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The effects of guanidine hydrochloride on the 'random coil' conformations and NMR chemical shifts of the peptide series GGXGG

Kevin W. Plaxco, Craig J. Morton, Shaun B. Grimshaw, Jonathan A. Jones, Maureen Pitkeathly, Iain D. Campbell and Christopher M. Dobson*

Oxford Centre for Molecular Sciences, University of Oxford, New Chemistry Laboratory, South Parks Road, Oxford OX1 3QT, U.K.

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Summary

The effects of the commonly used denaturant guanidine hydrochloride (GuHCl) on the random coil conformations and NMR chemical shifts of the proteogenic amino acids have been characterized using the peptide series Ac-Gly-Gly-X-Gly-Gly-NH_2. The ϕ angle-sensitive coupling constants, ROESY cross peak intensities and proline cis–trans isomer ratios of a representative subset of these peptides are unaffected by GuHCl, which suggests that the denaturant does not significantly perturb intrinsic backbone conformational preferences. A set of $^3J_{H^N H^\alpha}$ values is presented which agree well with predictions of recently developed models of the random coil. We have also measured the chemical shifts of all 20 proteogenic amino acids in these peptides over a range of GuHCl concentrations. The shifts exhibit a linear dependence on denaturant concentration and we report here correction factors for the calculation of 'random coil' ^{1}H chemical shifts at any arbitrary denaturant concentration. Studies of a representative subset of peptides indicate that ^{13}C and ^{15}N chemical shifts are also perturbed by the denaturant. These results should facilitate the application of chemical shift-based analytical techniques to the study of polypeptides in solution with GuHCl. The effects of the denaturant on the quality of NMR spectra and on chemical shift referencing are also addressed.

Introduction

Protein folding in the cell follows synthesis of the polypeptide chain on a ribosome. Refolding in vitro is more readily studied using a variety of techniques based on the denaturation and refolding of previously native proteins. Often these studies involve characterization of the refolding kinetics of chemically denatured proteins (Plaxco and Dobson, 1996), but other widely used approaches include the investigation of the hydrogen exchange properties (reviewed by Englander et al., 1996), partially folded conformations (Redfield et al., 1994; Ptitsyn, 1995; Schulman et al., 1995; Wang and Shortle, 1995; Kay and Baldwin, 1996) and unfolding kinetics (Hoeltzli and Frieden, 1995; Kiefhaber et al., 1995) of proteins under partially or fully denaturing conditions. An assumption implicit in many of these studies is that chemical denaturants unfold proteins in a manner that produces a distribution of conformations similar to that populated by unfolded polypeptides in vivo (Tanford, 1968). Other studies rely on comparisons of the structural or spectral characteristics of the unfolded state with the previously reported properties of 'unstructured' polypeptides in simple aqueous solutions. The chemical denaturants used to unfold proteins may, however, complicate the interpretation of many of these studies due to the effects they might have on both the intrinsic conformational preferences and spectral characteristics of unstructured polypeptides.

Significant insights into the mechanisms of protein folding have also been gained from studies of the residual structure present in denatured proteins (Dobson, 1992; Shortle, 1993; Wüthrich, 1994; Shortle, 1996; Smith et al., 1996). Of potential applicability to these studies are a number of chemical shift-based analytical techniques developed to characterize the structure and dynamics of polypeptides. These techniques include methods which

*To whom correspondence should be addressed.

employ chemical shift values to identify native secondary structural elements (Pastore and Saudek, 1990; Wishart et al., 1991a,1992; Ösapay and Case, 1994; Wishart and Sykes, 1994a,b; Asakura et al., 1995; Oldfield, 1995), to estimate the secondary structural preferences of short peptides (Rizo et al., 1993; Shin et al., 1993; Merutka et al., 1995), to monitor folding transitions (Reily et al., 1992; Oldfield, 1995) and to measure main-chain flexibility (Wishart et al., 1991b). Critical to these approaches has been the availability of data on the 'random coil' chemical shifts of the proteogenic amino acids. Random coil chemical shifts, defined as the chemical shifts of amino acid residues in the context of a polypeptide that is free to access all sterically allowed regions of conformational space, have been measured using short, simple peptides such as protected and unprotected Gly-Gly-X-Ala (Bundi et al., 1975; Richarz and Wüthrich, 1978; Bundi and Wüthrich, 1979; Braun et al., 1994), unprotected Gly-Gly-X-Gly-Gly (Keim et al., 1973,1974; Merutka et al., 1995) and Ac-Gly-Gly-X-Ala-Gly-Gly-NH_2 (Wishart et al., 1995a). ^{1}H chemical shift lists have been compiled for these peptides under a variety of solvent conditions, including water (Bundi and Wüthrich, 1979; Merutka et al., 1995), dimethyl sulfoxide (Masson and Wüthrich, 1973; Bundi et al., 1975), aqueous trifluoroethanol (Jiménez et al., 1992; Merutka et al., 1995) and aqueous urea (Jiménez et al., 1986; Wishart et al., 1995a); ^{13}C chemical shifts have been reported in aqueous trifluoroethanol and aqueous acetonitrile (Thanabal et al., 1994). To date, however, no compilation of random coil chemical shifts has been published for peptides in solutions containing the commonly used denaturant guanidine hydrochloride (GuHCl). This has limited the application of chemical shift-based analytical techniques to the study of partially or fully denatured polypeptides in solution with GuHCl, due to the difficulty of discriminating between chemical shift changes arising from potentially interesting residual structure and those due to the effect of GuHCl on random coil chemical shifts.

