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Gas-phase acidities of the 20 protein amino acids

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This manuscript is dedicated to the memory of Sharon Lias and her many valuable contributions to the thermochemistry of gas-phase ions and neutrals.

Abstract

The gas-phase acidities of the 20 protein amino acids (PAAs) have been determined using an electrospray ionization–quadrupole ion trap instrument. Three different methods were used to determine both the absolute acidities and the relative acidity ordering of the PAAs. The extended kinetic method was used to determine absolute acidities for all 20 PAAs with substituted carboxylic acids and substituted phenols as reference acids. Acidities were obtained with an average uncertainty of ±10 kJ/mol, which is large compared to some of the differences between amino acids with similar acidities. To determine the relative acidity ordering, single-reference kinetic method experiments were performed using both the reference acids from the absolute acidity studies and tryptophan and threonine as reference acids. Additional ordering information was obtained from kinetic method experiments in which proton-bound dimer ions comprising pairs of amino acids were generated and dissociated in the ion trap. The recommended acidity ordering is Gly < Ala < Pro < Val < Leu < Ile < Lys < Trp < Phe < Tyr < Met < Ser < Thr < Cys < Gln < Gln < Arg < Asn < His < Glu < Asp. Isodesmic acidity values were also obtained at the B3LYP/6-311++G**//B3LYP/6-31+G* level of theory with acetic acid as the reference acid. The theoretical acidities are in excellent agreement with the absolute acidities obtained from the extended kinetic method studies. The calculations predict that the preferred isomer for protonated cysteine and tyrosine is not a carboxylate anion, but rather a thiolate anion and a phenoxide anion, respectively.

Keywords: Gas-phase acidity; Amino acids; Extended kinetic method; Thermochemistry; Density functional theory calculations

1. Introduction

Recently, we have been studying the effects of systematic substitutions on the gas-phase thermochemical properties of amino acids by determining the intrinsic properties of non-protein amino acids (NPAAs) [1–3]. In addition to the biological relevance, the NPAAs serve as attractive candidates to study the subtle interplay between the structure and energetics of amino acids. We have been using a combined experimental–theoretical approach using the extended kinetic method in a quadrupole ion trap and high-level density functional theory calculations to determine various thermochemical properties of NPAAs [1,2]. These studies were made possible, in part, because a relatively consistent set of proton affinities for the 20 PAAs has been agreed upon [4–8].

In contrast to the wide array of proton affinity studies on amino acids, there have been relatively few determinations of the gas-phase acidities (ΔH_{acid})[1] of these species. Glycine is volatile enough that gas-phase equilibrium studies were performed by Locke and McIver [9], and by Kebarle and co-workers [10], giving rise to acidities of 1433 ± 8.8 and 1429 ± 8.8 kJ/mol, respectively. Locke and McIver also determined an equilibrium acidity for alanine of 1425 ± 8.8 kJ/mol [9]. In a collaboration with Kass and co-workers [11], we recently determined the acidity of cysteine using both the extended kinetic method in an ion trap mass spectrometer and the gas-phase equilibrium method with chloroacetic acid as the reference in an icr instrument to be 1395 ± 9 kJ/mol.

O'Hair, et al. [12] used the simple version of the single-reference kinetic method to determine gas-phase acidities for 18 of the 20 PAAs using glycine as the reference acid (aspartic acid

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[1] In this work, acidity is assumed to refer to ΔH_{acid} rather than ΔG_{acid} unless otherwise noted.

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and glutamic acid were too acidic for use with the single reference method). This remarkable study made use of a FAB source to create proton-bound dimers and until this year represented the only gas-phase acidity study for 16 of the 20 amino acids. Recently, Cassady and Dixon (unpublished work) and Afonso et al. (unpublished work) independently determined the gas-phase acidities of aspartic acid and glutamic acid, completing the set of PAA acidity measurements. Cassady and Dixon used bracketing experiments in an ICR to ΔG_{acid} values for Asp, Glu and their amides (unpublished work). Afonso et al. (unpublished work) determined ΔH_{acid} values for Asp and Glu using an extended kinetic method analysis similar to the one presented in this work.

In preparation for a systematic study of NPAA acidities, we first carried out a re-evaluation of the gas-phase acidities of all 20 protein amino acids. We report the results of several experimental studies: absolute acidities for all amino acids from the extended kinetic method using carboxylic acids and substituted phenols as reference acids and several studies of the relative acidity ordering for the amino acids with acidity differences that are smaller than the uncertainty of the absolute measurements. Importantly, the absolute acidities determined by the extended kinetic method experiments explicitly take entropic effects into account in deriving the final acidity values. Finally, the results of high-level hybrid density functional theory calculations are presented which give indications for the preferred sites of deprotonation in these species as well as the relative acidities of other sites. Interestingly, the calculations predict that the preferred gas-phase structure of deprotonated cysteine and tyrosine are not carboxylates, but rather a thiolate and a phenoxide, respectively.

2. Experimental

2.1. Experimental methods

All experiments were performed using a Finnigan LCQ Deca quadrupole ion trap equipped with an external electrospray ionization source (ESI). Dilute solutions of the amino acid of interest and a reference acid with known acidity were generated in slightly basic (1% NH_4OH) methanol:water solution, with mixing ratios varying from 50:50 to 80:20 (v:v). Solution concentrations were varied in order to maximize the production of proton-bound dimers of the deprotonated amino acid and the deprotonated reference acid and were usually in the range of 1×10^{-4}–5×10^{-5} M. Solutions were directly infused into the electrospray ionization source at flow rates of 5–20 μL/min. Electrospray and ion focusing conditions were also varied to maximize the ion count for the proton-bound heterodimer. The proton-bound dimer ions were isolated at $q_z = 0.250$ with a mass-width adjusted to maximize ion signal while still maintaining isolation. For some of the relative acidity determinations between light and heavy amino acids, the q_z value was lowered in order to allow for both deprotonated amino acid products to be stable in the ion trap. The isolated ions were allowed to undergo collision-induced dissociation with the background helium atoms. The ratio of the deprotonated reference acid to the deprotonated amino acid was obtained by performing an activation amplitude scan from 0 to 100% in steps of two activation amplitude percentage values. The final ion ratios are averages of at least three scans obtained on several different days.

