

## Are Carboxyl Groups the Most Acidic Sites in Amino Acids? Gas-Phase Acidities, Photoelectron Spectra, and Computations on Tyrosine, *p*-Hydroxybenzoic Acid, and Their Conjugate Bases

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**Abstract:** Deprotonation of tyrosine in the gas phase was found to occur preferentially at the phenolic site, and the conjugate base consists of a 70:30 mixture of phenoxide and carboxylate anions at equilibrium. This result was established by developing a chemical probe for differentiating these two isomers, and the presence of both ions was confirmed by photoelectron spectroscopy. Equilibrium acidity measurements on tyrosine indicated that  $\Delta G_{\text{acid}}^{\circ} = 332.5 \pm 1.5 \text{ kcal mol}^{-1}$  and  $\Delta H_{\text{acid}}^{\circ} = 340.7 \pm 1.5 \text{ kcal mol}^{-1}$ . Photoelectron spectra yielded adiabatic electron detachment energies of  $2.70 \pm 0.05$  and  $3.55 \pm 0.10 \text{ eV}$  for the phenoxide and carboxylate anions, respectively. The H/D exchange behavior of deprotonated tyrosine was examined using three different alcohols ( $\text{CF}_3\text{CH}_2\text{OD}$ ,  $\text{C}_6\text{H}_5\text{CH}_2\text{OD}$ , and  $\text{CH}_3\text{CH}_2\text{OD}$ ), and incorporation of up to three deuterium atoms was observed. Two pathways are proposed to account for these results, and all of the experimental findings are supplemented with B3LYP/aug-cc-pVDZ and G3B3 calculations. In addition, it was found that electrospray ionization of tyrosine from a 3:1 (v/v)  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  solution using a commercial source produces a deprotonated  $[\text{M} - \text{H}]^-$  anion with the gas-phase equilibrium composition rather than the structure of the ion that exists in aqueous media. Electrospray ionization from acetonitrile, however, leads largely to the liquid-phase (carboxylate) structure. A control molecule, *p*-hydroxybenzoic acid, was found to behave in a similar manner. Thus, the electrospray conditions that are employed for the analysis of a compound can alter the isomeric composition of the resulting anion.

### Introduction

It has been uniformly assumed that all 20 peptide-forming amino acids afford carboxylate ions in the gas phase upon deprotonation or negative-ion electrospray ionization.<sup>1</sup> This supposition is based upon analogy to the known behavior of these compounds in aqueous solution and the fact that the carboxyl group is the most acidic functional group in these molecules. However, when two or more substituents are present in a compound, as is the case in amino acids and peptides, they can interact, and their relative acidities may be altered.<sup>2</sup> This was recently shown to be the case for cysteine, where the most acidic site was found to be the thiol and the conjugate base is a thiolate rather than a carboxylate.<sup>3,4</sup> This conclusion was reached by Tian et al.<sup>3</sup> on the basis of acidity measurements,

hydrogen–deuterium exchange experiments, and high-level computations. Woo et al.<sup>4</sup> independently drew the same conclusion on the basis of photoelectron spectroscopy of the deprotonated  $[\text{M} - \text{H}]^-$  cysteine anion. These findings are mirrored in peptides in that thiolates play an important role in some enzyme-catalyzed processes.<sup>5</sup> They also raise the following question: is cysteine unique, or is the preferred anionic site something other than a carboxylate in other amino acids?

In this paper, we report the observation of both conjugate bases for tyrosine in the gas phase. We give the equilibrium proportions of the corresponding phenoxide and carboxylate anions along with a gas-phase equilibrium acidity determination of tyrosine and the results of hydrogen–deuterium exchange measurements<sup>6</sup> and a photoelectron spectroscopy study. The isomeric compositions of deprotonated tyrosine and *p*-hydroxybenzoic acid formed by electrospray ionization as well as data

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from high-level computations that supplement the experimental results are also reported.

## Experimental Section

All of the gas-phase reactions were studied in a dual-cell model 2001 Finnigan Fourier transform mass spectrometer (FTMS) equipped with a 3 T superconducting magnet and controlled by an IonSpec (now Varian) data system or a 3 T instrument using an IonSpec electrospray cart.

**Equilibrium Acidity Measurements.** Tyrosine equilibrium acidity determinations were carried out using benzoic acid and fluoroacetic acid in the dual-cell instrument by measuring forward and reverse proton-transfer rate constants. In both cases, tyrosine was admitted into the instrument via a heated solid probe inlet, and its  $[M - H]^-$  ion was produced by 2.5 eV electron ionization. After the anion was allowed to equilibrate with the neutral acid for  $\sim 1$  s, the resulting ions were transferred to the second cell and cooled with a pulse of argon, and the  $[M - H]^-$  ion ( $m/z$  180) was isolated using a stored-waveform inverse Fourier transform (SWIFT) excitation.<sup>7</sup> It was then allowed to react with benzoic acid or fluoroacetic acid as a function of time, and the reaction rate constant was obtained at three different pressures of the neutral acid. In the reverse direction, benzoate and fluoroacetate were generated by electron ionization (7.5 eV), and the neutral pressure of tyrosine in the reaction region had to be determined. This posed a problem because tyrosine is not very volatile and was added via the solid inlet probe, which was  $\sim 1$  cm from the reaction region but  $\sim 1$  m from the ionization gauge used to measure the pressure. This led to a pressure differential, which was addressed by measuring the reaction rates of tyrosine with hydroxide and fluoride, one after the other. These very exothermic proton-transfer reactions can safely be assumed to occur upon every collision,<sup>8</sup> thereby enabling pressure correction factors of 1.37 and 1.57, respectively, to be obtained. The average of these values ( $1.47 \pm 0.14$ ) was used in the subsequent determinations of the rate constants for the reactions between tyrosine and the carboxylate anions. The tyrosine dipole moment was needed in order to obtain the collision (ADO) rate constants<sup>9</sup> and determine the pressure differential; it was obtained by carrying out B3LYP/aug-cc-pVDZ calculations and assuming a Maxwell–Boltzmann distribution based on the enthalpies of the nine most stable conformers that were located. The resulting dipole moment was 5.28 D, but the equilibrium determination is insensitive to this value, and dipole moments spanning the range 5.0–6.0 D altered the acidity by no more than 0.1 kcal mol<sup>-1</sup>.

