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Biophysical Chemistry 96 (2002) 129–140

Biophysical Chemistry

www.elsevier.com/locate/bpc

^{1}H-NMR parameters of common amino acid residues measured in aqueous solutions of the linear tetrapeptides Gly-Gly-X-Ala at pressures between 0.1 and 200 MPa

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Received 6 March 2001; received in revised form 21 September 2001; accepted 24 September 2001

Abstract

For the interpretation of chemical shift changes induced by pressure in proteins, a comparison with random-coil data is important. For providing such a data basis, the pressure dependence of the ^{1}H-NMR chemical shifts of the amino acids *X* in the random-coil model peptides Gly-Gly-*X*-Ala were studied for the 20 common amino acids at two pH values (pH 5.0 and 5.4) at 305 K, in the pressure range from 0.1 to 200 MPa. The largest shift changes $\Delta\delta$ with pressure *p* can be observed for the backbone amide protons. The average linear pressure coefficient $\delta_{\Delta p}$ is 0.38 ppm GPa^{-1}, with a root mean square deviation of 0.2 ppm GPa^{-1}. In contrast to the downfield shift typical for amide protons, the H^{α}-resonances typically shift upfield, with a pressure coefficient of -0.025 ppm GPa^{-1} and a root mean square deviation of 0.05 ppm GPa^{-1}. The side chain resonances are only weakly influenced by pressure, on average they are shifted by 0.014 ppm GPa$^{-1,}$ with a root mean square deviation of 0.14 ppm GPa^{-1}. The exceptions are the side chain amide protons of asparagine and glutamine. Here, values of 0.214 (Asn $H^{\delta 21}$), 0.417 (Asn $H^{\delta 22}$), 0.260 (Gln $H^{\varepsilon 21}$) and 0.395 (Gln $H^{\varepsilon 22}$) ppm GPa^{-1} can be observed. In both cases, the pressure dependent shift is larger for the pro-E proton than for the pro-Z proton. Within the limits of error the equilibrium constant for the *trans*- and *cis*-conformers at the proline peptide bond is independent of pressure in the pressure range studied.

Keywords: Tetrapeptide; High pressure; NMR spectroscopy; Random coil; Chemical shift

1. Introduction

For many years, high-pressure NMR spectroscopy has been successfully used to investigate thermodynamic properties of solutions and relate them to structural properties of the molecules involved. In the past, only few of them dealt with proteins [1–5], mainly because sensitivity posed severe limitations to the possible applications.

Abbreviations: 2D: two-dimensional; TOCSY: total correlation spectroscopy; NMR: nuclear magnetic resonance spectroscopy

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0301-4622/02/$ - see front matter
PII: S0301-4622(02)00018-2

With the increased sensitivity and resolution of the new generation of high-field NMR-spectrometers, a number of detailed investigations of pressure effects on protein structure and folding were reported [6–12].

For a qualitative as well as quantitative analysis of the chemical shift data of proteins, the comparison with standard parameters from random-coil peptides or protein data bases allows the separation of specific structural features from unspecific factors characteristic for a given amino acid residue. The tetrapeptides Gly-Gly-*X*-Ala were introduced early as reference compounds for the random-coil structures by Bundi and Wüthrich [13–16]. They reported the ^{1}H-chemical shifts of these peptides in aqueous solution, with *X* representing one of the 20 DNA-coded amino acids. Later on, ^{13}C and ^{15}N chemical shifts of these peptides were published [17,18]. ^{1}H and ^{31}P chemical shifts are also reported for the phosphorylated forms of these peptides with *X*=P-His [19], P-Asp [20], P-Ser, P-Thr, P-Tyr [21,22] and P-Hyp [23].

The interpretation of chemical shift effects induced by pressure in proteins is still in its infancy, although some of the changes, at least, can be consistently interpreted in structural terms. The change of the H^N-chemical shifts could be associated with the change of the length of intramolecular hydrogen bond under pressure [9]. A data basis for the chemical shift changes with pressure for random-coil peptides is not available yet and will be reported in this paper.

2. Materials and methods

The tetrapeptides H-Gly-Gly-*X*-Ala, containing Gly, Ala, Arg, Glu, His and Pro at *X* position, were purchased from Bachem AG, Liestal. All other peptides were synthesised at the Universitätsklinikum Regensburg (A. Brunner, S. Modrow). For NMR experiments, a solution of 5 mM tetrapeptide, 50 mM phosphate buffer and 1 μM NaN_3 in 80% H_2O and 20% D_2O was used. 0.1 mM 4,4-dimethyl-4-silapentane-sulfonic acid (DSS) was added as internal reference for the proton chemical shifts. The pH was adjusted to pH 5.00 and 5.40, respectively. The pH values were measured with a combination glass electrode pH meter 761 Calimatic from Knick; the values given are not corrected for the deuterium isotope effect. For the observation of resonances, superimposed by the water resonance, additional experiments were performed in 99.9% D_2O solution. In this case, the original H_2O/D_2O solution containing the buffer was freeze-dried and then redissolved in the same amount of pure D_2O. A possible pH-change resulting from the transfer from H_2O to D_2O was not corrected.