2.2. The extended kinetic method

Ion affinities and entropy contributions are obtained from the extended kinetic method that has been described in detail elsewhere [13–16]. The final version of the extended kinetic method takes the form shown below, where ΔH_{Bi} is the gas-phase acidity of

$$\ln\left(\frac{I_{B_i}}{I_A}\right) \approx \frac{\Delta H_{B_i} - \Delta H_{avg}}{RT_{eff}} - \frac{\Delta H_A - \Delta H_{avg}}{RT_{eff}} + \frac{\Delta S_{B_i}}{R} - \frac{\Delta S_A}{R}$$

reference acid i and ΔH_{avg} is the average gas-phase acidity of the set of reference acids. Two plots are generated to obtain the final thermochemical information, the first of which (plot 1) is of $\ln[I(\mathrm{Ref}-H)^-/I(\mathrm{AA}-H)^-]$ versus $\Delta H_{Bi} - \Delta H_{avg}$. A best-fit line to the data in kinetic method plot 1, yields a slope equal to $1/RT_{eff}$ and a y-intercept equal to $-[(\Delta H_A - \Delta H_{avg})/RT_{eff} + \Delta S_A/\mathrm{R} - \Delta S_{Bi}/\mathrm{R}]$. Each activation energy yields a different slope and intercept, and plotting the negative of each of the intercepts versus the slopes from plot 1 gives kinetic method plot 2. From this plot the gas-phase acidity and a prediction for the entropy of deprotonation can be obtained (slope = $\Delta H_A - \Delta H_{avg}$; y-intercept = $\Delta S_{Bi}/R - \Delta S_A/R$) [2,16].

The use of the intercept from plot 2, which is derived from transition state activation entropy differences, as a quantitative measure for the thermodynamic protonation entropy has received considerable attention in the literature of late [2,16–23]. Drahos and Vekey [18] performed a series of simulations using the MassKinetics program and came to the conclusion that whereas enthalpies obtained from the kinetic method are generally in agreement with literature values, entropies are generally underestimated, and they recommended scaling the derived entropy term by 1.35. Three feature commentaries [19–21] on the Vekey paper were recently published in which the conclusions from the Vekey paper [18] were evaluated and additional data was presented in an effort to try to come to a consensus on how to handle entropy in the kinetic method. Vekey had the opportunity to comment on the commentaries [22] and came to the conclusions that (1) the extended kinetic method, rather than its simpler forms, *must* be used to determine thermochemical information for all but the simplest systems, (2) when the entropy difference is less than about 35 J mol^{-1} K^{-1}, the corresponding ion affinities should be accurate, and (3) if entropy effects are large (>35 J mol^{-1} K^{-1}) it is likely that the entropy values will be underestimated. In the studies presented here, entropy effects were generally small. With the exception of the Asp and Glu studies, the absolute values of the derived ΔS terms were less than 15 J mol^{-1} K^{-1}, and therefore the gas-phase enthalpies of deprotonation should be well represented.

2.3. Orthogonal distance regression analysis

Ervin and Armentrout [20] have recently developed a new approach to fitting kinetic method plots involving an orthogonal

distance regression approach. In this procedure, a regression algorithm is used to force n best-fit lines at m different energies to cross at a common point. The x-coordinate of this crossing point corresponds to the enthalpic term of interest and the y-coordinate corresponds to the entropic term. Kinetic method data for all amino acids were fit with both the traditional and ODR procedures and the ion affinities and derived entropy terms from the two fitting methods are virtually identical. The advantage of the ODR method is that it gives a more realistic estimation of the uncertainty of the derived values. Final uncertainties are obtained from Monte Carlo simulations in which random noise is generated within user-defined ranges of the uncertainties in the gas-phase acidity of each reference acid and in the experimental ion ratios. For these studies, uncertainty in the acidity of the reference acid was ±8 kJ/mol, and the uncertainty in the ln(ratio) values was ±0.05.

2.4. Theoretical procedures

Theoretical values for gas-phase acidities were obtained from hybrid density functional theory calculations using the B3LYP functional combinations [24,25]. All calculations were performed using PCModel and the Gaussian98 W suite of programs [26]. A conformational search is first performed using the GMMX algorithm in PCModel, and the lowest 30–50 structures for each molecule are then used as starting points for progressively increasing levels of ab initio or density functional theory calculations. Ultimately, geometries and harmonic vibrational frequencies for all amino acids and their deprotonated forms were calculated at the B3LYP/6-31+G* level. Total electronic energies were obtained from B3LYP/6-311++G** single point calculations at the B3LYP/6-31+G* geometries. Enthalpies at 298 K were calculated using ZPE and thermal corrections obtained from unscaled harmonic vibrational frequencies.

Predictions for the gas-phase acidities were computed from isodesmic reaction (1) with acetic acid (ΔH_{acid} = 1456 kJ/mol) [27] serving as the reference acid.

$$AA + OAc^- \rightarrow [AA - H]^- + HOAc \quad (1)$$

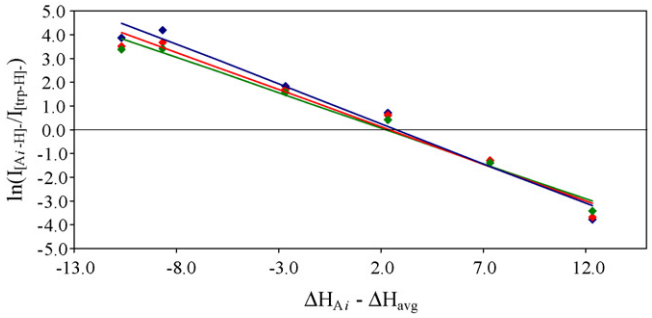

Fig. 1. Plot of $\ln(I[A_i - H]^-/I[Trp - H]^-)$ versus $\Delta H_{Ai} - \Delta H_{avg}$. Data shown at activation amplitudes 14% (blue diamonds), 22% (red diamonds), and 48% (green diamonds). Lines obtained from ODR fit as described in text.