We have investigated the effects of GuHCl on the conformations and chemical shifts of the proteogenic amino acids. We have done so in order to clarify the relationship between GuHCl-induced denatured states and the conformations of polypeptides unfolded in simple aqueous solution, and to facilitate the application of chemical shift-based analytical techniques to the study of proteins and peptides in GuHCl-containing solutions. We report here the effects of GuHCl on the ^{1}H chemical shifts of all 20 proteogenic amino acids in the context of the protected pentapeptide Ac-Gly-Gly-X-Gly-Gly-NH_2 (GGXGG) and provide correction factors that enable the interpolation of random coil chemical shifts at any arbitrary GuHCl concentration. The effects of GuHCl on ^{13}C and ^{15}N chemical shifts were determined for seven representative peptides. We also report evidence for the effects of GuHCl on intrinsic conformational preferences, on spectral quality and on chemical shift referencing.

Materials and Methods

Peptide synthesis

Peptides were synthesized on an Applied Biosystems 430A solid-phase peptide synthesizer using a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin. Couplings were achieved using single couple cycles and a 10-fold excess of the HBTU-activated Fmoc amino acids and DIEA. Analysis of the Fmoc deprotection mixtures indicates that coupling was quantitative. After completion of the synthesis, the peptides were amino-acylated using a mixture of 0.1 M acetic anhydride and 0.1 M pyridine in dimethylformamide. The blocked peptides were cleaved from the resin using trifluoroacetic acid in the presence of water, thioanisole and ethane dithiol as scavengers, except for the methionine peptide for which ethylmethylsulfide was also included. The aspartate peptide was synthesized in the presence of HOBT/piperidine to reduce aspartimide formation. The peptides were purified by diethyl ether precipitation of the reaction mixture, followed by repeated ether washes and lyophilization. Analytical HPLC and NMR indicated that most of the peptides were of sufficient purity to use without further work-up. Some peptides, however, were further purified of volatile protecting group and cleaving reagents by repeated lyophilization from 50% acetonitrile/water. The cysteine peptide was maintained under low pH and remained a mixture of the reduced and oxidized forms.

NMR spectroscopy of peptides

NMR measurements were carried out at 20 °C on peptides dissolved in 550 µl of a phosphate buffer consisting of 50 mM sodium phosphate, 10% D_2O and 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) containing the appropriate GuHCl concentration. ^{1}H chemical shift data were obtained for all 20 peptides at concentrations of 2, 4, 6 and 8 M GuHCl. Data were also obtained for several peptides at 0 M GuHCl. In order to observe solvent obscured H^α peaks, a small number of peptides were measured in the equivalent fully deuterated buffers. All samples were adjusted to a final pH value of 5.0 ± 0.1 (uncorrected meter reading). Final GuHCl concentrations were monitored by refractometry (Nozaki, 1972). Measurements were performed with peptide concentrations ranging from 5 to 140 mg/ml (high peptide concentrations were required to obtain high-quality natural abundance ^{13}C and ^{15}N spectra); no concentration-dependent effects were observed. Multidimensional and ^{1}H spectra were recorded at ^{1}H-resonant frequencies of 500 or 600 MHz on home-built spectrometers consisting of Oxford Instruments 11.7 or 14.2 T magnets operated by GE 1280 computers. ^{13}C spectra were recorded at 126 MHz using the

^{13}C decoupling coil of a triple-tuned ($^{1}H/^{13}C/^{15}N$) NMR probe. All spectra were recorded in the phase-sensitive mode with the carrier set to the centre of the spectrum. The chemical shifts of the peptide resonances were recorded at each of four GuHCl concentrations and fitted using a linear least-squares fitting algorithm (KaleidaGraph, Abelbek Software, Reading, PA, U.S.A.). Estimated errors are approximately ±0.5 ppb M^{-1} for GuHCl sensitivities ($\Delta\delta$) and ±0.02 ppm for extrapolated intrinsic (0 M GuHCl) chemical shift values.

The assignment of peptide ^{1}H resonances

Most peptides could be unambiguously assigned using 1D spectroscopic techniques. Ambiguous assignments were clarified using either ROESY (Bax and Davis, 1985) or DQF-COSY (Rance et al., 1983) 2D spectra. Discrimination between the resonances of oxidized and reduced cysteine was achieved by comparison with previously reported values (Wishart et al., 1995a). Initial spectra of aspartate samples indicated the presence of two species; analysis of the peptide by HPLC/MS, however, indicated that only species with the correct molecular mass were present (data not shown), suggesting that aspartimide formation may have occurred during synthesis. A discrepancy was observed between the experimental chemical shifts of the purified primary aspartate species in 0 M GuHCl, early reports of the random coil chemical shifts of aspartate (Richarz and Wüthrich, 1978; Bundi and Wüthrich, 1979) and those published in recent compilations (Merutka et al., 1995; Wishart et al., 1995a). To clarify this discrepancy, the aspartate isomer mixture was further analysed by a gradient selected natural abundance ^{1}H-^{13}C HSQC spectrum (Bruhwiler and Wagner, 1986; Kay et al., 1992). This allowed discrimination between the two species and the reported data are from the correct, α-peptide isomer. Sequential assignment of the glycine H^{α} resonances (Fig. 1) was made via a long-range ^{1}H-^{13}C HSQC (see below).

