Zwitterion formation in hydrated amino acid, dipole bound anions: How many water molecules are required?

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While the naturally occurring amino acids are not zwitterions in the vapor phase, they are in aqueous solutions, implying that water plays an important role in inducing zwitterion formation. Together, these observations inspire the question, ''How many water molecules are required to induce zwitterion formation in a given amino acid molecule?'' In this paper, we address this question in the context of mass spectrometric and size-selected photoelectron spectroscopic studies of hydrated amino acid anions. We utilize the facts that zwitterions possess very large dipole moments, and that excess electrons can bind to strong dipole fields to form dipole bound anions, which in turn display distinctive and recognizible photoelectron spectral signatures. The appearance of dipole-bound photoelectron spectra of hydrated amino acid anions, beginning at a given hydration number, thus signals the onset of greatly enhanced dipole moments there and, by implication, of zwitterion formation. We find that five water molecules are needed to transform glycine into its zwitterion, while four each are required for phenylalanine and tryptophan. Since the excess electron may also make a contribution to zwitterion stabilization, these numbers are lower limits for how many water molecules are needed to induce zwitterion formation in these amino acids when no extra (net) charges are involved. © *2003 American Institute of Physics.* [DOI: 10.1063/1.1620501]

INTRODUCTION

The hydration of biological molecules ranks among the most important problems in biology. At the macroscopic level, seasonal changes in biological phenomena are often triggered by the availability of water. At the microscopic level, one of the more intriguing examples of water's dramatic effects on biological molecules is zwitterion formation in the naturally occurring amino acids. The facts that these amino acids are not zwitterions when isolated in the gas phase,[1–6] but that they are in aqueous solutions, implies that hydration drives their transformation. Moreover, the observation that humidity alone can awaken biological activity in dried proteins[7] hints that the required number of water molecules per unit may be small.

Theory has addressed this problem, with several calculations on the minimum number of water molecules needed to form the glycine zwitterion having been reported. Computations by Krogh-Jespersen *et al.*[8] and Tunon *et al.*[9] both found one water molecule not to be enough. Gordon *et al.*,[10] however, reported that just two water molecules can stabilize the glycine zwitterion in a local minimum. Calculations by Kassab *et al.*[11] found that three water molecules are sufficient to lower the glycine zwitterion's energy into coincidence with that of its non-zwitterion form. Kokpol *et al.*[12] predicted that the first hydration shell of the glycine zwitterion consists of five water molecules. Novoa[13] suggested a number around five or six. A study by Siebrand *et al.*,[14] favored the number, six. In the calculations of Foerner *et al.*,[15] even twelve water molecules were considered. The effect of hydration on zwitterion formation in amino acids has also been investigated theoretically for the cases of alanine and tryptophan. Suhai *et al.*[16] found that four waters stabilized the L-alanine zwitterion. Also, Simons *et al.*[17] found that three water molecules were sufficient to bring the zwitterionic form of tryptophan down to an energy only slightly higher than its non-zwitterionic counterpart. Furthermore, extensive calculations by Gutowski[18–21] have dealt with the influence of an extra electronic charge on zwitterion formation.

Most experiments on zwitterion formation in gas-phase amino acids have not dealt with the role of hydration. Williams *et al.*[22–24] utilized black body infrared radiative dissociation (in a FT mass spectrometer) along with modeling to study cation–amino acid complexes. He found that the interaction of protons and heavy alkali cations with amino acids stabilized their zwitterionic forms. Beauchamp *et al.*[25] used quadrupole ion-trap mass spectrometry to investigate cationized aggregates of amino acids, finding zwitterion (salt bridge) formation in them. Also, Wesdemiotis *et al.*[26] utilized tandem mass spectrometry to study alkali cation-induced zwitterion formation in amino acids. Furthermore, Bowers *et al.*[27,28] employed the ion mobility technique to study protonated and sodiated oligoglycines, and Jarrold *et al.*[29] used the same method to study alkali-cationized polyalanine peptides. Experiments on hydrated amino acid clusters are less common. As an extension of his pioneering work on the spectroscopy of bare amino acids, Levy *et al.*[30] measured a resonant two-photon ionization (R2PI) spectrum associated with hydrated tryptophan clusters. A year later in 1989, Sulkes *et al.*[31] recorded the laser induced fluorescence (LIF) spectrum of the tryptophan–single water complex. Over a

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decade later, Williams *et al.*[32,33] studied hydrated lithium-cationized valine complexes and found that hydration promoted further stabilization, with three waters and a lithium cation leading to zwitterion formation. Also recently, Simons *et al.*[17,34] conducted R2PI, ultraviolet (UV) hole-burning, and infrared (IR) spectroscopy on hydrated tryptophan clusters, correlating the results with his calculations.

