Proton Affinities and Photoelectron Spectra of Phenylalanine and *N*-Methyl- and *N*,*N*-Dimethylphenylalanine. Correlation of Lone Pair Ionization Energies with Proton Affinities and Implications for N-Methylation as a Method to Effect Site Specific Protonation of Peptides

Sherrie Campbell,† Elaine M. Marzluff,† M. T. Rodgers,† J. L. Beauchamp,*,† Margaret E. Rempe,‡ Kimberly F. Schwinck,‡ and D. L. Lichtenberger‡

Contribution No. 8878 from the Arthur Amos Noyes Laboratory of Chemical Physics. California Institute of Technology. Pasadena. California 91125. and The Laboratory for Electron Spectroscopy and Surface Analysis. Department of Chemistry. University of Arizona, Tucson. Arizona 85721

Received October 25. 1993. Revised Manuscript Received March 31. 1994•

Abstract: A Fourier transform ion cyclotron resonance (FT-ICR) technique for measuring gas-phase proton affinities is presented which utilizes collisional dissociation of proton-bound clusters by off-resonance translational excitation. A simplified RRKM analysis relates unimolecular dissociation rates to proton affinities. This technique is used to measure values for the proton affinities of phenylalanine and *N*-methyl- and *N*,*N*-dimethylphenylalanine of 220.3, 223.6, and 224.5 kcal/mol, respectively (relative to the proton affinity of NH_3 = 204.0 kcal/mol). The proton affinity measured for phenylalanine is in excellent agreement with reported literature values. The photoelectron spectra of these three molecules are also presented and analyzed. Assignments of bands to specific ionization processes are aided by comparison with model compounds such as methyl-substituted amines and 2-phenylethylamines. These data are employed to examine the correlation of adiabatic nitrogen lone pair ionization energies with gas-phase proton affinities for phenylalanine, *N*-methylphenylalanine, and *N*,*N*-dimethylphenylalanine in comparison to correlations for other amino acids and selected aliphatic amines. Although amine nitrogen methylation increases the potential for localizing charge at the amine terminus of protonated peptides by increasing the gas-phase proton affinity, the present study establishes that the increase is not sufficient to compete with protonation of some of the more basic side chains in peptides.

Introduction

The number of investigations into chemical reactivities, proton affinities, and dissociation mechanisms of biological molecules in the gas phase has increased dramatically during recent years. This is due in part to the advances in experimental methodology for the desorption and ionization of thermally fragile high molecular weight molecules. Desorption of an amino acid or larger biological sample from a matrix using laser desorption,[1] plasma desorption,[2] or fast atom bombardment (FAB)[3] has become an efficient method for producing charged species. Additionally, electrospray[4] and thermospray[5] ionization techniques are used to produce multiply charged gas-phase biological species. The attachment of a proton or cation to a peptide may occur at a number of sites due to the many basic functional groups within the molecule. This is a complex process since folding of the molecule allows several basic sites to simultaneously interact with and solvate the charge center.[6,7] Covalent attachment of an ionized functional group (such as an ethyltriphenylphosphonium moiety or a quaternary nitrogen) is the only method currently available for positive ions with which one can *select* the specific site of ionization.[8,9]

The present study examines the effect of amine methylation on the proton affinity and ionization energies of phenylalanine. It is known that the attachment of a methyl group to a primary amine (such as ethylamine) increases the proton affinty by roughly 6 kcal/mol.[10] Attachment of a second methyl group increases the proton affinity by an additional 5 kcal/mol. Since the proton affinity of phenylalanine has been reported by several investigators to be between 219 and 221 kcal/mol,[11–15] *N*-methylation could potentially increase the basicity of phenylalanine to a level greater than many of the most basic unsubstituted amino acids, which typically have proton affinities less than 230 kcal/mol. Incorporation of this modified amino acid into the N-terminus of a peptide could effect the specific site of protonation in the molecule. Additionally, attachment of methyl groups to a primary amine decreases the ionization energy of the nitrogen lone pair orbital.[16] Amine methylation of phenylalanine should result in the

* Author to whom correspondence should be addressed.
† California Institute of Technology.
‡ University of Arizona.
• Abstract published in *Advance ACS Abstracts*, May 15, 1994.

(1) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299.
(2) Torgerson, D. F.; Skowronski, R. P.; Macfarlane, R. D. *Biochem. Biophys. Res. Commun.* **1974**, *60*, 616.
(3) Barber, M.; Bordoli, R. S.; Sedgewick, R. S.; Tyler, A. N. *J. Chem. Soc., Chem. Commun.* **1981**, 325.
(4) (a) Meng, C. K.; Mann, M.; Fenn, J. B. *Z. Phys. D.* **1988**, *10*, 361. (b) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.
(5) (a) Blakely, C. R.; Vestal, M. L. *Anal. Chem.* **1983**, *55*, 750. (b) Vestal, M. L.; Fergusson, G. J. *Anal. Chem.* **1985**, *57*, 2373.
(6) Meot-Ner, M. *J. Am. Chem. Soc.* **1984**, *106*, 278.
(7) Grese, R.; Cerny, R.; Gross, M. *J. Am. Chem. Soc.* **1989**, *111*, 2835.
(8) Watson, J. T.; Wagner, D. S.; Chang, Y. S.; Strahler, J. R.; Hanash, S. M.; Gage, D. A. *Int. J. Mass Spectrom. Ion Processes* **1991**, *111*, 191.
(9) Vath, J. E.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1990**, *100*, 287.
(10) Lias, S. G.; Bartmess, J. E.; Liebman, J. F.; Holmes, J. L.; Levin, R. D.; Mallard, W. G. *J. Phys. Chem. Ref. Data* **1988**, 17(1).
(11) Wu, Z.; Fenselau, C. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 403.
(12) (a) Locke, M. J.; McIver, R. T. *J. Am. Chem. Soc.* **1983**, *105*, 4226. (b) Locke, M. J. Ph.D. Thesis, University of California, 1983 (unpublished results).
(13) Bojesen, G. *J. Am. Chem. Soc.* **1987**, *109*, 5557.
(14) Gorman, G. S.; Speir, J. P.; Turner, C. A.; Amster, I. J. *J. Am. Chem. Soc.* **1992**, *114*, 3986.
(15) Proton affinity of NH_3 referenced at 204.0 kcal/mol.
(16) Kimura, K.; Katsumata, S.; Achiba, Y.; Yamazaki, T.; Iwata, S. *Handbook of He(I) Photoelectron Spectra of Fundamental Organic Molecules*; Halsted Press: New York, 1981.

0002-7863/94/1516-5257$04.50/0

destablization of the nitrogen lone pair orbital while leaving all other molecular orbitals unaffected. Identification of the nitrogen lone pair orbital in the photoelectron spectra of methyl-substituted phenylalanines should be easy, as it is shifted to lower energies.

A FT-ICR technique for measuring gas-phase proton affinities is introduced which utilizes collisional dissociation of proton-bound clusters by off-resonance translational excitation.[17] A simplified RRKM analysis is used to determine proton affinities from product ion abundances. This experimental methodology is similar to that pioneered by Cooks and co-workers[18] for the extraction of thermochemical data from competitive dissociation processes.

