptide bonds of the helix. The constants $\Delta\chi_1$ and $\Delta\chi_2$ were determined by non-linear least-squares fitting, so that they can reproduce the folding shifts of the amide protons reported in NMR literature for a variety of r, θ, and γ values reported in the Protein Data Bank, for α-helical parts of globular proteins. Calculations were performed on PDB crystal structures 193L, 1CHN, 1WEJ, 2MLT and 9ANT, with experimental shifts from BMRB IDs 1093, 3440, 1170, 245 and 1037, respectively.

Calculation of the structure under pressure based on experimental pressure-induced shifts of amide protons

Structural calculation was performed by the molecular dynamics program X-PLOR (Brünger, 1993), modified to include structural constraints given by a harmonic pseudopotential E_{shift} defined by Equation 2 (Kuszewski et al., 1995). The potential is proportional to the squares of differences between calculated chemical shifts, $\Delta\delta_{calc}$ and the experimentally determined pressure-induced shifts, $\Delta\delta_{exp}$. $\delta_{30\,bar,calc}$ is the sum of the magnetic anisotropy effect (Equation 1), ring current effect and electric field effect calculated from the starting structure at 30 bar.

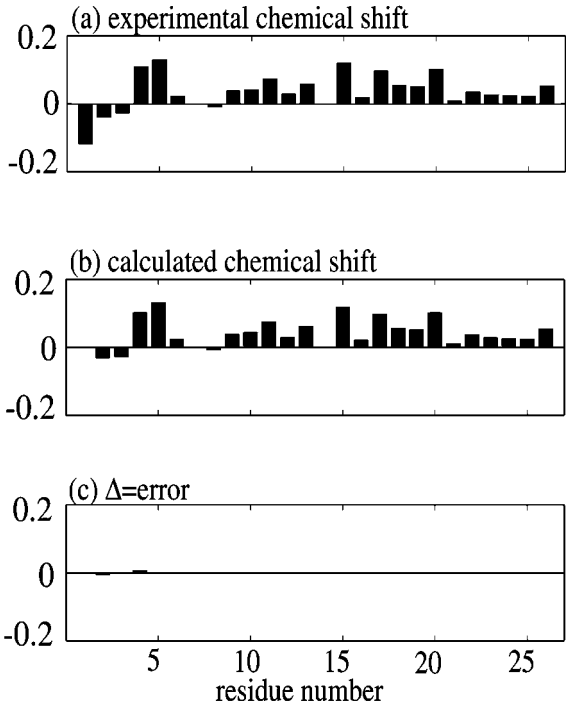

Figure 4. Amide ^{1}H NMR chemical shift changes in melittin between 30 and 2000 bar. (a) Experimental pressure-induced shifts, (b) calculated shifts obtained from X-PLOR, and (c) differences between (a) and (b).

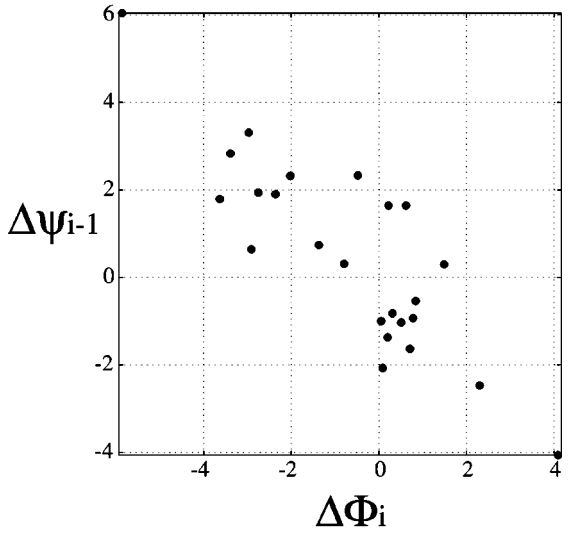

Figure 5. The correlation between the pressure-induced changes of the torsion angles ϕ_i and ψ_{i-1} (correlation coefficient $= -0.85$, using all residues except Gly-1 and Pro-14).