The effects of GuHCl on ^{13}C and ^{15}N chemical shifts

The effects of GuHCl on ^{13}C and ^{15}N chemical shifts were determined for selected peptides (alanine, arginine, glutamate, isoleucine, leucine, lysine, threonine, tyrosine and valine). Chemical shift values were determined for several of these peptides at 0 or 1.5, 3 and 6 M GuHCl in phosphate buffer containing ≈10 mM DSS using natural abundance ^{13}C and ^{15}N spectroscopy. As the effects of GuHCl on the carbon and nitrogen chemical shifts of these residues were found to be linear, additional residues were monitored at only two GuHCl concentrations. Carbon chemical shifts were obtained from 1D ^{13}C spectra with GARP decoupling (Shaka et al., 1985) applied to protons during both the relaxation and acquisition periods. Side chain and C^{α} resonances were assigned using a gradient-selected natural abundance ^{1}H-^{13}C HSQC spectrum (Bruhwiler and Wagner, 1986; Kay et al., 1992), while carbonyl resonances were assigned using an HSQC sequence optimized for the small $^{2}J_{H^{\alpha}C=O}$ and $^{3}J_{H^{\alpha}C=O}$ couplings. From these spectra the ^{1}H resonances of the four glycine residues in the peptide series were unambiguously

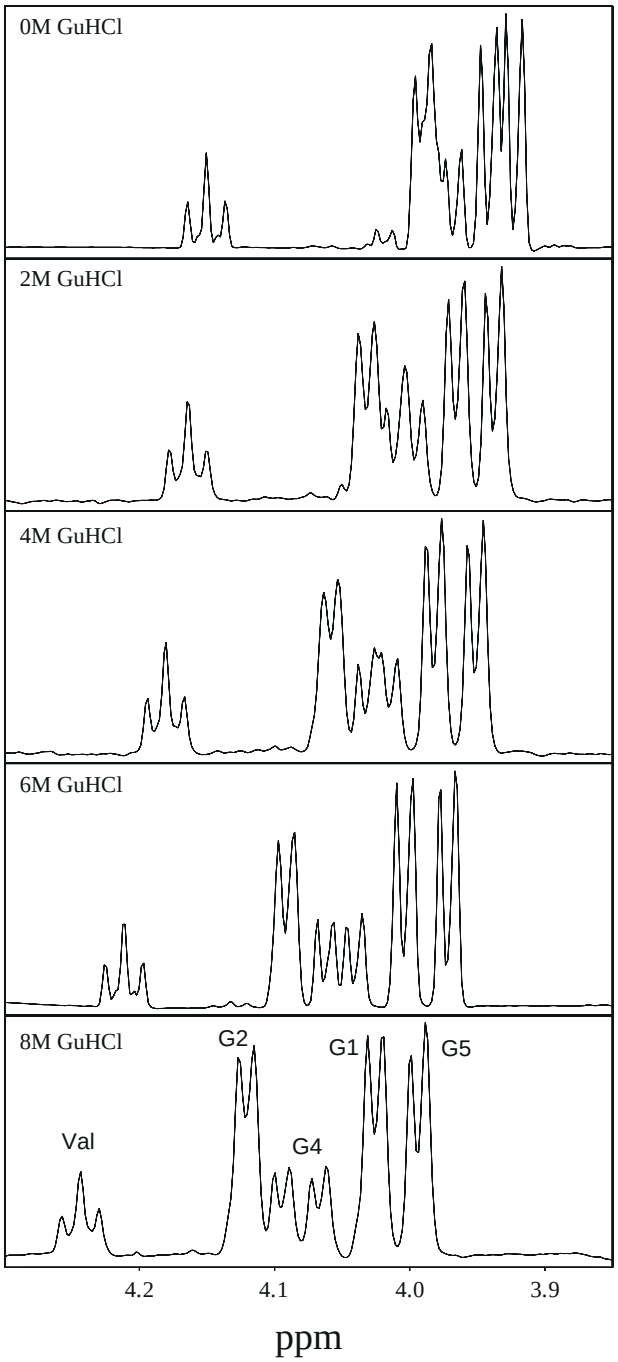

Fig. 1. The effects of GuHCl on the H^{α} ^{1}H chemical shifts of the valine pentapeptide. Illustrated are the H^{α} resonances of the peptide in 0, 2, 4, 6 and 8 M GuHCl. The H^{α} resonances of the valine and the four glycine residues (numbering from the amino terminus) are indicated. The increase in the splitting of the two H^{α} resonances of the glycine at position 4 is similar to the differential GuHCl sensitivities observed for some pairs of H^{β} protons (see text) and presumably reflects stereospecific electronic effects at this position.

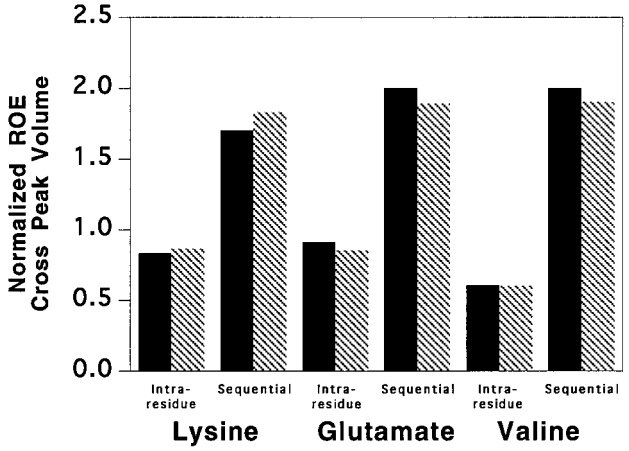

Fig. 2. Normalized sequential and intraresidue H^N-H^α cross peak volumes, reflecting interatomic distances sensitive to the ϕ and ψ torsional angles, respectively. Here the normalized cross peak volumes of representative peptides in 0 (filled) and 6 M (cross-hatched) GuHCl are identical to within experimental errors of ±5%. The r^{-6} distance dependence of ROE cross peak volumes makes this an extremely sensitive test of even small conformational changes and suggests that GuHCl does not perturb conformational preferences.

assigned. The effects of GuHCl on ^{15}N chemical shifts were determined for the same representative subset of residues using natural abundance ^1H-^{15}N HSQC spectra (Bruhwiler and Wagner, 1986; Kay et al., 1992). Assignments were made by comparison with the previously assigned proton spectra.