We have demonstrated that it is useful when assessing the protonation energetics of molecules to examine the correlations of proton affinities with adiabatic lone pair ionization energies of basic sites.[19,20] Such correlations can be made even when the site of protonation is not associated with the lowest ionization energy of the molecule. These correlations have been shown to have several applications. If the proton affinity and site of protonation are known, the correlation can be used to estimate the orbital ionization energy of the site, which is useful in assigning bands in complex photoelectron spectra. This is of particular interest for molecules such as phenylalanine, where the amine nitrogen lone pair and phenyl π orbitals give rise to unresolved overlapping bands. Similarly, if the lone pair ionization potential of a basic site in a molecule can be determined using photoelectron spectroscopy, then such correlations can be used to predict the intrinsic base strength of that site. The correlation of adiabatic nitrogen lone pair ionization energies with gas-phase proton affinities for phenylalanine, *N*-methylphenylalanine, and *N,N*-dimethylphenylalanine is presented with the purposes of identifying the effect of methyl substitution of the amine nitrogen on the base strength of the molecule and assessing the photoelectron band assignments.

Experimental Details

Photoelectron spectra were recorded at the University of Arizona on an instrument featuring a 36-cm radius hemispherical analyzer (10-cm gap) with customized sample cells, excitation sources, detection and control electronics, and data collection methods that have been previously described.[21] Since the amino acids sublime and then deposit on cooler parts of the instrument, the sensitivity and resolution progressively degrade with time during data collection. The data are represented analytically with the best fit of asymmetric Gaussian peaks (GFIT program).[22] The confidence limits of the peak positions and widths are generally ±0.02 eV. The confidence limit of the area of a band envelope is approximately ±5%, with uncertainties introduced from the base-line subtraction and fitting in the tails of the peaks. When peaks are strongly overlapping, as in the first ionization band of phenylalanine, the individual parameters of a peak are less significant since they are dependent on the parameters of other peaks in the band. The adiabatic ionization energy (energetic onset of each peak) is estimated to occur at the point twice the half-width at half-maximum from the vertical ionization energy on the low-energy side of the band.

The proton affinities of the samples were determined at Caltech using an external ion source Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. A detailed description of this instrument has been previously published.[23] The spectrometer incorporates an external Cs ion bombardment source, octopole ion guide for transferring ions into the high-field region of a 7-T superconducting magnet, a standard 2 × 2 × 3 in. detection cell, and the electronics required for data acquisition and processing in the Fourier transform mode. The instrument has three regions of differential cryogenic pumping, resulting in a residual background pressure of high 10^{-10} or low 10^{-9} Torr in the detection cell. A specially designed ionization gauge is an integral part of the ICR cell and is accurately calibrated using a capacitance manometer connected directly to the cell through a static port. Collision gases are delivered through stainless steel tubing directly to the ICR cell using computer controlled General Valve pulsed valves or manual Varian leak valves. Typical static N_2 gas pressures used in collision-induced dissociation experiments are (2–5) × 10^{-8} Torr.

Samples are typically prepared by dissolving small amounts of solid (~0.1 mg) directly into a 2–3-μL drop of a mixture of glycerol and trifluoroacetic acid spread onto a copper probe tip. For the generation of proton-bound clusters, equal amounts of both reference bases are thoroughly mixed together, and a small sample is used for the analysis. Several solid samples do not readily dissolve in the glycerol/trifluoroacetic acid matrix and must first be dissolved in water (typically, 0.5 mg/mL). For these mixtures, 1–2 μL of solution are mixed with the matrix on the probe tip. Desorption of proton-bound clusters from the probe tip is accomplished in the source region of the spectrometer using 6–8-keV Cs ions.

N,N-dimethylphenylalanine was synthesized using the technique described by Bowman and Stroud.[24] The product yield was adequate at 23%. Purity was confirmed by examination of the melting point (218 °C) and H^1-NMR of the product. All other samples were commercially available from Sigma Chemical Co. and used as provided without further purification.

Results and Discussions

Most of the naturally occurring amino acids are α-substituted glycine derivatives and can be represented by the general formula $H_2NRCHCOOH$, where R represents one of 19 different side chains. Proline is the only amino acid that does not fit this general formula, as it is a cyclic secondary amine. Differences that arise in the photoelectron spectra or gas-phase proton affinity values of amino acids primarily show the effect of changing the R group of the molecule. We have detailed in a previous paper the effects of varying the amino acid side chain on gas-phase proton affinities and nitrogen adiabatic lone pair ionization potentials.[20] The present study examines the gas-phase properties of the amino acid phenylalanine and its *N*-methyl derivatives. Differences in the photoelectron spectra and gas-phase proton affinities of these species result from the effect of methyl substitution on the amine nitrogen.

Photoelectron Spectra of Substituted Phenylalanines. Typically, the lowest ionization potential observed in the photoelectron spectra of the amino acids results from ionization out of the amine nitrogen lone pair orbital (n_N), which has a lower ionization potential by 1 eV than the next closest peak.[25] For amino acids with complex side groups which have orbital energies comparable to the nitrogen lone pair, such as aromatic rings or sulfur, nitrogen, or oxygen atoms, the lowest ionization potential may result from ionization out of a side-chain orbital. In these systems, the peaks for ionization from the n_N orbital and the side-chain orbital tend to overlap and are unresolved, making it difficult to interpret the photoelectron spectra of several of the amino acids. The amino acid phenylalanine is an α-substituted glycine compound with the side chain R = benzyl. The photoelectron spectrum is shown in Figure 1a. The broad band that spans the low ionization potential region between 8 and 10 eV results from the overlap of three peaks. These peaks result from ionization out of three high-energy molecular orbitals, one contributed from the amine nitrogen lone pair and two from the side-chain aromatic ring. Cannington and Ham first reported this feature in the photoelectron spectrum of phenylalanine, but were unable to assign ionization energies to the three orbitals, as the broad spectral

(17) Gauthier, J. W.; Trautman, T. R.; Jacobsen, D. B. *Anal. Chim. Acta* **1991**, *246*, 211.
(18) McLuckey, S. A.; Cameron, D.; Cooks, R. G. *J. Am. Chem. Soc.* **1981**, *103*, 1313.
(19) Staley, R. H.; Kleckner, J. E.; Beauchamp, J. L. *J. Am. Chem. Soc.* **1976**, *98*, 2081.
(20) Campbell, S.; Beauchamp, J. L.; Rempe, M.; Lichtenberger, D. L. *Int. J. Mass Spectrom Ion Processes* **1992**, *117*, 83.
(21) (a) Lichtenberger, D. L.; Kellogg, G. E.; Kristofzski, J. G.; Page, D.; Turner, S.; Klinger, G.; Lorenzen J. *Rev. Sci. Instrum.* **1986**, *57*, 2366. (b) Calabro, D. C.; Hubbard, J. L.; Blevins, C. H., II; Campbell, A. C.; Lichtenberger, D. L. *J. Am. Chem. Soc.* **1981**, *103*, 6839.
(22) Lichtenberger, D. L.; Fenske, R. F. *J. Am. Chem. Soc.* **1976**, *98*, 50.
(23) Marzluff, E. M.; Campbell, S.; Rodgers, M. T.; Beauchamp, J. L. Submitted for publication.
(24) Bowman, R.; Stroud, H. *J. Chem. Soc.* **1950**, 1342.
(25) Klasnic, L. *J. Electron. Spectrosc. Relat. Phenom.* **1976**, *8*, 161.

