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Apparent pK_a shifts of titratable residues at high denaturant concentration and the impact on protein stability

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Abstract

Urea and guanidine–hydrochloride (GdnHCl) are frequently used for protein denaturation in order to determine the Gibbs free energy of folding and kinetic folding/unfolding parameters. Constant pH value is applied in the folding/unfolding experiments at different denaturant concentrations and steady protonation state of titratable groups is assumed in the folded and unfolded protein, respectively. The apparent side-chain pK_a values of Asp, Glu, His and Lys in the absence and presence of 6 M urea and GdnHCl, respectively, have been determined by ^{1}H-NMR. pK_a values of all four residues are up-shifted by 0.3–0.5 pH units in presence of 6 M urea by comparison with pK_a values of the residues dissolved in water. In the presence of 6 M GdnHCl, pK_a values are down-shifted by 0.2–0.3 pH units in the case of acidic and up-shifted by 0.3–0.5 pH units in the case of basic residues. Shifted pK_a values in the presence of denaturant may have a pronounced effect on the outcome of the protein stability obtained from denaturant unfolding experiments.

Keywords: pK_a values; Protein folding; Protein stability; Urea; Guanidine–hydrochloride; ^{1}H-NMR

1. Introduction

Protein stabilities as well as folding/unfolding rate constants may be determined by linear extrapolation to zero molar denaturant of the energetic and kinetic parameters derived for a series of denaturant concentrations at constant pH [1]. Thereby, proteins are assumed to follow a two-state folding mechanism, hence, only the folded (N) and unfolded (U) form of the protein coexist. The charge occupancies of titratable groups in N and U, respectively, are believed to be independent of denaturant concentration. However, maintenance of equal extent of protonation at different denaturant concentrations is not guaranteed because of the effects of the denaturant on the pH meter readings, pH_r [2,3]. pH_r, defined as

$$pH_r = -\log(\gamma_{H^+}) - \log[H^+] + E_j \quad (1)$$

not only depends on the proton concentration $[H^+]$ but also on the activity coefficient γ_{H^+} and the liquid junction potential error E_j of the pH glass electrode. γ_{H^+} depends on inter-proton as well as proton–cosolvent interactions. Thus, at infinite dilution of $[H^+]$ γ_{H^+} will not reach unity because of the latter interactions. γ_{H^+} at the infinite $[H^+]$ dilution limit is also referred to as the 'primary medium effect' since it reflects solute–cosolvent interactions in the mixture compared to solute–solvent interactions in water. γ_{H^+} is related to the Gibbs free energy of transferring the proton from water to the mixture. The change in pH readings due to proton–cosolvent interactions and the liquid junction potential error E_j is often seen with mixed solvents [4] such as urea or guanidine–hydrochloride (GdnHCl) solutions: the pH measured is not representative of $[H^+]$ [2,3].

There are not only the 'primary medium effect' and E_j interfering with the correct pH meter readings, in addition,

Abbreviations: f_P, fraction protonated; GdnHCl, guanidine–hydrochloride; N, folded state; pK_a, logarithm of the proton association constant relating to activities; pK_a^*, pK_a relating to activities and using activity coefficients reflecting solute–solute interactions only; pK_a^C, pK_a relating to concentrations; U, unfolded state.

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changed activity coefficients of ions at high salt or denaturant concentration also affect apparent pK_a values of acids and bases: reduced ion activities at high salt result in up-shifted pK_a [5]. Likewise, apparent pK_a values of acids and bases are significantly up-shifted at high urea concentration, by comparison with the values measured in the absence of urea [6,7]. The effect of GdnHCl on the apparent pK_a values is less pronounced: a tendency for down-shifted pK_a of acids and up-shifted pK_a of bases is indicated [8]. Correction values for pH meter readings using a glass electrode, and thus for the apparent pK_a values obtained by titration, have been proposed [2,3].

Here, the apparent pK_a values of the Asp, Glu, His and Lys side chain functional groups in the presence and absence of 6 M denaturants urea and GdnHCl, respectively, are presented. The protonation states during the pH titrations were monitored by ^{1}H-NMR. NMR chemical-shift-derived ratios of protonated/deprotonated functional groups are independent of the activity coefficients and reflect 'true' concentration ratios. The glass electrode pH meter readings necessary for relating the NMR chemical shift data to pH, however, are affected by the proton–cosolvent interactions and E_j leading to apparent pK_a shifts. Keeping the pH meter readings constant at different denaturant concentrations alters the charge occupancies of the titratable groups possibly affecting the outcome of protein stability determined by denaturant unfolding experiments.

