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Arginine: Its pK_a value revisited

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Abstract: Using complementary approaches of potentiometry and NMR spectroscopy, we have determined that the equilibrium acid dissociation constant (pK_a value) of the arginine guanidinium group is 13.8 ± 0.1. This is substantially higher than that of ~12 often used in structure-based electrostatics calculations and cited in biochemistry textbooks. The revised intrinsic pK_a value helps explains why arginine side chains in proteins are always predominantly charged, even at pH values as great as 10. The high pK_a value also reinforces the observation that arginine side chains are invariably protonated under physiological conditions of near neutral pH. This occurs even when the guanidinium moiety is buried in a hydrophobic micro-environment, such as that inside a protein or a lipid membrane, thought to be incompatible with the presence of a charged group.

Keywords: protein electrostatics; pH titration; equilibrium acid dissociation constant; pKa value; guanidinium; tautomer; potentiometry; NMR spectroscopy

Introduction

The arginine side chain plays key roles in many essential biochemical processes. The planar guanidinium group often partakes in ionic and hydrogen bonding interactions critical to the structure and stability of proteins and protein complexes.[1] It is also involved in proton (H^+) transfer cascades in systems such as bacteriorhodopsin and cytochrome c oxidase. In these systems, arginine acts in its fully protonated state as a modulator of the pK_a values of the groups serving as proton donors and acceptors.[2–5] Furthermore, arginine has also been proposed to function as a general acid/base in a small number of enzymatic mechanisms,[6–8] and its guanidinium moiety is a nucleophile or electrophile for a variety of post-translational modifications.[9] Key to all of these functions is the charge state and hence equilibrium acid dissociation constant (pK_a value) of the arginine side chain. However, within a biological context, an arginine in a measurably populated neutral state has never been unambiguously observed, even when buried in a highly hydrophobic environment such as the interior of a protein or in the middle of a lipid bilayer.[10,11]

The unusual ability of the arginine side chain to retain its charge under all physiological conditions is not well understood. It is especially perplexing that an arginine remains cationic even when buried in the nonpolar hydrophobic core of a folded protein.[12] This is not what occurs with the ionizable side chains of lysine, histidine, tyrosine, cysteine, aspartic acid, and glutamic acid residues. For example, systematic studies have shown that the pK_a values of these residues at numerous internal positions in staphylococcal nuclease shift in the direction that promotes the neutral state. The shifts can be

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Table I. *pK_a Values Obtained from Potentiometric Titrations*

	pK_a values		
[Arg], m*M*	Arg guanidinium	Lys α-aminium	Lys ζ-aminium
125	13.50 ± 0.2	9.39 ± 0.1	10.91 ± 0.1
200	13.61 ± 0.2	9.34 ± 0.1	10.88 ± 0.1
500	13.83 ± 0.2	9.41 ± 0.1	10.97 ± 0.1
500	13.76 ± 0.2	9.39 ± 0.1	10.93 ± 0.1
500	13.85 ± 0.2	9.37 ± 0.1	10.89 ± 0.1
Avg[a]	13.8 ± 0.1	9.4 ± 0.1	10.9 ± 0.1

[a] Averages and standard deviations for the three replicas with 500 m*M* arginine.

substantial, such that lysines can have pK_a values below 6[13] and aspartic/glutamic acids above 10.[14,15] Consequently, these residues are usually neutral when buried within the interior of a protein under solution conditions with pH values near 7. This is consistent with the incompatibility between charged species and dry, hydrophobic environments. In contrast, the behavior of the arginine side chain is completely different.

Three main factors could determine the unusual ability of the arginine side chain to remain ionized, even in micro-environments that are normally considered to be incompatible with charges. First, the guanidinium moiety is large and its positive charge is delocalized over many atoms involved in a conjugated Y-π system.[16] Owing to this delocalization, the charge unit of the guanidinium moiety has a large effective radius, and therefore its hydration energy, which is inversely proportional to radius, is low.[17] The low hydration energy of the arginine side chain should mitigate the penalty for dehydration upon its transfer in the charged state to a hydrophobic environment. Note that this does not necessarily imply that the arginine side chain is not well hydrated. On the contrary, more crystallographic water molecules are usually found associated with the guanidinium moiety than with the primary amine of the lysine side chain.[12,18] Only the carboxylic oxygen atoms of aspartate/glutamate residues appear to have higher affinities for water.[19,20]

The second factor that might enhance the ability of the arginine side chain to remain charged in environments, such as the protein interior or the middle of a lipid bilayer, is the conformational flexibility of its long side chain. This enables the guanidinium group to search for polar atoms, including buried water molecules, to satisfy its propensity to donate up to five hydrogen bonds.[12]