$$E_{shift} = k_\delta \sum_{proton} \left(\Delta\delta_{calc} - \Delta\delta_{exp}\right)^2$$
$$\Delta\delta_{calc} = \left(\delta_{2kbar,calc} - \delta_{30bar,calc}\right) \quad (2)$$
$$\Delta\delta_{exp} = \left(\delta_{2kbar,exp} - \delta_{30bar,exp}\right)$$

k_δ is an energy constant and was chosen to have the large value of 4000 kcal/mol·ppm^2 which emphasizes the chemical shift restraints. One thousand steps at 0.002 ps/step of molecular dynamics and energy minimisation refinements involving 400 cycles of Powell's conjugate gradient process were performed with X-PLOR, using chemical shift restraints for all the amide protons except for those of Gly-1. No other NMR-derived restraints were used, the only other energies used being standard bonded and non-bonded interactions, with default values.

Results

Chemical shift assignments for melittin in methanol were made at low and high pressure. The shift values and NOEs are consistent with previous literature values (Bazzo et al., 1988; Pastore et al., 1989; Buckley et al., 1993) and with the expected structure of the peptide, which is helical throughout except for a break at residues 11–13. The changes in shift were linear with pressure for all protons and reversible, with the largest changes being in the HN (Figure 2). Moreover, except for a few N-terminal residues, the HN shifts are all toward low field, the average value for residues 5–26 being 0.047 ppm at 2 kbar. The chemical shift changes are listed in Table 1. They are smaller by an order of magnitude than the secondary shifts, consistent with the notion that pressure alters the conformation of the backbone, but that the overall helical structure is retained at 2 kbar. Chemical shift changes for side-chain protons generally decrease as one goes out along the side-chain, consistent with the major origin of the shifts being from magnetic anisotropy of the bonds in the main-chain backbone, and with melittin forming an isolated helix in solution, with no tertiary interactions.

The chemical shift changes were used as restraints in restrained molecular dynamics calculations using XPLOR. In order to obtain the most meaningful parameters for the calculation, a statistical analysis was carried out for HN chemical shifts in the α helical parts of globular proteins. This produced best-fit values of $\Delta\chi_1$ and $\Delta\chi_2$ of -11.5 and -41.5×10^{-30} ppm Å^{-3} for C=O and 12.5 and -35.0 for C–N, respectively. Standard values were used for the other relevant parameters (Williamson and Asakura, 1993). The calculated and experimental shift values are well correlated, with $R = 0.78$, and the quality of the fit is shown in Figure 3.

In order to calculate the change in structure with pressure, the crystal structure 2mlt (Terwilliger and Eisenberg, 1982) was used to calculate starting shifts, and the experimental change in chemical shift between 30 and 2000 bar was used as a restraint to change the structure. The experimental HN shift changes could be reproduced very well by the calculation: all shifts were reproduced to within 0.01 ppm, as shown in Figure 4. Covalent bond lengths and angles remained essentially unchanged. Hydrogen bond lengths were reduced by 0.021 ± 0.039 Å, changing nonuniformly over the sequence. Backbone dihedral angles ϕ and ψ also changed non-uniformly over the sequence to both positive and negative values, generally by no more than $\pm 3°$. Figure 5 shows that changes in ϕ_i and ψ_{i-1} are negatively correlated, which acts to maintain good hydrogen bond geometry by avoiding excessive distortion in the hydrogen bond angle between N−H and C=O in the helix.

The best-fit structure at 2 kbar is superimposed on the low-pressure structure in Figure 6. A helical structure with a kink in the middle (in residues 12–14) is retained, but the ϕ and ψ angles are changed for most residues within $\pm 3°$ with a concomitant decrease in hydrogen bond lengths by an average of 0.021 Å. Correspondingly, the total length of the helix, expressed by the distance from Cα of residue 1 to Cα of residue 26, shrinks by 0.4 Å (from 34.9 Å to 34.5 Å). The root-mean-squares atom displacement for the main chain is 0.11 Å with a largest displacement of 0.25 Å for some atoms. Some significant local structural changes are shown enlarged in the figure.