2. Materials and methods

Peptide NH_2–Gly-Gly-Glu-Ala–OMe and blocked amino acids Ac-Asp–NH_2, Ac-His–NHMe and Ac-Lys–NH_2 were purchased from Bachem (Bubendorf, Switzerland). NH_2–Gly-Gly-Glu-Ala–OMe was N-terminally acetylated.

For the pH titrations, samples were dissolved at concentrations between 22 and 30 mM using the following solvent compositions: (i) H_2O, (ii) 6 M urea in H_2O and (iii) 6 M GdnHCl in H_2O. The pH was adjusted to the starting value by addition of HCl or NaOH using a Mettler Toledo InLab 423 AgCl combination glass electrode. 14 or 15 1-dimensional ^{1}H-NMR experiments were acquired at 37 (Glu) or 25 °C (Asp, His, and Lys) in the pH range 2 to 8 (Asp, Glu), 3 to 9.5 (His) and 7 to 13.8 (Lys), respectively, on a Bruker DRX600 spectrometer applying external locking on D_2O. The pH was increased by adding small amounts of 5 or 0.5 M NaOH, respectively, after transfer of the sample to an Eppendorf tube and equilibration at the corresponding temperatures. NaOH was replaced by KOH in the case of Ac-Lys–NH_2 because of the lower affinity of K^+ to the glass electrode tip than Na^+, the latter possibly interfering with the pH meter readings at high pH. Glass electrode pH readings were repeated after data acquisition and were consistent within 0.05 pH units. During the 1.5 s relaxation delay, the carrier frequency was positioned on the urea and guanidine–NH_2 hydrogen resonance, respectively, for selective low-power irradiation. Water suppression was achieved with the 3-9-19 sequence [9].

A modified form of the Henderson–Hasselbalch equation [10,11] was applied to analyze the pH-dependence of the Asp H^{β}, Glu $H^{\beta/\gamma}$, His $H^{\delta 2/\varepsilon 1}$ and Lys $H^{\delta/\varepsilon}$ chemical shifts δ(pH):

$$\delta(\mathrm{pH}) = -\frac{\delta_a + \delta_b 10^{n(\mathrm{pH}-\mathrm{p}K_a)}}{1 + 10^{n(\mathrm{pH}-\mathrm{p}K_a)}}. \quad (2)$$

The chemical shift plateaus δ_a and δ_b at the acidic and basic pH limits, the pK_a and the Hill coefficient n, which probes for cooperativity, were non-linear least squares fitted to Eq. (2).

3. Results and discussion

3.1. Determination of pK_a values

pK_a values of the four titratable side chains in water, 6 M urea and 6 M GdnHCl, respectively, are listed in Table 1. Since the residues contain only one titratable charged group, pK_a values determined for the water solution closely resemble those of the corresponding model compounds in which intra-molecular charge–charge interactions are absent and which are entirely exposed to the solvent where unfavorable desolvation effects are reduced to a minimum [12]. In 6 M urea, up-shifts of the apparent pK_a of 0.3 to 0.5 pH units are observed for both, acidic and basic residues (Fig. 1). In contrast, 6 M GdnHCl effects down-shifts of the apparent pK_a of acidic residues by −0.2 to −0.3 pH units and up-shifts of the pK_a of basic residues by +0.3 to +0.5 pH units.

Correction values δpH for glass electrode pH readings have been published [2,3]. One may use the infinite-dilution standard for pH definition of the denaturant solution:

$$\mathrm{pH}^* = -\log(\gamma^*_{H^+}) - \log[H^+]. \quad (3)$$

The proton activity coefficient $\gamma^*_{H^+}$ neglects proton–cosolvent interactions and reaches unity at infinitely low $[H^+]$.

Table 1
Apparent pK_a values of ionizable amino acid side chains in water and in mixed solvents containing 6 M denaturant

Residue	Water[a]	6 M urea[a]	6 M GdnHCl[b]
Ac-Asp–NH_2[c]	4.08±0.03[d]	4.50±0.03	3.78±0.03
Ac-Gly-Gly-Glu-Ala–OMe[e]	4.45±0.03	4.91±0.03	4.27±0.03
Ac-His–NHMe[c]	6.38±0.03	6.78±0.03	6.69±0.03
Ac-Lys–NH_2[c]	10.31±0.03	10.61±0.03	10.82±0.03

[a] The ionic strength varied between 0.02 and 0.1 M during titration.
[b] Ionic strength of 6 to 6.1 M.
[c] Titrated at 25 °C.
[d] The error in pK_a reflects the uncertainty in the pH reading before and after NMR data acquisition.
[e] Titrated at 37 °C.