For species with more than one acidic site, separate sets of calculations were performed with the proton initially removed from each acidic site. For some anions, isomerization from initial structures to more stable final structures through proton transfer occurred. Gas-phase acidities were calculated for different sites in each amino acid based on the lowest energy final structures.

3. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis) and used without further purification.

4. Results

4.1. Experimental results

Absolute gas-phase acidities were determined for all 20 PAAs using the extended kinetic method in an ESI-quadrupole ion trap instrument. First and second kinetic method plots for the tryptophan study are shown in Figs. 1 and 2. Fig. 1 shows a plot of $\ln[I(Ref - H)^-/I(Trp - H)^-]$ versus $\Delta H_{ref} - \Delta H_{avg}$ at three selected collision energies. For this study, the following reference acids were used: *o*-chlorobenzoic acid ($\Delta H^\circ_{acid} = 1402 \pm 8$ kJ/mol) [28], *p*-chlorobenzoic acid ($\Delta H^\circ_{acid} = 1404 \pm 8$ kJ/mol) [28], *o*-fluorobenzoic acid ($\Delta H^\circ_{acid} = 1410 \pm 8$ kJ/mol) [28], *p*-fluorobenzoic acid ($\Delta H^\circ_{acid} = 1415 \pm 8$ kJ/mol) [28], *o*-toluic acid ($\Delta H^\circ_{acid} = 1420 \pm 8$ kJ/mol)

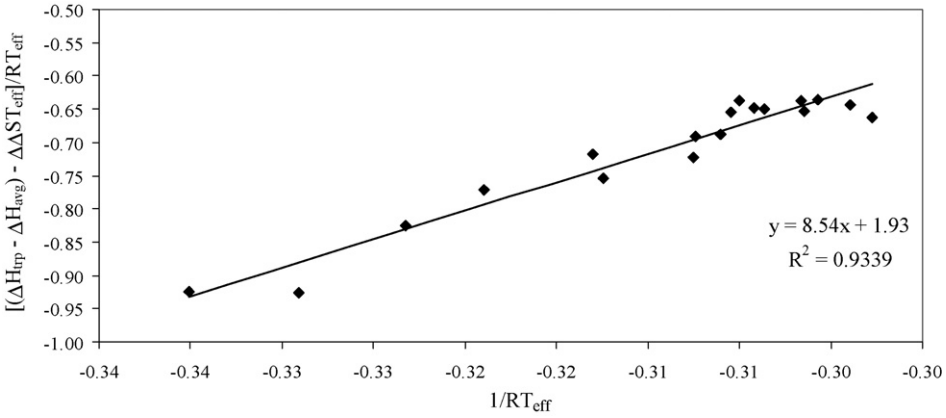

Fig. 2. $[(\Delta H_{Trp} - \Delta H_{avg}) - T_{eff}\Delta\Delta S]/RT_{eff}$ versus $1/RT_{eff}$.

[28], and *p*-toluic acid ($\Delta H^{\circ}_{acid} = 1425 \pm 8$ kJ/mol) [28]. A full list of reference acids and their gas-phase acidities is provided in Supporting Information. Ion ratios were obtained at varying activation amplitudes between 0 and 100%, corresponding to 0–5 V, in the laboratory frame. In the tryptophan study, activation amplitudes below 14% were not energetic enough to give ionic products, whereas activation amplitudes above 48% resulted in a leveling off of the collision energy due to collisional cooling of the activated ions in the ion trap. Therefore, activation amplitudes between 14 and 48 % were used to determine the gas-phase acidity of tryptophan. A separate best-fit line is generated for each activation amplitude (shown for 14, 22 and 48% in Fig. 1). The negative intercepts of these best-fit lines are plotted against their slopes to give the second kinetic method plot shown in Fig. 2. The desired thermochemical information is obtained from a best-fit line to the data in plot 2. The slope of this line corresponds to $\Delta H_{Trp} - \Delta H_{avg}$ and the intercept gives a prediction for $\Delta S/R$, the deprotonation entropy. In this case, combining the slope, 8.5 kJ/mol, with the average acidity of the six reference acids, 1412.7 kJ/mol gives an acidity for tryptophan of 1421.2 kJ/mol. A ΔS of 16 J mol^{-1} K^{-1} is derived from the intercept.

The new orthogonal distance regression method was used to determine the uncertainties for these derived values [20]. This method has been reviewed in detail elsewhere, and is generally deemed to give realistic error limits for derived thermochemical quantities from the extended kinetic method [20]. Importantly, the ODR method does not require the generation of a second kinetic method plot, which can introduce unwanted correlation between the derived enthalpic and entropic terms. For the tryptophan study, experimentally determined ion ratios at all 18 activation amplitudes between 14 and 46% were considered in the ODR method. The 18 best-fit lines were forced to cross at a single isothermal point corresponding to the desired enthalpy and entropy values. Monte Carlo simulations were then performed to determine the robustness of the isothermal point and to generate uncertainty values at the 95% confidence level. For tryptophan, the derived enthalpy is nearly identical to the traditional method, 1420.4 with an uncertainty of ±9 kJ/mol. The derived entropy term is 14 J mol^{-1} K^{-1} with an uncertainty of ±9 entropy units. All experimental values quoted in the remainder of the manuscript are ODR-derived values.