Discussion

The results obtained here agree both qualitatively and quantitatively with those from previous studies. It is worth noting that the NMR chemical shift restraints have produced a compression of the peptide structure. This in itself is a good indicator of the fundamental correctness of the method, since the only driver for the structural change is the chemical shift. We find a negative correlation ($R = -0.79$) between change in hydrogen bond distance and change in H^N chemical shift (Figure 7). A numerically similar correlation has

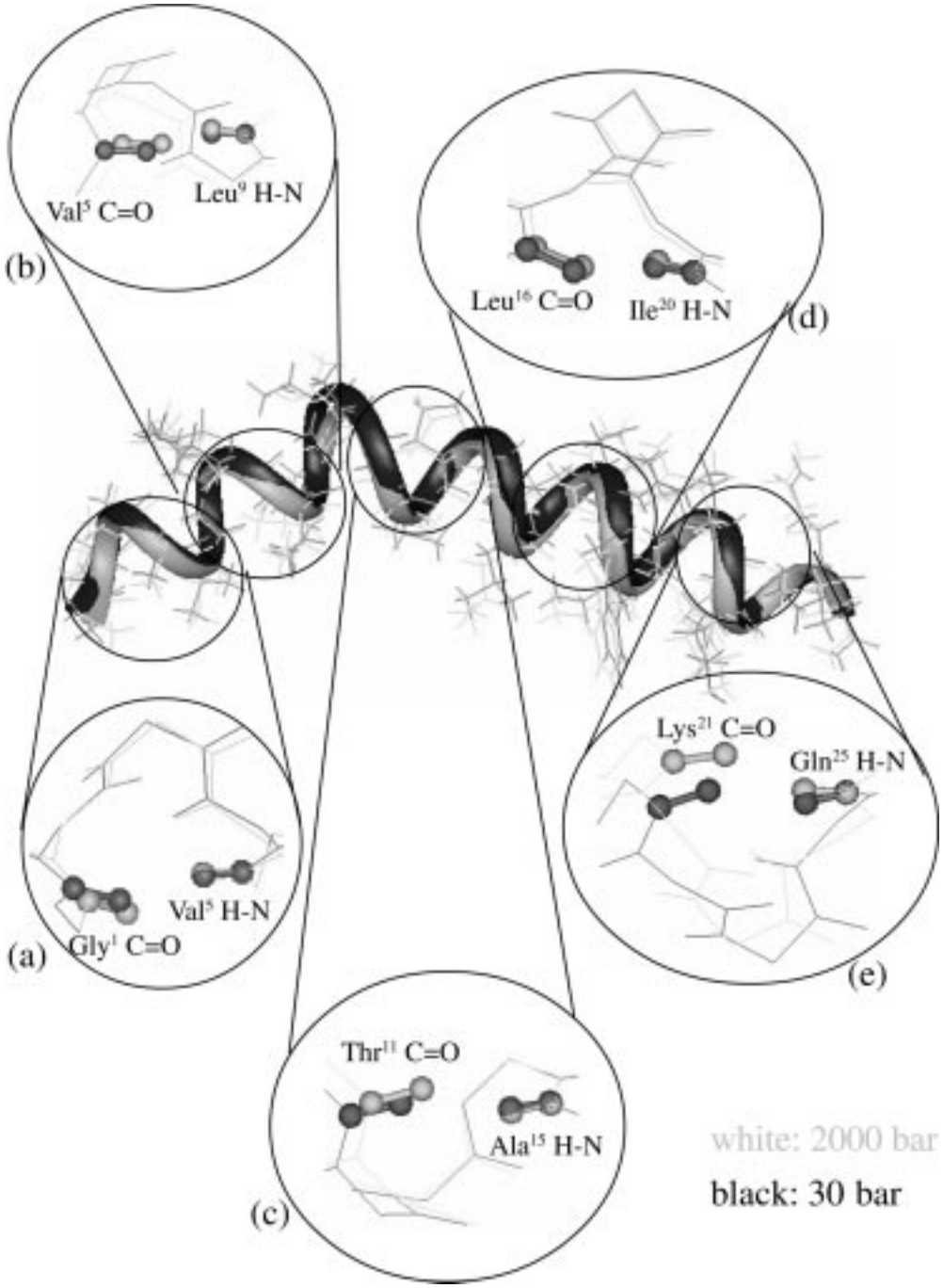

Figure 6. The conformational change of the melittin α-helix by pressure. In black is shown the structure at 30 bar, and in grey the structure at 2 kbar. (a) Pressure-induced conformation changes around the N-H atoms of Val-5 between 30 bar and 2 kbar: the relative orientation of the Gly-1 carbonyl group strongly affects the amide proton chemical shift of Val-5; (b–e): Pressure-induced changes in the relative orientation of the carbonyl group of the $n-4^{th}$ residue with respect to the amide group of the n^{th} residue for n = 9 (b),15 (c), 20 (d) and 25 (e). The position of the amide nitrogen is kept fixed in each expanded figure.

Table 1. Pressure-induced chemical shifts $\Delta = \delta$ (2 kbar) $- \delta$ (30 bar) for individual proton signals of melittin in methanol determined at 750 MHz. Chemical shifts were measured relative to the residual methyl proton signal of the solvent (99.8% deuterated methanol C^2H_3OH) at both pressures (3.317 $\pm$ 0.003 ppm from 3-trimethylsilyl-(3,3,2,2-^{2}H) propionate). Chemical shifts of non-equivalent protons are averaged

