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On the NMR analysis of pK_a values in the unfolded state of proteins by extrapolation to zero denaturant

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Abstract

Detailed knowledge of the pH-dependence in both folded and unfolded states of proteins is essential to understand the role of electrostatics in protein stability. The increasing number of natively disordered proteins constitutes an excellent source for the NMR analysis of pK_a values in the unfolded state of proteins. However, the tendency of many natively disordered proteins to aggregate via intermolecular hydrophobic clusters limits their NMR analysis over a wide pH range. To assess whether the pK_a values in natively disordered polypeptides can be extrapolated from NMR measurements in the presence of denaturants, the natively disordered backbone of the C-terminal fragment 75 to 105 of Human Thioredoxin was studied. First, assignments using triple resonance experiments were performed to confirm lack of secondary structure. Then the pH-dependence of the amides and carboxylate side chains of Glu residues (Glu88, Glu95, Glu98, and Glu103) in the pH range from 2.0 to 7.0 was monitored using 2D $^{1}H^{15}N$ HSQC and 3D C(CO)NH experiments, and the behavior of their amides and corresponding carboxyl groups was compared to confirm the absence of nonlocal interactions. Lastly, the effect of increasing dimethyl urea concentration on the pK_a values of these Glu residues was monitored. The results indicate that: (i) the dispersion in the pK_a of carboxyl groups and the pH midpoints of amides in Glu residues is about 0.5 pH units and 0.6 pH units, respectively; (ii) the backbone amides of the Glu residues exhibit pH midpoints which are within 0.2 pH units from those of their carboxylates; (iii) the addition of denaturant produces upshifts in the pK_a values of Glu residues that are nearly independent of their position in the sequence; and (iv) these upshifts show a nonlinear behavior in denaturant concentration, complicating the extrapolation to zero denaturant. Nevertheless, the relative ordering of the pK_a values of Glu residues is preserved over the whole range of denaturant concentrations indicating that measurements at high denaturant concentration (e.g. 4 M dimethyl urea) can yield a qualitatively correct ranking of the pK_a of these residues in natively disordered proteins whose pH-dependence cannot be monitored directly by NMR.

Keywords: NMR analysis; Denaturant; Unfolded state of proteins; pK_a values

1. Introduction

The large body of experimental and computational studies of well-structured proteins has provided extensive knowledge about the folded state, but information on the unfolded state is still limited. Central to the biophysical properties of proteins are hydrophobicity and electrostatics, but the latter is still incompletely understood. Although progress has been made by the recognition of ionizable groups on the surface of thermophilic proteins which confer high thermal stability, structure-based calculations of protein energetics [1,2] require more information about the unfolded protein state to explain the pH-dependence of protein stability [3,4].

A widely used model of the contribution of electrostatics to protein energetics predicts the pH-dependence of the ΔG for protein unfolding with the aid of the pK_a values of ionizable residues in both the folded and unfolded states [5,6]. A wealth of information has been accumulated on the pK_a values of ionizable residues in folded proteins via NMR [7–9] and mutagenesis analysis [10–16]. In contrast, NMR analysis of the pH-dependence of chemical shifts in the unfolded state of most well-structured proteins is challenging due to the inability to populate the unfolded state during the course of the pH-titration. This limitation has been overcome in a few cases: (a) unfolded protein segments [7,17,18], (b) the equilibrium mixture of the unfolded and folded states of an unstable protein [19], and (c)

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the unfolded monomers of a folded heterodimer [17,20]. In other cases, the pK_a values of ionizable residues in the unfolded protein have been indirectly determined using the pK_a values in the folded state, the pH-dependence of the ΔG for protein unfolding, and the "zero interaction model" [18,21–23]. In summary, few reports are available in the literature on the pK_a values of ionizable residues in unfolded protein states. Among them are the unfolded state of the N-terminal SH3 domain of the drk protein, which exchanges slowly with its folded state [19,24] and exhibits pK_a values for Asp (3.75 to 4.13) and Glu (4.08 to 4.45) with dispersions of 0.38 and 0.37 pH units, respectively [19,25]; the unfolded monomers of the heterodimeric Leucine zipper which show pK_a values for Glu (4.55 to 4.76) with a dispersion of 0.21 pH units [20,26]; the short natively disordered segments of the N-terminal domain of the ribosomal protein L9 [18,27] which exhibit pK_a values for Asp and Glu with dispersions of up to 0.38 and 0.52 pH units, respectively; and one of the large unfolded monomers of reassembled *E. coli* thioredoxin (ETrx) that shows pK_a values for Asp (3.77 to 3.94) and Glu (4.08 to 4.60) with dispersions of 0.17 and 0.52 pH units, respectively [17]. The accumulated results indicate that the pK_a values of Asp and Glu in the unfolded state of proteins show a dispersion of up to 0.5 pH units and that these values are within 0.5 pH units from those observed in host–guest tetrapeptides [28,29]. Whether this dispersion results from local and/or nonlocal interactions remains unclear and requires more mutagenesis analysis, as done for the N-terminal SH3 domain of drk [19,25].