Similar procedures were used to determine the absolute gas-phase acidities of the other 19 protein amino acids. Table 1 shows measured acidity values for all 20 protein amino acids as well as the derived entropy terms from the ODR procedure. First kinetic method plots for the other amino acid studies presented in the work are given in Supporting Information. Uncertainties from the ODR procedures varied from ±9 to ±22 kJ/mol but on average were on the order of ±12 kJ/mol. These represent 95% confidence uncertainties and are a factor of 2 larger than the ±1 σ-values that we have quoted in most of our previous studies.

In contrast to the proton affinities of the PAAs which span a relatively large range of basicity, the acidities of the 20 PAAs cluster into three groups: (1) eleven of the least acidic amino acids have acidities between 1407 and 1434 kJ/mol, (2) a cluster of six moderately acidic amino acids are found to have acidities between 1381 and 1394 kJ/mol, and (3) a cluster of 3 strongly acidic amino acids comprising His and the two dicarboxylic acids, Glu and Asp, with acidities in the range of 1345–1375 kJ/mol. Unfortunately, the gas-phase acidities of the reference acids are not known to an accuracy of better than 8 kJ/mol. This places a limit on the final uncertainties on the acidities derived from the kinetic method and makes a relative acidity ordering difficult to determine from the absolute values.

Table 1
Experimental and theoretical gas-phase acidities (kJ/mol/mol) for the 20 Protein amino acids

Acid	ΔH_{acid}	ΔH_{acid}, theory[a]	ΔH_{acid}, literature[b]
Alanine	1430 ± 8	1432	1425 ± 8.8[c]
Arginine	1381 ± 9	1387	1389 ± 13
Asparagine	1385 ± 9	1384	1388 ± 13
Aspartic acid	1345 ± 14	1345	1340[d]
Cysteine	1395 ± 9[e]	1396[e]	1393 ± 13
Glutamic acid	1348 ± 21	1349	1347[d]
Glutamine	1385 ± 11	1378	1388 ± 13
Glycine	1434 ± 9	1434	1433 ± 8.8[c]
Histidine	1375 ± 8	1374	1385 ± 13
Isoleucine	1423 ± 8	1426	1418 ± 13
Leucine	1419 ± 10	1428	1419 ± 13
Lysine	1416 ± 7	1415	1412 ± 13
Methionine	1407 ± 9	1412	1405 ± 13
Phenylalanine	1418 ± 18	1417	1408 ± 13
Proline	1431 ± 9	1430	1430 ± 13
Serine	1391 ± 22	1392	1392 ± 13
Threonine	1388 ± 10	1397	1390 ± 13
Tryptophan	1421 ± 9	1422	1410 ± 13
Tyrosine	1413 ± 11	1419	1408 ± 13
Valine	1431 ± 8	1430	1420 ± 13

[a] Values obtained from isodesmic reaction (1) with acetic acid (ΔH_{acid} = 1456 kJ/mol).
[b] Values obtained from reference [12] unless otherwise noted.
[c] Reference [9].
[d] Reference (Afonso et al., unpublished work).
[e] Reference [11].

4.2. Computational results

Extensive calculations were carried out in support of the experimental studies using hybrid density functional theory. The GMMX conformational searching routine in PCModel was used to generate starting structures, which were further investigated using the B3LYP functional combination with increasingly larger basis sets. Ultimately, geometries were obtained at the B3LYP/6-31+G* level of theory. Single point energy calculations at B3LYP/6-311++G** were then performed at the B3LYP/6-31+G* geometries. The total electronic energies were converted to enthalpies at 298 K using unscaled vibrational frequencies at the B3LYP/6-31+G* geometries. A table of electronic energies, thermal corrections, and enthalpies at 298 K is given in Supporting Information. In addition, pictures of the lowest-energy neutral and deprotonated forms for all amino acids are provided in Supporting Information. The basis sets in this study were chosen based on extensive studies from our initial proton affinity studies of proline and its analogs based on agreement with experimental PAs obtained from the extended kinetic

method. In these early studies, we did not use the conformational searching routines; rather we generated starting structures by hand based on likely hydrogen bonding interactions. The computational study described here is much larger in scope than our previous studies. The GMMX routine sorts through 50,000 random conformers based on free-rotation of all single bonds and generates a list of low-energy structures. We investigated at least 30 randomly-generated conformers for each neutral and each anion, and then used chemical intuition to try to construct lower energy structures by hand.

After our theoretical calculations were completed, an elegant theoretical study of the proton affinities of all 20 PAAs was published by Paizs and co-workers [5]. This study made use of a simulated annealing procedure [29–31] to generate starting structures and calculations at B3LYP/6-31+G** and G2MP2 levels of theory. We compared our final structures for the neutral amino acids with the Paizs structures and in all cases except the ones noted below, our final structures were identical with those of Paizs [5]. The fact that our conformational searching routines found the minimum energy structures for nearly all the neutral amino acids gives us confidence that the similar procedure should work for the anions, where hydrogen bonding is much stronger and leads to fewer low-energy conformations. Cartesian coordinates for the lowest energy conformers for each amino acid and anion are given in Supporting Information.

The lowest energy neutral structures for all the amino acids listed in the Paizs study contain a strong hydrogen bond between the OH group of the carbonyl group and the nitrogen lone pair of the amino group (OH–NH_2) [5]. We re-optimized the B3LYP/6-31+G** structures from the Paizs study at the B3LYP/6-31+G* level of theory and found that this minimum energy structure is the global minimum for all but three amino acids. We find that an alternative hydrogen bonding structure, involving the hydrogens on the amino group with the carbonyl oxygen (NH_2–O=C), is lower in energy for the simplest amino acid glycine. This energy difference is extremely small, less than 1 kJ/mol, however, the (NH_2–O=C) conformer has been shown to be the lowest energy conformer by a variety of levels of theory [32–37]. We also found that this conformer is lower in energy for alanine and leucine. For isoleucine, the OH–NH_2 structure is lower in energy at B3LYP/6-31+G*, but when zero-point and thermal corrections are included the NH_2–O=C structure is lower in energy. We also found this behavior for lysine (*vide infra*).