The third reason that arginine might always be charged under physiological conditions lies with its high intrinsic pK_a value. Based on historical experiments dating back almost a century,[21,22] a side chain pK_a value of ~12 is commonly cited in textbooks and used in structure-based electrostatics calculations. However, the accepted pK_a values of 13.6 and 13.4 for the conjugate acids of guanidine and N-methyl guanidine, respectively,[23,24] suggest strongly that the intrinsic pK_a of the arginine side chain is significantly higher than 12. If so, then even when buried in the driest and most hydrophobic environments available, it may not become deprotonated to any significant level at neutral pH, or even at pH values up to 10. That is, due to an energetic penalty of $\Delta G^o = 2.303RT(\Delta pK_a)$, the pK_a of an arginine might never be perturbed to a value as low as 7 because the host proteins would unfold owing to the associated loss of net stability.[17] In this respect, the properties of the lysine side chain are very different. With an intrinsic pK_a value near 10.4 in the context of a random coil polypeptide,[25,26] the pK_a values of lysines within folded proteins can be shifted below 7. Thus, it is energetically possible to have buried lysine side chains that are completely deprotonated and neutral at physiological pH.[13]

In this study, we used complementary potentiometric and NMR spectroscopic methods to reinvestigate the pK_a value of the arginine side chain. Our measured pK_a values of 13.8 ± 0.1 are indeed almost 2 log units higher than that commonly assigned to this residue. This finding helps explain why, unlike the other ionizable amino acids, arginines remain predominantly charged in proteins under physiological conditions.

Results

Potentiometry

We used potentiometry to measure directly the release of protons by arginine in response to the addition of KOH. The results of these potentiometric titrations are summarized in Table I and representative titration curves are shown in Figure 1. The blank used in these experiments consisted of a solution of lysine in water, allowing both calibration of the KOH titrant and control of the ionic strength without requiring the addition of supporting electrolytes. The pK_a values resolved for and attributed to the α-aminium (9.4 ± 0.1) and side chain ζ-aminium (10.9 ± 0.1) groups of free lysine are consistent with accepted data.[27,28]

The two titration events in a solution of lysine can be visualized without baseline correction. This is not the case for arginine. With charged α-carboxylate and guanidinium moieties and a neutral α-amine, the arginine titration begins at its isoionic point near pH 11 (i.e., the unadjusted pH of the initially prepared solution). Although masked by the steep rise in pH due to the necessary addition of large quantities of KOH, the guanidinium titration becomes apparent after the lysine/water blank is subtracted. As shown in the inset of Figure 1, inflection points in the difference curve can be seen for each titrating group. Fitting of this curve to a three-

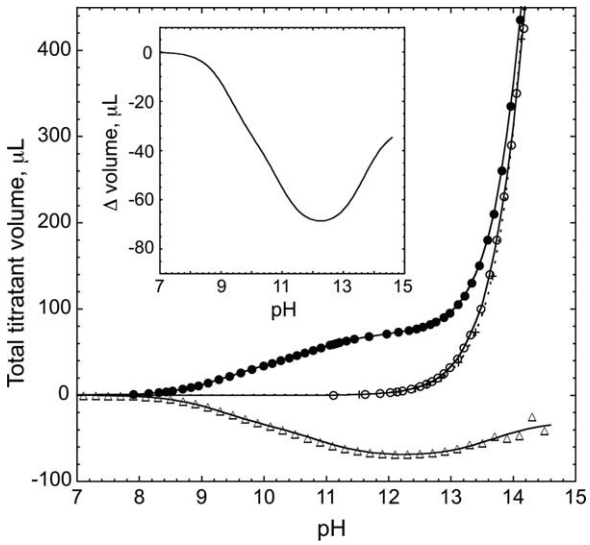

Figure 1. pK_a measurements by potentiometry. Shown are titration curves of lysine (solid circles), arginine (open circles), and a water blank (x with dashed line). The arginine versus lysine/water difference curve is represented with open triangles. The lines are provided to guide the eye. The inset is a close up of this difference curve showing the best fit to Eq. (2). Solutions were 200 m*M* in each amino acid with 100 m*M* KCl.

site binding isotherm yielded the desired pK_a value of the arginine side chain.

The pK_a value of the arginine guanidinium group obtained by analysis of the difference potentiometric titration curves is, on average, 13.8 ± 0.1 (Table I). This result is from multiple experiments performed over the course of five months with samples containing 500 m*M* arginine. Experiments were also carried out with lower concentrations of the free amino acid. Although yielding larger fitting errors due to the proportionally higher water background, a concentration-dependence was observed and a pK_a value of 13.50 ± 0.2 was measured with 125 m*M* arginine (Table I). This small variation may be related to ionic strength differences or perhaps to the known propensity of the charged arginine side chain to associate with itself.[29] However, for samples of arginine at 10 and 100 m*M*, similar high pK_a values were obtained by NMR methods.