Considering the increasing number of natively disordered proteins originated by the current proteomics efforts, it would be interesting to know whether the dispersion in the pK_a values of Asp and Glu residues can be greater than 0.5 pH units as a result of nonlocal interactions. Recent reports [17,30] suggest that medium range nonlocal interactions within the highly charged segment of a natively disordered C-fragment of ETrx produce significant discrepancies between the pH midpoints (pH_m) of the backbone amides of Asp and Glu and the pK_a of their own carboxylate side chains. In contrast, the moderately charged segment of the same fragment shows negligible discrepancies and provides no evidence for the presence of nonlocal interactions [17].

A complication in any attempt to enlarge the database of pK_a values in natively disordered proteins by NMR analysis arises from the tendency of amphipatic segments to aggregate via intermolecular hydrophobic clusters in certain pH range. To counteract this aggregating tendency we propose to use chemical denaturants in an attempt to increase the population of the monomeric state and obtain the pK_a values by an extrapolation to zero denaturant. To gain confidence that this proposal is feasible, we decided to start with the natively disordered C-terminal fragment encompassing residues 75 to 105 of Human Thioredoxin (HC74), a control system for which the pK_a values can be measured in the presence and absence of denaturants such as dimethyl urea (DMU). Our results indicate that in fragments in which nonlocal interactions are insignificant: (i) the addition of DMU produces upshifts in the pK_a values of Glu residues that are nearly independent of their position in the sequence, and consistent with previous reports [31–33]; and (ii) the upshifts in the pK_a values are not proportional to the DMU concentration, complicating the extrapolation to zero DMU; nevertheless, the relative ordering of the pK_a values of Glu residues is preserved over the whole range of DMU concentrations.

2. Experimental

2.1. NMR analysis of fragment HC74

The fragment HC74 was generated using ^{15}N–^{13}C-labeled HTrx and previously reported expression, isolation, CNBr cleavage and purification procedures [34–36]. pH measurements were performed with an Accumet combination electrode of 3 mm diameter and 1.5" flexible stem and used without correcting for either deuterium isotope effect [37] or denaturant effect. Titrations were conducted with solutions of HCl or NaOH. NMR experiments were performed on a Varian INOVA AS600 spectrometer with a triple resonance probe. The ^1H chemical shift was referenced to the temperature dependent H_2O frequency and corrected using sodium 2,2-dimethyl,2-silapentane,5-sulfonate (DSS) as an external reference. Since the addition of DMU in the strongly acidic regime affects the position of the water resonance, DSS samples were prepared at different pH s between pH 2 and 7 in the same buffer used for fragment HC74 that contains the same increasing concentrations of DMU (1 M, 2 M, 3 M and 4 M). The corresponding chemical shift corrections are available as Supplementary material. The ^{15}N and ^{13}C chemical shifts were indirectly referenced [38]. The temperature was calibrated using standard procedures with methanol [39]. A solution of 0.5 mM ^{15}N–^{13}C-labeled HC74 in 10 mM Potassium Phosphate buffer (KP_i), containing 10% D_2O and 4 M DMU at pH 6.44 and 25 °C was used to acquire the 3D HNCACB, C DIPSY, CBCACONH, and C(CO)NH [40] experiments with 512 (t_3), 38 (t_2), and 48 (t_1) complex points, spectral widths of 8000 (^1H), 1600 Hz (^{15}N), and 3400 Hz (^{13}C), and a variable number of transients (8 to 24). NMR data were processed and analyzed using NMRPipe [41] and SPARKY [42], respectively. The assignments of the backbone and side chain of fragment HC74 in the absence of DMU were obtained by transferring chemical shifts from high to low DMU concentrations (3 M, 2 M, 1 M and 0 M) via 2D $^1H^{15}N$ HSQC and 3D C(CO)NH spectra at pH 6.44 and 25 °C. The secondary chemical shifts of the amide proton (H^N), α-carbons (C^α) and amide nitrogen (N) of fragment HC74 in the absence of DMU were calculated using the random coil data base [43] and corrected for sequence [44] dependence.

2.2. pH-dependence of chemical shifts in the backbone amides and carboxylate side chains of fragment HC74

Solutions of 0.1 or 0.3 mM of fragment HC74 in 10 mM KP_i, 10% D_2O, and increasing DMU concentrations (0 M, 1 M, 2 M, 3 M, and 4 M) at pH 4.25 or pH 6.5 were titrated with solutions of HCl or NaOH between pH 2.0 and pH 7.0 at 25 °C to acquire NMR data. The pH-dependence in the chemical shifts of the

amides and $^{13}C^{\gamma}$ of Glu residues from ^{15}N–^{13}C-labeled fragment HC74 were monitored using 2D $^{1}H^{15}N$ HSQC and 3D C(CO)NH experiments, respectively. Acquisitions of the latter required 512 (t_3), 38 (t_2), and 48 (t_1) complex points, spectral widths of 8000 (^{1}H), 1600 Hz (^{15}N), and 3400 Hz (^{13}C), and a variable number of transients (8 to 24). The chemical shifts of fragment HC74 were corrected to account for the effect of DMU concentrations on the position of the water peak under strongly acidic conditions (see Supplementary material). Typically, these corrections produce upshifts in the pK_a values that amount to 0.01 and 0.03 pH units for 1 M DMU and 4 M DMU, respectively.