The goal of the experiments reported here was to determine the minimum number of water molecules necessary to induce zwitterion formation in several α-amino acids. Our approach was to form hydrated amino acid clusters, to provide them with low energy electrons in jet expansions, and then to look with both mass spectrometry and anion photoelectron spectroscopy for evidence of zwitterion formation. We conducted these experiments with glycine (Gly), phenylalanine (Phe), and tryptophan (Trp). Although highlights of our findings for all three of these will be presented in this report, for brevity, we will illustrate our results mainly through the case of glycine.

EXPERIMENT AND RESULTS

Glycine was heated to ~170 °C in the source's stagnation chamber which itself was pressurized at 1–2 atm. with water vapor and argon. This mixture was allowed to expand through a 25 μm diameter nozzle into high vacuum. There, electrons from a biased Th–Ir filament were injected into the jet in the presence of axial magnetic fields. Through their many cooling collisions, these electrons formed swarms of much lower energy secondary electrons, which we believe to be the main agents of electron attachment. The resultant negative ions were then extracted and mass analyzed with a magnetic sector mass spectrometer. Source conditions for phenylalanine and tryptophan were similar to those for glycine, only slightly hotter, i.e., ~180–185 °C.

The mass spectrum of hydrated glycine cluster anions exhibited no peak at the mass of the glycine parent anion (only fragment ions). No peak was detected for $[Gly(H_2O)_1]^-$. Neither was ion intensity detected for $[Gly(H_2O)_2]^-$, nor for $[Gly(H_2O)_3]^-$, nor for $[Gly(H_2O)_4]^-$. At the mass of $[Gly(H_2O)_5]^-$, however, a peak appeared, and mass peaks in the $[Gly(H_2O)_n]^-$ series continued to appear out through $n=10$, after which the ion signals were quite weak. Thus, $[Gly(H_2O)_5]^-$ was the smallest intact hydrated glycine cluster anion to be seen. This result marks a ''sea change'' in the stability of hydrated glycine anions at $n=5$, and it alone could be interpreted as being indicative of the onset of zwitterion formation in glycine.

The mass spectra of hydrated phenylalanine and hydrated tryptophan anions also displayed size onsets for the formation of intact cluster anions, these occurring at $n=4$ (rather than $n=5$) in both of these two cases. Again, such onsets are suggestive of zwitterion formation in and of themselves. We did observe, however, that the mass spectra of hydrated phenylalanine and hydrated tryptophan anions were more complicated than that of hydrated glycine anions. The relative complexity of these two systems compared to that of glycine is possibly related to the ease with which electrons attack aromatic moieties, thereby opening additional channels for electron attachment.

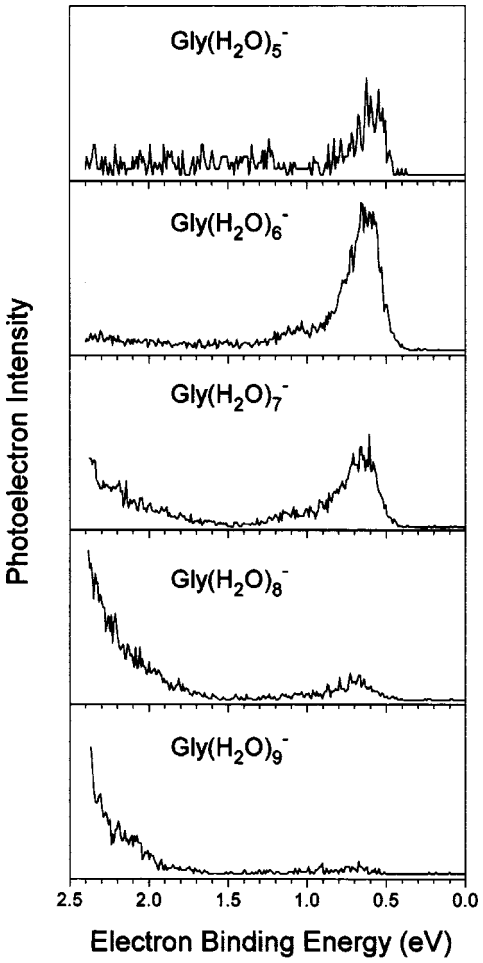

FIG. 1. The photoelectron spectra of hydrated glycine anions, $[Gly(H_2O)_n]^-$, $n=5-9$, recorded with 2.409 eV photons.