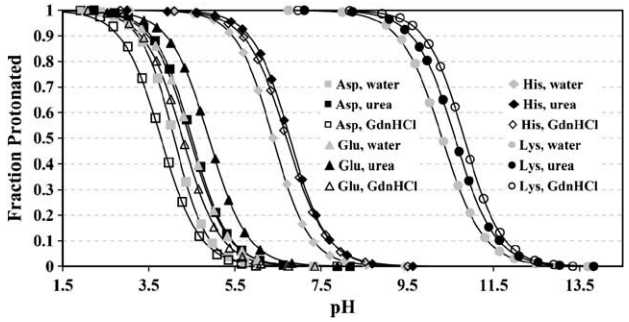

Fig. 1. pH titration curves of the ionizable side chains of Asp, Glu, His and Lys in water, in the presence of 6 M urea and in the presence of 6 M guanidine–hydrochloride, respectively. Continuous lines depict best-fits of Asp H^{β}, Glu H^{γ}, His $H^{\delta 2}$ or Lys H^{ε} chemical shift data according to Eq. (2). pH-dependent chemical shifts are normalized in order to represent the fraction protonated f_P: $f_P=[\delta_b-\delta(\mathrm{pH})]/[\delta_b-\delta_a]$, where δ_a and δ_b are the chemical shifts at acidic and basic pH limits, respectively.

This contrasts the definition of pH_r (see Eq. (1)) where the infinite dilution activity coefficient γ_{H^+} of the proton is different from unity because of the interactions of the proton with the cosolvent. In addition, pH_r includes the liquid junction potential error E_j. Hence, the pH meter reading pH_r has no clear physical meaning in terms of proton activity whereas pH* is more representative of the 'true' proton concentration and can be derived by applying the pH correction value δpH: $pH^*=pH_r+\delta pH$. δpH is composed of the 'primary medium effect' term $\log(\gamma_{H^+})$ at infinite dilution of $[H^+]$ and the liquid junction error term $-E_j$ of the pH glass electrode [4]. Reported values of δpH are −1.31 pH units for 6 M urea and +0.72 pH units for 6 M GdnHCl [2,3]. Apparent pK_a values observed in the presence of denaturant can be corrected by δpH according to

$$pK_a^* = pK_a + \delta pH \tag{4}$$

where

$$pK_a^* = \log(\gamma^*_{AH}[AH]/(\gamma^*_{A-}[A^-]\gamma^*_{H^+}[H^+])) \tag{5}$$

in the case of acidic and

$$pK_a^* = \log(\gamma^*_{AH^+}[AH^+]/(\gamma^*_{A}[A]\gamma^*_{H^+}[H^+])) \tag{6}$$

in the case of basic residues [2,3]. The activity coefficients of Eqs. (5) and (6) reflect solute–solute interactions and reach unity at infinite dilution of the reactants.

3.2. pK_a values in urea

Regarding the urea data of this study, pK_a up-shifts are observed for all residues suggesting that pH readings are dominated by the 'primary medium effects' and E_j, yielding apparently increased pK_as. Application of the published pH corrections of −1.31 pH units to the pK_a values measured in 6 M urea (Table 1) yields pK_a^* values significantly smaller than the ones measured in water. The uniform down-shifts of the corrected pK_a^* suggest stabilizing interactions of the dissociated reaction products consisting of the charged acidic and uncharged basic residues, respectively, with the cosolvent. Thus, dissociation is promoted upon transferring the ionizable groups from water to the urea solution. However, corrected pK_a^* values have to be considered with care because the pH correction value δpH includes term E_j which is specific for the type of glass electrode used. Differences in E_j may contribute to the pronounced pK_a^* down-shifts after pH correction.

The dielectric constant of 6 M urea is slightly higher than that of pure water [2] which could be another source for the pK_a shifts. Continuum electrostatic calculations reveal little change in the pK_a upon increasing the water dielectric constant in case of a fully exposed titratable group as used in this study (data not shown).

3.3. pK_a values in GdnHCl

In case of the pK_a data recorded in concentrated GdnHCl, application of the pH correction values δpH leads to up-shifted pK_a^* for both, acid and basic residues, by comparison to the pK_a values measured in water. This implies that the reaction products of the protonated uncharged acid and the protonated charged base, respectively, are more stabilizing in the denaturant solution than in water. As mentioned above, pH correction values have to be applied with care when different electrode types are used because of the differences in E_j.

