Acid/base Properties of α-methyl and Gem-Dimethyl Derivatives of Cysteine and Serine from the Extended Kinetic Method

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Abstract:

The gas-phase proton affinities (PA) for the non-protein α-methyl cysteine (**1**), gem-dimethyl cysteine (penicillamine) (**2**), α-methyl serine (**3**) and 3-methylthreonine (**4**) have been determined using the extended kinetic method in ESI-tandem mass spectrometers. Experimental proton affinities of 923.5 ± 9.8, 925.0 ± 8.5, 932.1 ± 10.1, and 924.5 ± 7.7 were determined for **1-4**, respectively. Gas-phase enthalpies of deprotonation (ΔH_{acid}) for **2**, **3** and **4** were also determined experimentally to be 1379 ± 11 and 1380 ± 9 and 1378 ± 11 kJ/mol. Hybrid density functional theory calculations at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) level of theory give predictions for the proton affinities of **1-4** and ΔH_{acid} for **2-4** that are in excellent agreement with the measured values. A computed ΔH_{acid} for **1** of 1389 kJ/mol was also determined Computed acidities for **2-4** were also determined and the agreement with the experimental acidity for **2** is excellent. The computed acidities for **3** and **4** are somewhat larger than the experimental acidities, but are within the experimental error limits, For **1** and **2**, the preferred deprotonation site is the SH group on the side chain rather than the COOH group similar to other cysteine analogs that have previously been studied in our lab.

Introduction

The introduction of soft ionization sources, such as electrospray ionization (ESI)[1] and matrix-assisted laser desorption ionization (MALDI),[2] have allowed the power of modern mass spectrometers to be brought to bear on non-volatile analytes. The sensitivity, high resolution, and high mass range of current mass spectrometers provide a platform for both qualitative and quantitative measurements of nearly every conceivable molecule type, from small-molecule pharmaceuticals, to organic, inorganic and biological polymers, to intact protein complexes.[3] In addition to these analytical applications, mass spectrometers can also be used to study the gas-phase ion chemistry of a host of different molecules. A variety of gas-phase techniques, including MS-MS studies,[4] hydrogen-deuterium exchange reactions,[5] and spectroscopic methods,[6] have allowed for direct and indirect measurements of ion structure. By studying chemical reactions in the absence of solvent, one can gain information on the intrinsic properties of gas-phase ions, including the kinetics, mechanisms, and energetics of different reactions. In addition, fundamental quantities including heats of formation, gas-phase acidities and basicities, ionization potentials, and electron affinities can be determined for both neutral and charged molecules using standard techniques such as the equilibrium method,[7] the bracketing method,[7] and the Cooks kinetic method.[8-13]

Among the most fundamental properties of a molecule are its gas-phase acidity and gas-phase basicity. Pioneering studies of the reversal of the gas-phase acidity ordering of simple alcohols from that in solution by Brauman and co-workers demonstrated the need for knowledge of intrinsic chemistry as a necessary step for a complete understanding of solution behavior.[14] Similarly, the mobile proton model[15,16] for peptide fragmentation that is the foundation for automated peptide sequencing algorithms relies fundamentally on the basicity of the different

amide sites along the peptide backbone. A fundamental knowledge of the acid-base properties of amino acids both as isolated molecules and as part of peptide chains is therefore required for a complete understanding of the peptide fragmentation process.

Pioneering work by Locke and McIver[17,18] Kebarle and co-workers,[19] Amster and co-workers,[20] Bojesen and co-workers,[21,22] Harrison and co-workers,[23] Tabet and co-workers,[24] O'Hair and co-workers, Gronert and Bowie,[25] Poutsma and co-workers,[26-28] Cassady and co-workers,[29,30] and Bouchoux and co-workers,[31-40] using a variety of experimental and theoretical methods, established gas-phase acid-base values for the protein amino acids (PAA), i.e., those 20 amino acids that are used to form proteins. In addition to these PAAs, the Poutsma group has also been studying acid-base properties for a class of compounds known as "non-protein amino acids" (NPAA).[27,28,41-45] NPAAs are not coded for by RNA, but are found throughout nature as secondary products of plant and fungi metabolism.[46] Many NPAAs are similar in structure to one or more of the PAAs and can compete with them in a variety of biological processes, including being mis-incorporated into peptides and proteins.[47-53] NPAAs have also found use in peptide drug discovery experiments though *in vitro* translation coupled to mRNA display. *In vitro* translation systems can be tailored to deliberately incorporate NPAAs while excluding PAAs to create custom exotic peptides with enhanced drug-like properties.[54]

In addition to their biological relevance, NPAAs serve as useful model compounds for studying the interplay between amino acid structure and thermochemical properties. Simple substitutions can cause dramatic effects in structure and thermochemical properties. For example, substitution of an oxygen atom for the epsilon CH_2 group in arginine (Arg) results in the NPAA canavanine (Cav), which is a potent insecticide.[49,50,55] The electron withdrawing nature of the oxygen atom leads to a decrease in proton affinity in Cav of more than 40 kJ/mol as compared to

Arg[42] and leads to differing stability of its zwitterionic form when complexed to alkali metal ions.[56] Similarly, increasing the size of the five-membered ring in proline (Pro) to a six-membered ring leads to the NPAA pipecolic acid (Pip), which has a slightly larger PA than Pro,[27] and a causes a different selective fragmentation effect when incorporated into peptides.[57-59]

In 2007, in preparation for NPAA acidity studies, Poutsma and co-workers measured the gas-phase acidities of the 20 protein amino acids using the extended kinetic method including entropy effects for the first time.[26] One of the exciting conclusions from that study, which was published separately in collaboration with Kass and co-workers, was the computational prediction that cysteine (Cys) prefers to deprotonate at the thiol group of the side chain rather than at the COOH group.[26,60] The suggestion that cysteine possessed a thiolate structure in the gas phase was first put forth by Wang and co-workers based on their photoelectron spectroscopy study of deprotonated cysteine.[61] Several theoretical studies have since been performed that all predict that the SH group is more acidic than the COOH group.[26,60,62,63] However, the experimental situation is more complicated and the structure of deprotonated cysteine depends greatly on the manner of its preparation. An infrared multiple photon ionization (IRMPD) study by Oomens and co-workers found no evidence for the thiolate in the IR spectrum.[64] Further exploration of their setup concluded that their ESI source forms primarily solution-like structures, which would be the carboxylate.[65] A recent study by DeBlase et al. using cryogenic ion vibrational pre-dissociation spectroscopy on cryogenically cooled H/D isotopologs of deprotonated cysteine showed a strong hydrogen bond in the anion.[66] Their calculated potential for proton migration between the two sites is so shallow and the strength of the hydrogen bonding interaction so strong that the distinction between the thiolate and carboxylate structures is in their words “somewhat semantic”. Nevertheless, the fact that cysteine is predicted to deprotonate on the S-H has motivated us to

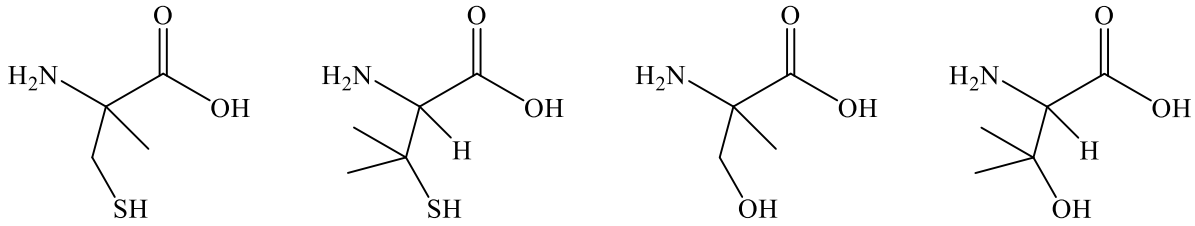
investigate other cysteine analogs to see how the deprotonation site preference and overall acid-base properties depend on the local structure of the side chain.