Data were fitted to a modified Hill equation [45]

$$\delta(\mathrm{pH}) = \frac{\delta_{\mathrm{base}} + \delta_{\mathrm{acid}} \times 10^{n(\mathrm{pH_m}-\mathrm{pH})}}{1 + 10^{n(\mathrm{pH_m}-\mathrm{pH})}}. \quad (1)$$

The acidic (δ_{acid}) and basic (δ_{base}) plateaus, Hill coefficient (n) and $\mathrm{pH_m}$ were left as floating parameters when fitting the amides pH-dependence. For the carboxyl side chains two separate fits were actually performed. In the first case all four parameters ($\mathrm{pH_m}$, n and the plateaus) were treated as free parameters and only the data in the absence of denaturant was used. In the second case, the plateaus were fixed to the values obtained from a "global" fit that considered all data, both in the absence and presence of DMU. The rationale for performing this second fit is that the chemical shifts of the $^{13}C^{\gamma}$ in both the protonated and unprotonated carboxyl group are thought to be insensitive to the aqueous organic solvent composition and thus the level of the plateaus should be independent of the DMU concentration.

3. Results

3.1. Secondary chemical shift analysis of fragment HC74

To determine the degree of disorder of fragment HC74 under nativelike conditions, the first step was to assign this fragment at high denaturant concentration and transfer the assignment to conditions with zero denaturant. The assignment of the amides was straightforward with the exception of some residues that exhibit similar chemical shifts (e.g.; F77 and K82, Q84 and E88, V86 and Q78, L104 and L97) or low signal/noise ratio (e.g., T76, G83, and S90 at pH close to 7). The second step was to determine the secondary chemical shifts (differences between the experimental values and those from a statistical random coil model) of the amide protons and α-carbons, which yielded absolute values of less than 0.3 ppm and 1.0 ppm, respectively (see Fig. 1). Inspection of these shifts reveals some segments (F77 to K81 and E95 to A99) where the secondary chemical shifts of the α-carbons are predominantly negative and consistent with a tendency to adopt β strands, while the secondary chemical shifts of the corresponding amide protons lack any particular preference. This lack of conformational preference is typical of natively disordered segments.

In summary, the secondary chemical shift analysis of fragment HC74 confirms its natively disordered state.

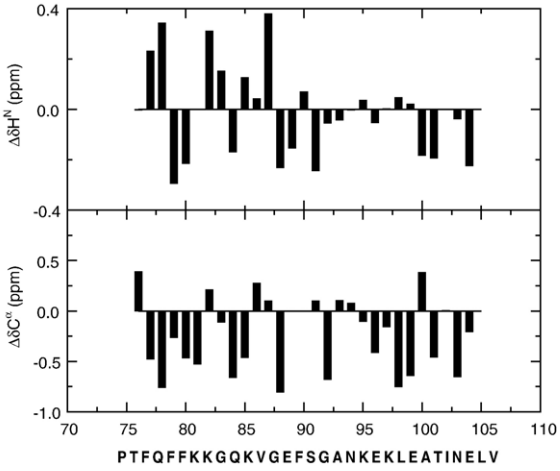

Fig. 1. Standard structural NMR analysis of the natively disordered HC74. The secondary chemical shifts of the H^N ($\Delta\delta\ H^N$) and C^{α} ($\Delta\delta\ C^{\alpha}$) are defined as the differences between their observed chemical shift and their corresponding random coil values. The $\Delta\delta\ H^N$ and $\Delta\delta\ C^{\alpha}$ are given at pH ≈ 6.44 and 25 °C in the top and bottom panel, respectively.

3.2. pH-dependence of amide chemical shifts in fragment HC74

Assessing the role of nonlocal interactions in natively disordered fragments using standard NMR methods is difficult. To assess their presence in the natively disordered fragment HC74, the pH-dependence of the amide chemical shifts of its ionizable and nonionizable residues were monitored in the acidic regime without the help of denaturants (see Fig. 2, Table 1 and Supplementary material) in search of correlations between the midpoint ($\mathrm{pH_m}$) values of Glu residues and its neighboring nonionizable residues [30].