Having generated hydrated glycine, phenylalanine, and tryptophan cluster anions, we next conducted anion photoelectron spectroscopy on each of them separately. Negative ion photoelectron spectroscopy is conducted by crossing a mass-selected beam of anions with a fixed-frequency photon beam and energy-analyzing the resultant photodetached electrons. It is governed by the energy-conserving relationship, $h\nu = EBE + EKE$, where $h\nu$ is the photon energy, EBE is the electron binding energy, and EKE is the measured electron kinetic energy. Photodetachment was accomplished with 514 nm photons (2.409 eV/photon) from an argon ion laser operated intra-cavity. Photoelectrons were analyzed with a hemispherical electron energy analyzer, having a resolution of ~25 meV.[35]

Figure 1 presents the photoelectron spectra of $[Gly(H_2O)_n]^-$, $n=5-9$. The first few spectra are dominated by single, relatively narrow [full width at half maximum (FWHM) ~0.2 eV] peaks at low EBE. This peak in the spectrum of $[Gly(H_2O)_5]^-$ exhibits a low signal-to-noise ratio and is centered at an EBE of 0.59 eV. In the spectrum of $[Gly(H_2O)_6]^-$, a very similar-looking peak is stronger and centered at an EBE of 0.62 eV. The much smaller peak immediately to its high EBE side is due to the excitation of a

molecular water stretching motion. Peaks analogous to those at EBE's of 0.59 eV and 0.62 eV in the foregoing cases also persist in the spectra of $[Gly(H_2O)_n]^-$, $n=7$, 8, and 9. While there is a slight shift to higher EBE (typically ~0.03 eV) in each case, it is remarkable that all of these peaks appear at essentially the same EBE in their respective spectra. Thus, as a practical matter, this peak hardly shifts with hydration number. In addition to this low EBE peak, a high EBE feature also appears in the spectrum of $n=7$ and continues to increase in prominence in the spectra of $n=8$ and 9 relative to their low EBE peaks.

The photoelectron spectra of intact hydrated phenylalanine and hydrated tryptophan cluster anions are very similar to those of intact hydrated glycine anions. The same low EBE peak seen in the glycine case also emerged in the photoelectron spectra of both of these cases. In the case of hydrated phenylalanine anions, this peak (at EBE=0.6 eV) appeared with weak intensity first in $[Phe(H_2O)_4]^-$. By $[Phe(H_2O)_5]^-$, however, this low EBE peak, i.e., at the same spectral location, dominated its spectrum. By $[Phe(H_2O)_6]^-$, the same low EBE peak (again at EBE=0.6 eV) was present but weak again, and a high EBE tail-like feature made its debut in this species. In the case of hydrated tryptophan anions, only one size displayed the low EBE peak (at 0.6 eV) described above, and that was $[Trp(H_2O)_4]^-$. It also displayed high EBE features.

INTERPRETATION AND DISCUSSION

In interpreting these spectra, let us first consider the two simplest explanations, i.e., $[A(H_2O)_n]^-$ might be $A(H_2O)_n^-$ or it might be $A^-(H_2O)_n$, where A is an amino acid. Accordingly, one might suppose that these spectra are due to the photodetachment of water cluster anions which have been solvated, in each case, by an amino acid molecule. We recorded the photoelectron spectra of $(H_2O)_n^-$ cluster anions some years ago,[36] and they do not resemble the spectra under discussion here. Alternatively, one might also suppose that these spectra arise from the multiple hydration of the (nonzwitterionic) amino acid molecular anion. All three of the amino acid anions studied here, however, are highly unstable, autodetachment-prone anions. Electron transmission spectroscopy has determined the electron affinity of canonical glycine, for example, to be -1.9 eV.[37] Still, one might imagine the effect of many solvents to be the lowering of the energy of an amino acid anion into stability. Previous studies[38,39] have demonstrated solvent-stabilization of otherwise unstable anions in the cases of molecular anions solvated by water molecules. Characteristic of this phenomena, however, are successive solvent shifts (typically, ~0.2 eV) in the ensuing spectra beyond the cluster anion size with the minimum number of solvent molecules necessary for achieving anion stability. Not only are five waters too few to stabilize the highly unstable glycine anion, the fact that sequential solvent shifts are absent among the low EBE peaks in the spectra of $[Gly(H_2O)_n]^-$ is even more decisive evidence against solvent stabilization of the glycine valence anion being an explanation. Thus, these two explanations are eliminated.