With this idea in mind, the gas-phase acid-base properties of homocysteine (hcys) and 5-mercaptonorvaline (hhcys), cysteine homologs with one and two additional methylene groups in the side chains, were determined.[45] Increasing the length of the side chain leads to an increase in proton affinity of 29 and 41 kJ/mol for hcys and hhcys due in part to an increase in overall polarizability of molecules as well as more favorable intramolecular hydrogen bonding in the cations. Calculations for deprotonated hcys and hhcys also predict that the S-H group is the preferred deprotonation site. Bearing in mind that strong intramolecular hydrogen bonding with the heteroatoms of the side chain plays a role in the unusual deprotonation behavior for Cys, hcys, and hhcys, the gas-phase acid-base properties of homoserine (hser) and 5-hydroxynorvaline (hhser), the analogous serine homologs, were also investigated. Intramolecular hydrogen bonding differences in the serine homologs were found to play a role in their proton affinities with increasing side-chain length leading to an increase in PA of 29 and 74 kJ/mol over Ser for hser and hhser, respectively. Changing the side chain length does not affect the acidity of the two serine homologs, nor does it affect the preferred site of deprotonation, which is predicted be to the COOH group for both molecules.

Backbone modified amino acids are of interest in peptide drug development as they conformational restrict secondary structures into predictable topologies. Specifically, α-methyl amino acids have been shown to constrict short peptides into an alpha helix folding pattern.[67] These properties have been exploited to create short peptide inhibitors of protein-protein interactions driven by helix-helix interactions.[68] In addition, chemical modifications on reactive side chains through “stapling” have been used to further constrict peptide secondary structure and add

proteolytic stability. Stapling is typically performed at sulfur-containing side-chain residues such as cysteine or cysteine analogs.[69] To this end, the Hartman group has been interested in incorporating cysteine[70] and serine[71] analogs into peptide discovery platforms to identify novel peptide inhibitors for protein-protein interactions.

Given the relevance of modified cysteine and serine analogs in drug discovery applications, an investigation of their intrinsic gas-phase acid-base properties is warranted. Here we present a combined kinetic method and density functional theory study of the gas-phase proton affinity and ΔH_{acid} for four serine/cysteine-based NPAAs, α-methylcysteine (amcys, **1**), gem-dimethylcysteine (dmcys, **2**), α-methylserine (amser, **3**) and gemdimethylserine (dmser, **4**).



amcys (**1**) dmcys (**2**) amser (**3**) dmser (**4**)

Experimental Section

Kinetic method experiments

All experiments were performed in a Thermo TSQ Quantum Ultra triple quadrupole instrument equipped with an IonMax ESI source. Full experimental details have been presented elsewhere.[27,43] For proton affinity studies, dilute solutions (*ca.* 1–10 x 10^{-3} M) of an amino acid and one of a series of reference bases in slightly acidified (1% formic acid) 50:50 methanol:water were directly infused (flow rates 5–15 μl/min) into the electrospray ionization source of the TSQ. Electrospray and ion focusing conditions were varied in order to maximize the ion count for the

proton-bound heterodimer $[A–H^+–B_i]^+$, where A is the analyte of interest and B_i is one of a series of reference compounds with known basicity. The proton-bound dimer ions were isolated in Q1 at a resolution of 0.7 – 1.0 amu and were allowed to pass into the rf-only collision cell (q2). The isolated ions were allowed to undergo collision-induced dissociation with argon gas maintained at a pressure of 0.5 mTorr. Product ion spectra were recorded at collision energies between 0 and 30 V (in the laboratory frame of reference). The intensities of each primary product ion and any secondary product ions were recorded and analyzed using standard extended kinetic method (KM) techniques.[8,10,12,13,72] Secondary product ion intensities were added to the corresponding primary product intensities before undergoing KM analysis. Experiments were repeated on at least three different days and were averaged to give the final ratios $\ln[B_iH^+/AH^+]$ for use in the KM workup. For gas-phase acidity studies, the method was the same except that basic (10% NH_4OH) 80:20 methanol:water solutions were used to form proton-bound dimer ions of the form $[A^––H^+–B_i^–]^–$.

Enthalpy (PA, ΔH_{acid}) and entropy contributions (ΔS_{prot}, ΔS_{deprot}) were obtained from the extended kinetic method (EKM) that has been described in detail elsewhere.[8,10,12,13,72] For proton affinity determinations, this method requires a plot of $\ln(I_{[BiH+]} / I_{[AH+]})$ vs. $PA_{Bi} – PA_{avg}$, where $I_{[BH+]}$ and $I_{[AH+]}$ are the intensities of the protonated reference base and amino acid products, PA_{Bi} is the proton affinity of the i^{th} reference base, and PA_{avg} is the average proton affinity of the set of *i* reference bases. For gas-phase acidity studies, the analysis is identical except that the ratios $\ln(I_{[ref – H]–} / I_{[A – H]–})$ are plotted versus $GA_{Bi} – GA_{avg}$ to make plot 1 (see Figure 4). Strictly speaking, the gas-phase acidity and gas-phase basicity of a molecule A refer to the free energy of deprotonation and negative free energy of protonation. The negative enthalpy of protonation is given the unique name proton affinity. Unfortunately there is no analogous unique term for the

deprotonation enthalpy ΔH_{acid}. In general discussions of acid/base behavior, when we use the term gas-phase acidity, we are referring to ΔH_{acid}, unless otherwise noted.

The Orthogonal Distance Regression (ODR) method as implemented in the ODR-pack program of Ervin and co-workers was used to extract proton affinities/deprotonation enthalpies and protonation/deprotonation entropies from the data.[72] In this method all ln(intensity ratios) for *m* reference bases at *n* collision energies are analyzed simultaneously. A total of *n* lines are generated and forced to cross at a single isothermal point, which gives the proton affinity/acidity and protonation/deprotonation entropy for the amino acid in question. This method also gives a realistic estimation of the errors in the measured quantities by using Monte Carlo simulations to determine isothermal points from randomly-perturbed intensity ratios. For these studies, we used a window of ± 8 kJ/mol in the reference acidity/basicity values and a window of ± 0.05 for the ln(ratio) values. Proton affinity/acidity values are reported with error bars corresponding to ± 1 standard deviation, as determined from the Monte Carlo simulations.