Residue	ΔNH	ΔαH	ΔβH	ΔγH	ΔδH	ΔεH	ΔζNH	ΔγMe (Ile)	ΔδMe
Gly1	−0.120	−0.024							
Ile2	−0.042	0.022	0.000	−0.040				−0.007	−0.009
Gly3	−0.029	0.016							
Ala4	0.109	0.025	0.007						
Val5	0.130	0.038	−0.037	−0.013					
Leu6	0.023	−0.003	−0.013	−0.015					0.008
Lys7	0.000	0.002	−0.041	−0.006	−0.047	−0.002	−0.094		
Val8	−0.012	0.036	−0.045	−0.011					
Leu9	0.039	−0.009	−0.028						0.004
Thr10	0.042	0.008	−0.019	−0.014					
Thr11	0.074	−0.006	−0.006	0.005					
Gly12	0.029	0.000							
Leu13	0.059	−0.030	−0.041	0.016					0.009
Pro14		−0.015	−0.027	0.012	0.007				
Ala15	0.120	−0.022	−0.021						
Leu16	0.019	−0.023	−0.003	−0.034					0.002
Ile17	0.097	−0.021	0.003	−0.018				0.002	0.000
Ser18	0.055	−0.019	−0.001						
Trp19	0.052	−0.074	−0.003						
Ile20	0.102	−0.020	−0.024	−0.004				0.002	0.002
Lys21	0.009	−0.019	−0.059	−0.016	−0.025	−0.015	−0.050		
Arg22	0.035	−0.021	−0.033	−0.017	−0.026		−0.044		
Lys23	0.027	0.054	−0.061	−0.059	−0.046	−0.015	−0.102		
Arg24	0.025	−0.022	−0.026	−0.030	−0.009		0.001		
Gln25	0.022	−0.030	−0.015	−0.009					
Gln26	0.053	−0.050	−0.030	−0.005					
$CONH_2$	0.003								
	0.099								

been seen previously and was used by us in a preliminary analysis of the effect of pressure on BPTI (Li et al., 1998).