Analysis of peptide conformational preferences

Peptide conformational preferences were monitored using a variety of techniques. The conformationally sensitive amide proton-H^α coupling constant ($^3J_{H^NH^\alpha}$) values were obtained by peak fitting 1D amide proton (H^N) doublets, H^N-H^α DQF-COSY cross peaks or the appropriate natural abundance ^1H-^{15}N HMQCJ (Kay and Bax, 1990) cross peaks using the fitting routines in FELIX 2.3 (Biosym Inc., San Diego, CA, U.S.A.) and in-house software (Smith et al., 1991). The signal-to-noise ratio and the variation between repeated experiments indicate that these measurements have a precision of about ±0.1 Hz. The coupling constants of the lysine, glutamate and valine peptides were determined in both 0 and 6.0 M GuHCl from DQF-COSY data. Conformationally sensitive ROE cross peak intensities were measured on the lysine, glutamate and valine peptides using 2D ROESY spectroscopy. These experiments were conducted at ^1H resonant frequencies of 600 MHz (lysine and glutamate) or 500 MHz (valine) on ≈25 mg/ml peptide samples in 0 and 6.0 M GuHCl solutions. Changes in ROE cross peak intensities due to variations in sample concentration and viscosity were removed by normalizing cross peak volumes relative to the volume of the fixed-distance acyl blocking group-NH pair. The estimated errors in relative ROE cross peak measurements were determined by measuring the volumes of featureless regions of the 2D spectra and are about ±5%. The effects of GuHCl on the conformational preference of the proline ω bond were determined by monitoring the relative areas of integrated cis and trans H^β peaks in 1D spectra collected with a relaxation delay of 10 s. The reported value and error bars represent the mean and standard deviation of measurements made at 0, 2, 4, 6, and 8 M GuHCl.

Chemical shift referencing

The possibility of differential effects of GuHCl on the chemical shifts of referencing compounds indicates that care must be taken with the referencing of spectra collected from samples containing this denaturant. In order to avoid this problem, the ^1H and ^{13}C values reported in this work are all relative to internal DSS, which was assigned a chemical shift of 0.00 ppm under each of the conditions employed. Indirect methods were used for ^{15}N chemical shift referencing (Wishart et al., 1995b). In order to further characterise the effects of GuHCl on ^1H referencing, additional samples were made by adding 2 mM dioxane to the relevant GuHCl-containing buffers. While dioxane proved resistant to GuHCl effects ($\Delta\delta = 1.5$ ppb M^{-1}), both water ($\Delta\delta = -44$ ppb M^{-1}) and GuHCl itself ($\Delta\delta = 15$ ppb M^{-1}, extrapolated 0 M $\delta = 6.69$ ppm) reveal substantial changes in chemical shift with changes in GuHCl concentration.

Results and Discussion

The effects of GuHCl on spectral quality

Considering the important role GuHCl has played in our understanding of the kinetics and thermodynamics of protein folding, the literature contains relatively few reports of NMR spectroscopy conducted on GuHCl-containing solutions. This may be at least partially due to a belief that the high conductivity and viscosity of concentrated GuHCl solutions significantly degrades spectrometer performance. It is our experience, however, that the spectroscopy of GuHCl-containing solutions is not significantly more difficult than spectroscopy of simple aqueous solutions.

While the inclusion of GuHCl does not make spectroscopy qualitatively more difficult, some reduction in total signal strength is observed. Adequate sample shimming and receiver coil tuning and matching may be easily achieved, but the increased sample conductivity inevitably results in a reduction in the receiver coil quality factor, Q. In our experience Q is reduced by ≈35% in 8 M GuHCl, leading to a corresponding loss in signal intensity (Lu, 1997). Similarly, radio-frequency induced sample heating of these highly conducting solutions is not a significant problem, except for the most demanding experiments involving long spin-lock or decoupling sequences. Thus, excellent NMR spectra can easily be obtained for proteins

TABLE 1
EXPERIMENTAL AND PREDICTED[a] $^3J_{H^NH^\alpha}$ COUPLING CONSTANTS OF THE PROTEOGENIC AMINO ACIDS IN THE CONTEXT OF A GGXGG PEPTIDE IN 6 M GuHCl AT 20 °C, pH 5.0

Residue	$^3J_{H^NH^\alpha}$ (Hz)	
	Experimental	Predicted
Ala	6.1	6.1
Arg	6.9	7.2
Asn	7.7	7.4
Asp	7.8	7.2
Cys (ox)	7.7	–
Cys (red)	7.3	6.8
Gln	7.1	7.1
Glu	6.7	6.8
His	7.8	7.2
Ile	7.1	7.6
Leu	6.8	7.1
Lys	7.0	7.1
Met	7.1	7.3
Phe	7.3	7.5
Ser	7.0	6.7
Thr	7.9	7.6
Trp	7.0	6.9
Tyr	7.8	7.3
Val	7.2	7.7

[a] Predicted random coil values from Smith et al. (1996). These values are based on the distribution of ϕ angles in the protein data base and represent an average over all the different adjacent amino acids, rather than for residues with adjacent glycines.

and peptides, even in highly concentrated GuHCl solutions (Fig. 1).

The effects of GuHCl on peptide conformation

Disordered conformations of proteins have traditionally been studied under conditions that cause proteins to remain unfolded, such as in the presence of chemical denaturants, in a manner assumed to produce a distribution of conformations similar to that populated by unfolded polypeptides under more physiologically relevant solvent conditions (Tanford, 1968). NMR evidence suggests that different denaturants induce different conformational distributions (Arcus et al., 1995; Kotik et al., 1995), which may be a result of differential effects on the intrinsic backbone conformational preferences, although this in turn may be due to differential disruption of cooperative side-chain interactions. We have investigated the effect of GuHCl on probes of the conformational preferences of ϕ, ψ and the proline ω dihedral angles.