NMR spectroscopy

In parallel, we attempted to measure with NMR spectroscopy the pK_a value of the arginine side chain in the context of a blocked tripeptide, acetyl-Gly-Arg-Gly-amide. As part of a study to define the pH-dependent chemical shifts of all the common ionizable amino acid side chains, this compound was chosen to mimic a random coil polypeptide and to avoid possible complications due to the deprotonation of the terminal α-aminium and α-carboxylic acid moieties.[26] Unfortunately, under highly alkaline conditions, the tripeptide slowly hydrolyzed over the course of the titrations, which required ~1 day due to detection of ^{13}C at natural abundance. A similar hydrolysis problem precluded potentiometric studies of blocked arginine derivatives. As a result, reliable pH-dependent chemical shift changes could only be measured for the arginine side chain $^{13}C^{\zeta}$ and $^{1}H^{\delta}$ nuclei in the intact peptide [Fig. 2(A)]. Based on these data, an average pK_a value of 13.8 ± 0.1 was obtained. However, this could be an underestimation as the maximum sample pH value was only 14.5, and thus the plateau chemical shifts of the deprotonated species were not well defined. Also, according to the manufacturer's electrode specifications, interference from the ~1 *M* Na^+ added during the titration may have reduced the apparent sample pH by 0.1 to 0.2 log units.

Subsequently, we monitored via NMR spectroscopy the titration of numerous reporter nuclei in free $^{13}C_6/^{15}N_4$-arginine. In this case, KOH was used to reduce possible electrode alkaline errors.[30] Also, a higher final pH of 15.25 was attained. As shown in Figure 2(B), two distinct titration events were observed and could be confidently attributed to the α-aminium and guanidinium moieties. Under all pH values examined, the arginine α-carboxylic acid, with a known pK_a value of ~ 2,[27,31,32] is unquestionably deprotonated. Based on the individually fit titration curves of these nuclei in data sets recorded with both 10 m*M* and 100 m*M* $^{13}C_6/^{15}N_4$-arginine, the average pK_a value of the α-aminium in the context of charged α-carboxylate and guanidinium moieties is 9.15 ± 0.05. This is consistent with previously reported pK_a values of 9.0 to 9.3.[27,31,32] Most importantly, the average of the pK_a values fit individually for several guanidinium reporter nuclei in the presence of a charged α-carboxylate and neutral α-amine is 13.9 ± 0.15. The similarity of results for the acetyl-Gly-Arg-Gly-amide tripeptide and free arginine also indicates that electrode errors (K^+ versus Na^+) are minor and that the presence of the negatively charged carboxylate does not significantly increase the pK_a value of the guanidinium group.

Discussion

Originating from pioneering studies in 1924[21] and 1930,[22] the pK_a value of the guanidinium group in free arginine is typically cited as 12 to 12.5. However, values ranging from as low as 11.4 to as high as 13.6 have been reported over the years.[27,28,31–42] Although this may in part result from differences in experimental conditions, the pK_a value of the arginine side chain appears to decrease only slightly with increasing temperature.[28] Thus, the substantial variation in published pK_a values most likely reflects the great experimental difficulty in measuring multi-step acid-base equilibria under highly alkaline conditions.

Given the importance of knowing an accurate reference acid dissociation constant for arginine, we