The ultimate goal of the computational study was to give predictions for the gas-phase acidities for the amino acids. The B3LYP/6-311++G**//B3LYP/6-31+G* methodology gives proton affinities for simple amines that are within 4–6 kJ/mol of established literature values. In contrast, this method gives an absolute acidity (1446 kJ/mol) for acetic acid that is 10 kJ/mol too low (ΔH_{acid} = 1456 kJ/mol) [28]. For the amino acids, similar results are found. We therefore chose to use an isodesmic approach (reaction (1)) which corrects for the deficiencies of the theoretical method. As this method is a relative acidity determination, the uncertainty in the derived acidities is less than it would be for the direct acidities. The actual uncertainty is unclear, but we assume that it is on the order of ±8–10 kJ/mol.

Table 1 shows derived isodesmic acidities for all 20 amino acids. In general, the agreement between theory and experiment is excellent. All theoretical acidities are within the experimental error bars of the experimental acidities obtained from the extended kinetic method, and vice versa. This excellent agreement between theoretical and experimental acidities lends support to both sets of values. Further, the theoretical acidities are also in excellent agreement with the experimental acidities of OBG [12] and with recent results from Tabet and co-workers (unpublished work) (*vide infra*).

5. Discussion

Absolute experimental and theoretical acidities were obtained for all 20 PAAs. The following sections highlight trends in acidity values, comparisons with previous studies, and finally, several determinations of the relative acidity ordering of the 20 amino acids.

5.1. Lysine

Recently, Paizs and co-workers [5] and Williams and co-workers [38] independently showed that the lowest-energy conformer for neutral lysine from our 2002 proton affinity study is not the global minimum. Our lowest-energy neutral conformer was found to be extended, whereas the lowest-energy cation was found to form a strong hydrogen bond. Both Paizs and Williams located the same global minimum structure for lysine with a (OH–NH_2) hydrogen bond [5,38]. Using the conformational searching routine we located both this cyclic structure and an extended conformation with a (NH_2–O=C) hydrogen bonding motif as the two lowest energy conformers at the B3LYP/6-31+G* level of theory. The extended structure is much lower in energy than the one from our 2002 paper. The cyclic structure is found to be *ca.* 2.5 kJ/mol lower in energy than the extended structure. However, upon including zero-point corrections the stability reverses and the extended structure is more stable by *ca.* 3 kJ/mol. The difference in 298 K enthalpies reduces to 1 kJ/mol with the extended conformer being lower in energy. The isodesmic acidity for lysine using the extended conformer is 1415 kJ/mol, which is in excellent agreement with our kinetic method value of 1416 kJ/mol.

For lysine, we found 12 different conformers within 10 kJ/mol of the global minimum, some of which are extended and some of which are cyclic with strong hydrogen bonding with the side chain. All of the deprotonated structures involve strong hydrogen bonding between both amino groups and the carboxylate. The lowest energy structure has a strong hydrogen bond between the hydrogens on the side chain amino group and one of the carboxylate oxygens (r = 1.96 Å). Presumably, the proton-bound dimer between deprotonated lysine and a carboxylate anion will involve the deprotonated lysine molecule in a cyclic form. Dissociation to an extended form of the neutral should therefore involve a large entropy change. The extended kinetic method has been used to measure these large entropy changes for bifunctional molecules such as diamines and diols [2,39–42]. The measured entropy term for lysine was rather

small, $-9 \pm 10\,J\,mol^{-1}\,K^{-1}$, which suggests that the transition state for dissociation of the proton-bound dimer anion involves a cyclic neutral lysine structure. Given the relatively small difference in energy between the various conformers, this behavior seems reasonable.

5.2. Aspartic acid and glutamic acid

At the time of the writing of this manuscript, there have been no published values for the acidities of glutamic or aspartic acids. Afonso et al. recently reported unpublished results from extended kinetic method studies in which they determined acidities of 1340 and 1347 kJ/mol for aspartic and glutamic acids, respectively (Afonso et al., unpublished work). Using the methods described above, we determined an absolute acidity of 1345 ± 14 kJ/mol for Asp in excellent agreement with the value of Afonso et al. The derived entropy value is moderately large, $-14 \pm 14\,J\,mol^{-1}\,K^{-1}$ indicating some degree of structure change between the neutral and deprotonated forms. The lowest energy form of Asp is the same as the one found in the Paizs study [5] with a strong (OH–NH_2) hydrogen bond ($r = 1.93$ Å) and a weaker interaction between the amino hydrogens and the side-chain carbonyl oxygen ($r = 2.62$ Å) as shown in Fig. 3a. Both the backbone and side chain carboxylic acid groups are strongly acidic, with the backbone carboxylic acid group being more acidic at the B3LYP/6-31+G* level of theory (Fig. 3b and c). The OH–O=C hydrogen bonding motif results in a much stronger hydrogen bond ($r = 1.46$ Å) in the anion, and hence the geometry change. A theoretical prediction for the gas-phase acidity of Asp of 1345 kJ/mol is obtained from isodesmic reaction (1) for the backbone carboxylic acid group. The entropy value derived from the kinetic method experiment, $-14 \pm 14\,J\,mol^{-1}\,K^{-1}$, would correspond to a ΔS_{acid} of $94\,J\,mol^{-1}\,K^{-1}$, which would lead to a prediction for ΔG_{acid} of 1316 kJ/mol. This value is somewhat lower than a recent unpublished icr bracketing study by Cassady and Dixon of 1323 ± 13 kJ/mol (unpublished work).