All NMR experiments were either carried out on a Bruker DRX-500, operating at a proton frequency of 500 MHz or a Bruker DRX-600, operating at a proton frequency of 600 MHz. For ^{1}H 1D spectra, a 1-s low-power water suppression pulse was applied. ^{1}H-^{1}H 2D TOCSY spectra were recorded according to Braunschweiler and Ernst [24], using an MLEV-17 decoupling sequence [25] with 50 ms isotropic mixing time. Chemical shifts are referenced to the signal of DSS contained in the samples. Spectral analysis and peak picking were performed using the program XWIN-NMR 2.6 (Bruker). The magnetic field was locked to D_2O. All measurements were done at 305 K. It was calibrated by measuring the chemical shift difference $\Delta\delta$ of the two methanol resonances in high-pressure capillaries, according to [26]. Over the range 250–320 K, the temperature is given by Eq. (1) where $\Delta\delta$ is the difference in chemical shift [ppm] between the methyl and hydroxyl resonance of 100% methanol.

$$T(K) = 403.0 - 29.53\ \Delta\delta - 23.87\ (\Delta\delta)^2 \qquad (1)$$

To apply high-pressure to the peptide solution, sample tubes of the type introduced by Yamada [27] were used. The actual system is depicted in Fig. 1. All details of the design and cell preparation are given in [28]. The glass cells are drawn from borosilicate glass capillaries. The outer diameter of the end of the sample cell is adapted to the dimensions of the probeheads of Bruker DRX-600 and DRX-500 spectrometers. The active volume proper has an inner diameter of 1 mm and an outer diameter of 5mm and the length of the active part is 35–40 mm. Prior to use, the capillaries are etched with 3% HF (aq.) on both the inner and outer surfaces. The capillary is glued (Eccobond

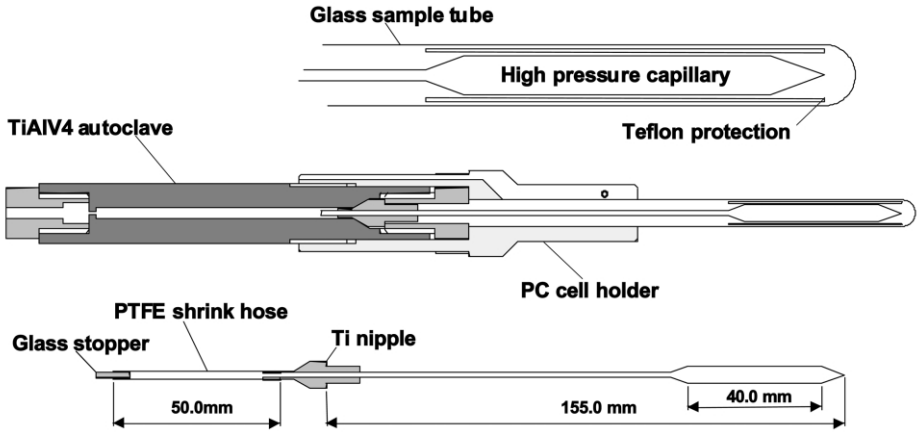

Fig. 1. High-pressure capillary and autoclave system: the NMR probehead is protected against the high-pressure capillary by a Teflon tube and a thick walled glass sample tube. The high-pressure capillary is connected with a cone-ended nipple to the autoclave. A flexible PTFE tube allows the pressure to be transferred from the pressure fluid to the sample fluid.

104, Grace Electronic Material, Heidelberg) into the bore of TiAlV4-nipple with one conical end. A piece of polytetraflouroethene (PTFE) shrink-hose is attached to the open end of the capillary. After filling the cell, the shrink hose is sealed with a glass stopper. The flexible PTFE tubing allows the pressure to be transferred from the pressure fluid to the sample fluid. Before use in the spectrometers, all capillaries are pressure-tested, normally up to 250 MPa. Some of the capillaries withstand pressures up to 400 MPa. Highest pressures used in DRX-500 and DRX-600 spectrometers were 200 MPa, hitherto.

To protect the spectrometer probehead from damage, the glass capillaries are placed into a short polished Teflon tube (50 mm length, 5 mm inner diameter, 0.5 mm walls). In addition, the Teflon helps to adjust the capillary in the centre of a thick walled 10-mm sample glass tube (Wilmad 513-7PPH). This tube presents the outer burst shield for the high-pressure capillaries and prevents, in case of an explosion, fluid from penetrating the probehead. The field homogeneity is not perturbed much by the high-pressure setup and line widths smaller than 2 Hz can be achieved without special effort.

The pressurising fluid (methylcyclohexane/mehylcyclopentane 50:50) is compressed with a manually operated piston cylinder compressor. The pressure is controlled with a Bourdon pressure gauge. Pressure is transferred to the sample via a high strength steel capillary. Pressure changes are performed slowly enough to keep the temperature in equilibrium.

3. Results and discussion

3.1. Pressure effects on chemical shifts

Generally, the pH of a solution is dependent on pressure. Since a pressure-induced pH shift of the solvent can also lead to shifts of the resonances, possible pH-dependent shifts have to be separated from ‘pure’ pressure-induced shifts. The pH of a phosphate buffered solution is known to decrease with pressure, since dissociation is generally accompanied by a negative reaction volume [29]. In the buffering range of the phosphate buffer, the negative dissociation volume leads to a decrease of approximately 0.4 pH units when increasing the pressure from 0.1 to 100 MPa. [30]. Therefore, we measured the pressure dependence of the chemical shifts of the amino acid *X* in the tetrapeptides Gly-Gly-*X*-Ala, with two different pH-values separated by 0.4 pH units. At 0.1 MPa, the pH of the samples was adjusted to 5.0 and 5.4, respectively, before varying the pressure. In some cases the data were completed by experiments at pH 7.0.

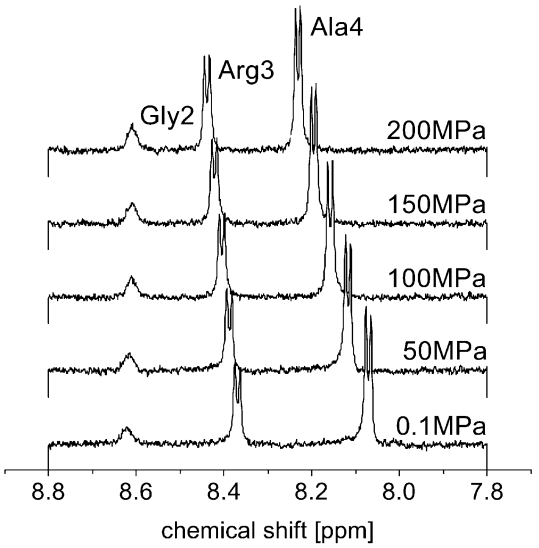

Fig. 2. 1D ^{1}H NMR spectra of Gly-Gly-Arg-Ala at various pressures. The sample contained 5 mM Gly-Gly-Arg-Ala in 50 mM phosphate buffer and 1 μM NaN_3 in 80% H_2O and 20% D_2O. Only the downfield part of the spectra is shown. The data were recorded in the pressure range from 0.1 to 200 MPa at 305 K. The pressure was varied in steps of 50 MPa. The total recording time at 600 MHz was 19 min.