It is evident that some kind of transformation has occurred by $n=5$ in $[Gly(H_2O)_n]^-$, and that it can not be explained in terms of a simple solvated anion model. The interaction that gives rise to this species must have a significant cooperative aspect. The interpretation most consistent with our results is zwitterion formation. As a consequence of their internal charge separations, zwitterions possess large dipole moments. For isolated amino acid zwitterions, the magnitude of this dipole moment has been variously estimated by theory[18,40] to be between 11 and 16 D. There are two ways in which an excess electron could attach itself to such a charge-separated, yet net neutral species. One would be to form its valence anion. Anions of salt molecules are well known, e.g., alkali halide anions,[41] but if zwitterionic $Gly(H_2O)_5$ were to form such a valence anion, solvation by additional water molecules would surely cause successive spectral shifts, and these are not observed among the low EBE spectral peaks.

The other opportunity for electron attachment is dipole-binding. In that case, an excess electron is bound by the dipolar field of a neutral molecule or cluster, forming its anion. Among the conditions that such a neutral species must meet is that it possess a dipole moment of ~2.5 D or greater. Some time ago, we found that anions in which the excess electron is dipole-bound display distinctive and therefore recognizable photoelectron spectral signatures.[42] These spectra are dominated by single, narrow peaks located at relatively low EBE, often with weaker spectral features to their high EBE sides, these latter features being due to the molecular vibrations of their components. To illustrate the characteristic photoelectron signature of dipole bound anions, several examples from our work are shown in Fig. 2. (All of the spectra shown are for species where the excess electron interacts with a single net dipole, except for $(HF)_3^-$, where the electron is thought to interact separately with both dimeric and molecular moieties.[43]) As a rule of thumb, the EBE of the dipole-bound peak is expected to increase with the dipole moment of the neutral molecule or cluster, while the width of the peak broadens with the internal mode complexity of the system. Figure 2 demonstrates that the spectrum of $[Gly(H_2O)_5]^-$ fits nicely into a progression of dipole-bound photoelectron spectra, where their EBE's as well as their widths gradually increase in going from the top of the figure to its bottom. This comparison shows that dipole-binding is a natural explanation for the spectrum of $[Gly(H_2O)_5]^-$ and the analogous low EBE peaks of the other $[Gly(H_2O)_n]^-$ species. The low EBE spectral profiles of the $[Gly(H_2O)_n]^-$ cluster anions look like those of dipole bound anions.

For all the species studied here, their low EBE peaks occur at ~0.6 eV. This EBE is large in comparison to most known dipole bound systems, and it suggests electron binding to relatively high dipole moments. It is interesting to estimate whether this value of EBE implies a dipole moment which is reasonable for a zwitterion, i.e., a consistency check. Using a calibration curve based on the pseudopotential calculations of Desfrancois and Schermann[44] and assuming their "large" molecule limiting case, implies that a dipole-bound EBE of 0.6 eV corresponds to a dipole moment

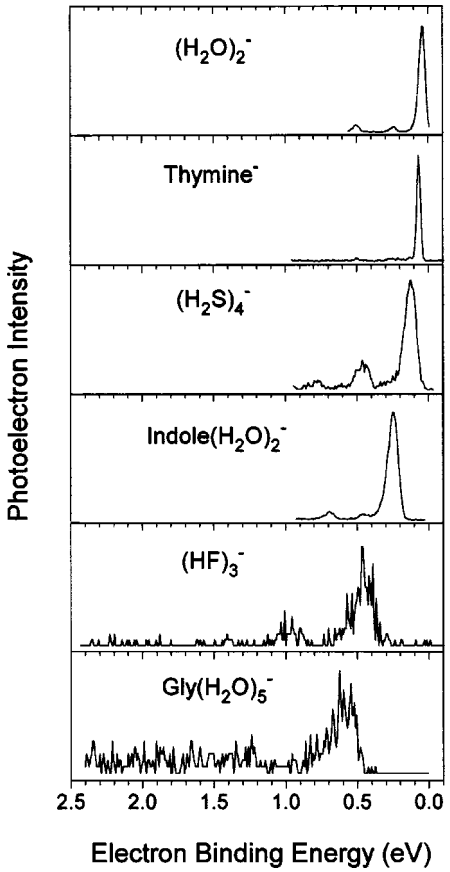

FIG. 2. A comparison of selected dipole-bound electron photoelectron spectra.