Similar results were found for glutamic acid. Our experimental acidity value of 1348 ± 11 kJ/mol is in excellent agreement with the acidity of Afonso et al. (1347 kJ/mol) (unpublished work). Neutral Glu has hydrogen bonds of the (OH–NH_2) type, ($r = 1.85$ Å) and a weaker interaction between the amino hydrogens and the side-chain carbonyl atom ($r = 2.00$ Å) as shown in Fig. 4. Upon deprotonation, the hydrogen bonding scheme changes to that of aspartate with a strong (OH–O=C) interaction ($r = 1.49$ Å) and an additional NH_2–O=C interaction ($r = 2.28$ Å), Fig. 4b. As with Asp, the backbone carboxylic acid group of Glu is found to be more acidic than the side-chain carboxylic acid group, Fig. 4c. A theoretical acidity of 1349 kJ/mol is found from isodesmic reaction (1) for backbone deprotonated Glu, in excellent agreement with the experimental acidity. As with Asp, the change in geometry upon deprotonation manifests itself in the measured entropy term from the kinetic method experiment, which is on the same order as that from the Asp study, $-20\,J\,mol^{-1}\,K^{-1}$. It should be noted that the quality of the Glu kinetic method data was not as good as the Asp data and therefore the uncertainties in the derived acidity and entropy terms are larger (±21 kJ/mol and $\pm 41\,J\,mol^{-1}\,K^{-1}$). Using the derived entropy term to give a prediction for ΔS_{acid} of $88\,J\,mol^{-1}\,K^{-1}$ leads to a derived ΔG_{acid} of 1321 kJ/mol. This value is lower than the acidity found in Cassady's bracketing study of 1335 ± 14 (C.J. Cassady, D.A. Dixon, unpublished work).

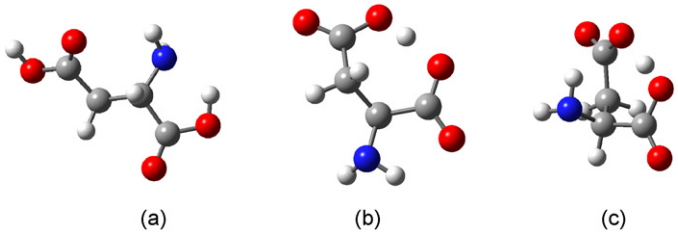

Fig. 3. Lowest energy structures for (a) Asp, (b) backbone deprotonated Asp, and (c) side-chain deprotonated Asp at B3LYP/6-31+G*.

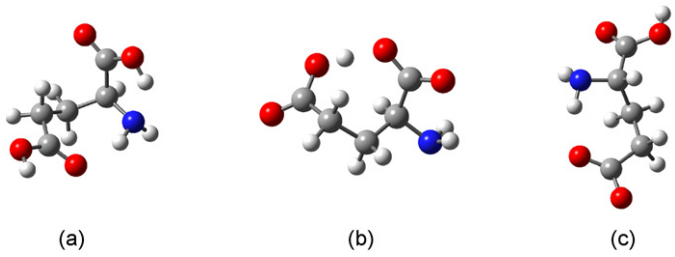

Fig. 4. Lowest energy structures for (a) Glu, (b) backbone deprotonated Glu, and (c) side-chain deprotonated Glu at B3LYP/6-31+G*.

Given the large uncertainty in the absolute acidity value for Glu, and the relatively small difference between the measured acidities of Glu and Asp, we performed relative acidity measurements using proton-bound dimers between deprotonated Asp and deprotonated Glu. Assuming that entropy effects should be roughly the same in these two species, the dissociation ratios indicate that Asp is a stronger acid than Glu (*vide infra*). As mentioned above, the agreement between the theoretical and experimental acidities for all amino acids is excellent (Table 1). Given that the theoretical and experimental values for Glu and Asp are in accord, we feel confident that the absolute acidity values for Asp and Glu are in the correct order.

5.3. Cysteine and tyrosine

One of the advantages to the combined experimental–theoretical approach is the ability to determine information about higher energy isomers that are either not present or are present in small amounts in gas-phase samples from theoretical calculations. DFT calculations were used to determine the relative acidity of different sites for amino acids with more than one acidic group. For example, we recently reported that the preferred site of deprotonation for cysteine is the thiol side chain group rather than the backbone acid group [11]. In general, carboxylic acids are more acidic in the gas-phase than simple thiols, ($\Delta\Delta H^{\circ}_{acid}$ ($CH_3CH_2CH_2SH$–$CH_3CH_2CO_2H$) = 28 kJ/mol) [28]. However, in the case of cysteine, theoretical calculations predict

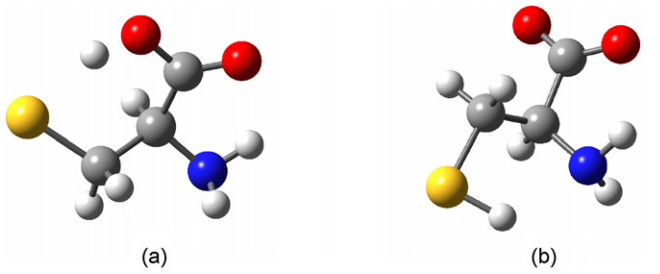

Fig. 5. Lowest energy structures for the (a) thiolate form of deprotonated Cys and (b) carboxylate form of deprotonated Cys.