Continuum electrostatic calculations carried out at high and low ionic strength on solvent-exposed acidic and basic model compound residues using the non-linear Poisson-Boltzman approach do not reveal changes in pK_a upon increasing the ionic strength (data not shown). This is not against expectation since solvent-exposed model compound residues are used as reference state in such calculations. Residues on which calculations were performed contain only a single ionizable group and are solvent-exposed. Thus, there are no electrostatic interactions established which could be screened by the salt. In addition, calculations of the electrostatic energy terms are performed on a single molecule and do not reflect changes in pK_a due to altered activity coefficients at high ionic strength.

The observed down- and up-shifts of the apparent pK_a of acidic and basic residues, respectively, may be rationalized by qualitative calculations of pK_a and pK_a^C as a function of the activity coefficients (Fig. 2). GdnHCl behaves like a salt effecting reduced activity coefficients of ions.

$$pK_a = \log(\gamma_{AH}[AH]/(\gamma_{A-}[A^-]\gamma_{H^+}[H^+])) \tag{7}$$

in case of acidic and

$$pK_a = \log(\gamma_{AH^+}[AH^+]/(\gamma_{A}[A]\gamma_{H^+}[H^+])) \tag{8}$$

in case of basic residues. At the titration midpoint where pH equals pK_a, $\gamma_{AH}[AH]=\gamma_{A-}[A^-]$ and $\gamma_{AH+}[AH^+]=\gamma_A[A]$, respectively. pK_a^C, which reflects 'true' concentrations is

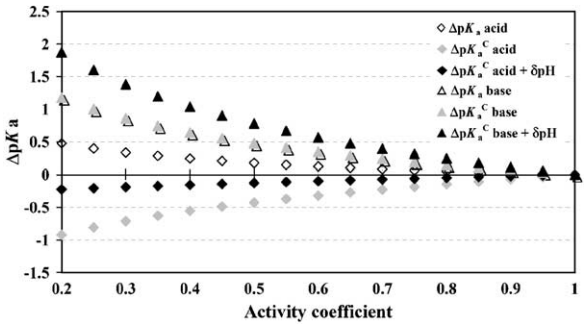

Fig. 2. Qualitative assessment of the pK_a shifts induced in acids and bases by changes in the activity coefficients of ions H^+, A^- and AH^+. For acids, γ_{A-} and γ_{H+} were varied between 0.2 and 1 whereas γ_{AH} was fixed at 1. In the case of bases, γ_{AH+} and γ_{H+} were varied between 0.2 and 1 and γ_A was set to 1. ΔpK_a were calculated according to pK_a(ion activity coeff. <1) − pK_a(ion activity coeff. $=1$). The pH correction accounting for the reduced proton activity was obtained by $\delta pH=[H^+]-\log(y_{H+}[H^+])$. ΔpK_a is relating to activities and ΔpK_a^C is relating to concentrations (see Results and discussion for more details).

defined according to

$$pK_a^C = \log([AH]/([A^-][H^+])) \quad (9)$$

in the case of acidic and

$$pK_a^C = \log([AH^+]/([A][H^+])) \quad (10)$$

in the case of basic residues. pK_a^C can be calculated for the condition $\gamma_{AH}[AH]=\gamma_{A-}[A^-]$ and $\gamma_{AH+}[AH^+]=\gamma_A[A]$, respectively, and considering the total residue concentration C_{tot}:

$$pK_a^C = \left(\gamma_{A-}^2 + \gamma_{A-}\gamma_{AH}\right)/\left(C_{tot}\gamma_{AH}^2\right) \quad (11)$$

for acidic and

$$pK_a^C = \left(\gamma_A^2 + \gamma_A\gamma_{AH^+}\right)/\left(C_{tot}\gamma_{AH^+}^2\right) \quad (12)$$

for basic residues. Since the averaged NMR chemical shift of a chemically exchanging compound is sensitive to concentrations rather than to activities, it is pK_a^C and not pK_a which is derived from the NMR pH titrations (Fig. 2). Thus, $[AH]=[A^-]$ and $[AH^+]=[A]$ at the titration midpoint. However, pH meter readings are sensitive to the reduced activity of $[H^+]$ represented by the curve $\Delta pK_a^C + \delta pH$ of Fig. 2. Noteworthy, ΔpK_a^C is independent of C_{tot}. By reducing the activity coefficients of the ions, pK_a^C of the acidic residue is expected to decrease whereas pK_a^C of the basic residue should increase, which is indeed seen in the pH titration experiments in the presence of GdnHCl. pK_a on the other hand, which is not detected by the NMR pH titration, is increasing for both, acidic and basic residues, after reduction of the ion activity coefficients (Fig. 2).