In contrast to similar studies of natively disordered polypeptides [30], the pH-dependence of the backbone amides in the ionizable residues of HC74 is rather weak yielding differences between the chemical shift of the unprotonated and protonated Glu residues ($\Delta\delta$) which are rather close to the cutoff values (0.8 ppm for nitrogens and 0.08 ppm for the protons). In one case (amide proton of E88) the $\Delta\delta$ even falls below the signal-to-noise ratio suggesting unexpected weak interactions with its carboxyl group. With the exception of this residue, for which the fittings failed, analysis of the titration curves for Glu residues in terms of a modified Hill equation [45] (see Eq. (1)) yields $\mathrm{pH_m}$ values with significant dispersion (0.60 ± 0.10 pH units) in both amide nitrogens and protons. In all cases the Hill coefficients (n) are around 0.9 (within experimental uncertainties), revealing strong intra-residue interactions between the carboxyl groups and the corresponding amides.

Moreover, the titration curves of these amide protons, with the exception of E88, show the typical trend found for a Glu residue in model tetrapeptides [29]: the acidic plateau is lower than the basic, which has been previously interpreted [29] as evidence for the presence of H-bonds between the carboxyl group and the amide. As previously shown [30], natively disordered polypeptides encompassing Asp and/or Glu residues

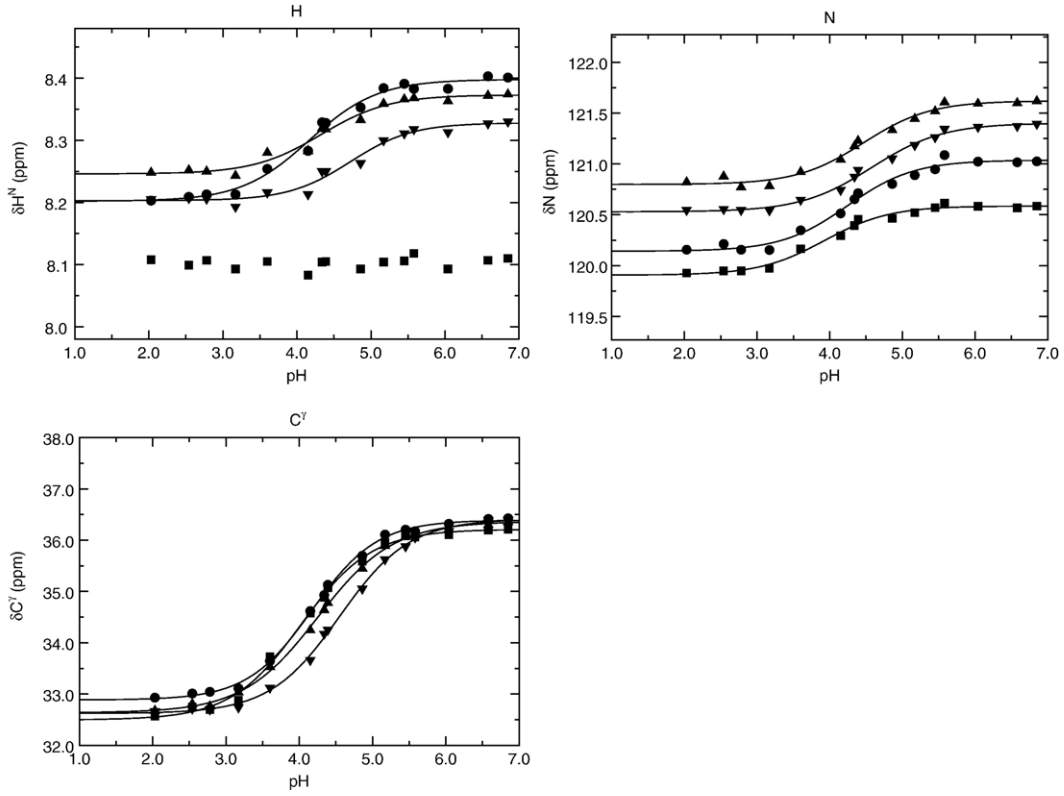

Fig. 2. pH-dependence in the chemical shifts of Glu residues from the natively disordered fragment HC74. pH-dependence in the chemical shifts of H^N (top left), amide N (top right), and $^{13}C^{\gamma}$ (bottom) for E88 (squares); E95 (circles); E98 (up triangles); E103 (down triangles). The solid lines show the results of the nonlinear fittings to the modified Hill equation.

may also show pH-dependent nonionizable residues which constitute candidates for local and/or nonlocal interactions with the ionizable residues. In this case, the amides of ten nonionizable residues surrounding E88, E95, E98 and E103 (K85, V86, K96, L97, T100, I101, N102, L104) are very weakly pH-dependent; only the C-terminal V105 shows a strong dependence (see Supplementary material for fits to the Hill equation for selected residues). Therefore is not possible to distinguish the effect of short-, medium- or long-range interactions.

In summary, there is no evidence for strong nonlocal interactions involving the Glu residues and nonionizable residues. The weak pH-dependencies observed in the latter are more plausibly explained by local interactions with nearby Glu residues.