Specific NMR probes exist for both the ϕ and ω dihedral angles of the peptide backbone. The $^3J_{H^NH^\alpha}$ coupling constant is sensitive to the dihedral angle ϕ and thus its measurement provides a convenient probe of the distribution of conformations about the N-C^α bond. The coupling constants observed for three representative GGXGG peptides (where X represents lysine, glutamate or valine) exhibit coupling constants that are identical (to within <0.2 Hz) when measured in 0 or 6 M GuHCl. The peptide bond dihedral angle, ω, lacks significant conformational freedom except in the context of X-Pro peptide bonds. Proline cis–trans isomerization ratios, however, also remain unchanged between 0 and 8 M GuHCl (at $11.9 \pm 1.5\%$ cis). These data suggest that GuHCl does not perturb the intrinsic conformational preferences about either the ϕ or ω dihedral angles.

Sequential and intraresidue H^N-H^α ROE cross peak intensities can provide an indirect probe of the distribution of conformations about the three backbone angles ϕ, ψ and ω (e.g. Fiebig et al., 1996). Analysis of the ROE cross peak patterns of representative peptides (containing lysine, glutamate and valine) in 0 and 6 M GuHCl indicates no significant change in the normalized volumes of the sequential and intraresidue H^α-H^N (Fig. 2) or H^β-H^N (data not shown) cross peaks. This demonstrates that neither ϕ nor ψ (nor χ_1) angle preferences are altered by the presence of the denaturant. Thus, $^3J_{H^NH^\alpha}$ coupling constants, proline cis–trans ratios and sequential ROE data all suggest that GuHCl does not perturb the distribution of random coil conformations adopted by unfolded polypeptides.

In recent years, several models have been developed of the distribution of conformations that comprise the random coil state (Serrano, 1995; Smith et al., 1996). The lack of a consistent compilation of random coil $^3J_{H^NH^\alpha}$ coupling constants (see the discussion in Merutka et al., 1995) has limited tests of these models to the comparison of predicted coupling constants and NOE patterns with data compiled from chemically denatured proteins and small peptides (Serrano et al., 1995; Fiebig et al., 1996; Smith et al., 1996). A set of $^3J_{H^NH^\alpha}$ coupling constants for GGXGG peptides in 6 M GuHCl are presented in Table

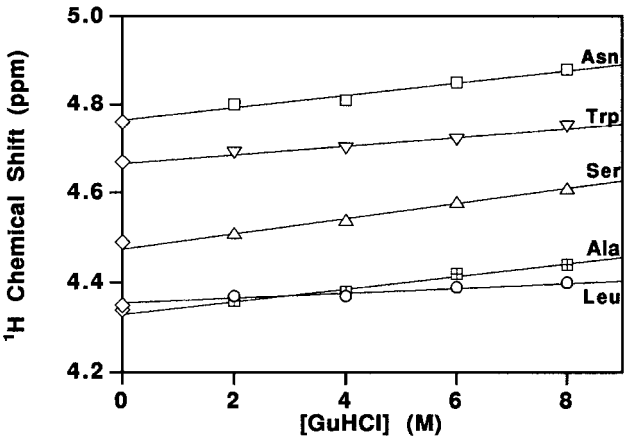

Fig. 3. The effect of GuHCl on the chemical shifts of a representative set of H^α resonances. The data are well fitted by linear functions and extrapolation of these fits to 0 M GuHCl predicts values very similar to random coil values previously published for a similar peptide series and indicated with diamonds (Merutka et al., 1995). Residue names are denoted with the standard three-letter code.