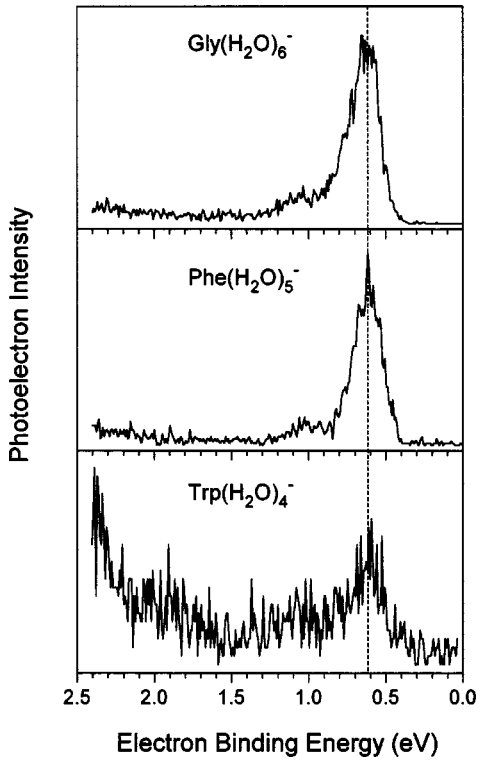

FIG. 3. A comparison of representative anion photoelectron spectra taken from our results with hydrated glycine anions, hydrated phenylalanine anions, and hydrated tryptophan anions.

of roughly 12 D. Twelve Debye is certainly a reasonable value for the dipole moment of an isolated zwitterion. However, the zwitterion species described here have several water molecules associated with them, and while we do not know their structural arrangement in relation to the charge-separated portion of the complex, they are probably oriented so as to at least partially counteract the dipole moment of an isolated zwitterion. Thus, we have only a rough idea of what the resulting dipole moment value should be. Likewise, methods for estimating dipole moment values from measured EBEs are not well developed. All in all, however, a dipole bound spectral peak at EBE=0.6 eV is plausible as a hydrated zwitterion feature.

Remarkably, the low EBE peaks seen in the spectra of hydrated phenylalanine and hydrated tryptophan anions are all located at essentially the same EBE values as in hydrated glycine anions, which themselves all appear at essentially the same EBE values. This striking finding is shown in Fig. 3, where the spectra of $[Gly(H_2O)_6]^-$, $[Phe(H_2O)_5]^-$, and $[Trp(H_2O)_4]^-$ are compared. $Gly(H_2O)_6^-$ was selected to represent the glycine case because it displays the highest quality, least complicated spectrum (see Fig. 1). The same reason applied to the selection of the $[Phe(H_2O)_5]^-$ spectrum to represent the phenylalanine case. For $[Trp(H_2O)_4]^-$, there was only one choice. The sameness of the low EBE peaks in each of the three amino acid systems studied supports our interpretation, because the R-group (residue) of an amino acid is spatially distinct from its charge-separated, zwitterion-forming portion. The low EBE peaks are insensitive to the differences between the R-groups. The excess electron is clearly interacting with the charge-separated portion, this being common to all three systems. Thus, it is understandable that different amino acid zwitterions should have essentially the same spectral (dipole-bound) signals.

Having established that five waters are necessary to induce zwitterion formation in the hydrated anions of glycine, while four are required in the hydrated anions of both phenylalanine and tryptophan, we next speculate as to the role of water molecules in zwitterion formation. Certainly, they serve to lower the energy of the zwitterionic form of a given amino acid in a thermodynamic sense. Beyond this, however, two specific functions present themselves. (1) The water molecules may provide a pathway for proton transfer[14,45] from the -COOH end of the amino acid to its $-NH_2$ end; (2) once formed, the water molecules may also stabilize one or both of the zwitterion's separated charges. Together, these two roles suggest a significant degree of orientation for those water molecules which were essential in initially forming zwitterion threshold species, e.g., those in $Gly(H_2O)_5^-$, $Phe(H_2O)_4^-$, and $Trp(H_2O)_4^-$.

These particular water molecules provide the microscopic environment for a given amino acid that makes proton transfer and zwitterion formation possible, and as such, they are in a category apart from other waters that may subsequently be added to the complex. In this light, the threshold size of a given hydrated amino acid complex can be seen as its core zwitterionic species. This may explain why the low EBE, dipole-bound peaks in the spectra of $Gly(H_2O)_n^-$ and

$Phe(H_2O)_n^-$ shift so little. Once the zwitterionic core is established, additional water molecules interact less strongly and have less need to be strictly oriented, producing relatively small changes in the dipole moment of the system and thus in its dipole-bound EBE. Subsequent water solvents simply hydrate the established core. Furthermore, extrapolation of empirical data suggests that the addition of even one Debye's worth of dipole moment to a pre-existing, large dipole moment of say, 10 D can be expected to change its dipole binding EBE by only ~45 meV.