For the tetrapeptide Gly-Gly-*X*-Ala, the pK_a values of the side chains were determined by NMR-spectroscopy [14]. They are 3.9 for Asp, 4.3 for Glu, 10.3 for Tyr and 11.1 for Lys. The pK_a value of His of 7.0, clearly deviates from the value of 6.0 reported in textbooks (see, e.g. Lehninger [31]). The pK_a-value of the C-terminal carboxyl group is approximately 3.3 and varies slightly when the amino acid in position *X* is exchanged [14]. Since only the pK_a-values of the side chains of Glu and His are close to the pH values of 5.0 and 5.4 used in our experiments, specific pH-effects (if any) could only be expected for these two residues.

As an example of the spectra obtainable, the downfield part of a proton spectrum of Gly-Gly-Arg-Ala is shown in Fig. 2 at different pressures. The resolution obtained in our set-up and spectral quality is good enough for experiments on peptides and proteins. In proteins, the chemical shifts can usually be represented as a linear function of pressure, although for some residues in the proteins studied so far, a non-linear pressure dependence has clearly been observed. This is also true for the random-coil peptides, only for a few samples significant deviations from linearity can be observed (Fig. 3). From a direct inspection of the data, only two exceptions can be identified, the backbone amide proton resonance of glutamate and the side chain NH-resonance $H^{\varepsilon 1}$ of tryptophan. However, for a complete description of the data, the dependence of the chemical shifts δ on the pressure *p* was fitted with two models, with the linear relationship

$$\delta = \delta_0 + \delta_{\Delta p}\ (p - p_0) \tag{2}$$

and a second order polynomial

$$\delta = \delta_0 + \delta'_{\Delta p}\ (p - p_0) + \delta_{\Delta 2p}\ (p - p_0)^2 \tag{3}$$

Here, δ_0 is the chemical shift at atmospheric pressure p_0 of 0.1 MPa, $\delta_{\Delta p}$ the linear pressure coefficient, $\delta'_{\Delta p}$ and $\delta_{\Delta 2p}$, the first and second order pressure coefficients. The chemical shifts δ_0 at atmospheric pressure and the linear pressure coefficients δ_Δ obtained from a regression analysis are listed in Tables 1 and 2, for all proton resonances of the 20 common amino acids *X*. In addition, the first and second order coefficients defined in Eq. (3) are summarised for the backbone amide resonances in Table 1.

3.2. Pressure-induced shifts of backbone resonances

In general, the largest pressure dependent shift changes are observed for the backbone amide protons (Table 1). The mean linear pressure coefficient $\langle \delta^{HN}_{\Delta p} \rangle$ for the backbone amide resonances is 0.38 ppm GPa^{-1}, with a root mean square deviation of 0.20 ppm GPa^{-1}. The mean value is close to the values observed in proteins. When calculating $\langle \delta^{HN}_{\Delta p} \rangle$ from the data reported for BPTI by Li et al. [9], one obtains 0.38 ppm GPa^{-1} at 309 K , exactly the value observed on our random-coil peptides. In HPr protein from *S. carnosus* [11] and in gurmarin [8], the mean values of the linear pressure coefficients $\langle \delta^{HN}_{\Delta p} \rangle$ are smaller than in the random-coil peptides, but of the same sign. They