Table 1
Fitting parameters for Glu residues in the natively disordered fragment HC74 (see Eq. (1))

Residue	pH_m	n	$\Delta\delta$	pH_m (const)
E88 N	3.93±0.12	0.97±0.22	0.70±0.1	
E88 C^{γ}	4.02±0.05	0.91±0.10	3.76±0.2	4.06±0.01
E95 H	4.17±0.10	0.94±0.21	0.21±0.1	
E95 N	4.25±0.11	0.96±0.25	0.90±0.1	
E95C^{γ}	4.18±0.03	0.98±0.08	3.50±0.1	4.20±0.01
E98 H	4.33±0.14	0.95±0.33	0.13±0.1	
E98 N	4.47±0.11	0.95±0.23	0.80±0.1	
E98C^{γ}	4.25±0.03	0.85±0.06	3.74±0.1	4.31±0.01
E103 H	4.72±0.15	1.04±0.35	0.12±0.1	
E103 N	4.58±0.08	0.89±0.14	0.86±0.1	
E103 C^{γ}	4.55±0.04	0.91±0.07	3.81±0.1	4.61±0.01

The pH_m, n and $\Delta\delta$ parameters are the result of fitting to the data in the absence of denaturant, with all parameters left floating. The last column gives the pH_m values from a fitting to the same data but constraining the plateaus to have the same values for all DMU concentrations.

3.3. pH-dependence of carboxylate side chain chemical shifts in fragment HC74

To assess whether the pH-dependence of the backbone amides of Glu residues reflect that of their corresponding carboxylates as expected in a natively disordered segment where nonlocal interactions do not play a significant role [17], the chemical shifts of its Glu ($^{13}C^{\gamma}$) residues were monitored in the pH range from 2 to 7 using 2D $^{1}H^{15}N$ HSQC and 3D C(CO)NH experiments in the absence of chemical denaturants. The titration curves show a simple sigmoidal dependence (see Fig. 2) and were again fitted to Eq. (1), see Table 1. These analysis indicate a dispersion in the pK_a values of 0.54±0.10 pH units, although they are within 0.3 pH units of those found in model tetrapeptides [29] (see Fig. 3). For the carboxylates two separate fits were actually performed (see Experimental): one unconstrained (all parameters free) and one constraining the plateaus to be the same for all concentrations of denaturant. The results of the latter fit, which differ from the unconstrained fit by

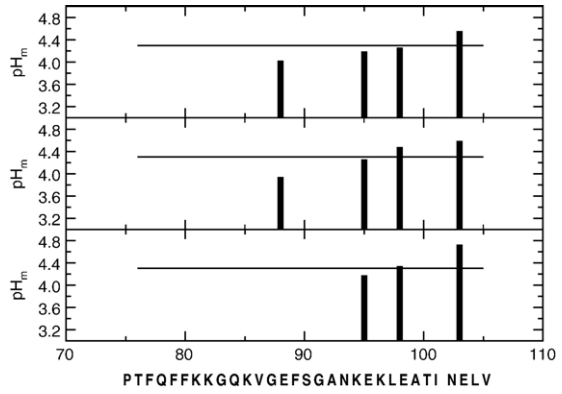

Fig. 3. pH-dependence in the carboxylate side chains and backbone amides of Glu residues from the natively disordered fragment HC74. Top: pH_m of the carboxylate side chains of Glu shown by vertical bars. Middle: pH_m of backbone amide nitrogen of Glu shown by vertical bars. Bottom: pH_m of backbone amide proton of Glu shown by vertical bars. The pK_a of Glu in model host–guest tetrapeptides [28] are shown by solid horizontal lines. The sequence of HC74 is shown underneath the bottom panel.

about 0.06 pH units, are shown in the rightmost column in Table 1 and will be used in the following discussions.

In summary, the observed differences between the pH_m of the amide (proton and/or nitrogen) of Glu residues and its corresponding carboxylate's pK_a are small, further confirming the lack of significant nonlocal interactions.

3.4. Denaturant effect on the pH-dependence of carboxylate side chain chemical shifts in fragment HC74

To determine whether the pK_a values of carboxylate side chains from Glu residues can be extrapolated from their values in the presence of denaturants, dimethyl urea (DMU) was selected as a denaturant instead of the more commonly used Guanidine HCl or urea for two reasons: (i) DMU is chemically less reactive than urea and less prone to modify amino and imino groups under strongly acidic conditions; and (ii) DMU is expected to have less effect than guanidine HCl on charge screening.

The chemical shifts of the $^{13}C^{\gamma}$ of the carboxylate were easily monitored for E88, E98, and E103; however, monitoring E95 was more complicated due to overlapped cross-peaks throughout the covered pH range in the C(CO)NH experiments, which become more pronounced as the DMU concentration increases (see Supplementary material). The resulting titration curves display simple sigmoidal pH-dependence (see Fig. 4) and they were analyzed using the modified Hill equation (Eq. (1)) just as the data in the absence of DMU.