Strictly speaking, the entropy term from the EKM analysis is an average difference in activation entropy for the two dissociation channels. Numerous studies have shown that this quantity correlates strongly with the difference in protonation entropies. [10,73-78] The entropy values from ODR analysis are highly dependent on the actual deprotonation entropies of the reference bases and the uncertainty range in PA/GA of the Monte Carlo simulations. Rather than reporting the ΔS values as absolute protonation entropies, we prefer to use them as *semi-quantitative* estimates of the average difference in protonation entropy between the amino acid and reference bases/acids. The reference bases for this study were chosen partially because they have only one basic site and do not have the ability to form strong intramolecular hydrogen bonds when protonated. A large entropy difference between the amino acid and the reference bases can

therefore be used as a probe for an increase in intramolecular hydrogen bonding in the protonated amino acid versus the neutral (or the anion and the neutral in acidity measurements). The ODR workup also generates effective temperature values for each activation energy, which are used to generate the kinetic method plots shown in this manuscript.

Computational Methods

Predictions for proton affinities and ΔH_{acid} for all amino acids studies were also obtained from hybrid density functional theory calculations using the B3LYP functional combinations.[79,80] All *ab initio* and density functional theory calculations were performed using the Gaussian09 suite of programs.[81] The GMMX conformer searching routing in PCModel[82] was used to find conformations within 40 kJ/mol of the minimum-energy structure for all neutral, cationic, or anionic species. These structures are used as starting points for a series of *ab initio* and density functional theory calculations of progressively higher levels of theory. Ultimately, geometries and harmonic vibrational frequencies for the amino acids, their N-protonated forms, and their OH/SH-deprotonated forms were calculated at the B3LYP/6-31+G(d) level. Zero-point energy (ZPE) and thermal corrections were obtained from un-scaled harmonic vibrational frequencies. Total electronic energies were obtained using single-point energy calculations at the B3LYP/6-311++G(d,p) level and are combined with ZPE, thermal corrections, and a PV work term (= RT) to give 298 K enthalpy values. Total entropies were taken from the Gaussian09 output without scaling. Gibbs free energies were calculated by adding the "thermal correction to Gibbs Free Energy" obtained from B3LYP/6-31+G(d) frequency calculations to the B3LYP/6-311++G(d,p) single-point energies.

Predictions for the proton affinities for the amino acid homologs were computed directly from calculated enthalpies at 298 K according to reaction 1. For all of the amino acids in this study,

$$AAH^+ \longrightarrow AA + H^+ \quad (1)$$

we were able to locate multiple low-energy conformers. Thermochemical values presented here are Boltzmann-weighted enthalpy values for the different conformers obtained by determining relative gas-phase populations based on G_{298}. We chose the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) level of theory based on previous work on proton affinities of amino acids[27,28,42] in which the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) method gave nearly quantitative agreement with experimental PAs for a variety of nitrogenous bases including dimethyl amine, isopropyl, ethylene diamine and glycine.[83] Despite the excellent agreement for absolute proton affinities with literature values, we report here predictions for PA from isodesmic reaction 2, using glycine as a reference base with known proton affinity of 886.5 kJ/mol.[83]

$$AAH^+ + Gly \longrightarrow AA + GlyH^+ \quad (2)$$

For the gas-phase acidity studies, raw deprotonation enthalpies were computed from equation 3. For the cysteine analogs two different deprotonation sites were investigated, the COOH

$$AAH \longrightarrow [AA - H]^- + H^+ \quad (3)$$

terminus and the SH group of the side chain. During some of the geometry optimizations, proton transfer occurred between the two groups as their acidities are quite close. Final structures are identified in Figures 1, S1, and S2 and Table S2 with the site of deprotonation indicated.

Whereas the B3LYP/6-311++G(d,P)//B3LYP/6-31+G(d) method gives proton affinities in quantitative agreement with experimental values, it does not perform as well for calculated acidities of even the simplest carboxylic acids, such as acetic acid. The B3LYP/6-311++G(d,p)//6-31+G(d) method gives an raw acidity for HOAc that is 10 kJ/mol too low (1446 vs. 1456 kJ/mol).[84] In our PAA acidity study, we found that while this method does not give absolute acidity values that agree with experimental results, the *relative* acidity values predicted from this method are in excellent agreement with experimental acidities.[26] Therefore, in this study we used reaction

$$AAH + CH_3COO^- \longrightarrow [AA - H]^- + CH_3COOH \quad (4)$$

4 to give an isodesmic prediction for acidity of the amino acids in this study, with acetic acid used as the reference acid.

Materials

Amino acids were purchased from MP Biochemicals (α-methyl serine), Sigma (gem-dimethyl cysteine), Combi-Blocks (gem-dimethyl serine), and Nagase Chemical Company (α-methyl cysteine). All reference acids and bases were purchased from Sigma-Aldrich and were used without further purification. All solutions are made with HPLC grade methanol (Sigma-Aldrich) and 18 MΩ H_2O (Millipore).

Results and Discussion

Proton Affinities

The proton affinities of **1-4** were obtained from the extended kinetic method using the techniques described above. The reference bases used in the four studies and their proton affinities are given in Table 1. Experimental proton affinities for **1-4** are given in Table 2 along with

computationally predicted PAs. The lowest-energy computed structures for neutral and protonated **1** and **2** are shown in Figure 1 and those of neutral and protonated **3** and **4** are shown in Figure 2. Additional low-energy conformers and relative 298 K free energy values for **1-4** are shown in Figures S1 – S6.