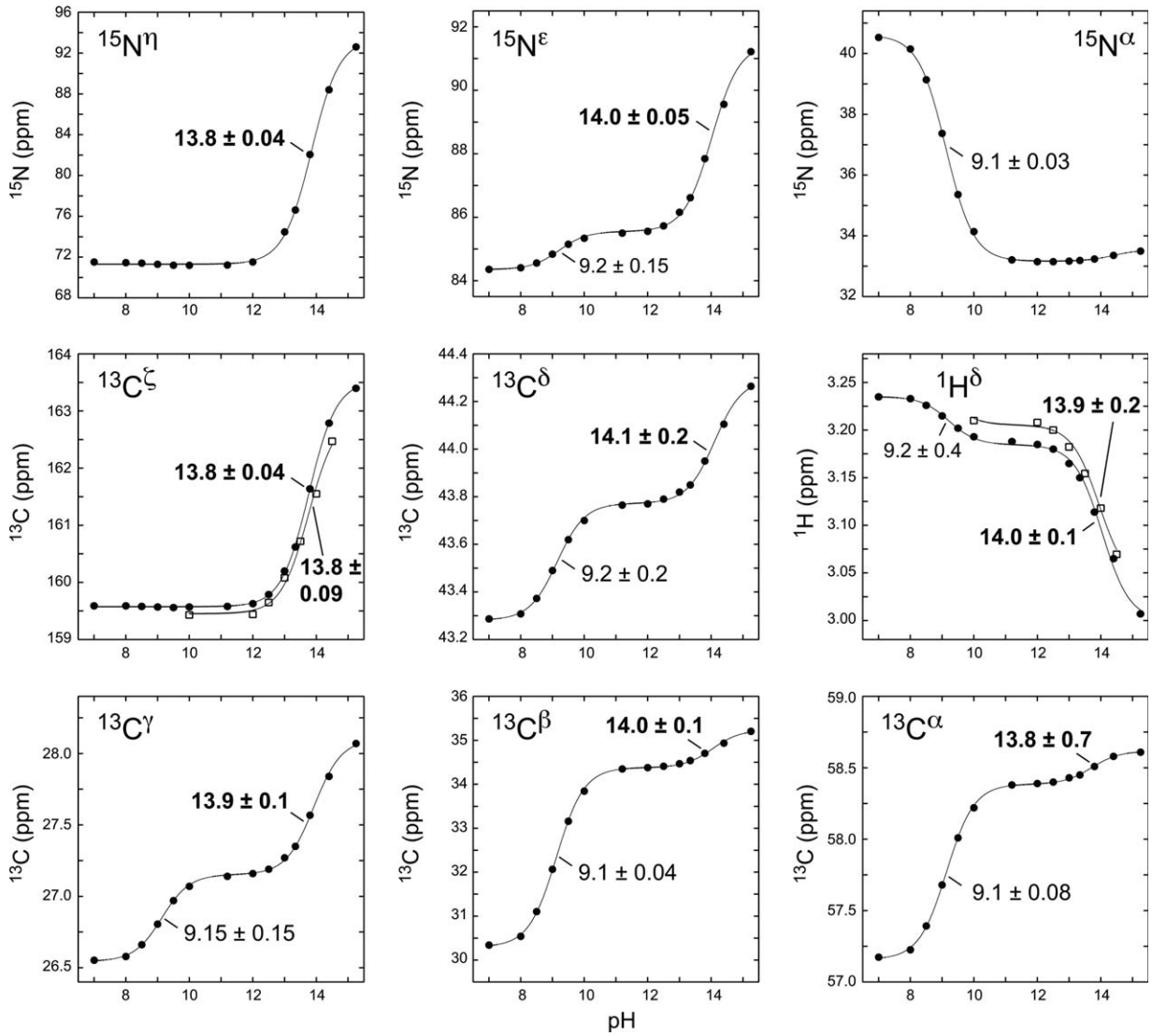

Figure 2. Arginine pK_a measurements by NMR spectroscopy. Shown are titration curves of the arginine $^{13}C^{\zeta}$ and $^{1}H^{\delta}$ nuclei in the 10 m*M* unlabeled blocked tripeptide acetyl-Gly-Arg-Gly-amide (open squares), and the side chain ^{13}C and ^{15}N nuclei in 100 m*M* $^{13}C_6/^{15}N_4$-arginine (solid circles). The two $^{15}N^{\eta}$ nuclei yield one degenerate signal. The curves and listed pK_a values (bold for guanidinium) are the best nonlinear least squared fits to equations describing one or two sequential acid dissociation equilibria. Not shown are data for the arginine ^{13}CO (reporting pK_a 9.13 ± 0.05) and $^{1}H^{\alpha}$ (pK_a 9.15 ± 0.04). Note that the titration data for each nuclei were fit separately and the small variations in pK_a values shown in each panel of the figure reflect the sensitivity to factors such as experimental noise and the extrapolated end-point chemical shifts. Since the tautomeric forms of the neutral arginine side chain will rapidly interconvert in a pH-independent equilibrium, only a single overall macroscopic pK_a value can actually be measured from the averaged chemical shift changes reported by these nuclei. Also, although arginine is following a predominantly sequential deprotonation pathway with increasing sample pH, the biphasic titration curves indicate that the chemical shifts of several reporter nuclei are sensitive to the ionization state of both the α-aminium and guanidinium groups (see Ref. 46 for further discussion).

have revisited this fundamental question using complementary potentiometric and NMR spectroscopic approaches. Our measurements were carried out independently by different personnel in two separate laboratories using different techniques and instrumentation. Collectively, we have found that the arginine guanidinium moiety has a pK_a value of 13.8 to 13.9 in both the context of the free amino acid and a blocked tripeptide.

Our results confirm that the value of ~12 commonly used for arginine in structure-based pK_a calculations significantly overestimates the very weak acidity of the guanidinium group. Most importantly, a value closer to 14 is consistent with the observation that arginine side chains are always predominantly charged in proteins over a wide range of pH values spanning from physiological to as high as 10. This is the case even when the arginine side chains are buried in apparently dehydrated, hydrophobic micro-environments.[12]

For the arginine side chain to be neutral under physiological conditions of pH, a shift in its pK_a value

from 13.8 down to at least 7 would be necessary. This is equivalent to a Gibbs free energy change of almost 10 kcal/mol. Any protein bearing an arginine with such a shifted pK_a would be destabilized by this full energetic penalty. Although not as large as the hydration energies of small ions, 10 kcal/mol exceeds the thermodynamic stability of most globular proteins in their folded versus unfolded states and is comparable with that of very tight ligand binding events.[43] In contrast, with intrinsic pK_a values of ~10.4, 3.9, and 4.3,[25,26] the side chains of lysine, aspartic acid, and glutamic acid, respectively, can be tolerated in their neutral forms within the context of a folded protein. That is, pK_a values below 6 for the ζ-aminium group of lysine, and as high as 10 for the carboxylic acid groups of aspartic and glutamic acids have been measured.[13–15]

Even in the lipid bilayer, which in principle is more stable than most proteins, local instabilities would likely lead to water penetration and reorganization of polar head groups. This would almost certainly preclude maintenance of the strictly hydrophobic micro-environments that would be required to keep the arginine side chain in the neutral state. Thus, most biological environments are probably too unstable to tolerate the burial of an arginine side chain in its neutral form at physiological pH.