Analysis of the constrained (to same plateaus) fitting of the family of titration curves for each individual Glu residue at increasing DMU concentration (see Table 2) indicates upshifts in the apparent pK_a values of Glu residues (about 0.3 to 0.5 pH units at 4 M DMU) and complex effects on the Hill coefficients

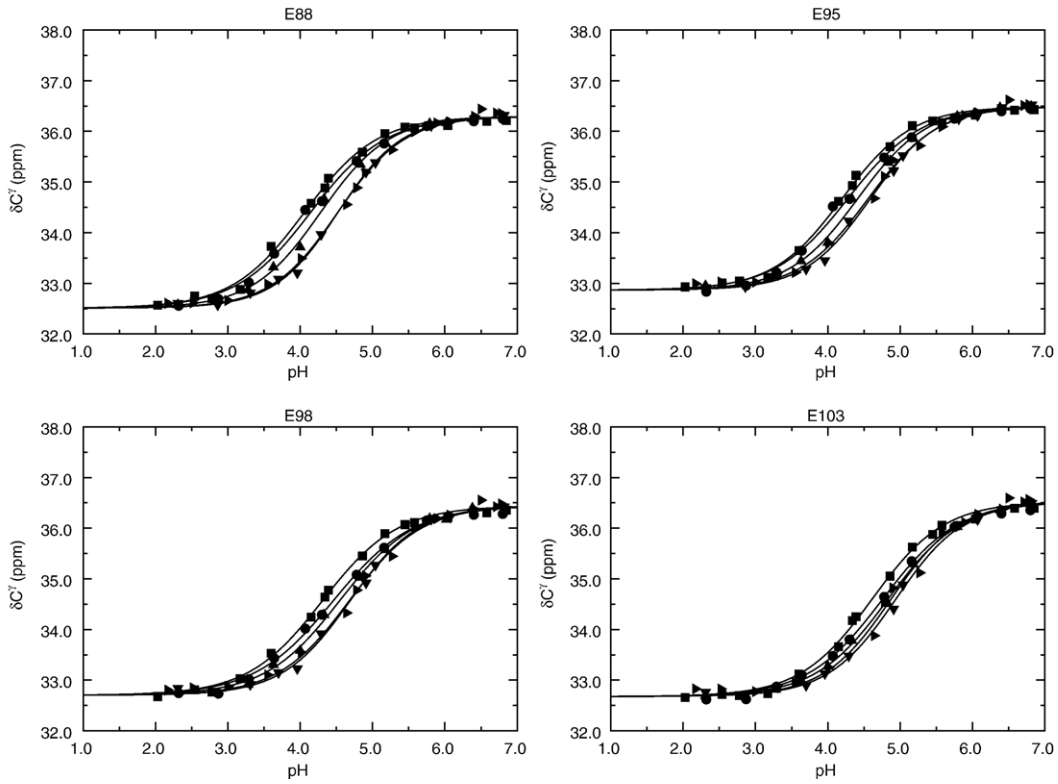

Fig. 4. Effect of DMU on the pH-dependence in the carboxylate side chains of Glu residues from the natively disordered fragment HC74. The pH-dependence of the chemical shifts of $^{13}C^{\gamma}$ of E88 (left top panel); E95 (right top panel); E98 (bottom left panel); E103 (bottom right panel) is depicted at zero (squares), 1 M (circles), 2 M (up triangles), 3 M (down triangles), and 4 M (right triangles) DMU concentrations. The solid lines show the result of the nonlinear fittings to the modified Hill equation, constraining the plateaus to be independent of the concentration of denaturant.

Table 2
Fitting parameters for Glu residues in the natively disordered fragment HC74 under increasing DMU concentrations

	E88		E95		E98		E103	
	pK_a	n [a]	pK_a	n [a]	pK_a	n [a]	pK_a	n [a]
0 M	4.06±0.01	0.87	4.20±0.01	0.91	4.31±0.01	0.84	4.61±0.01	0.88
1 M	4.15±0.01	0.84	4.28±0.01	0.86	4.46±0.01	0.80	4.76±0.01	0.84
△(1, 0)	0.09±0.02		0.08±0.02		0.15±0.02		0.15±0.02	
2 M	4.28±0.01	0.98	4.42±0.01	0.92	4.55±0.01	0.84	4.83±0.01	0.87
△(2, 1)	0.13±0.02		0.14±0.02		0.09±0.02		0.07±0.02	
3 M	4.52±0.01	0.94	4.59±0.01	0.95	4.69±0.01	0.93	4.94±0.03	0.93
△(3, 2)	0.24±0.02		0.17±0.02		0.14±0.02		0.11±0.02	
4 M	4.52±0.01	0.95	4.56±0.01	0.92	4.68±0.01	0.88	4.88±0.01	0.93
△(4, 3)	0.00±0.02		−0.03±0.02		−0.01±0.02		−0.06±0.02	

The fittings were obtained by constraining the plateaus to have the same values for all DMU concentrations. The △(i, j) columns show the difference between the pK_a values at jM and iM DMU; if the dependence of △(i, j) on the concentration of DMU were linear all of them should have the same value.
[a] Uncertainties are ±0.01.