that the thiol group is about 8 kJ/mol more acidic than the carboxylic acid group. This acidity enhancement for the thiol can be understood by examining the structures of the lowest energy carboxylate and thiolate forms of deprotonated cysteine shown in Fig. 5. The lowest energy structure that we reported for cysteine contains three strong hydrogen bonds and is the same structure as in the Paizs study [5]. The thiolate isomer forms two strong hydrogen bonds, one of the (NH_2–O=C) type ($r = 2.27$ Å) and a stronger interaction between the sulfur anion and the COOH hydrogen ($r = 1.93$ Å). The carboxylate isomer has hydrogen bonds of the (NH_2–O=C) type ($r = 2.21$ Å) and a weaker hydrogen bond between the thiol hydrogen and the amino nitrogen ($r = 2.4$ Å). We reported experimental acidities of 1394 ± 14 kJ/mol from the extended kinetic method, and 1399 ± 9.2 from an icr equilibrium study with chloroacetic acid [11]. We also reported theoretical acidities from isodesmic reactions with acetic acid of 1396 kJ/mol, in excellent agreement with both experimental determinations. Since the experimental acidity is in excellent agreement with the theoretical value for the thiolate, this suggests that the proton-bound dimer ion contains the thiolate for of deprotonated cysteine despite the fact that the carboxylate form would form a stronger hydrogen bond with the reference acids.

In this study, we found that the preferred site of deprotonation for tyrosine is the phenol group on the side chain rather than the backbone carboxylic acid group. The lowest energy neutral isomer contains a strong hydrogen bond of the (OH–NH_2) type ($r = 1.89$ Å), and is the same structure as found in the Paizs study [5]. The phenoxide isomer of deprotonated tyrosine contains the same strong (OH–NH_2) hydrogen bond ($r = 1.87$ Å), whereas the carboxylate isomer contains a weaker (NH_2–O=C) interaction ($r = 2.01$ Å). The difference in acidity between these two isomers is small, about 4 kJ/mol at the B3LYP/6-311++G**//B3LYP/6-31+G*. The isodesmic acidity of the phenol side chain is predicted to be 1419 kJ/mol. The extended kinetic method gives a value of 1413 ± 11 kJ/mol, in excellent agreement with the theoretical acidity.

5.4. Comparison with literature values

The fourth column of Table 1 shows the recommended literature values of the 18 amino acids for which experimental values have been determined. As can be seen, the agreement between our extended kinetic method values and the literature values is excellent. Our value for the acidity of glycine, 1434 ± 9 kJ/mol is excellent agreement with the value from Locke and McIver [9], 1433 ± 8.8, and is slightly higher than that of Kebarle and co-workers [10], 1429 ± 8.8 kJ/mol, but is well within the error limits Our acidity for alanine, 1430 ± 8 kJ/mol is slightly higher than Locke and McIver's acidity of 1425 ± 8.8 kJ/mol [9] but again the error limits overlap substantially.

The majority of the amino acid acidity measurements come from a single paper by O'Hair, et al. [12], in which they used the single-reference kinetic method to obtain acidities (ΔG_{acid}) for 17 amino acids relative to glycine. Combining the relative values with Locke and McIver's value for the gas-phase acidity of glycine [9] led to absolute values for ΔG_{acid}. The free energy values were converted to ΔH_{acid} acidities using a constant entropy term of -10 J mol^{-1} K^{-1}. As has been noted, our measured entropy terms from the extended kinetic method are, in general, of this order and are relatively constant across the set of amino acids, with the exception of glutamic and aspartic acids. Thus, entropy is not playing a large role in the kinetic method experiments and the agreement between our values and the acidities of OBG is excellent.

5.5. Relative gas-phase acidity ordering

Due to the fact that the uncertainties in the absolute acidities are larger than the differences between some amino acid pairs, a relative ordering of the acidities of the 20 amino acids is difficult to obtain from the absolute acidities. In order to determine a relative acidity ordering for all 20 PAAs several approaches were used. The first makes use of the data used in the absolute acidity studies. If a reference acid was used in more than one acidity study, the logarithm of the product ion ratios can be used to give an indication for the relative acidity of the different amino acids. These ratios reflect a difference in apparent acidity (related to ΔG_{acid}), that is, they do not account for entropy effects. But, as already noted, with the exception of aspartic acid and glutamic acid, entropy effects were found to be both small and relatively constant among the amino acids. Therefore, the relative acidities determined from comparing the ln(ratio) values should be reflective of the actual ΔH_{acid} ordering. A summary of the measured dissociation ratios at 30% collision energies is given in Supporting Information. Table 2 shows the relative acidity order obtained from (a) the absolute measurements, (b) the isodesmic acidities from B3LYP/6-31++G**/B3LYP/6-31+G*, (c) the single reference measurements from OBG, and (d) the relative acidity determinations from the absolute measurements. Columns (e) and (f) contain data from the additional relative acidity determinations described below. Column (g) in Table 2 contains our recommended acidity ordering for all 20 amino acids based on the discussion below.

The second relative acidity measurement is a single reference approach in which a single amino acid was chosen as a calibrant similar to that performed by OBG. Proton-bound dimer ions are generated between the amino acid of interest and the calibrant. The values of the ln(ratio) can be used in a similar manner to that described above to give an indication of the relative acidities (ΔG_{acid}) of the different amino acids. As with the previously described relative acidity measurements, we assume that since