It is possible to compare the reduction in hydrogen bond length observed here with results from other experimental techniques. One of the best estimates comes from Raman and infrared studies. For example, Shimizu et al. (1988) observe a reduction in C=O stretch frequency of 1.5 cm^{-1}/kbar, consistent with the range of values (1.0–2.0 cm^{-1}) seen by Goossens et al. (1996), and which they ascribe to weakening of the C=O bond arising from increased electron density in the hydrogen bond. This change in vibrational frequency can be related to changes in hydrogen bond length using the observation that there is a reduction in C=O stretch frequency of 20 cm^{-1} on going from α-helix (average H-bond length 2.06 Å) to β-sheet (average H-bond length 1.96 Å; Baker et al., 1984). The relationship is thus approximately 0.005 Å/cm^{-1}. The result of Shimizu et al. (1988) therefore corresponds to a change in hydrogen bond length of ca. 0.015 Å over the 2 kbar used here. Our results, i.e. a mean reduction in hydrogen bond length of 0.02 Å compare well with this relationship.

The total length of the helix, expressed by the distance from Cα of residue 1 to Cα of residue 26, shrinks from 34.9 Å to 34.5 Å, a compression of 1.15% or 6×10^{-6} bar^{-1}. This falls well within the range of adiabatic compressibilities measured for proteins (Gekko and Hasegawa, 1986), is close to the static compressibility of 7×10^{-6} bar^{-1} observed in the α-helical domain of lysozyme at 1 kbar using X-ray crystallog-

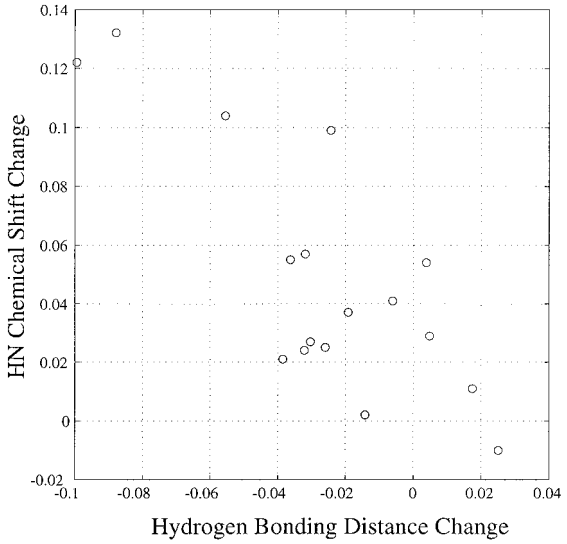

Figure 7. Correlation between the experimental HN chemical shift change and the calculated H^N- - -O hydrogen bond distance change in melittin as a result of pressure (2 kbar). The slope is −1.0 ppm/Å. All H-bonded residues were used (i.e. not HN of residues 1–4, 10–14).

raphy (Kundrot and Richards, 1987), and is also similar to the value of 9×10^{-6} bar^{-1} obtained from normal mode analysis of myoglobin (Yamato et al., 1993). Finally, the magnitude of changes seen in the backbone dihedral angles with pressure is similar to those deduced in BPTI using ^{15}N shift changes (Akasaka et al., 1999), and also to those observed in the crystal structure of lysozyme (Kundrot and Richards, 1987). The structural changes calculated using only the HN shift changes therefore closely match to the available experimental evidence. Although the structural change depicted in Figure 6 is not very large, it would be sufficient to cause a significant change in the activity of an enzyme if this happens close to its active centre.

In this paper, ^{1}HN NMR chemical shifts were used as sole experimental restraints to elucidate the change in structural coordinates of atoms of a small protein at high pressure, to a precision of ca. 0.02 Å (20% of ca. 0.1 Å). The strategy was to calculate *changes* of coordinates based on *changes* in chemical shift, rather than utilizing the absolute values of chemical shift. Although the present calculation deals with a simple peptide comprising only helical structure and the simulation was performed only on amide protons, the predicted structural change is reasonable, which encourages us to explore the further use of chemical shift for structural determination in proteins. Calculations using other groups of protons (e.g. Hα) suggest that these protons carry compatible but complementary information.

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