(oscillating between 0.80 and 0.98). Somewhat unexpectedly (see Table 2), the upshifts depend on the particular Glu residue, although this dependency is small, and seem to reach saturation between 3 and 4 M DMU.

In summary, the upshifts in the apparent pK_a values of Glu produced by DMU as denaturant are nonlinear in the DMU concentration and complicate the extrapolation to zero DMU concentration.

4. Discussion

4.1. pH-dependence in the carboxylate side chains and backbone amides of Glu residues in fragment HC74

Previous studies [17,30] indicate that the signatures for nonlocal interactions in disordered polypeptides are: (i) significant differences (>0.15 pH units) between the pK_a of the carboxylates and the pH_m of their amides; and (ii) the presence of nonionizable neighbors with a strong pH-dependence. Fragment HC74 does not show either signature (see Table 1). First, the differences are slightly larger than 0.15 pH units, but the experimental uncertainties are of the same order, weakening their significance. These sizable uncertainties are partly due to the fact that the differences between the acidic and basic plateaus for the amides of E88, E95, E98, and E103 are too close to the cutoff values (0.8 ppm for nitrogens and 0.08 ppm for the protons). As for the second signature, the nonionizable neighbors of the Glu residues (K85, V86, S90, L97, T100, I101, N102, and L104) show at most a weak pH-dependence.

Although nonlocal interactions are ruled out, this weak pH-dependence might lead us to explain the dispersion of 0.54±0.10 pH units in the pK_a values of the Glu residues. In the case of E88, whose amide proton shows no pH-dependence and which has the lowest pK_a, it is possible that H-bond interactions between its carboxyl group and its corresponding amide are masked by interactions between the same carboxyl group and the long side chain of K85. An unambiguous determination of the interactions responsible for this dispersion might require mutagenesis analysis.

4.2. Effect of the denaturant on the pK_a values of Glu residues in fragment HC74

According to previous reports on pH measurements in mixed aqueous organic solvents using a glass electrode [31,32], the upshifted pK_a values obtained at high concentration of DMU need to be corrected for both the liquid potential of the electrode and the so-called "primary medium effect;" the latter refers to the expected proton–DMU interactions with the concomitant effect on the proton activity coefficient. Further support for this correction comes from a recent study on the denaturant effect on the pK_a of Glu in a natively disordered tetrapeptide [33]. Since this putative correction depends only on the nature of the electrode and the proton–denaturant interactions, changes in DMU concentration should (i) not affect the level of the chemical shift plateaus in the family of titration curves and just shift the titration curve along the pH axis, and (ii) produce shifts in the apparent pK_a values of Glu residues that are independent of its position in a polypeptide chain. Regarding the first point, unconstrained fittings for Glu residues of fragment HC74 that include the plateau levels as fitting parameters for each separate DMU concentration result in very slight variations (less than 3% of the difference between the acidic and basic plateaus). This confirms that the chemical shifts of $^{13}C^{\gamma}$ in its protonated and unprotonated states are rather insensitive to the denaturant concentration. As for the second point, Table 2 shows that the upshifts in the apparent pK_a values are dependent on the Glu's location, but the dependency is small enough to consider them nearly independent. Unfortunately, the unambiguous determination of the interactions (e.g., residue–DMU interactions) responsible for the small observed dependency requires a large data set. In this work, this small dependency is neglected and the upshift in the apparent pK_a value is considered nearly independent of the Glu's location.

The situation is more complicated for the amide nitrogens and protons. Further inspection of the effect caused by varying the DMU concentration on the chemical shifts for ionizable and nonionizable residues (see Supplementary material and Fig. 5), indicates unexpected shifts suggesting that more than just proton–DMU interactions are at play. First, the chemical shift