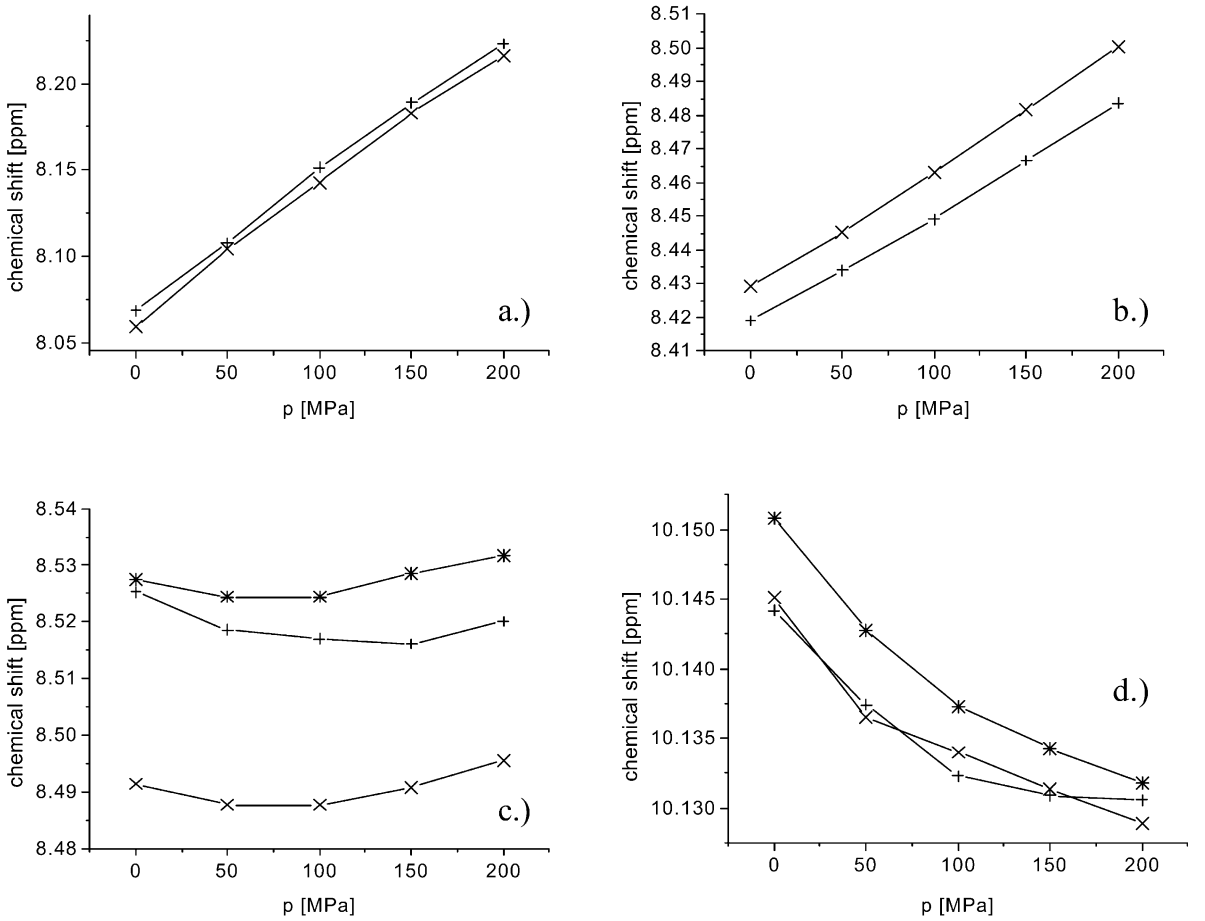

Fig. 3. Pressure dependence of chemical shifts of selected tetrapeptides Gly-Gly-*X*-Ala. The chemical shifts δ [ppm] are plotted as function of the pressure *p* [MPa] at different pH-values. The amino acid *X* is Cys (a), Asp (b), Glu (c), and Trp (d). Measurements were performed at pH 5.0 (×), pH 5.4 (+), and pH 7.0 (*).

are 0.25 ppm GPa^{-1} at 313 K and 0.28 ppm GPa^{-1} at 298 K, respectively. However, the spread in pressure coefficients is clearly larger in proteins than in random-coil peptides. In BPTI [9], they vary between −0.11 and 1.39 ppm GPa^{-1}, in gurmarin between −0.65 and 1.05 ppm GPa^{-1} [8] and in HPr between −1.08 and 1.48 ppm GPa^{-1} [11]. In peptides, the minimum value is 0.0 ppm GPa^{-1} (Glu) and the maximum value is 0.78 ppm GPa^{-1} (Cys).

Whereas for amide protons, in proteins, on average, downfield shifts are observed with increasing pressures; for the H^{α}-resonances, upfield shifts are usually observed. In BPTI $\langle \delta^{H\alpha}_{\Delta p} \rangle$ is −0.07 ppm GPa^{-1}, the pressure coefficients range between −0.42 and 0.26 ppm GPa^{-1} [10]. In gurmarin, an average value of −0.12 ppm GPa^{-1} has been found with minimum and maximum shift coefficients of −0.51 and 0.10 ppm GPa^{-1}, respectively [8]. In our random-coil peptides, the average shift is −0.025 ppm GPa^{-1} and ranges from −0.08 (Ala) to 0.11 (Glu) ppm GPa^{-1}. Again, the average values in native proteins are close to that of the random coil peptides, but the variation of the pressure coefficients are much larger.

Table 1
Chemical shifts and pressure coefficients of the amide and H^{α}-protons of amino acid *X* in Gly-Gly-*X*-Ala at 305 K in aqueous solution at pH 5.4[a]

*X*3	First order model			Second order model			First order model		
	δ_0^{HN} [ppm]	$\delta_{\Delta p}^{HN}$ [ppm GPa^{-1}]		δ_0^{HN} [ppm]	$\delta'_{\Delta 2p}^{HN}$ [ppm GPa^{-1}]	$\delta_{\Delta 2p}^{HN}$ [ppm GPa^{-2}]	$\delta_0^{H\alpha}$ [ppm]	$\delta_{\Delta p}^{H\alpha}$ [ppm GPa^{-1}]	
Ala	8.283	0.537	±0.018	8.281	0.63	−0.44	4.326	−0.080	±0.024
Arg	8.376	0.338	±0.006	8.376	0.35	−0.06	4.361	−0.077	±0.006
Asn	8.503	0.307	±0.018	8.504	0.26	0.34	4.720[c]	−0.046	±0.006
Asp	8.418	0.323	±0.006	8.419	0.29	0.18	4.634[c]	0.026	±0.006
Cys	8.409	0.383	±0.024	8.409	0.38	0.00	4.565	0.010	±0.006
Gln	8.440	0.335	±0.012	8.440	0.33	0.05	4.349	−0.068	±0.006
Glu	8.522	−0.026	±0.024	8.521	−0.13	0.53	4.293	0.110	±0.012
Gly	8.403	0.133	±0.006	8.403	0.12	0.05	4.044[b]	0.045	±0.012
His	8.367	0.502	±0.018	8.364	0.60	−0.51	4.713[c]	−0.020	±0.006
Ile	8.204	0.501	±0.024	8.200	0.63	−0.66	4.189	−0.058	±0.036
Leu	8.319	0.436	±0.030	8.317	0.53	−0.47	4.345	−0.088	±0.006
Lys	8.403	0.337	±0.018	8.403	0.36	−0.25	4.301	−0.075	±0.006
Met	8.428	0.383	±0.018	8.426	0.45	−0.34	4.479	−0.089	±0.012
Phe	8.301	0.489	±0.018	8.301	0.47	0.10	4.614	−0.042	±0.006
Pro_{cis}							4.568	0.024	±0.006
Pro_{trans}							4.416	−0.007	±0.006
Ser	8.356	0.396	±0.006	8.352	0.48	−0.34	4.495[c]	0.010	±0.006
Thr	8.251	0.633	±0.018	8.249	0.72	−0.44	4.353	0.016	±0.006
Trp	8.181	0.457	±0.018	8.178	0.57	−0.57	4.652[c]	−0.068	±0.006
Tyr	8.194	0.336	±0.006	8.193	0.38	−0.20	4.590	−0.011	±0.006
Val	8.191	0.517	±0.018	8.189	0.61	−0.45	4.146	−0.035	±0.006