TABLE 2
PROTON CHEMICAL SHIFT PARAMETERS AS A FUNCTION OF GuHCl CONCENTRATION AT 20 °C, pH 5.0

Residue	Resonance	Experimental 6.0 M GuHCl (ppm)	Extrapolated 0.0 M GuHCl (ppm)	Δδ/Δ[GuHCl] (ppb M^{-1})	Residue	Resonance	Experimental 6.0 M GuHCl (ppm)	Extrapolated 0.0 M GuHCl (ppm)	Δδ/Δ[GuHCl] (ppb M^{-1})
Ala	H^N	8.32	8.35	−5.0	Leu	H^N	8.24	8.27	−5.0
	H^α	4.42	4.33	14.0		H^α	4.39	4.36	5.5
	H^β	1.45	1.45	0.5		H_2-β	1.63	1.63	0.0
Arg	H^N	8.34	8.34	−3.5		H^γ	1.63	1.63	0.0
	H^α	4.43	4.36	12.0		CH_3-δ	0.88	0.88	0.0
	H^β	1.84	1.82	3.5		CH_3-δ	0.94	0.93	2.0
	H^β	1.92	1.93	2.0	Lys	H^N	8.31	8.35	−5.0
	CH_2-γ	1.67	1.67	2.0		H^α	4.39	4.32	13.5
	CH_2-δ	3.25	3.22	6.5		H^β	1.73	1.80	3.0
	NH-ε	7.23	7.18	8.5		H^β	1.90	1.89	0.5
Asn	H^N	8.46	8.50	−5.0		CH_2-γ	1.46	1.45	2.0
	H^α	4.85	4.77	14.0		CH_2-δ	1.73	1.69	7.0
	H^β	2.85	2.80	10.0		CH_2-ε	3.04	3.01	5.0
	H^β	2.94	2.88	10.5	Met	H^N	8.34	8.40	−6.5
	NH_2-γ	7.00	6.92	14.0		H^α	4.53	4.56	5.0
	NH_2-γ	7.60	7.63	−3.5		H^β	2.08	2.05	0.5
Asp	H^N	8.39	8.40	−2.0		H^β	2.15	2.14	0.5
	H^α	4.74	4.63	15.5		CH-γ	2.58	2.56	3.5
	H_2-β	2.76	2.70	10.5		CH-γ	2.66	2.65	2.0
Cys	H^N	8.38	8.43	−8.0		CH_3-ε	2.12	2.11	3.0
(reduced)	H^α	4.64	4.55	15.5	Phe	H^N	8.26	8.25	1.5
	H^β	3.01	2.98	5.5		H^α	4.68	4.59	16.5
	H^β	3.04	2.96	12.5		H^β	3.06	3.06	1.5
Cys	H^N	8.49	8.53	−5.0		H^β	3.23	3.19	6.5
(oxidized)	H^α	4.78	4.64	15.0		C(2,6)H	7.30	7.29	3.0
	H^β	3.09	3.02	13.0		C(3,5)H	7.39	7.39	0.0
	H^β	3.34	3.29	7.5		C(4)H	7.34	7.34	0.0
Gln	H^N	8.41	8.44	−5.0	Pro(*trans*)	H^α	4.49	4.46	6.5
	H^α	4.42	4.35	12.0		H_2-β	2.32	2.31	1.5
	H^β	2.05	2.02	4.0		CH_2-γ	2.06	2.06	0.0
	H^β	2.19	2.18	2.0		CH-δ	3.67	3.65	3.0
	CH_2-γ	2.43	2.38	8.5		CH-δ	3.72	3.68	5.0
	NH_2-δ	6.91	6.85	10.5	Pro(*cis*)	H^α	4.71	4.65	11.5
	NH_2-δ	7.52	7.57	−8.0		H_2-β	2.42	2.41	1.5
Glu	H^N	8.43	8.50	−9.0		CH_2-γ	1.73	1.76	−3.5
	H^α	4.39	4.31	12.5		CH_2-δ	3.54	3.53	1.5
	H^β	2.00	1.97	4.0	Ser	H^N	8.37	8.40	−4.5
	H^β	2.13	2.11	5.0		H^α	4.58	4.48	17.0
	CH_2-γ	2.35	2.31	7.5		H^β	3.93	3.89	7.0
Gly	H^N	8.40	8.43	−3.5		H^β	3.95	3.91	7.0
	H_2-α	4.08	4.00	13.5	Thr	H^N	8.17	8.22	−8.0
His	H^N	8.52	8.52	−3.0		H^α	4.49	4.35	25.0
	H^α	4.86	4.75	18.5		H^β	4.37	4.32	8.5
	H^β	3.26	3.20	10.0		CH_3-γ	1.25	1.21	5.5
	H^β	3.41	3.35	10.0	Trp	H^N	8.16	8.16	0.5
	C(2)H	8.70	8.59	18.5		H^α	4.72	4.67	10.0
	C(4)H	7.40	7.32	13.5		H^β	3.28	3.26	3.5
Ile	H^N	8.09	8.14	−6.5		H^β	3.38	3.35	6.5
	H^α	4.24	4.19	11.5		C(2)H	7.32	7.31	3.0
	H^β	1.92	1.92	0.0		C(4)H	7.66	7.66	0.0
	CH-γ	1.21	1.20	2.0		C(5)H	7.18	7.18	0.0
	CH-γ	1.49	1.47	3.5		C(6)H	7.26	7.26	0.0
	CH_3-δ	0.88	0.88	0.0		C(7)H	7.54	7.53	2.0
	CH_3-γ	0.94	0.93	2.0		N(1)H	10.08	10.17	−13.0

The first column is the experimentally observed chemical shift value for the various protons in 6.0 M GuHCl. The second column contains chemical shift values extrapolated to 0 M GuHCl. These do not differ significantly from experimentally observed 'random coil' values in water (see text). The third term denotes the change in chemical shift as a function of the concentration of GuHCl and can be used in conjunction with column 2 to calculate chemical shifts at any arbitrary GuHCl concentration. Two values for geminal protons are included where these were resolved (for example the H^β protons of tyrosine); where the two values could not be resolved (due to overlap or because of the complexity of the multiplets involved) a single value estimated to correspond to the center of the multiplet is reported. Residues are denoted using the standard three-letter code and the atom nomenclature is from Wüthrich (1986).

TABLE 2
(continued)

Residue	Resonance	Experimental 6.0 M GuHCl (ppm)	Extrapolated 0.0 M GuHCl (ppm)	Δδ/Δ[GuHCl] (ppb M^{-1})	Residue	Resonance	Experimental 6.0 M GuHCl (ppm)	Extrapolated 0.0 M GuHCl (ppm)	Δδ/Δ[GuHCl] (ppb M^{-1})
Tyr	H^N	8.22	8.22	−1.0	Val	H^N	8.06	8.14	−10.0
	H^α	4.62	4.52	16.0		H^α	4.22	4.13	14.0
	H^β	2.99	2.98	3.5		H^β	2.16	2.15	2.0
	H^β	3.14	3.10	7.0		CH_3-γ	0.96	0.95	1.5
	C(2,6)H	7.18	7.15	5.0		CH_3-γ	0.97	0.96	2.5
	C(3,5)H	6.88	6.87	1.5					

1. The equivalence observed for the coupling constants of peptides in 0 and 6 M GuHCl (see above) suggests that this compilation might provide a valuable data set of intrinsic ϕ distributions for comparison with the predictions of these models.

The effects of GuHCl on ^1H chemical shifts

GuHCl alters the chemical shifts of H^α, amide proton and some side-chain resonances (Fig. 1). The induced changes in chemical shift are a linear function of GuHCl concentration over the 2 to 8 M range observed, with a median linear correlation coefficient, r^2, of 0.90 (Fig. 3). Extrapolation of the chemical shifts of carbon-bound hydrogens to 0 M GuHCl (Fig. 3) predicts aqueous shifts correct to within ≤0.01 ppm for representative peptides studied at 0 M GuHCl (including lysine, glutamate and valine) and to ≤0.04 ppm of chemical shifts previously published for a related peptide series lacking terminal blocking groups (Merutka et al., 1995). The linearity of this behaviour allows us to report correction terms for the prediction of random coil chemical shifts at any arbitrary GuHCl concentration (Table 2).