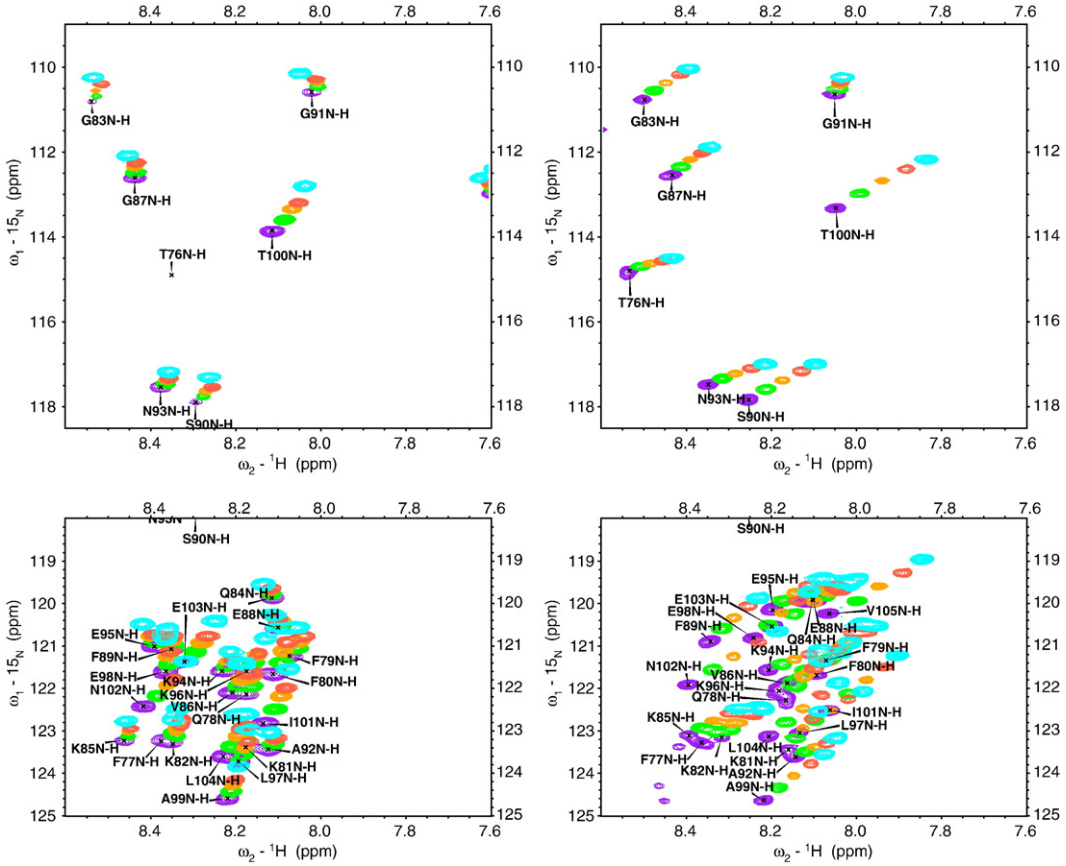

Fig. 5. DMU-dependence in the backbone amides of selected residues from the natively disordered fragment HC74. Top and bottom left: overlay of portions of $^1H^{15}N$ HSQC spectra at pH 6.44. Top and bottom right: overlay of portions of $^1H^{15}N$ HSQC spectra at pH 2.0. The panels show the cross-peaks from uncorrected $^1H^{15}N$ HSQC spectra at zero (purple), 1 M (green), 2 M (orange), 3 M (red), and 4 M (cyan) DMU concentrations (see methodology). The labeled cross-peaks correspond to $^1H^{15}N$ HSQC spectra at zero DMU concentration.

plateaus of the titration curves for the amides of ionizable residues show a strong dependency on the concentration of DMU. This effect is quite pronounced, especially when considered as a percentage of the difference between the basic and acidic regime plateaus, reaching values of up to 150% (compared with just 2–3% for the carboxylates). Second, the behavior of the chemical shifts amides of nonionizable residues upon changes in the concentration of DMU depends on the residue's identity and location in the sequence (e.g.; G87 versus T100 or A99 versus N102; and G87 versus G91) as shown in Fig. 5.

The strong dependency of the plateaus of the amide's titration curves on the DMU concentration suggests interactions between the amides and the denaturant (possibly H-bonds), while the fact that there is a pH-dependence at all reflects interactions between these amides and their corresponding carboxyl groups, presumably through H-bonds [29]. It is unclear which of these, if any, predominates, since the changes in the amide's acidic and basic plateaus are of the same order of magnitude as the difference between them. In contrast, the acidic and basic plateaus of the carboxylate side chains show a small dependence on denaturant concentration, suggesting low sensitivity to possible H-bond interactions between the carboxyl group and the denaturant.

Although the upshifts in the pK_a's of the Glu residues can be considered independent of their location, they are not linear in the DMU concentration (see Table 2), complicating the extrapolation from high to zero concentration of the denaturant. Nevertheless, these upshifts appear to reach saturation between 3 M and 4 M DMU and the addition of 4 M DMU produces upshifts in the apparent pK_a values of Glu of about 0.37±0.10 pH units. Interestingly, these upshifts are close to the ones found for a Glu residue in a host–guest tetrapeptide in an aqueous solution of 6 M urea[33].

5. Conclusions

The addition of denaturant (DMU) produces the expected upshifts in the pK_a values of Glu residues that are nearly independent of their position in the sequence. However, these upshifts are not linear in the DMU concentration and seem to reach saturation between 3 and 4 M DMU, complicating the extrapolation to zero DMU. This nonlinearity is in marked contrast with the behavior found in simpler systems [31–33], possibly reflecting the complexity of interactions among Glu residues, neighboring nonionizable residues, and the surrounding aqueous organic solvent.

Nevertheless, the relative ordering of the pK_a values of Glu residues is preserved over the whole range of DMU concentrations indicating that measurements at high denaturant

concentration (such as 4 M DMU) can yield a qualitatively correct ranking of these residues in natively disordered polypeptides. In summary, a correction of −0.37 pH units to the apparent pK_a values at 4 M DMU can provide estimates within 0.10 pH units.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2007.06.004.

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