[a] The chemical shift δ_0^{HN} at 0.1 MPa, the linear pressure coefficient $\delta_{\Delta p}^{HN}$, the first order and second order pressure coefficients $\delta'_{\Delta 2p}^{HN}$ $\delta_{\Delta 2p}^{HN}$ were obtained by fitting the data to Eq. (2) and Eq. (3), respectively. The H^{α}-chemical shifts were only fitted with the linear model (Eq. (2)).

[b] The two H^{α}-resonances of glycine are degenerated.

[c] The H^{α}-chemical shifts were measured in D_2O.

3.3. Pressure-induced shifts of side chain resonances

An inspection of the linear pressure coefficients $\delta_{\Delta p}$ for the side chain resonances reveals that, in general, the pressure-induced shifts are rather small. The only exceptions are side chain protons, which can be engaged in hydrogen bonds. If one calculates the mean values $\langle\delta_{\Delta p}^{sCH}\rangle$ and $\langle\delta_{\Delta p}^{sNH}\rangle$ for the two groups in the tetrapeptides, one obtains −0.04 and 0.21 ppm GPa^{-1} with rmsds of 0.10 and 0.21 ppm GPa^{-1}. In gurmarin, one obtains for $\langle\delta_{\Delta p}^{sCH}\rangle$, −0.09 ppm GPa^{-1} [8]; in BPTI, −0.074 ppm GPa^{-1} for $\langle\delta_{\Delta p}^{sCH}\rangle$ and 0.17 ppm GPa^{-1} for $\langle\delta_{\Delta p}^{sNH}\rangle$ [10]. Again, the spread of the values are much larger in the proteins, minimum and maximum values of $\langle\delta_{\Delta p}^{sCH}\rangle$ are −0.80 and 0.05, and −1.06 and 1.78 ppm GPa^{-1} for the gurmarin and BPTI, respectively.

Rather large chemical shift changes $\langle\delta_{\Delta p}^{sNH}\rangle$ are observed for the side chain protons, which are part of polar or charged groups. These protons are presumably involved in hydrogen bonds to water. Interestingly, side chain amide protons of Asn and Gln show different pressure coefficients for the two, stereospecifically different protons. The pro-Z proton shows always a larger dependence on pressure (Table 2). From proteins, only a few data are available. In HPr from *S. carnosus,* some of the side chain amide protons were stereospecifically assigned. At 298 K the mean linear pressure coefficients are 0.27 and 0.53 ppm GPa^{-1} for the pro-E and the pro-Z protons, respectively [11]. The reason for this different behaviour is not

Table 2
Chemical shifts and pressure coefficients of the side chain protons of amino acid X in Gly-Gly-X-Ala at 305 K in aqueous solution at pH 5.4[a,b]