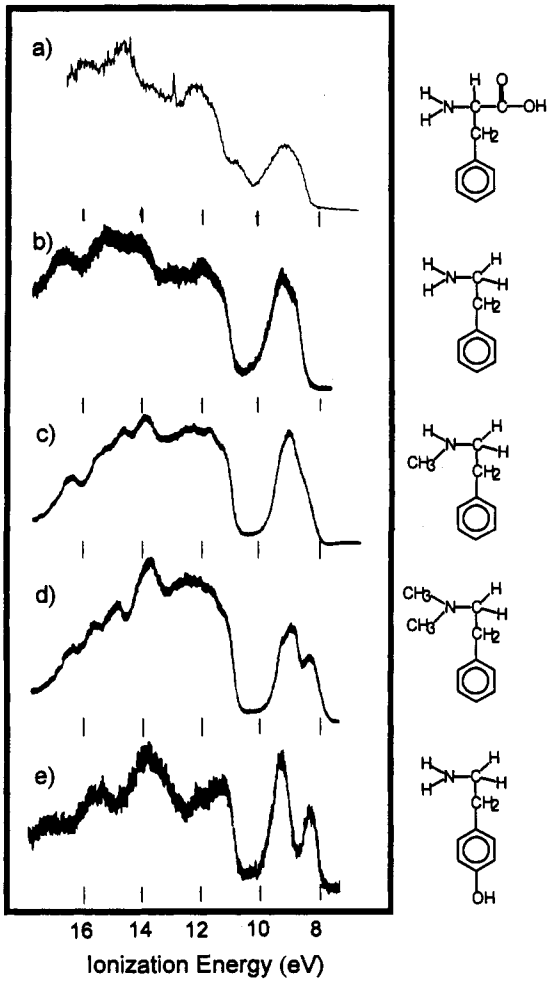

Figure 1. He I photoelectron spectra of (a) phenylalanine, (b) 2-phenylethylamine, (c) *N*-methyl-2-phenylethylamine, (d) *N,N*-dimethyl-2-phenylethylamine, and (e) *p*-hydroxy-2-phenylethylamine. The broad band in the 8–10-eV region observed in each spectrum results from the overlap of three peaks from aromatic π_3, π_2, and nitrogen lone pair (n_N) molecular orbitals. Methyl substitution on the amine nitrogen of c and d shifts the ionization energy of the n_N orbital out from under the broad band, allowing it to be easily identified. Para hydroxylation of the phenyl ring in e shifts only the π_3 orbital, leaving the peaks for the π_2 and n_N orbitals in the region of 9.3–9.5 eV. Spectra b–e were recorded by Domelsmith *et al.*[28]

band was unresolved.[26] An expanded view of the first ionization band of phenylalanine is shown in Figure 2a. This band displays shoulders on the low- and high-energy sides, requiring at least three peaks to suitably model the band profile.

The peaks in the low ionization potential region of phenylalanine can be identified by comparison to appropriate model compounds. In benzene, the degenerate e_{2g} orbital contributes a single peak in this region of the photoelectron spectrum. The vertical ionization potential of the e_{2g} orbital is reported to be 9.25 eV.[27] Substitution of a methyl group on the benzene causes a change in molecular symmetry, creating symmetric (π_3) and antisymmetric (π_2) orbitals. The π_3 orbital is destabilized with respect to the π_2 orbital and yields a lower vertical ionization potential of 8.83 eV, while the π_2 orbital ionization potential of 9.36 eV is slightly higher than that of the e_{2g} orbital of benzene.[27] These ionizations account for two of the three low-energy peaks in the phenylalanine spectrum.

A comprehensive study of the photoelectron spectra and ionization potentials for several substituted 2-phenylethylamines

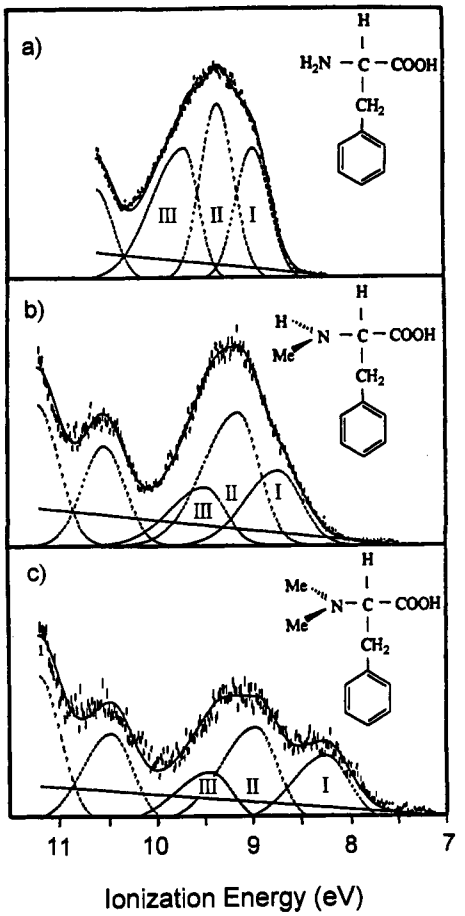

Figure 2. Expansion of the low ionization energy region in the photoelectron spectra of (a) phenylalanine, (b) *N*-methylphenylalanine, and (c) *N,N*-dimethylphenylalanine. The nitrogen lone pair orbital peak in phenylalanine (peak II in a) shifts to lower energy upon methylation (peaks I in b and I in c). Peak energy values and identifications are listed in Table 1.

has been reported by Domelsmith *et al.*[28] The aromatic π orbitals of 2-phenylethylamine, as well as those of phenylalanine, are quite similar to those of toluene. The π_3 and π_2 orbitals are not easily distinguished in the photoelectron spectrum of 2-phenylethylamine (Figure 1b), since the amine nitrogen lone pair orbital appears in the same energy region and creates an unresolved broad band similar to that in the photoelectron spectrum of phenylalanine. Two peak maxima can be identified at 8.99 and 9.35 eV. Determination of the location of the n_N orbital peak, as well as the location of the π_3 and π_2 peaks, is aided by methylation of the amine nitrogen of 2-phenylethylamine. It is expected that the ionization potential of the n_N orbital decreases with each methyl substituent, while the aromatic ionization potentials remain unaffected. This trend in the n_N orbital energies is predicted from the vertical ionization potentials of methylamine, dimethylamine, and trimethylamine decreasing from 9.64 to 8.97 to 8.44 eV, respectively.[16] The n_N peak maximum lies near 8.7 eV in the photoelectron spectrum of *N*-methyl-2-phenylethylamine (Figure 1c), and this peak is well resolved in the *N,N*-dimethyl-2-phenylethylamine spectrum at 8.35 eV (Figure 1d). Since secondary amines are predicted to have ionization potentials 0.6–0.8 eV lower than those of primary amines, the n_N orbital peak in the 2-phenylethylamine spectrum should lie in the region between 9.3 and 9.5 eV. The peak near 8.99 eV in the 2-phenylethylamine spectrum is identified as the π_3 orbital, as substitution of a hydroxyl group at the para position of the ring

(26) Cannington, P.; Ham, N. J. *J. Electron Spectrosc. Relat. Phenom.* **1983**, *32*, 139.
(27) Turner, D.; Baker, C.; Baker, A.; Brundle, C. *Molecular Photoelectron Spectroscopy*; Wiley-Interscience: London, 1970.
(28) Domelsmith, L. N.; Munchausen, L. L.; Houk, K. N. *J. Am. Chem. Soc.* **1977**, *99*, 4311.