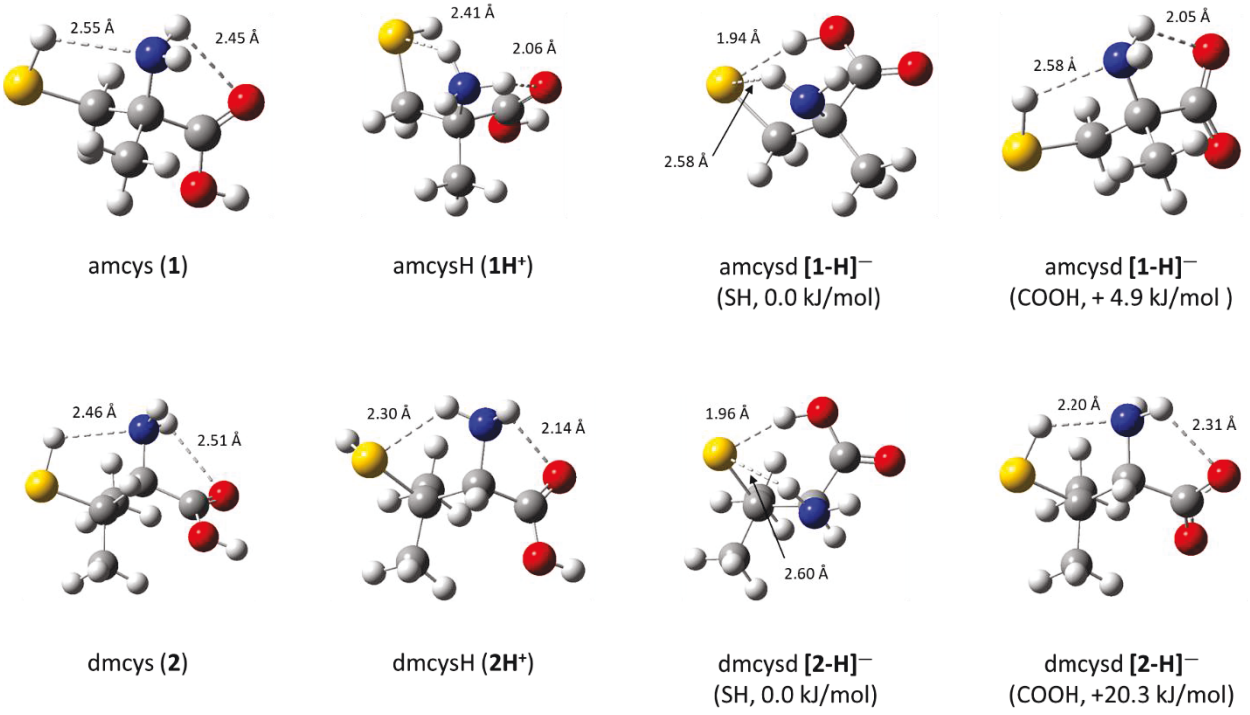


Figure 1. Lowest-energy conformations for neutral, protonated, SH-deprotonated, and COOH-deprotonated 1 and 2. Relative free energies at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d,p) level in kJ/mol.

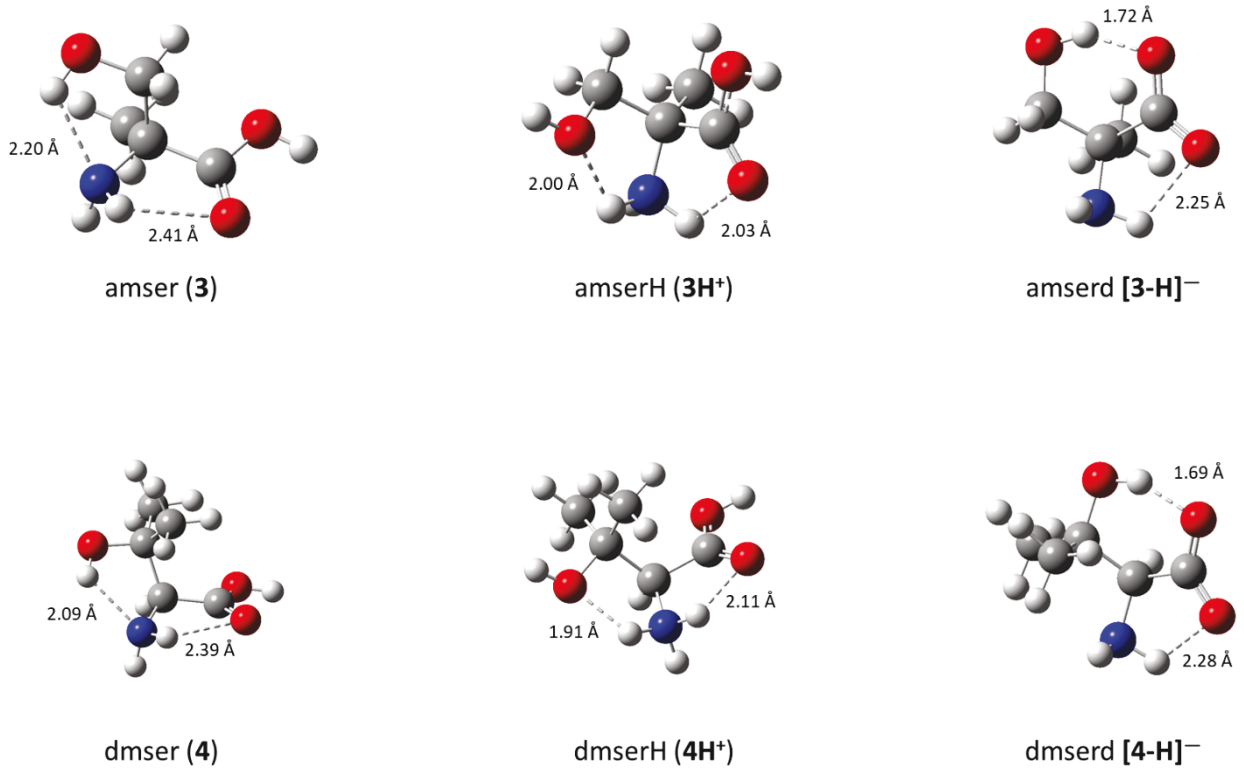


Figure 2. Lowest-energy conformations for neutral, protonated, and COOH-deprotonated 3 and 4.

For α-methyl cysteine (**1**), five reference bases were used in the kinetic method study with PAs ranging from 892.2 kJ/mol (benzamide) to 917.8 kJ/mol (n-propyl amine). Figure 3 shows the first kinetic method plot of $\ln(\mathbf{1H}^+/\mathrm{ref}_i\mathrm{H}^+)$ vs $\mathrm{PA\text{-}PA}_{avg}$ for the five reference bases at eight collision energies ranging from 3 to 24 eV (lab) in steps of 3 eV. The orthogonal distance regression (ODR) method was used to give a proton affinity of 923.5 ± 9.8 kJ/mol.