*X*3	Atom	δ_0 [ppm]	$\delta_{\Delta p}$ [ppm GPa^{-1}]		Atom	δ_o [ppm]	$\delta\Delta p$ [ppm GPa^{-1}]	
Ala	$H^{\beta 2}$	1.400	−0.031	±0.012				
Arg	$H^{\beta 2}$	1.885	−0.097	±0.006	$H^{\gamma 2/\gamma 3}$	1.661	−0.083	±0.006
	$H^{\beta 3}$	1.773	−0.035	±0.006	$H^{\delta 2/\delta 3}$	3.211	−0.082	±0.006
					H^{ε}	7.236	−0.204	±0.012
					$H^{\eta 11/\eta 12/\eta 21/\eta 22}$	6.680	−0.090	±0.042
Asn	$H^{\beta 2}$	2.842	0.009	±0.006	$H^{\delta 21}$	7.573	0.214	±0.012
	$H^{\beta 3}$	2.755	0.032	±0.006	$H^{\delta 22}$	6.966	0.417	±0.006
Asp	$H^{\beta 2}$	2.729	0.128	±0.006				
	$H^{\beta 3}$	2.592	0.089	±0.006				
Cys	$H^{\beta 2}$	2.977	−0.050	±0.012				
	$H^{\beta 3}$	2.926	0.014	±0.042				
Gln	$H^{\beta 2}$	2.124	−0.071	±0.006	$H^{\gamma 2/\gamma 3}$	2.380	−0.032	±0.006
	$H^{\beta 3}$	2.000	−0.023	±0.006	$H^{\varepsilon 21}$	7.532	0.260	±0.006
					$H^{\varepsilon 22}$	6.929	0.395	±0.012
Glu	$H^{\beta 2}$	2.086	−0.023	±0.006	$H^{\gamma 2/\gamma 3}$	2.301	0.089	±0.006
	$H^{\beta 3}$	1.949	−0.077	±0.018				
His	$H^{\beta 2}$	3.284	−0.025	±0.006	$H^{\delta 2}$	7.311	0.089	±0.006
	$H^{\beta 3}$	3.210	0.006	±0.006	H^{ε}	8.546	0.243	±0.018
Ile	H^{β}	1.872	0.027	±0.018	$H^{\gamma 12}$	1.458	−0.243	±0.024
					$H^{\gamma 13}$	1.190	−0.065	±0.018
					$(H^{\gamma 2})_3$	0.923	−0.058	±0.006
					$(H^{\delta 1})_3$	0.870	−0.078	±0.006
Leu	$H^{\gamma/\beta 2/\beta 3}$	1.624	−0.041	±0.018	$H^{\gamma/\beta 2/\beta 3}$	1.626	−0.041	±0.018
					$(H^{\delta 1})_3$	0.931	−0.060	±0.006
					$(H^{\delta 2})_3$	0.888	−0.061	±0.006
Lys	$H^{\beta 2}$	1.845	−0.096	±0.012	$H^{\gamma 2/\gamma 3}$	1.437	−0.174	±0.030
	$H^{\beta 3}$	1.772	−0.075	±0.012	$H^{\delta 2/\delta 3}$	1.688	−0.109	±0.006
					$H^{\varepsilon 2/\varepsilon 3}$	3.004	−0.073	±0.006
					$(H^{\xi})_3$	7.260	0.368	±0.018
Met	$H^{\beta 2}$	2.109	−0.058	±0.006	$(H^{\varepsilon})_3$	2.109	−0.058	±0.006
	$H^{\beta 3}$	2.013	−0.007	±0.006	$H^{\gamma 2/\gamma 3}$	2.590	−0.079	±0.006
Phe	$H^{\beta 2}$	3.121	−0.035	±0.006	$H^{\delta 1/\delta 2}$	7.287	−0.029	±0.006
	$H^{\beta 3}$	3.056	0.031	±0.006	$H^{\varepsilon 1/\varepsilon 2}$	7.386	0.015	±0.006
					H^{ξ}	7.328	0.025	±0.006
Pro_{cis}	$H^{\beta 2}$	2.384	−0.056	±0.006	$H^{\gamma 3}$	1.906	−0.095	±0.006
	$H^{\beta 3/\gamma 2}$	2.188	−0.083	±0.006	$H^{\beta 3/\gamma 2}$	2.188	−0.083	±0.006
					$H^{\delta 2/\delta 3}$	3.558	−0.113	±0.006
Pro_{trans}	$H^{\beta 2}$	2.266	−0.020	±0.006	$H^{\delta 2/\delta 3}$	3.632	−0.077	±0.006
	$H^{\beta 3/\gamma 2/\gamma 3}$	2.015	−0.082	±0.006	$H^{\beta 3/\gamma 2/\gamma 3}$	2.015	−0.082	±0.006
Ser	$H^{\beta 2/\beta 3}$	3.874	−0.024	±0.006				
Thr	H^{β}	4.241	−0.003	±0.006	$(H^{\gamma 2})_3$	1.213	−0.036	±0.006
Trp	$H^{\beta 2}$	3.281	−0.047	±0.012	$H^{\varepsilon 2}$	7.271	0.059	±0.006
	$H^{\beta 3}$	3.268	0.026	±0.006	$H^{\varepsilon 2}$	10.141	−0.071	±0.018
					$H^{\varepsilon 3}$	7.654	−0.078	±0.006
					$H^{\zeta 2}$	7.179	−0.011	±0.006
					$H^{\zeta 3}$	7.515	0.021	±0.006
					$H^{\eta 2}$	7.251	−0.006	±0.006

Table 2 *(Continued)*

*X*3	Atom	δ_0 [ppm]	$\delta_{\Delta p}$ [ppm GPa^{-1}]		Atom	δ_o [ppm]	$\delta\Delta$p [ppm GPa^{-1}]	
Tyr	$H^{\beta 2}$	3.120	−0.050	±0.006	$H^{\delta 1/\delta 2}$	7.146	−0.019	±0.006
	$H^{\beta 3}$	2.915	0.033	±0.006	$H^{\varepsilon 1/\varepsilon 2}$	6.849	0.000	±0.006
Val	H^{β}	2.114	−0.026	±0.006	$(H^{\gamma 1})_3$ $(H^{\gamma 2})_3$	0.943	−0.068	±0.006

[a] The nomenclature is according to IUPAC recommendations 1998 [37]. The chemical shift δ_0 at 0.1 MPa and the linear pressure coefficient δ_Δ were obtained by fitting the data to Eq. (2).

[b] The pressure-induced chemical shifts are clearly non-linear. Fitting the data with Eq. (3) gives 10.143 ppm, −0.137 ppm GPa^{-1} and 0.332 ppm GPa^{-2} for $\delta_0^{H\zeta 1}$, $\delta_{\Delta p}^{H\zeta 1}$, and $\delta_{\Delta 2p}^{H\zeta 1}$, respectively.

known, but the random-coil data show that they are not due to specific structural features of the HPr-protein.

3.4. Non-linear dependence of chemical shifts on pressure

In most proteins, the pressure-induced changes of chemical shifts can be fitted well by a linear dependence, as given in Eq. (1). However, there are also exceptions, where non-linear dependencies were clearly observed. They were usually interpreted as indications for a conformational equilibrium shifted by pressure. In HPr from *S. carnosus*, strong deviations from the linearity were observed in the active-centre loop around His15 and in the regulatory helix b [11]. These parts of the structure are assumed to exist in more than one state at atmospheric pressure, since they have to be able to adapt to different surfaces when interacting with other proteins. In the Ras-binding domain of RalGDS, non-linearities suggested the exchange with a folding intermediate that was stabilised by pressure [32].

In our random-coil peptides, the non-linearities were vanishing for most of the residues. This indicates that the above interpretation is true, since larger deviations from the linearity do not occur in unstructured peptides. Only the backbone amide of Glu and the side chain NH of Trp showed some non-linear behaviour (Fig. 3). However, the quantitative analysis shows that the contribution is very small (Tables 1 and 2). In fact, when using a second order model for fitting the data of all residues *X* in the tetrapeptide second order, pressure coefficients $\delta_{\Delta 2p}$ in the same range are obtained. This suggests that small $\delta_{\Delta p}$ may also contribute to other residues, but may not be observed because the linear term dominates. For glutamate, the first order coefficient is partially independent of the pH. This is probably due to internal hydrogen bonds of the carboxyl group of peptide amides. A quantitative analysis by Bundi and Wüthrich [13] suggested that in the dynamical equilibrium 20% of the side chains form an internal hydrogen bond with the amide of Gly2 and 50% to Glu3. This means that the amide proton of Glu3 is shielded from water on average 70% of the time.