Stated conversely, the fact that charged arginine side chains are found within the interiors of stably folded proteins indicates that they do not undergo dramatic pK_a shifts. Thus, proteins appear to have evolved to accommodate a buried guanidinium cation. In contrast to other ionizable side chains, the guanidinium group can participate in numerous stabilizing noncovalent interactions, including ion pairing, π-cation interactions, hydrogen bonding, and even association with another guanidinium cation. These effects could mitigate the cost of maintaining a buried side chain in the charged state despite being secluded from bulk water.[29,44,45] Thus, nature appears to resort to arginine when it requires the presence of a charged species in the hydrophobic and dehydrated environment of a biological setting.

Arginine pK_a measurements by potentiometry

Potentiometry is traditionally the method of choice for determining the pK_a values of small molecules because it involves a direct measurement of a pH titration curve. This means that the amplitudes of the titrations determined potentiometrically reflect the number of proton binding sites. However, the resulting pK_a values are macroscopic and, in the absence of any other information, not assignable to specific ionizable groups in the molecule. Furthermore, the standard potentiometry approach involves use of a glass electrode to measure pH values. At high pH, this method is fraught with difficulties caused primarily by the cation error and the sensitivity of the glass surface to these conditions.[30] Fortunately, these problems were minimized in this study by using a modern glass electrode designed specifically for work under highly alkaline conditions. Also, a difference titration against a lysine/water blank enabled titrant calibration and, in effect, provided an internal reference for the data fitting. Collectively, this strongly supports the accuracy of the potentiometrically determined pK_a values summarized in Table I for the arginine side chain.

Arginine pK_a measurements by NMR spectroscopy

NMR spectroscopy is often the method of choice for measuring polypeptide and protein pKa values because it provides site-specific information in the form of pH-dependent chemical shifts of nuclei associated with constituent ionizable groups.[26] However, chemical shift changes may arise from additional ionization events, and the resulting pK_a values are still macroscopic in the sense that they reflect the ensemble average of the pH-independent microscopic equilibria between all possible forms of a protein with a given total protonation level.[46] In the case of the free amino acid arginine, the pK_a values of its ionizable functional groups are sufficiently different that it follows one very dominant sequential deprontonation pathway with increasing sample pH (α-carboxylic acid then α-aminium then guanidinium). Thus, the fit pK_a value of 9.15 ± 0.05 can be assigned to arise almost exclusively to the α-aminium group in the context of the charged guanidinium and α-carboxylate moieties, and that of 13.9 ± 0.15 to the guanidinium group in the context of the neutral α-amine and charged α-carboxylate.

The small difference in pK_a values determined by NMR methods for the blocked acetyl-Gly-Arg-Gly-amide tripeptide (13.8 ± 0.1) and free arginine (13.9 ± 0.15) suggests that the ionization equilibrium of the guanidinium group is not substantially perturbed by the presence of the negatively charged α-carboxylate. This is expected given the length of the arginine side chain and the high ionic strength of a solution at pH ~ 14. Consistent with this observation, a comparison of the ionization equilibria of arginine versus citrulline in solutions of $0.3 - 2$ M ionic strength indicated that the pK_a values of the α-carboxylic acid and α-aminium groups are perturbed by only ~ −0.1 and +0.2 pK_a units, respectively, due to electrostatic interactions with the positively charged guanidinium versus neutral carbamido moieties (i.e., in the directions expected for favorable or unfavorable electrostatic effects).[32,41] Parenthetically, using internal reference compounds for pH calibration, the authors of this study also reported a pK_a value of 13.54 ± 0.01 for the arginine side chain by ^{1}H-NMR methods.[32]

pH-dependent chemical shifts

We are unaware of a case in which the pK_a value of an arginine side chain in a protein has been measured unambiguously (although see, for example, Refs. [47] and [48]). Nevertheless, such studies are important to pursue for many reasons, including understanding electrostatic interactions in proteins and testing postulated roles of arginines in catalytic mechanisms and in proton transfer pathways. Therefore, the reference chemical shift data summarized in Figure 3 provide a benchmark for studying the ionization states and pK_a values of arginines in polypeptides and proteins by NMR spectroscopy. In particular, the $^{13}C^{\zeta}$, $^{15}N^{\epsilon}$, and $^{15}N^{\eta}$ nuclei should all report coincident titration curves with $\Delta\delta$ values similar in magnitude and sign to those listed in this figure. This is required to be reasonably confident that any pH-dependent chemical shifts result directly from the deprotonation of the arginine and not indirectly due to structural or electrostatic changes from the deprotonation of other groups, such as amino or phenol moieties, in the protein. Of course, the protein must be confirmed to remain stably folded and well behaved under highly alkaline conditions.