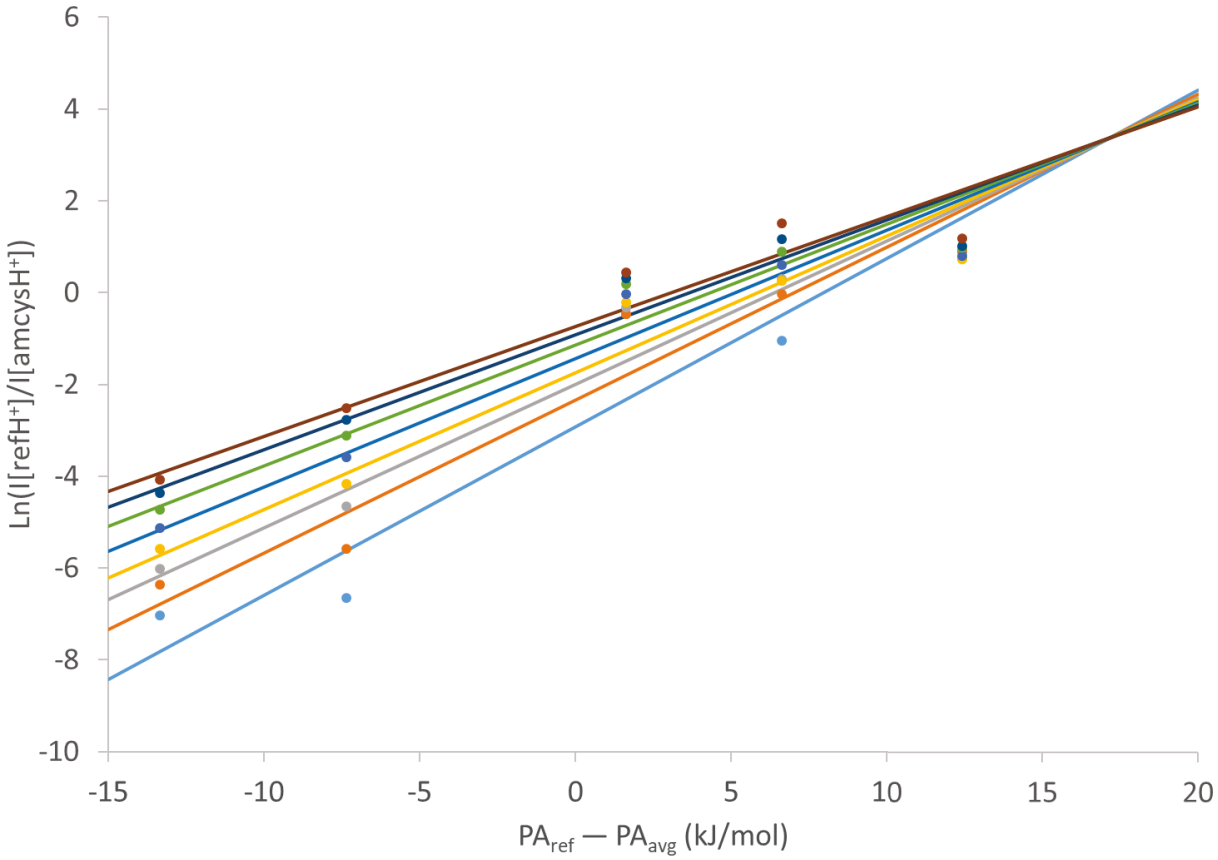


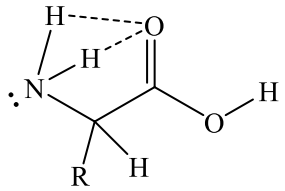
*Figure 3. First kinetic method plot for determination of proton affinity of **1**. Plot is of ln(RefH$^+$/1H$^+$) vs. PA – PA$_{avg}$. Collision energies range from 3 (blue) to 24 (maroon) eV lab in steps of 3 eV.*

Similar experimental procedures were used to determine proton affinities for **2-4**. The first kinetic method plots are shown in Figures S7-S9. For **2**, five reference bases with proton affinities in the range of 898.0 kJ/mol (N-ethylacetamide) to 922.7 kJ/mol (benzylamine) were used. For **3**, four reference bases with PAs between 912.0 kJ/mol (ethylamine) and 924.8 kJ/mol (isobutylamine) were used, and finally, four bases with PAs between 908.0 kJ/mol (N,N-dimethylacetamide) and 924.8 kJ/mol (isobutylamine) were used of the kinetic method study for **4.** The ODR workups gave PAs of 925.0 ± 8.5, 932.2 ± 10.2, and 924.5 ± 7.7 kJ/mol for **2-4**.

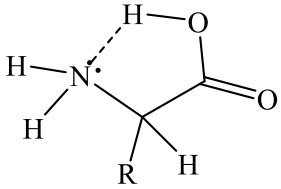
In addition to the proton affinity values, the ODR analysis gives a protonation entropy value. This quantity is the average difference in protonation entropy between the analyte and the set of reference bases used in the kinetic method study. An entropy difference of –29 J mol^{-1} K^{-1}

was determined for **1**, which indicates a moderate amount difference in hydrogen bonding between **1H**$^+$ and **1**. For comparison, the α–ω-diamines[85-87] and lysine homologs[28] have protonation entropies that are in the range of –50 to –100 J mol^{-1} K^{-1}. Protonation entropies of –33, –25, and –4 J mol^{-1} K^{-1} were determined for **2-4**, which also indicate a moderate increase in hydrogen bonding between the cations and the neutrals for **2** and **3** and very little increase in hydrogen bonding between **4** and **4H**$^+$. These entropy values can be rationalized by a careful examination of the geometries predicted for **1-4** and **1H**$^+$**-4H**$^+$ from density functional theory calculations (see below)

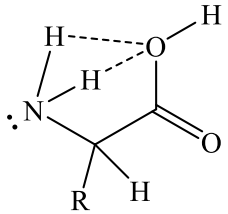
All amino acids have at least some degree of intramolecular hydrogen bonding in their neutral forms as the amino group can interact with the oxygen atoms of the carboxylic acid group. If the strength of the interaction increases upon protonation, one would expect to measure a large protonation entropy. Previous studies have identified three general hydrogen bonding motifs between the amino and COOH groups in neutral amino acids.[31,33-36,88-91] Using the nomenclature of Snoek,[88] motif type *i* involves symmetric hydrogen bonds between the two amino hydrogen



motif i



motif ii



motif iii

atoms and the carbonyl oxygen of the carboxylic acid group. In addition, the OH group of the COOH group is in the *syn* arrangement. There is also a variation of motif *i* in which the amino group is rotated such that only one hydrogen bond is formed with the carbonyl oxygen. Motif *ii* involves an *anti*-arrangement of the OH with respect to the C=O group and a hydrogen bond

between the OH hydrogen atom and the amino nitrogen. Motif *iii* is very similar to type-i except that the amine hydrogen atoms hydrogen bond with the OH oxygen atom of the carbonyl group. Using free energy as a comparison, motif *i* is preferred for the amino acids with aliphatic side chains (Ala, Gly, Ile, Leu, Phe,[35] and Val).[36,91] This H-bonding motif also allows for additional hydrogen bonding interactions between the amino nitrogen and alcohol side chains (Ser, Thr, Tyr[35])[91], sulfur-containing side chains (Cys and Met)[91], carboxylic acid side chains (Asp[91], Glu[34,91]), and the side chains of Arg, Gln, and Lys,[91] and is the preferred motif for these AAs. It is interesting to note that the lowest free energy conformer for both cysteine and serine is of type *i*, whereas the lowest enthalpy conformer is of type *ii* for both amino acids.[60,64,66,90] According to free energy, motif *ii* is the preferred H-bonding motif for Asn,[91] His,[91] Pro,[36,91] and Trp.[91]

The lowest-energy conformer for **1** is of type *i* with a hydrogen bond of 2.45 Å between one of the amino hydrogen atoms and the carbonyl oxygen and an interaction (2.55 Å) between the hydrogen on the sulfur and the amino nitrogen as shown in Figures 1 and S1 (in Figure S1, this conformer is labelled as amcys_001, where our naming scheme refers to the ranking of all unique conformers by 298K free energy). Interestingly this structure is slightly different from the preferred conformer for cysteine in which the S-H interaction is with the OH of the carboxylic acid.[91] Conformers with motifs *ii* and *iii* were located lying 3.1 and 4.2 kJ/mol higher in energy. In addition a conformer with an interaction between an amino hydrogen and the sulfur atom in the side chain was located lying 7.0 kJ/mol higher in energy. Six additional conformers were located with 298 K free energies greater than 7.0 kJ/mol above the lowest-energy conformer, of type *i*, but with no additional interaction of the side chain SH group with the termini. In the Supporting Figures, we only include the lowest-energy conformer for each unique hydrogen bonding arrangement found in the conformational searches. A complete list of all unique conformers for

neutral, protonated and deprotonated **1-4** along with their free energies and enthalpies is given in Table S1 of Supporting Information. Total electronic energies, thermal corrections, 298 K enthalpies, free energy corrections, and 298 K free energies (hartrees) for the lowest free energy conformers of neutral, protonated, deprotonated amino acids **1-4** at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) level of theory are given in Table 3.