A comprehensive interpretation of our results also requires an explanation for the high EBE spectral tails seen in the spectra of $Gly(H_2O)_n^-$ from $n=7-9$, of $Phe(H_2O)_n^-$ at $n=6$, and of $Trp(H_2O)_n^-$ at $n=4$. The shapes of these features at the edge of our electron energy window suggest that their full profiles must be quite broad. We tentatively interpret these features as being due to the photodetachment of hydrated zwitterion, valence anions. The strong interaction between an excess electron and a zwitterion salt causes the structure of the resulting valence anion to differ substantially from its corresponding neutral, inducing a broad Franck–Condon profile. (This is in contrast to the relatively narrow profile exhibited by dipole-bound systems, where the structures of the anion and its neutral are similar.) In the past, we have seen the competition between dipole-bound and valence cluster anions in their race for relative thermodynamic stability,[46] and that is probably what we are seeing here. For example, in the cases of $Gly(H_2O)_n^-$ at $n=5$ and 6, their valence anion manifestations sit higher in energy than their dipole-bound forms and thus go unseen, while at $n=7$, the valence form has caught up and the two have comparable energies. There, both are well populated and thus seen. By $n=8$ and 9, however, the valence forms are winning the race for relative stability, and the intensities of the dipole-bound forms are diminishing by comparison.

It is characteristic of dipole-bound electrons that they tend to reside outside the molecular framework of the dipole that tethers them. Since our results were gathered via dipole bound species, it is tempting to interpret them as pertaining to the formation of *net neutral zwitterions*, which themselves were simply tagged by their abilities to bind excess electrons through their dipole moments. In this picture, the excess (dipole bound) electron is only a spectator and a marker. This view is consistent with the fact that our results for the numbers of waters needed are in reasonable accord with most theory on neutral, hydration-induced zwitterion formation in simple amino acids. An alternative interpretation, however, is that the excess electron is an active participant, along with the water molecules, in inducing zwitterion formation. It is clear that positive charges can induce zwitterion stabilization, as has been seen in several studies involving amino acids with cations attached.[22–29] The interaction strength of a diffuse, dipole bound electron is, however, much weaker and thus is not analogous to that of an attached cation or anion with their much higher charge densities.

Nevertheless, in our case the excess (dipole bound) electron probably plays a role as a weak participant in zwitterion stabilization. The relatively high EBE of the dipole bound peaks observed in our spectra suggest a stronger and thus closer-in interaction of the excess electron with the framework of its polar complex than is often seen in weaker dipole bound anions, such as $(H_2O)_2^-$. Furthermore, calculations by Gutowski,[19] show that a dipole-bound electron can lower the energy of bare glycine's zwitterion, even though it still remains well above the energy of its non-zwitterionic form in that case. In addition, his calculations have found that an excess electron can stabilize the zwitterions of both arginine[20,21] and betaine[18] as dipole bound anions without hydration or other stabilizing agents. (Recent work in our lab confirms both of these predictions, qualitatively and quantitatively.) While arginine, with the highest proton affinity among the naturally occurring amino acids, was already on the edge for becoming a zwitterion and did not need much stabilization to do so, and while bare betaine is a zwitterion by itself, Gutowski's results nevertheless show the ability of dipole bound, excess electrons to participate actively in zwitterion stabilization. Thus, in the case of our present results, dipole bound excess electrons are likely helping the water molecules to stabilize the zwitterions, although we suspect that the role of the excess electrons is minor compared to that of hydration. Thus, we interpret five water molecules to be the minimum number required to induce zwitterion formation in glycine by hydration alone, i.e., without the influence of a net charge, while in phenylalanine and tryptophan the corresponding minimum number is four each. In light of this, we conclude that we have determined lower limits for the number of water molecules required, in these particular cases, for zwitterion formation in wholly neutral amino acids. The question of whether the stabilization provided by the excess electron is equivalent to that supplied by another water molecule is an interesting one. We speculate that it is not, and that the lower limits are probably also equal to the actual numbers of water molecules required for zwitterion formation in net neutral amino acid–water encounters.

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