In the absence of any detectable pH-dependent chemical shift changes for reliable reporter nuclei (i.e., $^{13}C^{\zeta}$, $^{15}N^{\epsilon}$, or $^{15}N^{\eta}$), one cannot a priori conclude whether an arginine is always charged or always neutral over the conditions examined. Of course, given an intrinsic pK_a value of almost 14, one expects (and finds) that the vast majority of arginines are charged in proteins. The observation of chemical shifts within the range of those shown in Figure 3 for the ionized side chain would certainly support this conclusion. In contrast, only the $^{15}N^{\eta}$ nuclei with dramatically different chemical shifts of ~71 ppm and 93 ppm in charged versus neutral arginine might serve as an indicator of its ionization state in the absence of any additional information. However, an arginine with an unusually low pK_a value will most certainly be in a very unusual environment, which may also lead to strongly perturbed chemical shifts. Indeed, the guanidino moiety shows substantially smaller chemical shift differences between its charged and neutral forms within nonpolar solvents than in an aqueous environment.[49] Unfortunately, this adds a further complication to consider when attempting to determine the ionization states of arginines in proteins by NMR spectroscopy.

Arginine tautomers and a route for pK$_a$ perturbations

In contrast to the planar guanidinium ion, the neutral side chain guanidine moiety is nonplanar and can adopt five possible tautomeric forms, each corresponding to the loss one of the five nitrogen-bonded protons.[16] All of these neutral tautomers exist in a pH-independent equilibrium and can interconvert via bond rotations and/or proton transfer. As such, the observed acid dissociation constant of an arginine side chain is actually the sum of the five microscopic acid dissociation constants leading to each of these tautomers.

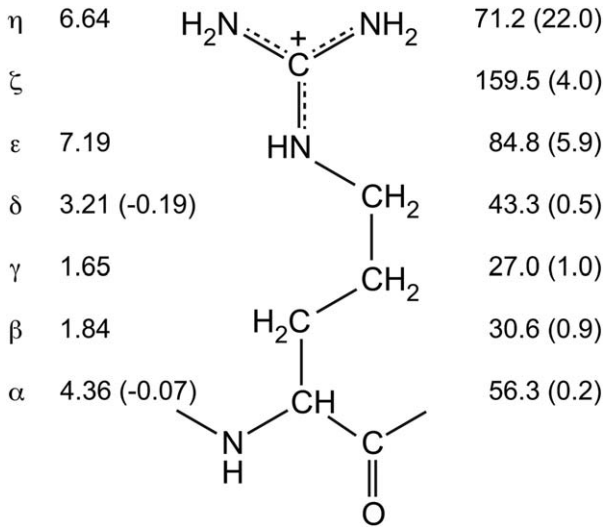

Figure 3. Summarized are the chemical shifts (^{1}H left, ^{13}C/^{15}N right; ppm) of positively-charged arginine at neutral pH in the blocked tripeptide acetyl-Gly-Arg-Gly-amide. Shifts of prochiral ^{1}H nuclei are averaged. Also shown in parentheses are the corresponding chemical shift changes upon deprontonation ($\Delta\delta = \delta_{\text{high pH}} - \delta_{\text{low pH}}$) of the guanidinium moiety. The latter were obtained from the pH titration curves of $^{13}C_6$/$^{15}N_4$-arginine shown in Figure 2 using the fit limiting chemical shifts for the guanidinium moiety in the presence of the neutral α-amine, and thus assumed to be representative of a polypeptide. In the case of free arginine, the $^{15}N^{\alpha}$ shifts upfield by −7.5 ppm upon deprontonation (not shown). The $\Delta\delta$ values for the $^1H^{\beta}$ and $^1H^{\gamma}$ are smaller in magnitude than 0.1 ppm, but could not be obtained reliably because of spectral overlap. Due to fast rotation about the N^{ϵ}-C^{ζ} and C^{ζ}-N^{η} bond, average values are observed for the $^1H^{\eta}$ and $^{15}N^{\eta}$ nuclei.[54] Although consistent with earlier studies, the $\Delta\delta$ values are generally larger than previously published, possibly due to better extrapolation to the high pH plateau chemical shifts.[42,49,51,57–60]