3.4. Effect of the pK_a shifts on protein stability

The shift in pK_a values at high denaturant concentration may have a pronounced impact on the outcome of the analysis of equilibrium or kinetic denaturant unfolding/folding experiments. Fig. 3 illustrates the pH-dependent difference in the charge occupancies of the titratable residues dissolved in 6 M denaturant and water, respectively. Positive and negative amplitudes represent loss and gain of negative charge, respectively, located on acidic residues, and gain and loss of positive charge, respectively, located on basic residues at high denaturant concentration. It is evident from Fig. 3 that the charge differences for non-interacting acidic residues are most pronounced at around pH 4.5. The residues are less negatively charged in urea and more negatively charged in GdnHCl solutions than they are in water, the charge differences between urea and GdnHCl amounting to +0.35 charge units. Assuming a stabilizing charged acidic residue in the folded state which coexists with the unfolded state in the two-state unfolding model of proteins, the folded protein would be substantially more stabilized in GdnHCl compared to water and urea solutions if the pH is kept constant at a pH meter reading of ~4.5. The same applies to the unfolded state, which can be pronouncedly stabilized/destabilized by residual charge–charge interactions [13]. In case of the basic residues His and Lys, the charge differences are highest at around pH 6.6 and 10.5, respectively. Because of the pK_a up-shift at high denaturant concentration, stabilizing charge effects are more pronounced in the presence of denaturant than they are in water due to higher charge occupancy at constant pH meter readings in the above ranges.

To keep the charge occupancies constant in the course of increasing denaturant concentration individual adjustment of the pH for each denaturant concentration is of order. However, this is commonly not done since the dependence on denaturant concentration of the apparent pK_as of the titratable groups is unknown and constant pH meter reading is used instead. In addition, the linear extrapolation method applied in the evaluation of denaturant unfolding data should

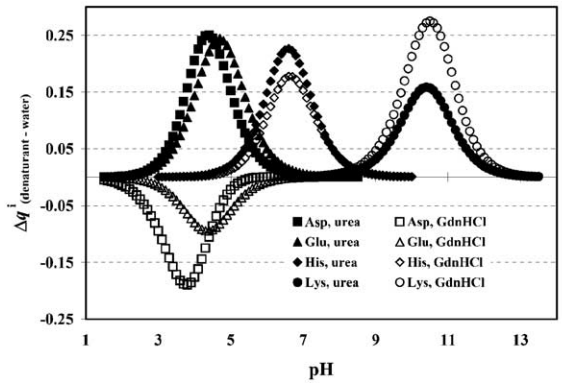

Fig. 3. pH-dependent charge differences $\Delta^i q$ of the ionizable side chains of Asp, Glu, His and Lys dissolved in 6 M denaturant and in water, respectively. $\Delta^i q$ was calculated on the basis of the fraction protonation f_P according to $\Delta^i q(pH)=f_{P,denaturant}(pH)-f_{P,\ water}(pH)$. Positive amplitudes indicate less negatively charged acidic residues and more positively charged basic residues, respectively, when dissolved in denaturant compared to water. The negative amplitudes of acids represent more negatively charged residues in the presence of denaturant.

correct for the denaturant concentration-dependent charge occupancies of titratable groups in a particular folding state [1]. Nonetheless, the change in the charge occupancies in the presence of denaturant might not be entirely compensated with the above method possibly contributing to the frequently occurring difference in the Gibbs free energy derived from urea and GdnHCl unfolding experiments [14], despite the fact that GdnHCl acts like a salt and screens charge interactions whereas urea does not. In addition, changed charge occupancies with increasing denaturant concentration and the concomitant effects on the stability of the F and U states will affect the *m*-value, the slope in the plot of the Gibbs free energy versus the denaturant concentration when the linear extrapolation method is applied. The *m*-values are representative of the protein surface exposed during denaturant unfolding [15]. Although the *m*-values of GdnHCl and urea denatured proteins are not directly related to each other, contributions due to denaturant concentration dependent charge occupancies should be considered as well in the interpretation of the *m*-values derived from unfolding data with the two denaturants.

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Appendix A. Supplementary material

Supplementary material consisting of four tables listing the parameters obtained by fitting the pH-dependent chemical shift data of the four residues to the Henderson-Hasselbalch equation can be found online at http://www.sciencedirect.com

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