The chemical shifts of H^α resonances are significantly perturbed by GuHCl and exhibit a mean Δδ of 13 ± 5 ppb M^{-1} (Fig. 4). The threonine H^α resonance moves 0.2 ppm upfield in 8 M GuHCl, and most other H^α resonances exhibit shifts of ≈0.1 ppm. These chemical shift changes are large enough to distort chemical shift-based conformational analysis, as a consistent deviation of ≥0.1 ppm from previously established random coil values is considered evidence of secondary structure formation (e.g. Wishart et al., 1992). Presumably, use of the data compiled here should significantly improve the accuracy of such analytical techniques when applied to polypeptides in GuHCl-containing solutions and may aid in the assignment of the spectra of GuHCl-denatured proteins.

Amide proton chemical shifts are also significantly altered by the presence of GuHCl. Most H^N proton chemical shifts move downfield upon the addition of the denaturant (mean Δδ = −5 ± 4 ppb M^{-1}) (Fig. 4) except for phenylalanine and tryptophan, both of which exhibit small positive Δδ values. Unlike hydrogens bound to carbon, the GuHCl perturbed chemical shifts of amide protons do not extrapolate to 0 M to give values consistent with previously published aqueous chemical shifts, even after the appropriate temperature corrections are applied (Merutka et al., 1995). Several test cases (including lysine, glutamate and valine), however, indicate that the H^N chemical shifts at 0 M GuHCl of the protected pentapeptides used in this study differ significantly from the chemical shifts reported previously for amide protons in a related free amino/carboxylate peptide series (Merutka et al., 1995). Values obtained for these test cases are all within 0.01 ppm of amide proton chemical shifts extrapolated to 0 M GuHCl.

Most of the ^1H resonances of amino acid side chains are only slightly altered by the presence of GuHCl. In 8 M GuHCl the observed changes in the chemical shifts of carbon-bound hydrogens range up to 0.15 ppm, but in many cases GuHCl does not alter chemical shifts at all. Some of the largest perturbations in side-chain chemical shifts are for ^1H atoms bonded to carbons adjacent to side-chain carbonyls (in aspartate, glutamate, asparagine and glutamine). These resonances exhibit GuHCl sensitivities very similar to H^α resonances that are also adjacent

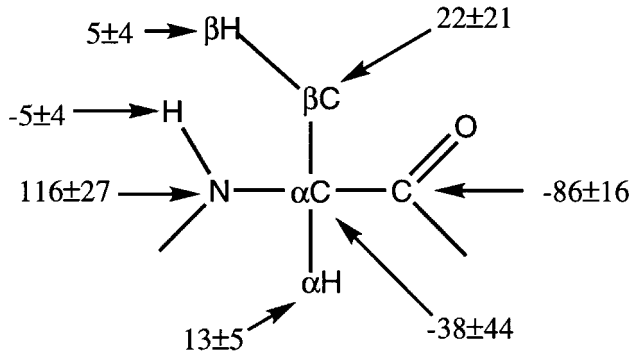

Fig. 4. The mean sensitivity of peptide resonances to GuHCl-induced chemical shift perturbations. All measurements are in ppb M^{-1}. The ^1H values reported represent the mean and standard deviation of the values of all 20 proteogenic amino acids. The ^{13}C and ^{15}N values represent the mean and standard deviation of a representative subset of seven amino acids (see text). The pattern of sensitivities suggests that the effects of GuHCl on chemical shift may be due to a mixture of charge induction effects and paramagnetic neighbouring group effects driven by a GuHCl-induced change in the hydrogen bonding of water.

TABLE 3
CHEMICAL SHIFT GuHCl CORRECTION FACTORS FOR THE ^{13}C AND ^{15}N RESONANCES OF SELECTED MEMBERS OF THE PEPTIDE SERIES Ac-GGXGG-NH_2, MEASURED AT 20 °C, pH 5.0

Residue	Resonance	Δδ/Δ[GuHCl] (ppb M^{-1})	Residue	Resonance	Δδ/Δ[GuHCl] (ppb M^{-1})
Ala	N	125		C^{γ}	−10
	C	−114		C^{δ}	2
	C^{α}	−41		C^{δ}	5
	C^{β}	45	Lys	N	121
Arg	N	137		C	−67
	C	−70		C^{α}	17
	C^{α}	−5		C^{β}	10
	C^{β}	10		C^{γ}	37
	C^{γ}	22		C^{δ}	7
	C^{δ}	37		C^{ε}	13
	N^{ε}	233	Thr	N	131
	C^{ζ}	−73		C	−67
Glu	N	118		C^{α}	−53
	C	−77		C^{β}	27
	C^{α}	−32		C^{γ}	17
	C^{β}	58	Tyr	N	127
	C^{γ}	98		C	−90
	C^{δ}	73		C^{α}	−27
Ile	N	101		C^{β}	33
	C	−100		C(1)	−23
	C^{α}	−27		C(2,6)	−30
	C^{β}	10		C(3,5)	−27
	$C(H_2)$-γ	10		C(4)	−37
	$C(H_3)$-γ	−7	Val	N	50
	C^{δ}	30		C	−93
Leu	N	134		C^{α}	−42
	C	−93		C^{β}	8
	C^{α}	−33		C^{γ}	13
	C^{β}	−6		C^{γ}	13