The observation of degenerate shifts for the two $^{15}N^{\eta}$ nuclei, as well as similar shifts for the $^{15}N^{\epsilon}$ and $^{15}N^{\eta}$ nuclei (Figs. 2 and 3), indicates that the neutral arginine tautomers are approximately isoenergetic and interconvert rapidly on the chemical shift timescale (i.e., $k_{ex} > 10^3$ s^{-1}). Otherwise, one would expect significantly different shifts for non-interconverting sp^2 (C=N bonded) and sp^3 (C—N bonded) hybridized nitrogens.[50] However, the microscopic pK_a values associated with each tautomer, and hence their equilibrium population distributions in the neutral form of the arginine side chain, remain uncertain. Based on an extrapolation of

temperature-dependent chemical shifts recorded for neutral arginine and methylguanidine analogs in DMSO/H_2O cosolvents, a distribution with ~2/3 protonated at N^{ϵ} has been estimated.[51] In contrast, theoretical calculations suggested that the two lowest energy tautomers arise from deprotonation of either the N^{ϵ} or one specific N^{η}.[52] Also, the observation that the hydroxide-catalyzed rate constant for hydrogen exchange of the arginine $N^{\epsilon}H$ is two-fold higher than the $N^{\eta}H$ suggests a lower microscopic pK_a value for N^{ϵ} deprotonation and hence preferential formation of the tautomer lacking a proton at this nitrogen.[53,54] A caveat to this argument is that steric factors could affect the relative rates of the diffusion-limited exchange reactions.

If a neutral arginine was indeed present in a folded protein, perhaps under highly alkaline conditions, then the relative populations of its tautomeric forms could certainly change in response to structure-dependent intermolecular interactions, such as hydrogen bonding. Moreover, it has been proposed that the pK_a value of an arginine side chain could be perturbed by interactions that twist the guanidinium group towards a nonplanar conformation.[6] Such structural-induced perturbations might enable the transient formation of a neutral guanidine moiety to serve, for example, as a general base in an enzymatically catalyzed reaction.

Materials and Methods

Potentiometric titrations

Solutions of the unmodified amino acids lysine and arginine (Sigma, >99.5% pure) were prepared volumetrically under an N_2 atmosphere (CO_2 free) with deionized water (degassed extensively by boiling under vacuum) made to 100 m*M* KCl. The stock solutions of lysine and arginine were initially at 500 m*M* and sealed under an N_2 atmosphere until use. The correct volumes of sample and dilutant were withdrawn with a Hamilton syringe from the sealed containers and injected into the isolated titration vessel, also held under an N_2 atmosphere. The water blanks were prepared in a similar manner. Total volumes used were in the range of 2.0 – 2.5 mL. The saturated KOH solution used as titrant was made from KOH pellets (Fisher), freshly dissolved in 100 m*M* KCl (prepared with degassed, deionized water).

Direct potentiometric titrations were performed using a glass electrode (pHG2HE8 from Radiometer America) that is inherently insensitive to cation error at pH values as high as 14.[30,55] The electrode chain was completed with a calomel reference electrode (REF401 from Radiometer America) connected to a PHM95 pH meter (Radiometer America). The calibration of electrodes was performed with IUPAC series certified pH 9.180 and 12.45 standards (Radiometer, NIST) just prior to each experiment and rechecked after completion. Between use these buffers were stored in tightly sealed containers at 4 °C. Titrations were performed with a Dosimat E665 (Metrohm) delivering volumes as low as 1 μL. The temperature of the samples was kept at 25 ± 0.1 °C with a water-jacketed titration vessel. Titrations began immediately following initial temperature equilibration, and were monitored continuously with a thermistor probe to ensure re-equilibration after the heating that results from the exothermic dilution of highly concentrated KOH titrant.

Potentiometric titration analysis

The titration of free lysine was used to calibrate the titrants. This involved fitting the pH titration difference curve (titration of a water blank subtracted from the titration of lysine) to a two-site model describing the deprotonation of the α-aminium and side chain ζ-aminium groups. The KOH titrant concentrations were thereby determined to range from 13.7 to 14.5 *M*. The error in titrant concentration was estimated to be ± 0.3 *M*, resulting in a propagated error for the fit pK_a values of ± 0.15 if > 12 and ± 0.03 if lower. Titrations were done multiple times to reduce these experimental errors.

To isolate the titration of the guanidine moiety of the arginine side chain, it was necessary to account for the water background (i.e., the steep rise in pH under highly alkaline conditions due to the increasing amounts of concentrated KOH). This requires taking the difference between the titration of the arginine sample and that of a suitable blank. The difference titration gives the volume of titrant, ΔVol_a, needed to achieve a given pH in the presence of the arginine side chain relative to that blank. The blank can be the aqueous solution void of arginine or, for practical reasons, a solution of lysine to help account for ionic strength effects. The difference curves were calculated by first interpolating the titration curves of both the arginine solution and the lysine solution, followed by their subtraction at equivalent pH values. The resultant difference curve is a convolution of three single-site binding isotherms: one for the guanidinium group of the arginine, one for the lysine side chain ζ-aminium, and one for the lysine α-aminium group. The arginine α-amino group, with a $pK_a \sim 9$ is not a factor because, unlike the case of the lysine sample, the titration of the arginine sample starts at pH ~ 11 and thus it is essentially fully deprotonated. Each isotherm was modeled according to the fundamental equation:

$$\bar{x} = \frac{\Delta Vol_a\,[KOH]}{n_M} = \frac{1}{1+10^{m(pK_a - pH)}} \qquad (1)$$

where $\bar{x}$ is the fraction of protons bound or released at each point of the titration, ΔVol_a is the total

differential volume of base added at point a, n_M is the initial number of moles of the molecule M, and [KOH] is the titrant molarity. The term m is a Hill coefficient used to evaluate the fitting, but fixed to 1 for the final analyses. The pK_a values were determined from fitting the difference titration curves to the following equation:

$$\Delta \mathrm{Vol_a} = \frac{n_{\mathrm{Arg}}}{[\mathrm{KOH}]}\left(\frac{1}{1+10^{\mathrm{m}\left(\mathrm{pK}_a(\mathrm{Arg_{guan}})-pH\right)}}\right) - \frac{n_{\mathrm{Lys}}}{[\mathrm{KOH}]}\left(\frac{1}{1+10^{\mathrm{m}(\mathrm{pK}_a(\mathrm{Lys}_\alpha)-pH)}} + \frac{1}{1+10^{\mathrm{m}(\mathrm{pK}_a(\mathrm{Lys}_\zeta)-pH)}}\right) \quad (2)$$

where $\mathrm{Arg_{gaun}}$ refers to the side chain guanidinium group of arginine, and Lys_α and Lys_ζ to the α-aminium and side chain ζ-aminium groups of lysine, respectively. The parameters were resolved by fitting all simultaneously, or by first analyzing the lysine-water difference curve and then fixing those parameters while fitting the remaining terms for the arginine−lysine difference curve.

NMR-monitored titrations

The blocked tripeptide acetyl-Gly-Arg-Gly-amide (>95% purity by HPLC) was purchased from Biometik. The tripeptide was initially at ~ 10 m*M* in 50 m*M* NaCl with 5% D_2O for lock and 1 m*M* DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as a pH-independent internal reference. The pH adjusted by addition of small aliquots of ~ 0.1 *M*, 1 *M*, or solid NaOH. Uniformly $^{13}C_6/^{15}N_4$-labeled L-arginine (Sigma-Aldrich) was initially at 10 m*M* or 100 m*M* in 50 m*M* NaCl with 5% D_2O and 1 m*M* DSS, and the pH adjusted by addition of 0.1 *M*, 1 *M*, or solid KOH. The solid hydroxide was used to obtain the highest pH values. Sample pH values were measured at room temperature (~20 °C) using a Thermal Scientific Orion* 3-Star pH meter and an Orion ROSS micro pH electrode (8220BNWP), calibrated with standard pH 7, 10, and 12.5 (Oakton) reference buffers. According to vendor's specifications, the electrode has a precision of ± 0.01 log units over a pH range of 0 to 14.

Spectra were recorded at 25 °C using a cryoprobe-equipped Bruker Avance III 500 MHz spectrometer. The ^{1}H and ^{13}C signals were referenced directly to internal DSS at 0.00 ppm, and ^{15}N referenced indirectly via magnetogyric ratios.[56] Chemical shifts for the unlabeled tripeptide were obtained from standard 1D ^{1}H-NMR and 2D magnitude-mode ^{13}C-HMBC spectra. Chemical shifts for the $^{13}C_6/^{15}N_4$-arginine were obtained from 1D ^{13}C-decoupled ^{1}H-NMR, ^{1}H/^{15}N-decoupled ^{13}C-NMR (with ^{1}H NOE), and ^{1}H/^{13}C-decoupled ^{15}N-NMR (with ^{1}H NOE) spectra. Samples were in 5 mm diameter NMR tubes, except at very high pH and ionic strength conditions, for which 3 mm tubes were used to enable tuning. Also, a 100 m*M* sample of $^{13}C_6/^{15}N_4$-arginine was required for the ^{15}N-NMR measurements because the Bruker TCI CryoProbe is designed for direct detection of ^{1}H and ^{13}C, but not ^{15}N signals.

Spectra were processed using Topspin 3. The pH-dependent chemical shifts were fit with GraphPad Prism and Matlab to published equations for one or two sequential titrations in order to obtain pK_a and limiting chemical shift values.[46] The standard deviations (fit precision) for the individual titrations shown in Figure 2 were obtained using a Monte Carlo approach with data points randomly varied by assumed standard deviations of 0.05 units for the sample pH, and 0.01/0.05/0.1 ppm for the ^{1}H/^{13}C/^{15}N chemical shifts. The pK_a values discussed in the text are the averages ± standard deviations of the means of the individually fit values for all nuclei reporting a given titration.

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