Similar results are seen in **2-4**, in which the lowest energy conformers are all of type *i* with the added stability of an interaction of the hydrogen atom on the side chain (SH for **2**, Figure 1 and OH for **3** and **4**, Figure 2) with the nitrogen atom of the amino group. Of note is the hydrogen bonding distance between the amino hydrogen atoms and the carbonyl oxygen atom which is predicted to be 2.51, 2.41, and 2.39 Å for **2-4**. An additional SH---N interaction of 2.46 Å is also predicted for **2**. In the serine analogs **3** and **4**, the hydrogen bond between the OH hydrogen of the side chain and the amino nitrogen atom is even stronger than the NH----O=C interaction with H-bonding distances of 2.20 and 2.09 Å, respectively.

The amino group is the preferred site of protonation for $\mathbf{1H}^+$ and it adopts a conformation with the hydrogen atoms on the protonated amino group interacting with the carbonyl oxygen atom. The interaction strengthens to a H-bonding distance of 2.06 Å. A new H-bond is formed between the hydrogen atom on the protonated amine and the sulfur atom of the side chain (2.41 Å) as shown in Figure 1. The increase in hydrogen bonding interaction is consistent with the stronger charge-dipole interaction of the protonated amino group and is consistent with the protonation entropy term of –29 J mol^{-1} $K^{-1}$from the EKM experiments. Protonated $\mathbf{2H}^+$ adopts a similar type-i-like structures with a strong hydrogen bond between the amino hydrogen atom and the carbonyl oxygen (2.03 Å) and a weaker interaction between an amino hydrogen and the sulfur

atom of the side chain (2.41 Å) as shown in Figure 1. Figure 2 shows that the protonated serine analogs **3H**$^+$ and **4H**$^+$ also form type-*i*-like structures with strong hydrogen bonds between amino hydrogen atoms and both the carbonyl oxygen atom and the oxygen atom of the hydroxyl group on the side chain. As in the neutrals, the interaction between the side chain OH and the amino nitrogen atom is the stronger interaction. The increase in hydrogen bonding interactions in the cations of **2** and **3** is also consistent with the entropy terms of –33 and –25 J mol^{-1} K^{-1}. That is, the formation of a more conformationally- constrained structures upon protonation leads to the large negative entropies. The relatively small value for the experimentally-measured entropy for **4** of –4 J mol^{-1} K^{-1} deserves comment. The decrease in NH---O=C H-bond length from neutral to cation in amino acids **1-4** is 0.39, 0.37, 0.38, and 0.28 Å, respectively, so the smaller change in geometry upon protonation may account for the smaller entropy for **4**.

As shown in Figures S1-S6, Additional higher-lying cations were located in our conformational search for **1H**$^+$ **– 4H**$^+$. Type-*iii* like structures with the protonated amine hydrogens interacting with the OH oxygen atom of the COOH group and the heteroatom of the side chain were located lying 10.1, 11.0, 11.2, and 19.0 kJ/mol above the global minima for **1H**$^+$**-4H**$^+$. Other higher-lying conformers with unique hydrogen bonding arrangements were located for the cations all of which lie more than 20 kJ/mol above the respective global minimum structures and are not expected to contribute to gas-phase populations at room temperature.

Using the 298 K free energy as a weighting factor, Boltzmann-weighted enthalpy values were obtained for neutral and protonated **1-4**. These enthalpies lead to predictions for raw proton affinities of **1-4** of 925.1, 919.0, 929.0, and 927.6 kJ/mol. In our previous work,[27,28,42] we have shown that the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) method gave nearly quantitative agreement with experimental PAs[83] for a variety of nitrogenous bases including dimethyl amine,

isopropyl amine, ethylene diamine and glycine. In addition, we have demonstrated that using isodesmic reactions at this level of theory gives predictions for proton affinities for amino acids that are within ± 4 – 8 kJ/mol of our experimental values.[27,28,42,44,45] Using isodesmic reaction 2 with glycine (PA = 886.5)[83] serving as a reference base gives Boltzmann-weighted predicted PAs of 927.0, 920.9, 931.0, and 929.6 kJ/mol for **1-4**. These predictions are in excellent agreement with the experimental proton affinities from the kinetic method studies with a mean absolute deviation of 3.4 kJ/mol.

According to the NIST website,[84] the proton affinities of cysteine and serine are 903.2 and 914.6 kJ/mol as tabulated in the 1998 Hunter and Lias proton affinity review.[83] These values are in quantitative agreement with more recent computations study by Gronert using G3MP2 theory.[91] Comparison with these values with our experimental results indicates that α-methylation increases the proton affinity of cysteine by *ca*. 20 kJ/mol and of cysteine by *ca*. 17 kJ/mol. These are slightly larger than the difference between the PAs of alanine and glycine (15.1 kJ/mol)[84] or for that of α-methylalanine (α-aminoisobutanoic acid, Aib) and alanine (11.4 kJ/mol).[84,92] Gem-dimethylation on the side chain also increases the proton affinity of cysteine by *ca.* 22 kJ/mol and of serine by a smaller amount, *ca*. 10 kJ/mol. The relevant comparison for gem-dimethylation is valine and alanine, which have a difference in PA of 9 kJ/mol.[84] This difference is on the same order as our measured difference for gem-dimethylation of Ser (10 kJ/mol) and about half as large as our difference for gem-dimethylation for Cys (22 kJ/mol). The increase in PA with α-methylation and gem-dimethylation is consistent with the greater overall polarizability of the methylated amino acids as well as the inductive effects of the added methyl groups.

Gas-Phase Acidities

Similar procedures were used to measure the gas-phase acidities (ΔH_{acid}) for **1-4**. Deprotonated **1** is not stable in the basic water:methanol solutions needed for kinetic method studies as it forms a dimer with a disulfide bond readily under these conditions. Consequently, we were only able to measure ΔH_{acid} values for **2-4**. Reference acids used in these kinetic method studies are given in Table 4 and the experimental acidities are listed in Table 2 along with predictions for deprotonation enthalpies at the COOH group and for deprotonation of the S-H groups of **1** and **2**.