Table 1. Vertical and Adiabatic Ionization Potentials for Peaks in the Photoelectron Spectra of Phenylalanine, *N*-Methylphenylalanine, and *N*,*N*-Dimethylphenylalanine

species	peak	vertical IP (eV)	adiabatic IP (eV)	orbital assignment
phenylalanine	I	8.9	8.5	π_3
	II	9.3	8.8	n_N
	III	9.7	9.2	π_2
N-methylphenylalanine	I	8.7	8.2	n_N
	II	9.1	8.5	π_3
	III	9.4	8.9	π_2
N,*N*-dimethylphenylalanine	I	8.2	7.7	n_N
	II	8.9	8.5	π_3
	III	9.3	9.0	π_2

changes only the lowest vertical ionization potential from 8.99 to 8.41 eV and leaves the broad band at 9.35 eV unaffected (Figure 1e).

The photoelectron spectrum of phenylalanine is very similar to that of 2-phenylethylamine. Both have a broad band in the low ionization potential region contributed from aromatic π_3, π_2, and amine nitrogen lone pair n_N orbital peaks. It is anticipated that methyl substitution on the amine nitrogen of phenylalanine will shift the n_N ionization potential to lower values, without having a significant effect on the phenyl π ionization potentials. The stacked photoelectron spectra of all three methylated phenylalanine species with an expanded low ionization potential region are shown in Figure 2. Table 1 lists the vertical and adiabatic ionization potentials for the numbered peaks in each photoelectron spectrum of Figure 2. The spectrum of *N*,*N*-dimethylphenylalanine (Figure 2c) is the easiest to interpret, as substitution of two methyl groups has changed the spectrum dramatically from that of the unsubstituted parent molecule. Peak I, with a vertical ionization potential of 8.2 eV, is contributed from the n_N orbital as the destabilization has lowered its ionization potential and shfited the peak from under the broad band. The π_3 and π_2 vertical ionization potentials (8.9 and 9.3 eV, respectively) are relatively unaffected by amine methylation and are similar to the analogous ionization potentials in toluene.

The n_N orbital can be assigned in the *N*-methylphenylalanine spectrum (Figure 2b) using the following arguments. Secondary amines are expected to have ionization potentials 0.4–0.5 eV higher than tertiary amines; therefore, the n_N orbital peak is predicted to lie in the region between 8.6 and 8.7 eV. The peak with the lowest ionization potential (I) in Figure 2b must arise from the n_N orbital. Table 1 confirms that the vertical and adiabatic ionization potentials of the π_3 and π_2 orbitals (peaks II and III, respectively) remain relatively constant from *N*-methyl- to *N*,*N*-dimethylphenylalanine. Methyl substitution at the amine has little effect on these orbitals.

Comparing the trends in the photoelectron spectrum of phenylalanine to those of 2-phenylethylamine, it is possible to identify the vertical ionization potential for the n_N orbital. Primary amines are expected to have ionization potentials 0.6–0.8 eV higher than secondary amines, so the vertical ionization potential for the n_N orbital of phenylalanine should lie in the region between 9.2 and 9.5 eV. This identifies peak II at 9.3 eV as the n_N orbital. The peak with the lowest ionization potential (I) in Figure 2a is identified as the π_3 orbital, as substitution of a hydroxyl group at the para position of the ring changes only the lowest vertical ionization potential from 8.9 to 8.5 eV and leaves the broad band at 9.3 eV relatively unaffected.[26] The π_2 orbital has the highest ionization potential in all three substituted phenylalanines and is always peak III.

The trends in the vertical ionization potentials of the substituted phenylalanines are best summarized in Figure 3. The effect of lowering the n_N ionization potential upon methyl substitution on a primary amine (methylamine) is clearly shown in Figure 3a. This trend is also observed in the n_N ionization potential of

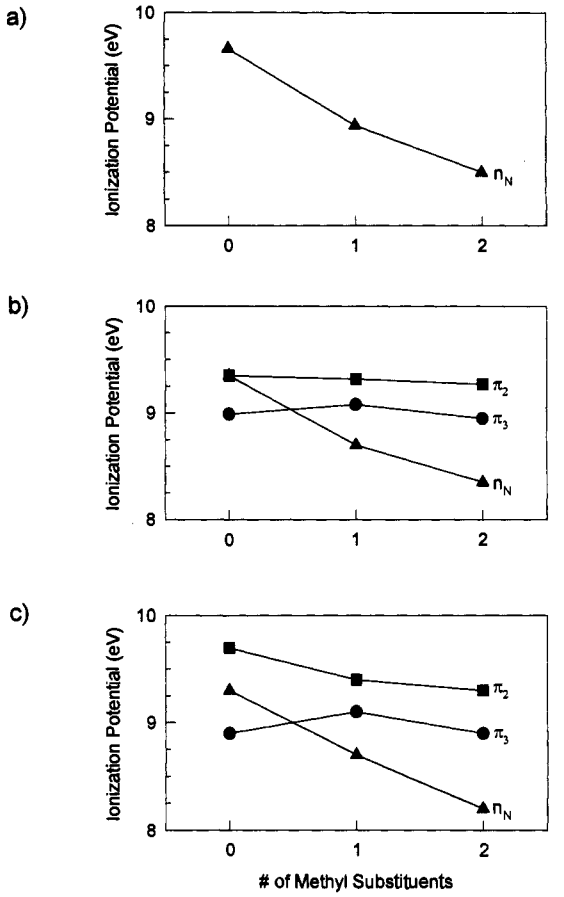

Figure 3. Trends in the vertical ionization potentials for *N*-methyl-substituted (a) methylamines, (b) 2-phenylethylamines, and (c) phenylalanines. The nitrogen lone pair (n_N) ionization potential decreases steadily in methyl-substituted amines, while the aromatic π orbitals remain relatively unaffected. Peak assignments for phenylalanine are confirmed by evaluating the trends in the n_N orbital.

substituted 2-phenylethylamines (Figure 3b), while the π_3 and π_2 orbitals remain relatively unchanged. Substituted phenylalanines display the same trends in the n_N and π orbitals as does 2-phenylethylamine, which facilitates the assignment of these orbitals.

Proton Affinities of Substituted Phenylalanines. The gas-phase proton affinity of phenylalanine has been determined by numerous investigators to be between 219 and 221 kcal/mol.[11-15] The proton affinity values for methyl-substituted phenylalanines have not been previously reported.

Several experimental methodologies exist for the determination of gas-phase proton affinities. Proton-transfer reactions which reach equilibrium at a fixed temperature yield gas-phase basicities which can give proton affinities if the entropy of protonation can be estimated using statistical thermodynamics.[29,30] Unfortunately, the use of equilibrium techniques with biomolecules is restricted by their low volatility. The kinetic method, pioneered by Cooks and co-workers,[18] has held the most promise to date for determining proton affinities of relatively nonvolatile molecules. The proton affinity is correlated to the ratio of the product ion abundances in the dissociation of proton-bound dimers. These ions are typically produced by metastable decay of the clusters in the field-free region of a multisector mass spectrometer, but more recently have been generated by low-energy collision-induced dissociation of proton-bound clusters in four-sector instruments.[31]

(29) Aue, D. H.; Bowers, M. T. *Gas Phase Ion Chemistry*; Academic Press: New York, 1979; Vol. 2.
(30) Henderson, W. G.; Taagepera, M.; Holtz, D.; McIver, R. T., Jr.; Beauchamp, J. L.; Taft, R. W. *J. Am. Chem. Soc.* **1972**, *94*, 4728.
(31) Cheng, X.; Wu, Z.; Fenselau, C. *J. Am. Chem. Soc.* **1993**, *115*, 4844.