For Trp in Gly-Gly-Trp-Ala, structural details were not reported. However, the non-linearity may be indicative of an internal hydrogen bond to a peptide carbonyl group or the C-terminal carboxyl group. Pressure would then shift the relative populations of internally or externally (water) hydrogen-bonded side chain $H^{\zeta 1}$. The pH-variation almost excludes the interaction with the C-terminal group of Ala4. No significant pH-induced shift of the methyl group of Ala4 was observed in the pH-range from pH 5.0 to 5.4 (data not shown). Nevertheless, the pressure coefficient of −0.13 ppm GPa^{-1} of the methyl group of Ala4 is almost one magnitude larger than for the other peptides (Table 3). This could reflect a change of the relative orientation of the tryptophan ring relative to the methyl group of Ala4, thus changing the ring current shift of this resonance.

3.5. Proline cis-trans isomerisation

The peptide bond preceding a proline residue usually occurs as a blend of *cis*- and *trans*-conformations. In NMR spectroscopy, the two conformations can be distinguished on the base of

Table 3
Chemical shifts and pressure coefficients of the resonances of Ala4 in Gly-Gly-*X*-Ala at 305 K in aqueous solution at pH 5.4[a]

*X*3	δ_0^{HN} [ppm]	$\delta_{\Delta p}^{HN}$ [ppm GPa^{-1}]		$\delta_0^{H\alpha}$ [ppm]	$\delta_{\Delta p}^{H\alpha}$ [ppm GPa^{-1}]		$\delta_0^{H\beta}$ [ppm]	$\delta_{\Delta p}^{H\beta}$ [ppm GPa^{-1}]	
Ala	8.365	0.383	±0.012	4.285	−0.076	±0.018	1.392	−0.021	±0.006
Arg	8.069	0.796	±0.024	4.132	−0.121	±0.006	1.335	−0.006	±0.006
Asn	8.349	0.485	±0.012	4.296	−0.055	±0.006	1.404	−0.037	±0.006
Asp	7.919	0.798	±0.018	4.131	−0.076	±0.006	1.334	0.003	±0.006
Cys	8.070	0.781	±0.012	4.144	−0.106	±0.006	1.343	−0.012	±0.006
Gln	8.389	0.498	±0.018	4.294	−0.065	±0.012	1.401	−0.031	±0.006
Glu	7.984	0.852	±0.024	4.149	−0.104	±0.006	1.335	0.000	±0.006
Gly	7.848	0.769	±0.030	4.167	−0.103	±0.012	1.335	−0.009	±0.006
His	8.248	0.550	±0.024	4.156	−0.087	±0.006	1.355	−0.040	±0.006
Ile	8.383	0.564	±0.030	4.302	−0.065	±0.006	1.393	−0.021	±0.006
Leu	8.300	0.587	±0.018	4.290	−0.075	±0.006	1.395	−0.031	±0.006
Lys	8.364	0.509	±0.018	4.309	−0.079	±0.012	1.398	−0.027	±0.006
Met	8.351	0.533	±0.030	4.301	−0.060	±0.006	1.400	−0.022	±0.006
Phe	8.301	0.489	±0.018	4.246	0.068	±0.006	1.327	−0.041	±0.006
Pro_{cis}	8.148	0.817	±0.018	4.123	−0.108	±0.056	1.360	−0.017	±0.006
Pro_{trans}	7.975	0.961	±0.030	4.172	−0.004	±0.012	1.332	0.003	±0.006
Ser	8.013	0.706	±0.018	4.160	−0.092	±0.006	1.342	−0.007	±0.006
Thr	8.373	0.522	±0.018	4.322	−0.067	±0.006	1.409	−0.028	±0.006
Trp	8.097	0.534	±0.024	4.175	−0.150	±0.006	1.216	−0.134	±0.024
Tyr	7.876	0.868	±0.024	4.110	−0.077	±0.006	1.316	−0.016	±0.006
Val	8.394	0.553	±0.018	4.302	−0.094	±0.006	1.396	−0.030	±0.006

[a] The chemical shift δ of 0.1 MPa and the linear pressure coefficient δΔ were obtained by fitting the data to Eq. (2).

their characteristic chemical shifts. From the integration of their resonances lines, the relative populations and hence the equilibrium constant $K = [trans]/[cis]$ can be determined. It has been calculated in our spectra from the intensities of the well-resolved resonances. At 305 K one obtains a value of $K = 3.9 \pm 0.2$ in 99.5% D_2O. Within the limit of errors, this value is unchanged in the pressure range between 0.1 and 200 MPa. This finding is in accord with the general observation that simple conformational equilibria, which are not accompanied by the production or redistribution of local charges, do show at best a fairly weak pressure dependence of their conformational equilibria [33–36]. *N*-acetyl-L-proline-NH-methylamide in unbuffered aqueous solutions shows between 330 and 335 K at pressures up to 150 MPa a *T*, *p* independent ratio of the *cis-trans* equilibrium with $x_{trans} = 0.76 \pm 0.02$. Also, in glycylsarcosine this equilibrium is within the precision of the experimental data independent of *p* [34].

The chemical shifts of the two conformers show a slightly different sensitivity towards pressure. The largest effect can be observed for the H^{δ}-resonances, where $\delta_{\Delta p}$ is larger for the *cis*-conformer.

3.6. Pressure dependence of J-coupling constants

In ^{15}N-enriched HPr from *S. carnosus* [11], a significant pressure dependence of the $^1J_{HN-N}$ coupling constants was described. At least, for the easily observable $^3J_{HN\text{-}H\alpha}$ coupling constant, no significant pressure dependence could be observed within the limits of error. Possible effects should be smaller than 2 Hz GPa^{-1}.