Table 2
Relative acidity ordering of the amino acids

ΔH-derived quantities		ΔG-derived measurements				
(a) Absolute exp.	(b) Theory	(c) OBG[a]	(d) Relative 20%[b]	(e) Single ref.[c]	(f) Binary[d]	(g) Recommended
Gly (1434)	Gly (1434)	Gly	Gly	Gly	Gly	**Gly**
Pro (1431)	Ala (1432)	Ala	Ala	Ala	Ala	**Ala**
Val (1431)	Pro (1430)	Pro	Pro	Pro	Pro	**Pro**
Ala (1430)	Val (1430)	Val	Val	Val	Val	**Val**
Ile (1423)	Leu (1428)	Leu	Leu	Leu	Leu	**Leu**
Trp (1421)	Ile (1426)	Ile	Ile	Ile	Ile	**Ile**
Leu (1419)	Trp (1422)	Lys	Lys	Lys	Lys	**Lys**
Phe (1418)	Tyr (1419)	Trp	Trp	Trp	Trp	**Trp**
Lys (1416)	Phe (1417)	Phe	Met	Phe	Phe	**Phe**
Tyr (1413)	Lys (1415)	Tyr	Phe	Tyr	Met	**Tyr**
Met (1407)	Met (1412)	Met	Tyr	Met	Tyr	**Met**
Cys (1395)	Thr (1397)	Cys	Cys	Ser	Thr	**Ser**
Ser (1391)	Cys (1396)	Ser	Ser	Thr	Ser	**Thr**
Thr (1388)	Ser (1392)	Thr	Thr	Cys	Cys	**Cys**
Asn (1385)	Arg (1387)	Arg	Arg	Gln	Asn	**Gln**
Gln (1385)	Asn (1384)	Asn	Gln	Arg	Gln	**Arg**
Arg (1381)	Gln (1378)	Gln	Asn	Asn	Arg	**Asn**
His (1375)	His (1374)	His	His	His	His	**His**
Glu (1348)	Glu (1349)		Glu		Glu	**Glu**
Asp (1345)	Asp (1345)		Asp		Asp	**Asp**

[a] Ordering from reference [12].
[b] Ordering obtained from comparing dissociation ratios for references acids that were used for more than one amino acid study, as described in text.
[c] Ordering obtained from single reference kinetic method using Trp (Gly–Met) and Thr (Tyr–His) as reference acids, as described in text.
[d] Ordering obtained from dissociation ratios of binary combinations of two amino acids, as described in text.

entropy effects were found to be small and constant across the amino acids, that the relative ordering for ΔG_{acid} is reflected in the relative ordering of ΔH_{acid}. In this study, the relative acidities of the less acidic amino acids, glycine to methionine, were found using tryptophan as the calibrant and those of the more acidic amino acids, phenylalanine to aspartic acid, were determined using threonine as the calibrant. The results of this study are given in Supporting Information and the relative ordering obtained from these results are shown in column (e) in Table 2 for a collision energy of 30%.

Finally, dimer ions of binary amino acid combinations for all amino acids with measured absolute acidities within 5 kJ/mol of each other were generated and dissociated at a collision energy of 20% in order to sort out apparent discrepancies in relative acidities. These results are shown in Supporting Information and the relative acidity ordering from these studies is given in column (f) of Table 2.

It is clear that the relative ordering of the eight least acidic amino acids is Gly < Ala < Pro < Val < Leu < Ile < Lys < Trp. This ordering indicates that the absolute acidity for Lys is probably too low. Interestingly, theory agrees with the extended kinetic method in terms of lysine's absolute acidity and its position in the acidity order. A theoretical acidity that is too low suggests that we have not located the global minimum for neutral lysine. Given the extensive discussion above, it is unlikely that we are missing a neutral isomer that is 7–9 kJ/mol lower in energy than the lowest energy neutral structures found by ourselves, Paizs [5], and Williams [38]. The EKM gives values for Ile and Leu that are reversed in terms of relative acidity. This is the only combination that we can't perform the binary experiment on, but theory and the other relative acidity experiments support the fact that Ile is a stronger acid than Leu.

The next three amino acids, Phe, Tyr, and Met have acidities that are essentially the same. The binary experiments indicate that Met is slightly more acidic than Tyr and that Tyr and Phe have the same acidity within the ±0.05 error limits of the logarithm of our measured ratios. Theory and the other single-reference experiments support this ordering. The ordering from the reference acid study is based on 4-fluorobenzoic acid. However, the logarithm of all three ratios are the same within our experimental error. Given the large uncertainty in the absolute acidity for Phe, the absolute values do not contradict this ordering.

There is a large drop in acidity of *ca.* 7 kJ/mol between the eleven least acidic amino acids and the cluster of six moderately acidic amino acids. These six cluster into two groups, Ser, Thr, and Cys, which have essentially the same acidities and Gln, Arg, and Asn, which are slightly more acidic than the first group, but have the same acidities. Notably, all of these species contain functional groups, (OH, NH_2, SH, and $C(=O)NH_2$) that can hydrogen bond in the deprotonated form, leading to enhanced acidities. The relative acidity experiment for Ser, Thr, and Cys led to differing results. We feel that the single reference experiment with Thr as the calibrant is the most reliable. This study indicates that the ordering is Ser < Thr < Cys. The next cluster of Gln, Asn, and Arg have by far the closest acidities of any of the amino acids. We chose the single reference method with threonine as the most reliable relative acidity measurement since

the binary experiments gave conflicting results (with ratios all essentially equal to 1:1). The recommended ordering is therefore Gln < Arg < Asn.

Finally, the three most acidic amino acids, His, Glu, and Asp have orderings that are the same from all methods, His < Glu < Asp. Histidine has an enhanced acidity due to the strong hydrogen bonding between both the imidizole and amino hydrogens and the carboxylate oxygens. The acidities of glutamic and aspartic acids are also significantly enhanced by hydrogen bonding in the anion.

6. Conclusions

The absolute gas-phase acidities for all 20 protein amino acids have been re-determined in an electrospray ionization–quadrupole ion trap instrument using the extended kinetic method with full entropy analysis. The absolute acidities are in excellent agreement with previous literature acidities. Theoretical, isodesmic predictions for the absolute acidities of all 20 amino acids from the B3LYP/6-311++G**/B3LYP/6-31+G* level of theory were also determined and were found to be in excellent agreement with the experimental acidities. Several relative acidity studies were carried out to determine a relative acidity ordering for the amino acids, since the uncertainties in the absolute acidities of some amino were larger than the differences between adjacent pairs. Notably, the preferred structure for the conjugate bases of Cys and Tyr were found to be a thiolate and a phenoxide, respectively rather than carboxylates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2007.02.018.

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