To determine the proton affinity of a base B_2 using the kinetic approach, a proton-bound dimer is formed from a reference base B_1 of known proton affinity and the base B_2. The dissociation of the cluster is dominated by two unimolecular reactions (eq 1).

$$[B_1\text{–}H^+\text{–}B_2] \begin{cases} \rightarrow B_1H^+ + B_2 & (1a) \\ \rightarrow B_1 + B_2H^+ & (1b) \end{cases}$$

Two assumptions are made in analyzing data from these experiments: (1) if species B_1 and B_2 are structurally similar, the difference in the entropy of protonation ($\delta\Delta S$) for forming the products is small and (2) the dissociation reactions have zero or very small reverse activation energies. With these assumptions, the difference in activation energies for reactions 1a and 1b (δE_0) is equal to the difference in proton affinities $PA(B_1) - PA(B_2)$.

The kinetic method employs transition–state theory to model unimolecular dissociation rates of the proton-bound dimers. As a result of this approach, dimers are described with a singular effective temperature (T). In reality, both Cooks type experiments and our own deal with a nonthermal internal energy distribution, and the dissociation kinetics are more appropriately analyzed using RRKM theory[32] and averaging over the energy distribution. We start with the simplified RRK[33] unimolecular dissociation rate described by eq 2, in which ν is the frequency factor for the

$$k(E) = \nu\left(\frac{E - E_0}{E}\right)^{s-1} \qquad (2)$$

reaction, E is the total energy of the excited molecule with s vibrational degrees of freedom, and E_0 is the activation energy. If there are two competing dissociation channels for the molecule, as there are for the proton-bound clusters, then the rates of channels 1 and 2 are related by eq 3. Simple mathematical

$$\frac{k_1(E)}{k_2(E)} = \frac{\nu_1}{\nu_2}\left(\frac{E - E_0(1)}{E - E_0(2)}\right)^{s-1} \qquad (3)$$

manipulation of eq 3 yields an expression which allows the proton affinity of the unknown base to be extracted from the ratios of the fragment ion abundances (eq 4), provided δE_0 is small

$$\ln\frac{k_1(E)}{k_2(E)} = \ln\left(\frac{\nu_1}{\nu_2}\right) - (s-1)\left(\frac{\delta E_0}{E - E_0(2)}\right) \qquad (4)$$

compared to $E - E_0$. As stated above, the dissociation rates are proportional to the product ion abundances and the differences in activation energies are equal to the differences in proton affinities. Plotting the natural log of the ratio of the product ion abundances versus the reference base proton affinity for a series of bases allows the proton affinity of the unknown species to be determined. The proton affinity of the unknown base is found where the dissociation rates for the reference and unknown base are equal (ln ratio = 0). An example of such a plot is shown for *N*-methylphenylalanine with a series of reference bases later in this section (Figure 6). It should be noted that the inverse of the slope of the plot is equal to the excess internal energy per degree of freedom in the activated complex and can be related to the temperature of the cluster.

Experimental methods which utilize different mass spectrometry techniques have distinctively different time scales and therefore sample ions of differing internal energies. To detect dissociation products using FT-ICR mass spectrometry, molecules must fall apart on a time scale of 100 ms to 1 s, corresponding to rates of 1–10 s^{-1}. In faster MS/MS type experiments of tandem mass spectrometers, molecules dissociate on a time scale of 10^{-4}–10^{-5} s, yielding rates of 10^4–10^5 s^{-1}. RRKM analysis shows that for *similar frequency factors and activation energies*, dissociation rates for systems of varying size are approximately the same when the excess energy per vibrational degree of freedom in the activated complex is comparable. This analysis explains why the kinetic method works as well as it does, since plots of the experimental results give straight lines regardless of the size of the reference base used. Big molecules with large numbers of degrees of freedom require higher internal excitation to dissociate on the same time scale as small molecules. Variation in frequency factors which result from entropic effects obviously complicates the analysis. Hence, it is best to choose reagent bases which are structurally similar, monodentate, and rigid.[31]

Gas-phase proton affinities of methyl-substituted phenylalanines were determined by FT-ICR mass spectrometry using the technique described above and have an average uncertainty of ±1 kcal/mol. Proton-bound clusters of reference bases and substituted phenylalanines were generated in the FAB source and transferred into the trapped ion cell of the FT-ICR mass spectrometer. Parent cluster ions were initially isolated by applying resonant frequency ejection pulses to all other species in the cell. The clusters were then translationally excited with sustained off-resonance radio frequency excitation[17] for several seconds and collisionally dissociated against a static pressure of nitrogen (2×10^{-8} Torr).

Ion populations generated in the FAB source and transferred to the detection cell have a wide distribution of energy, since without translational excitation, product ions are observed for dissociation against a static N_2 gas pressure which produces typically one or two collisions per second. Collisional dissociation appears to occur on a time scale faster than collisional or radiative cooling of the cluster ions. Using sustained off-resonance translational excitation, collision conditions are varied from 0 to 4 eV in the center of mass frame to determine if the average relative kinetic energy of the cluster has an effect on fragment product ion distributions. The kinetic energy of a trapped ion obtained from sustained off-resonance radio frequency excitation is described by eq 5, where q is the charge of the ion, E_0 is the

$$E_{\text{ion,lab}}(t) = \frac{q^2E_0^{\,2}}{2m(\omega - \omega_c)^2}\sin^2\left[\frac{(\omega - \omega_c)t}{2}\right] \qquad (5)$$

amplitude of the applied electric field, m is the mass of the ion, and $(\omega - \omega_c)$ is the difference between the applied excitation frequency and natural cyclotron frequency of the ion. The time-averaged center of mass kinetic energy of the trapped ion is determined by eq 6. A plot of product ion abundances versus

$$\langle E_{\text{ion,cm}}\rangle = \frac{m_{\text{gas}}}{m_{\text{gas}} + m_{\text{ion}}}\left[\frac{q^2E_0^{\,2}}{4m(\omega - \omega_c)^2}\right] \qquad (6)$$

center of mass kinetic energies for the proton-bound dimer of *N*-methylphenylalanine with 2-aminopyridine is shown in Figure 4. Increasing the center of mass kinetic energy to 2.6 eV produces nearly complete dissociation of the dimer into protonated 2-aminopyridine or protonated *N*-methylphenylalanine. Both species are produced with similar intensities since they have nearly identical proton affinities. Although the relative ion abundance of each product increases with increasing kinetic energy, the ratio of the product ion abundances is unaffected. A plot of the natural log of the ratio of product ion abundances versus the relative kinetic energy for proton-bound dimers of *N*-methylphenylalanine with proline, 2-aminopyridine, and trimethylamine is shown in Figure 5. Off-resonance collisional activation is conducted under multiple collision conditions, with sequential encounters resulting in accumulation of sufficient internal excitation to produce dissociation. Increasing the relative kinetic energy allows for more internal excitation to be accumulated per collision, but does

(32) (a) Marcus, R. A.; Rice, O. K. *J. Phys. Colloid Chem.* **1951**, *55*, 894. (b) Marcus, R. A. *J. Chem. Phys.* **1952**, *20*, 359.
(33) (a) Rice, O. K.; Ramsperger, H. C. *J. Am. Chem. Soc.* **1928**, *50*, 617. (b) Kassel, L. S. *J. Phys. Chem.* **1928**, *32*, 1065.