3.7. Neighbourhood effects on the pressure coefficients

It is well documented that the type of amino acid preceding or following in the sequence to a given amino acid has subtle but significant effects on the chemical shifts. For the tetrapeptide Gly-Gly-*X*-Ala, this has been described in detail by Bundi and Wüthrich [14]. At least a part of this

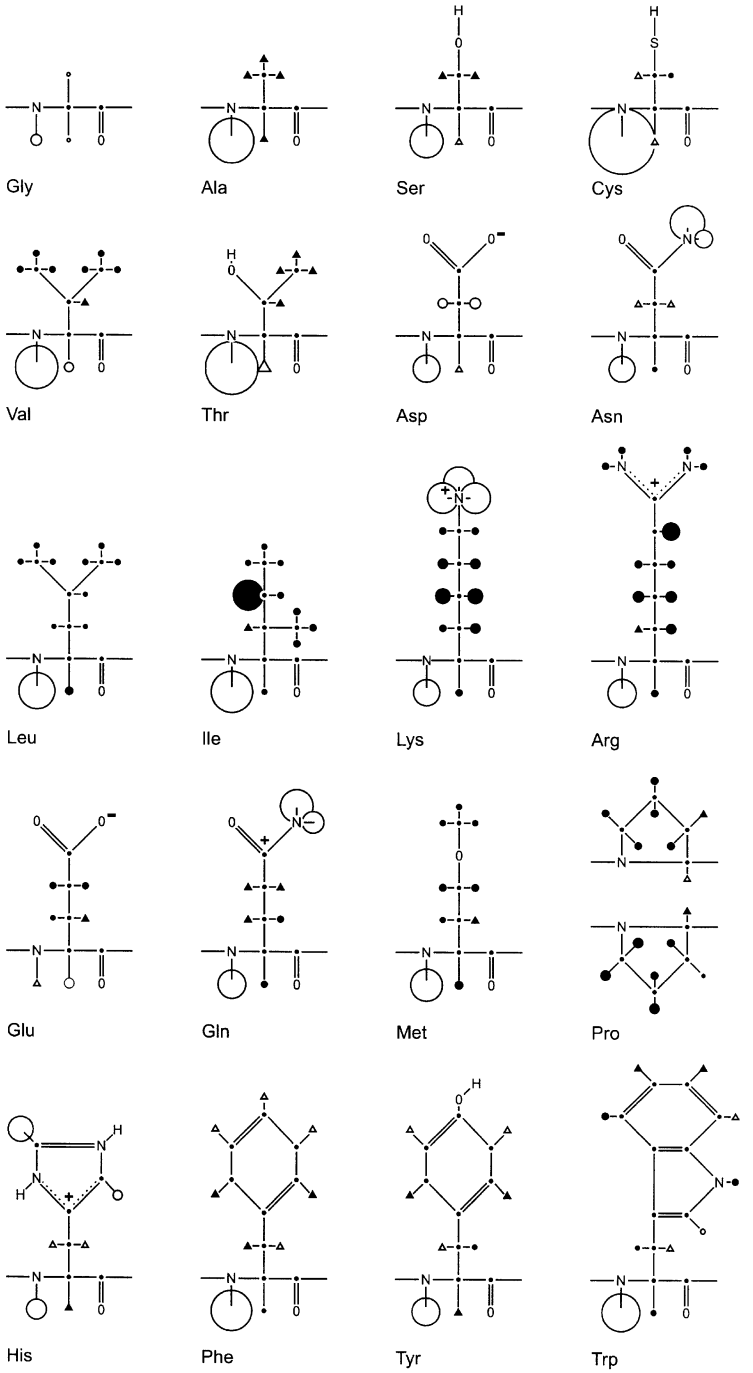

Fig. 4. Schematic view of the pressure effects observed. The magnitude of the linear pressure coefficients $\delta_{\Delta p}$ of the amino acid *X* in the tetrapeptides Gly-Gly-*X*-Ala is depicted schematically in the structural formula of *X*. The radius of the circles is proportional to the magnitude of $\delta_{\Delta p}$. Filled symbols symbolise a negative sign of $\delta_{\Delta p}$, empty symbols a positive sign. Very small coefficients are represented by triangles.

effect is caused by a small change of the conformational equilibrium, which in turn could also cause changes in the pressure coefficients. Since Ala4 is present in all peptides studied here, this would be a good candidate for testing such a hypothesis. The corresponding data are summarised in Table 3. Indeed, the linear pressure coefficients change with the amino acid *X* in position 3. For H^N, the smallest coefficient is observed for Ala with 0.34 ppm GPa^{-1} and the largest for Pro in *trans*-conformation with 0.96 ppm GPa^{-1}. For the H^α of Ala4, the values vary in the range of -0.15 (Trp) to 0.07 (Phe) ppm GPa^{-1}, for H^β in the range of -0.13 (Trp) to 0.003 (Asp) ppm GPa^{-1}. This means that the conformational equilibria of the random-coil peptides are sequence dependent and are influenced by pressure differentially.

4. Conclusions

The pressure dependent shifts in the model-peptides Gly-Gly-*X*-Ala are relatively small, as to be expected for non-structured peptides, but are characteristic for specific atoms in a given amino acid (for an overview, the pressure coefficients are represented in Fig. 4 in the structural formulas of the amino acids). The largest shift changes are observed for protons of groups involved in hydrogen-bonding to water. The data presented here provide a solid basis for the interpretation of pressure-induced chemical shift changes in proteins and allow the separation of specific effects, such as pressure-induced conformational changes of the protein from unspecific effects caused by the pressure dependent direct interaction between the solvent molecules and solvent exposed residues.

Acknowledgments

We are grateful to S. Modrow and A. Brunner for the synthesis of tetrapeptides, to E. Treml and S. Heyn for the preparation of the high-pressure capillaries and to the staff of the mechanical workshop, especially G. Niesner, for their support.

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