For the kinetic method study for **2**, we used five reference acids ranging in ΔH_{acid} from 1388 kJ/mol (2-nitrobenzoic acid) to 1406 kJ/mol (3-fluorobenzoic acid). Figure 4 shows the first kinetic method plot for the determination of the gas-phase acidity of **2** showing data for the five reference acids and eight collision energies ranging from 3 to 24 V lab. ODR analysis gives a value for the deprotonation enthalpy of 1380 ± 9 kJ/mol and a deprotonation entropy

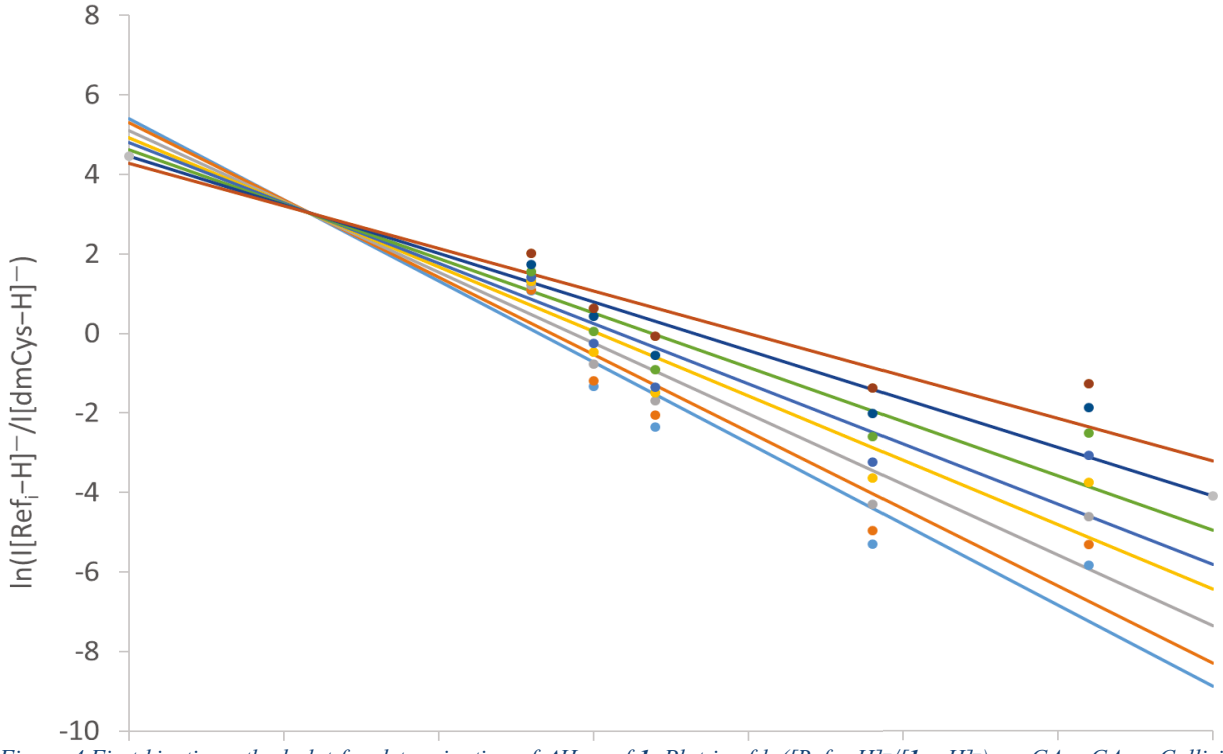


*Figure 4.First kinetic method plot for determination of ΔH_{acid} of **1**. Plot is of ln([Ref – H]$^-$/[**1** – H]$^-$) vs. GA – GA_{avg}. Collision energies range from 3 (blue) to 24 (maroon) eV lab in steps of 3 eV.*

of – –24 J mol^{-1} K^{-1}. For **3** and **4**, the same four reference acids were used: triflurobenzoic acid, 4-hydroxybenzophenone, 3-nitrophenol, and 4-fluorobenzoic acid. First kinetic method plots are shown in Figure S10 and S11. Deprotonation enthalpies of 1379 ± 12 and 1378 ± 12 were determined - for **3** and **4** using ODR analysis. Deprotonation entropies of –26 and –15 J mol^{-1} K^{-1} were also determined for **3** and **4** indicating a moderate difference in hydrogen bonding between the neutral amino acids and their anions.

As with our previous work with the gas-phase acidities of cysteine,[26,60] homocysteine (hcys),[45] and 5-mercaptonorvaline (hhcys)[45] the kinetic method gives no indication of which site is being deprotonated. As shown in Figure 1, the preferred site of deprotonation at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) level of theory for both **1** and **2** is the S-H group of the side chain. In both anions, the lowest-energy conformers have hydrogen bonding-interactions between the COOH hydrogen atom and the thiolate anion indicating some degree of sharing of the proton similar to cysteine and thus, that the acidities of the two sites are quite close to each other. The lowest energy conformer for **1-H**$^-$ that contains a deprotonated acid group and an S-H in the side chain lies 4.9 kJ/mol higher in free energy and has the same hydrogen bonding arrangement as neutral **1** (type *i*). The analogous structure for **2-H**$^-$ lies 20.3 kJ/mol higher in free energy and is again the lowest energy conformer for **2-H**$^-$ with a formally deprotonated COOH group. The formation of the short-strong hydrogen bond between the OH hydrogen atom and the thiolate sulfur atom in both **1-H**$^-$ (1.94 Å) and **2-H**$^-$ (1.96 Å) indicates that deprotonation should be entropically disfavored and is consistent with the entropy terms from the KM experiments of –24, –26, and –15 J mol^{-1} K^{-1}.

For amino acids **3** and **4**, the situation is more straightforward as the carboxylic acid group is the only strongly acidic site in these molecules. Figure 2 shows that the preferred conformers

for both **3-H**$^-$ and **4-H**$^-$ involves hydrogen bonding interactions between the carboxylate oxygen atoms with both the OH group of the side chain and one of the amino hydrogens. The OH---O=C interaction in the anions (1.72 Å, 1.69 Å) of **3-H**$^-$ and **4-H**$^-$ is much stronger than the OH---NH_2 interaction in the neutrals (2.20 Å, 2.09 Å), again suggests that deprotonation should be entropically disfavored, which is consistent with the measured entropy terms from the EKM experiments.

Raw deprotonation enthalpies were derived from Boltzmann-weighted free energies of all deprotonated amino acid conformers located in our conformational searches. Predictions of 1380, 1372, 1379, and 1381 kJ/mol were obtained for ΔH_{acid} for **1-4**. As mentioned in the theoretical methods section, the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) method underestimates the acidity for acetic acid by ~10 kJ/mol. Using equation 4 with acetic acid as the reference acid (ΔH_{acid} = 1456.0) gives isodesmic predictions for ΔH_{acid} of 1390 , 1382, 1388, and 1390 kJ/mol. The isodesmic prediction for **2** is in excellent agreement with the experimental acidity of 1380 ± 9 kJ/mol. The agreement between predicted and experimental acidities for **3** and **4** are in less good agreement with differences of 9 and 12 kJ/mol, respectively.