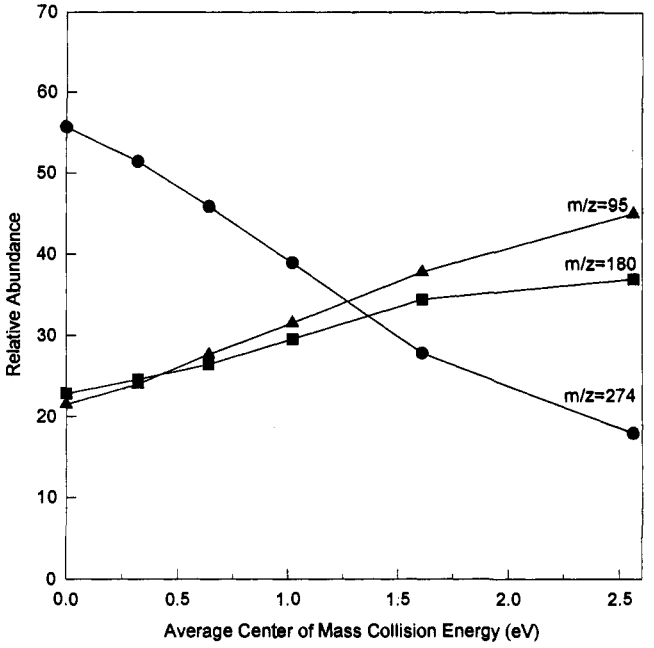

Figure 4. Variation in relative product ion abundance with center of mass collision energy observed in the collision-induced dissociation of the proton-bound dimer of *N*-methylphenylalanine and 2-aminopyridine (m/z = 274). Protonated *N*-methylphenylalanine (m/z = 180) and protonated 2-aminopyridine (m/z = 95) are produced with similar intensities since they have nearly identical proton affinities. Off-resonance excitation is performed for 600 ms using N_2 as the collision gas (2×10^{-9} Torr). Center of mass collision energies vary over the range 0–2.6 eV.

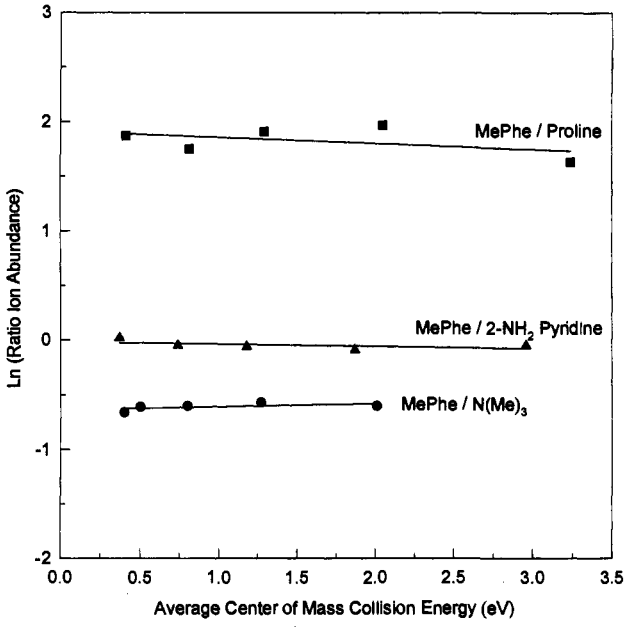

Figure 5. Ratio of product ion abundances for the collision-induced dissociation of proton-bound dimers of *N*-methylphenylalanine and reference bases, unaffected by the average relative kinetic energy. Flat slopes in this plot indicate that proton affinities obtained using this technique are independent of collision energies and structural entropic effects.

not alter the level of internal excitation which produces dissociation. This is in contrast to single-collision excitation, where internal energies considerably in excess of those required to produce dissociation may result from an individual encounter. As a result, product distributions used to derive proton affinities in these FT-ICR experiments are relatively insensitive to the ion kinetic energies used.

The dissociation technique described above is similar to one

Table 2. Summary of the Ratios of Product Ion Distributions from the Collision-Induced Dissociation of Proton-Bound Clusters Containing Substituted Phenylalanines and Various Reference Bases

proton-bound dimer	no. of trials	reference base proton affinity[a]	average ratio x-Phe/base	average ln ratio
Phe with				
Ile	12	219.3	4.5	1.5
Met	4	221.8	0.1	–2.0
Me-Phe with				
Pro	11	220.2	6.2	1.8
6-Me-purine	10	223.0	1.2	0.2
2-NH_2-pyridine	15	223.8	0.9	–0.1
$(Me)_3N$	16	225.1	0.6	–0.5
Gln	3	227.4	0.1	–2.0
Me_2-Phe with				
Me-Phe	3	223.6	1.8	0.6
$(Me)_3N$	4	225.1	0.7	–0.4

[a] Proton affinities in kcal/mol.

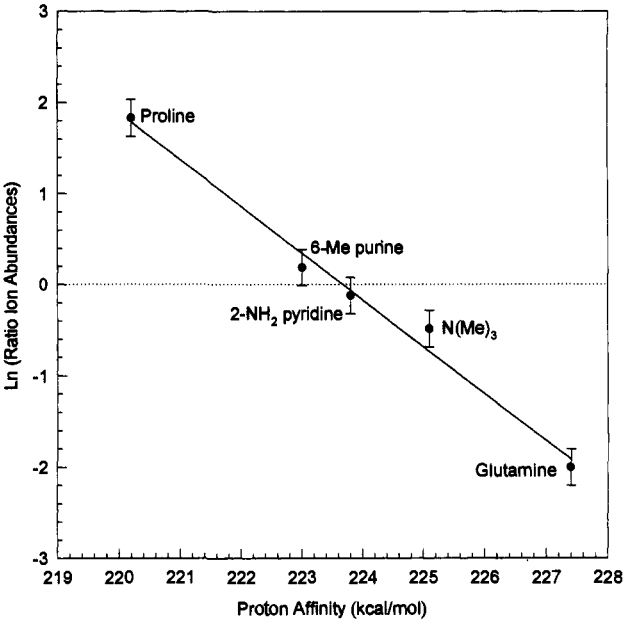

Figure 6. Natural logarithm of (protonated *N*-methylphenylalanine/protonated reference base) versus reference base proton affinity for five proton-bound dimers. The proton affinity for *N*-methylphenylalanine extracted from the plot is 223.6 kcal/mol. Data for these experiments are found in Table 2.

we recently developed using CW CO_2 laser irradiation, in which the proton-bound dimer of a reference base and a molecule of interest is subjected to infrared multiphoton dissociation (IRMPD) in an FT-ICR trapped ion cell.[34] Clusters are slowly energized until cleavage occurs selectively along the lowest energy pathway. This process also dissociates the cluster into two intact products, with the species of higher basicity preferentially retaining the proton.