to carbonyl carbons. Relatively large shifts are also observed for one of the two β hydrogens of the aromatic residues. Oddly, the second β hydrogen is significantly less sensitive to GuHCl than the downfield proton. A similar dispersion of geminal hydrogens is observed for the H^{α} resonances of the fourth glycine residue in most of the peptides (Fig. 1). Side-chain hydrogens bound to carbons adjacent to nitrogen atoms and hydrogens directly bound to nitrogen are significantly more sensitive to the presence of GuHCl than most aliphatic hydrogens. The 4H and 2H ring protons of histidine, adjacent to one and two nitrogens respectively, exhibit the largest GuHCl sensitivities of any side-chain carbon-bound hydrogens (18.5 and 13.5 ppb M^{-1}, respectively). Both glutamine and asparagine contain one side-chain amide proton that shifts upfield upon addition of the denaturant ($\Delta\delta = 10.5$ and 13.5 ppb M^{-1}, respectively) and one that shifts downfield ($\Delta\delta = -8.0$ and −3.5 ppb M^{-1}, respectively). The two other slowly exchanging side-chain H^N protons observed in this study, the $N^{\varepsilon}H$ of arginine and the tryptophan indole H^N, also exhibit significant GuHCl-induced perturbations ($\Delta\delta = 8.5$ and −13.0 ppb M^{-1}, respectively). No clear correlation between the GuHCl sensitivity of a residue and properties such as secondary structure preference is apparent.

The effects of GuHCl on ^{13}C and ^{15}N chemical shifts

Values of the mean ^{13}C and ^{15}N GuHCl sensitivities are illustrated in Fig. 4. Peptide ^{15}N chemical shifts are more sensitive to GuHCl than carbon chemical shifts, but exhibit relatively little residue-specific dispersion. The ^{15}N resonances of the representative set of peptides we have observed exhibit a mean GuHCl sensitivity of 116 ± 27 ppb M^{-1} (Table 3). For the carbon resonances, the largest GuHCl sensitivity is exhibited by carbonyl carbons but, as with the ^{15}N resonances, these show relatively little residue-specific dispersion (mean $\Delta\delta = -86 \pm 16$ ppb M^{-1}, Table 3). The effect of GuHCl on aliphatic carbon chemical shifts is relatively small (Fig. 4) and exhibits a very strong residue dependency; the mean ^{13}C $\Delta\delta$ of a representative set of C^{α} resonances is -38 ± 44 ppb M^{-1}. Side-chain ^{13}C resonances are similarly robust to GuHCl but also display a marked residue dependency, with a mean C^{β} $\Delta\delta = 22 \pm 21$ ppb M^{-1}. Even the largest of the changes in C^{α} and C^{β} chemical shifts predicted for 8 M GuHCl solutions are substantially less than the 0.7 ppm changes rep-

resentative of secondary structure (Wishart et al., 1994a). These data suggest that carbon and nitrogen GuHCl chemical shift corrections are relatively less important than the corresponding proton correction factors.

The origins of GuHCl-induced denaturation

The mechanism by which GuHCl denatures proteins remains unknown. It has been postulated to involve either unspecific changes in the solvation properties of water or the binding of GuHCl to specific binding sites on the polypeptide backbone (Pace, 1986). Our data are most consistent with the first suggestion: in particular, the lack of apparent effects of GuHCl on the backbone conformation of the peptides studied here does not support the hypothesis of specific binding sites.

It might be suggested that the changes in chemical shifts which we observe are simply a consequence of the increased ionic strength of GuHCl solutions. However, the insensitivity of proton chemical shifts to the presence of high concentrations of sodium chloride (C.J. Morton and K.W. Plaxco, unpublished results) indicates that this is not the case. The pattern of chemical shift changes (Fig. 4) could reflect changes in hydrogen-bonding interactions between the carbonyl oxygen, the amide proton and surrounding water molecules. A reduction in hydrogen bonding should result in an increased electron density at the carbonyl carbon, together with an increase in the double bond character of the carbon–oxygen bond. An increased electron density at the carbonyl carbon is consistent with the reduction in chemical shifts observed for this resonance with increasing GuHCl concentration (Pople, 1957a,b). A chemical shift reduction of reduced magnitude but the same sign for C^{α} resonances may reflect inductive effects originating at the carbonyl carbon. Simultaneously, an increase in the carbonyl bond order should increase the size of the paramagnetic shift at the neighbouring H^{α}, H^{β} and C^{β} positions in a potentially stereospecific manner (Pople, 1957a,b), as is observed. A reduction in hydrogen bonding to the NH group will decrease electron density on the amide nitrogen and amide hydrogen. Consistent with this, the GuHCl sensitivity of the amide nitrogen chemical shift is relatively large and positive. The negative $\Delta\delta$ value observed for H^{N} resonances is also consistent with this model because, while in general complex, the chemical shifts of hydrogen-bonded protons often decrease with decreasing hydrogen bonding (Pople et al., 1959).

The large perturbations observed for hydrogens adjacent to side-chain carbonyl carbons are qualitatively consistent with the model discussed above and further suggest that the influence of GuHCl on chemical shifts is mediated through changes in the hydrogen-bonding potential of water. This effect may well play a role in the ability of GuHCl to unfold proteins, as a decrease in the hydrogen-bonding potential of water could significantly reduce the hydrophobic effects thought to be a major contributor to the folding of most proteins.

Conclusions

We have characterized the effects of the commonly used denaturant GuHCl on the conformational preferences of the proteogenic amino acids in an effort to improve the understanding of the relationship between GuHCl-induced denatured conformations and the conformations of proteins unfolded under more biologically relevant solvent conditions. In addition, we have determined the effects of GuHCl on the random coil chemical shifts of the proteogenic amino acids to aid the application of chemical shift-based analytical techniques to the characterization of denatured state conformations and we have also compiled a set of random coil $^{3}J_{H^{N}H^{\alpha}}$ coupling constants for comparison with the predictions of models of the random coil state. We anticipate that the results presented here will aid ongoing efforts to assign denatured state NMR spectra and to monitor the presence of residual structure in the denatured state and thereby improve our understanding of the role such structure might play in the thermodynamics and kinetics of protein folding.

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