Given the difference between the experimental and computed acidities for **3** and **4**, we decided to perform some additional calculations on these species to see if changing either the method or the basis set of the single point energy calculations had a large effect on the computed acidities. We carried out single-point energy calculations at the B3LYP/6-311++G(3df,2p) and MP2/6-311++G(d,p) levels and the results are given in Table S2. The raw Boltzmann-weighted acidity for **3** at these levels actually increases from 1379 kJ/mol using B3LYP/6-311++G(d,p) single points to 1383 and 1386 kJ/mol at the B3LYP/6-311++G(3df,2p) and MP2/6-311++G(d,p) levels, respectively, which are in worse agreement with the experimental acidity. Boltzmann-

weighted isodesmic acidities of 1389 and 1386 kJ/mol are derived for **3** at the B3LYP/6-311++G(3df,2p) and MP2/6-311++G(d,p) levels, which are within 2 kJ/mol of the Boltzmann-weighted isodesmic acidity using B3LYP/6-311G(d,p) single point energies (1388). In addition to the single-single point energy calculations, we also re-optimized the geometries of all neutral and anion conformers for **3** using the B3LYP/6-311++G(d,p) basis set at the B3LYP, M06-2X, and MP2 levels of theory. As can be seen in Table S2, the Boltzmann-weighted isodesmic acidities differ by less than 3 kJ/mol from the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d,p) level. The lowest energy conformer for both the neutral and anion are the same at each level and Figures S3 and S4 show that the relative ordering of the conformers is relatively consistent across the methods.

Similar results were seen for **4** in which we performed additional single point energies at the B3LYP/6-31+G(d) geometries and re-optimized the geometries with 6-311++G(d,p) basis sets, though only at B3LYP and M06-2X levels. As with **3**, the Boltzmann-weighted isodesmic acidities differ by less than 2 kJ/mol from the original calculations. Given the agreement between the computed acidities from the different levels of theory, it appear that the experimental acidities for **3** and **4** may be somewhat low. It should be noted that the computed acidity values for both **3** and fall within the uncertainty ranges of the measured acidities (1379 ± 12 kJ/mol and 1378 ± 12 kJ/mol). For completeness, we also performed similar calculations for protonated **3** and **4** and determined Boltzmann-weighted proton affinities. Table S2 shows that the density functional theory-based calculations all give PAs within 2 kJ/mol of each other and the MP2 calculations give proton affinities that are on the order of 5 kJ/mol lower. All computed PA values are within the error bars of the measured experimental affinities.

As has been seen in our previous work on the gas-phase acidity of other NPAAs,[43-45] substitutions generally have a much smaller effect on the gas-phase acidity that they do on the proton affinities. This is mostly true in this study. Whereas α-methylation and gem-dimethylation increase the PA of **1-4** on the order of 10-20 kJ/mol over their PAA counterparts, the increase in acidity is somewhat smaller than that. For the cysteine analogs, the increase in acidity is 6 and 13 kJ/mol for **1** and **2** over cysteine (ΔH_{acid}=1395 ± 9 kJ/mol),[26,93] again consistent with the inductive effects of the added methyl groups and the overall increased polarizability of the analogs. Using the raw experimental acidities for **3** and **4** indicates an increase in ΔH_{acid} of *ca*. 12 kJ/mol, over serine (ΔH_{acid} =1391 ± 22 kJ/mol)[26,93], though given the uncertainties in both the acidity of serine and of the analogs, the effect is probably quite small.

Conclusions

Gas-phase proton affinities for **1-4** were determined using the extended kinetic method and density functional theory calculations. α-methylation and gem-dimethylation increases the proton affinity of cysteine and serine by around 10-20 kJ/mol. Smaller effects were seen for these substitutions on the gas-phase acidity of **1-4** versus their PAA counterparts, consistent with other serine and cysteine-analogs that were previously determined in our lab. Calculations predict that the preferred deprotonation sites for **1** and **2** are the S-H group on the side chain. Future studies are planned to investigate these anions by gas-phase hydrogen-deuterium exchange and by infrared multiple photon dissociation spectroscopy in order to gain a more complete picture of their deprotonation chemistry.

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Tables

Table 1. Reference bases used in kinetic method studies

Reference Base	PA (kJ/mol)	amcys (**1**)	dmcys (**2**)	amser (**3**)	dmser (**4**)
benzamide	892.1	X			
N-ethylacetamide	898.0	X			
thiazole	904.0		X		
pyridazine	907.2	X	X		
N,N-dimethylacetamide	908.0		X		X
ethyl amine	912.0	X		X	X
4-chloropyridine	916.1			X	
n-propyl amine	917.8	X		X	X
benzyl amine	922.7		X		
i-butyl amine	924.8			X	X

Table 2. Measured and calculated Boltzmann-weighted proton affinities and gas-phase acidities (kJ/mol) for amino acids **1-4**

molecule	PA (exp.)	PA (theor)[a]	GA (exp.)	GA (theor., SH)[b]	GA (theor., COOH)[b]
amcys (**1**)	923.5 ± 9.8	927.0	---	1389	1408
dmcys (**2**)	925.0 ± 8.5	920.9	1380 ± 9	1383	1408
amser (**3**)	932.1 ± 10.1	931.0	1379 ± 12	---	1388
dmser(**4**)	924.5 ± 7.7	929.6	1378 ± 12	---	1390

[a] isodesmic to glycine (PA = 886.5 kJ/mol). [b] isodesmic to acetic acid (ΔH_{acid} = 1456 kJ/mol)

Table 3. Total electronic energies, thermal corrections, 298 K enthalpies, free energy corrections, and 298 K free energies (hartrees) for the lowest free energy conformers of neutral, protonated, and deprotonated amino acids **1-4** at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) level of theory.

Molecule	$E_{electronic}$	H corr.	H^0_{298}	G corr.	G^0_{298}
amcys (**1**)	-761.393157	0.146272	-761.246885	0.100491	-761.292666
amcysH^+	-761.757189	0.160557	-761.596632	0.115850	-761.641339
amcys-S-deprot$^-$	-760.858817	0.134553	-761.724264	0.091681	-760.767136
amcys-O-deprot–	-760.853075	0.132754	-760.720321	0.087800	-760.765275
dmcys (**2**)	-800.715327	0.176063	-800.539264	0.128228	-800.587099
dmcysH^+	-801.075698	0.190120	-800.885578	0.141884	-800.933814
dmcys-S-deprot$^-$	-800.182828	0.164221	-800.018607	0.118714	-800.064114
dmcys-O-deprot	-800.168176	0.162470	-800.009081	0.115175	-800.056378
amser (**3**)	-438.423072	0.151228	-438.271844	0.107512	-438.315560
amserH^+	-438.788444	0.165508	-438.622936	0.122149	-438.666295
amser-O-deprot$^-$	-437.886268	0.137653	-437.748615	0.095378	-437.790890
dmser (**4**)	-477.751973	0.180526	-477.571447	0.134256	-477.617717
dmserH^+	-478.116369	0.194679	-477.921690	0.148206	-477.968163
dmser-O-deprot$^-$	-477.214392	0.166898	-477.047494	0.121290	-477.093102

Table 4. Reference acids used in kinetic method studies

Reference Acid	ΔH_{acid} (kJ/mol)	dmcys (**2**)	amser (**3**)	dmser (**4**)
4-fluorobenzoic acid	1410		X	X
3-fluorobenzoic acid	1406	X		
3-nitrophenol	1399	X	X	X
4-hydroxybenzophenone	1392	X	X	X
trifluoromethylbenzoic acid	1390	X	X	X
2-nitrobenzoic acid	1388	X		

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