To demonstrate the methodology employed for determining proton affinities, we present a detailed investigation for *N*-methylphenylalanine. The proton affinity of *N*-methylphenylalanine was determined by collisionally dissociating proton-bound clusters of five different reference bases with *N*-methylphenylalanine and evaluating each of their product ion distributions. The data for each experiment is listed in Table 2. The proton affinity of *N*-methylphenylalanine is bracketed between the values of 223.0 kcal/mol for 6-methylpurine and 223.8 kcal/mol for 2-aminopyridine.[10] A more precise estimate of the value can be made from the data plot in Figure 6. The proton affinity of *N*-methylphenylalanine extracted from the plot is 223.6 kcal/

(34) Campbell, S.; Beauchamp, J. L. *Proc. SPIE-Int. Soc. Opt. Eng.* **1992**, *1636*, 201.

mol, which is not the median of the bracketed energy difference but is closer to that of 2-aminopyridine.

Since the proton affinity of phenylalanine has been published by numerous investigators, phenylalanine can be used as a model compound to test the accuracy of our experimental methods. Proton-bound clusters of phenylalanine with isoleucine and with methionine are isolated and dissociated in the FT-ICR trapped ion cell. In agreement with the *N*-methylphenylalanine results, the ratios of product ion abundances remain constant over a range of collision energies. A plot of the natural log of the ratio of ion abundances versus the proton affinty of the reference base was generated using the values of 219.3 and 221.8 kcal/mol for the proton affinities of isoleucine and methionine, respectively (data in Table 2).[12] The plot yields a proton affinity for phenylalanine of 220.3 kcal/mol, in excellent agreement with the previously reported range of 219–221 kcal/mol. Although determining proton affinities with this technique in theory is quite simple, it can be difficult to generate a variety of proton-bound clusters. Several reference bases with a range of proton affinities were used in the attempt to generate dimers, but most failed to produce any detectable clusters. For reasons not yet understood, we found it easier to generate proton-bound dimers of reference bases with *N*-methylphenylalanine.

The proton affinity of *N*,*N*-dimethylphenylalanine was determined by collisionally dissociating the proton-bound clusters of *N*,*N*-dimethylphenylalanine with *N*-methylphenylalanine and with trimethylamine. As with the above phenylalanine example, *N*,*N*-dimethylphenylalanine forms proton-bound dimers with a very select group of reference bases which do not encompass a large range of proton affinities. The proton affinity of *N*,*N*-dimethylphenylalanine is bracketed between 223.6 and 225.1 kcal/mol (data in Table 2), and an analysis for these dissociations similar to Figure 6 yields a proton affinity of 224.5 kcal/mol.

Correlations of Adiabatic Nitrogen Lone Pair Ionization Potentials with Proton Affinities of Substituted Phenylalanines. The proton affinity of a molecule is related to the homolytic bond energy in the conjugate acid $D(B^+ - H)$, as indicated by eq 7. If

$$PA(B) = IP(H) - IP(B) + D(B^+ - H) \qquad (7)$$

the homolytic bond dissociation energy is constant for a particular functional group, the proton affinity will exhibit a linear correlation with the quantity IP(H) – IP(B). We have previously reported that a linear correlation exists between the proton affinities and the adiabatic nitrogen lone pair ionization energies for amino acids which protonate on the amine group.[20] Many of the amino acids correlate well with data for simple primary amines, but the amino acids proline and sarcosine do not. These species, as expected, best fit the correlation for secondary amines. The correlation of the nitrogen lone pair ionization energy of the amine group with the proton affinity of numerous amino acids, including phenylalanine and *N*-methyl- and *N*,*N*-dimethylphenylalanine, is shown in Figure 7. Included in the figure for comparison is the correlation observed for several primary, secondary, and tertiary amines. Deviations from the correlation have been previously explained in detail.[20]

The point for phenylalanine in Figure 7 does not precisely fit the correlation for primary amines. Although it is difficult to interpret the photoelectron spectrum of phenylalanine (as described above), we are reasonably confident with the assignment of the nitrogen lone pair as the second band in the photoelectron spectrum. The similar trends observed in the simple amine and 2-phenylethylamine ionization energies resulting from amine methylation support our conclusions (Figure 3). It is noteworthy to mention though, if the peak for the lowest ionization potential (I) in the photoelectron spectrum of phenylalanine is chosen instead of the second peak (II) for the adiabatic nitrogen lone pair ionization potential, the point for phenylalanine in Figure 7 resides slightly above the correlation line for the primary amines and appears to be in better agreement with many aliphatic amino acids.

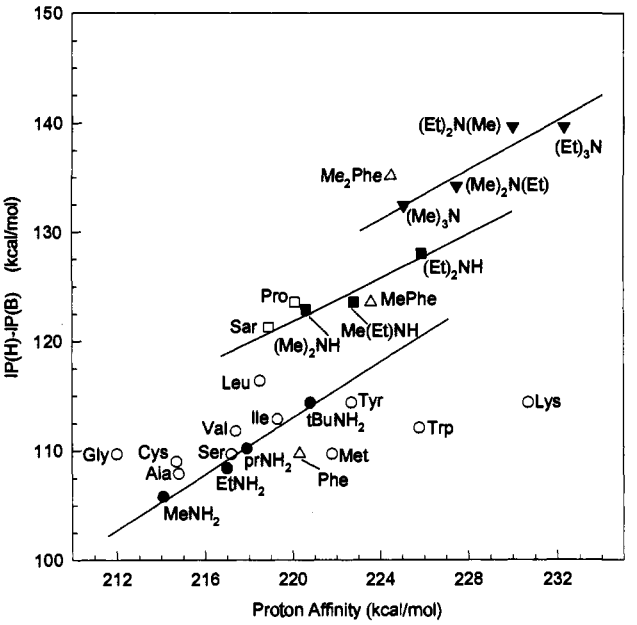

Figure 7. Correlation of the amine nitrogen lone pair adiabatic ionization energy with the proton affinity of numerous amino acids (open circles). Most of the amino acids fit the correlation with the primary amine data (filled circles), which is consistent with the amine group being the preferred site of protonation. Proline, sarcosine, and *N*-methylphenylalanine fail to fit the correlation for primary amines and instead fit the correlation for secondary amines (filled squares). As expected, *N*,*N*-dimethylphenylalanine fits the correlation for tertiary amines (filled triangles). Deviations from the correlations are explained in the text. The lines through the points are least-squares fits to the reference aliphatic amine data.

Deviation of the phenylalanine point below the primary amine correlation line of Figure 7 implies the proton affinity is larger than the value expected from simple protonation on the amine nitrogen. This enhanced basicity is proposed to result from stabilizing cation–π interactions between the proton on the amine nitrogen and the aromatic ring in the side chain. These interactions are believed to be primarily electrostatic, and previous studies have established that interactions between quaternary ammonium groups and π electrons of aromatic groups (such as benzene) are highly stabilizing.[35,36] Interaction energies for benzene with protonated ammonia, methylamine, and trimethylamine have been reported as 19.3, 18.8, and 15.9 kcal/mol, respectively.[35,36] The large, polarizable ring in the side chain of phenylalanine adopts a geometry which interacts with protons on the amine nitrogen. The net stabilization is not as large as the energies reported above since geometric constraints prevent an optimum interaction and entropic effects due to cyclization must be considered. Recent molecular mechanics calculations by Gorman and Amster[37] indicated that dipeptides containing phenylalanine in the C-terminus position displayed considerable stabilization between the protonated N-terminus and the aromatic side chain, yet indicated no significant stabilizing interaction between the proton and aromatic side chain for the isolated amino acid. In contrast, semiempirical calculations using AM1 methodology[38,39] indicate sizable interactions between the protonated

(35) Deakyne, C. A.; Meot-Ner, M. *J. Am. Chem. Soc.* **1985**, *107*, 474.
(36) MP2/6-31G** calculations of NH_4^+/C_6H_6 give a binding energy of 17.9 kcal/mol. Private communication with D. A. Dougherty.
(37) Gorman, G. S.; Amster, I. J. *J. Am. Chem. Soc.* **1993**, *115*, 5729.
(38) AM1 calculations were performed using the Hyperchem Computational Chemistry Software Package, Release 3, Autodesk, Inc., 1992.
(39) Dewar, M. J.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. *J. Am. Chem. Soc.* **1985**, *107*, 3902.

amine and the aromatic side chain of phenylalanine. Geometry optimizations yield two of the amine hydrogens simultaneously pointing directly at the aromatic side chain, polarizing the ring charge distribution to maximize the electrostatic interactions. Fractional charges of 0.272 and −0.137 on two of the amine hydrogens and phenyl carbons, respectively, result in hydrogen bonds that constrain the phenylalanine geometry. The fractional charges on the amine hydrogens decrease upon methylation from 0.272 for phenylalanine to 0.261 for *N,N*-dimethylphenylalanine. This is accompanied by a decrease in the charge induced on the phenyl carbons. The calculated proton affinities for phenylalanine and *N*-methyl- and *N,N*-dimethylphenylalanine are 210.7, 213.8, and 213.1 kcal/mol, respectively, roughly 10 kcal/mol lower than the experimental values. It should only be considered significant that the calculations predict small methyl substituent effects on the proton affinity of phenylalanine. Similar calculations exploring methyl substituent effects on the proton affinities of *n*-alkyl amines correctly reproduce the observed trend but underestimate the magnitude of observed effects.[40]

The point for phenylalanine is displaced horizontally from the correlation curve of Figure 7 by roughly 3.0 kcal/mol, implying an intrinsic proton affinity (neglecting intramolecular interactions) of 217.3 kcal/mol. Using the technique of high-pressure mass spectroscopy, Meot-Ner *et al.* determined the proton affinity of phenylalanine to be 216.8 kcal/mol.[41] This measurement was performed at a higher temperature, which would tend to mask stabilizing intramolecular interactions, and is in good agreement with the value predicted by the correlation curve.

The point for *N*-methylphenylalanine fits the correlation in Figure 7 for secondary amines reasonably well. As with phenylalanine, the point is slightly below the correlation line but is only displaced horizontally by approximately 1.8 kcal/mol. Since the proton affinity of *N*-methylphenylalanine has not previously been published, there is no high-temperature value available for comparison. Addition of a methyl group on the amine nitrogen increases the proton affinity of that site, but appears to weaken the intramolecular cation–π interaction, as the displacement from the correlation line is not as great as in phenylalanine. This observation follows the previously mentioned trend in the interactions of benzene with protonated amines, where interaction energies decreased upon methylation from ammonia to methyl- and trimethylamine. As may be expected from electrostatic considerations, interaction energies decrease as the fractional charge on the amine hydrogens decreases.

The point for *N,N*-dimethylphenylalanine, in contrast to the above examples, lies to the left of the correlation line for the tertiary amines. As noted above, the addition of a second methyl group on the nitrogen reduces the electrostatic charge on the amine hydrogen and thus reduces the stabilizing effect of the phenyl substituent. Although this molecule does not necessarily fit the tertiary correlation well, it follows the trend observed for the majority of aliphatic amino acids which fall to the left of the correlations for aliphatic amines.

An additional argument is offered to support the postulate that the proton affinity of phenylalanine reflects cation–π interactions. Substitution of methyl groups onto the nitrogen of primary amines increases the proton affinity substantially, whereas substitution of methyl groups onto the amine nitrogen of phenylalanine does very little to increase the proton affinity. The addition of one methyl group to ethylamine increases the proton affinity by 5.8 kcal/mol. The difference in the measured proton affinities of phenylalanine and *N*-methylphenylalanine, 3.4 kcal/mol, is not as large as expected. If the intrinsic proton affinity estimated from Figure 7 is used for phenylalanine (217.3 kcal/mol), the anticipated proton affinity of *N*-methylphenylalanine should be 223.1 kcal/mol (217.3 + 5.8). This value is in reasonable agreement with that observed experimentally (223.6 kcal/mol) and suggests that the protonated phenylalanine has additional stabilization.

(40) Dewar, M. J.; Dieter, K. M. *J. Am. Chem. Soc.* **1986**, *108*, 8075.
(41) Meot-Ner, M.; Hunter, E. P.; Field, F. H. *J. Am. Chem. Soc.* **1979**, *101*, 686.

Conclusions

This study presents a quantitative approach for extracting gas-phase proton affinities from cluster dissociation data. A simplified RRKM analysis is used to determine proton affinities from product ion abundances. This methodology is similar to that introduced by Cooks and co-workers[18] for the extraction of thermochemical data from competitive dissociation processes, but varies in the interpretation of the results.

The photoelectron spectra of amino acids with complex side groups can be difficult to interpret since the peaks for ionization from the amine nitrogen lone pair orbital and side-chain orbitals tend to overlap and are unresolved. The spectrum for phenylalanine clearly displays this problem as the nitrogen lone pair and aromatic phenyl π orbitals have virtually the same ionization energies. Methyl substitution on the amine nitrogen destabilizes the lone pair orbital and shifts the peak in the spectrum to a lower ionization potential. This technique is useful in general for identifying orbital peaks which are unresolvable in complex photoelectron spectra.

Methyl substitution onto the amine nitrogen of phenylalanine has also been shown to increase the basicity of the molecule. Although the increase in proton affinity is less than that observed for methylation of simple amines, complete methylation increases the proton affinity of phenylalanine by approximately 4 kcal/mol. This small net difference in proton affinities results from stabilizing intramolecular cation–π interactions which are present in phenylalanine (and to a much lesser extent *N*-methylphenylalanine) and cause the proton affinity to be higher than if simple protonation occurred on the amine. The proton affinity of *N,N*-dimethylphenylalanine does not reflect these additional stabilizing interactions and accounts for the small net difference in proton affinities between the parent and dimethylated species.

Due to the varying strengths of intramolecular interactions for the remaining amino acids, it is difficult to predict the effect of amine methylation on proton affinities. The increase could range from a few kilocalories/mole to roughly 11 kcal/mol, that observed for methylation of simple amines. Incorporation of a methylated amino acid into the N-terminus of a peptide will potentially increase the basicity of that site, but in most cases the increase will not be sufficient to compete with protonation of highly basic side chains or internally solvated amide oxygens.

Acknowledgment. J.L.B. gratefully acknowledges the Beckman Foundation and Institute for the initial funding and continuing support of the research facilities, and the National Science Foundation for their funding (CHE-9108318). We acknowledge the financial support of S.C. from a NIH-NRSA traineeship in Biotechnology, E.M.M. from a NIH-NRSA Human Genome traineeship and Rainin Fellowship, and M.T.R. from a California Institute of Technology Consortium grant. D.L.L. acknowledges support of the Department of Energy (Division of Chemical Sciences, Office of Basic Energy Sciences, Office of Energy Research; Contract No. DE-FG02-86ER13501), the National Science Foundation for assistance in support for instrumentation (CHE-9300841) and for support of K.F.S. under the REU program (CHE-376800), and the Materials Characterization Program (Arizona).