**Hydrogen–Deuterium Exchange.** A 200  $\mu$ M solution of tyrosine dissolved in a 3:1 (v/v) mixture of methanol and water with enough lithium hydroxide added to make the solution slightly basic was injected at a flow rate of 10  $\mu$ L min<sup>-1</sup> into a Micromass Z-spray electrospray ionization (ESI) source. The resulting ions were accumulated in a hexapole to improve the signal-to-noise ratio and subsequently were transported into the FTMS cell via a radio frequency (rf)-only quadrupole ion guide. They were then cooled with a pulse of argon and stored in the reaction region by the dynamically assisted gated trapping technique.<sup>10</sup> The deprotonated  $[M - H]^-$  ion of tyrosine was isolated using an arbitrary waveform excitation, and its H/D exchange behavior was monitored using EtOD (99.5+ atom % D), CF<sub>3</sub>CH<sub>2</sub>OD (99 atom % D), or C<sub>6</sub>H<sub>5</sub>CH<sub>2</sub>OD (96+ atom % D). The deuterated reagent was leaked into the vacuum system to pressures of  $1\text{--}5 \times 10^{-7}$  Torr for at least 5 h before the behavior of the tyrosine  $[M - H]^-$  ion was probed. This precaution resulted in effective deuterium contents of  $\geq 95\%$  for EtOD and CF<sub>3</sub>CH<sub>2</sub>OD and  $\geq 90\%$  for C<sub>6</sub>H<sub>5</sub>CH<sub>2</sub>OD,

as determined by examining the H/D exchange of isophthalate [*m*-C<sub>6</sub>H<sub>4</sub>(CO<sub>2</sub>H)CO<sub>2</sub><sup>-</sup>]. This is a suitable reaction for this purpose, since it undergoes exchange at the collision-controlled limit with all three deuterated reagents used in this work and only one deuterium is incorporated into the product in each case. The final  $d_1/d_0$  ratio consequently gives the effective deuterium content of the exchange reagent in the reaction region.<sup>11</sup>

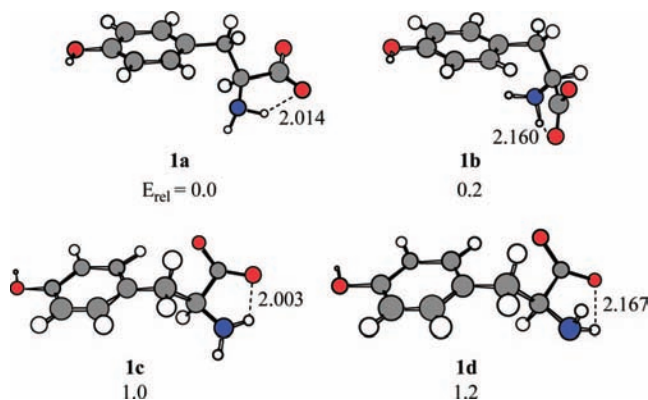
Pseudo-first-order bimolecular H/D exchange reaction rate constants were determined from the decay of the reactant ( $d_0$ ) ion and the amount of alcohol in the reaction region without correcting for the small amount of residual protic material. Excellent linear fits of the data were obtained in each case ( $r^2 \geq 0.998$ ). Apparent rate constants also were obtained by using Rate Calculator, a kinetics program developed by He and Marshall.<sup>12</sup> In the latter case, the deuterium content of the reagent was taken into account in the rate determination.

**Computational Methods.** The Becke three-parameter hybrid exchange plus Lee–Yang–Parr correlation density functional (i.e., B3LYP) was used along with Dunning's augmented correlation-consistent double- $\zeta$  basis set (i.e., aug-cc-pVDZ) in carrying out geometry optimizations on tyrosine, its conjugate bases, and the corresponding radicals.<sup>13,14</sup> These computations were performed on workstations at the Minnesota Supercomputer Institute using Gaussian 03.<sup>15</sup> Both phenoxide and carboxylate ions as well as radicals were examined, and a variety of conformations were probed in the following way: Monte Carlo calculations using the MMFF force field were run on a Macintosh PowerPC G4 computer using Spartan 04, and the 10 most stable structures for tyrosine and both of its  $[M - H]^-$  ions and radicals were reoptimized using density functional theory (DFT).<sup>16</sup> Vibrational frequencies for the optimized structures also were computed, and unscaled values were used to obtain the zero-point energies (ZPEs) and thermal corrections to 298 K. Low-frequency modes that contribute more than  $RT/2$  to the temperature adjustment to 298 K were replaced in all cases by 0.3 kcal mol<sup>-1</sup>. For select species, G3B3 calculations (a variant of G3 theory) were carried out as well.<sup>17,18</sup> All of the resulting relative energies, acidities, and adiabatic detachment energies (ADEs) are given as enthalpies at 298 K, with the exception of the vertical detachment energies (VDEs), which were computed in the usual way (i.e.,  $E_{\text{radical}} - E_{\text{anion}}$ ).

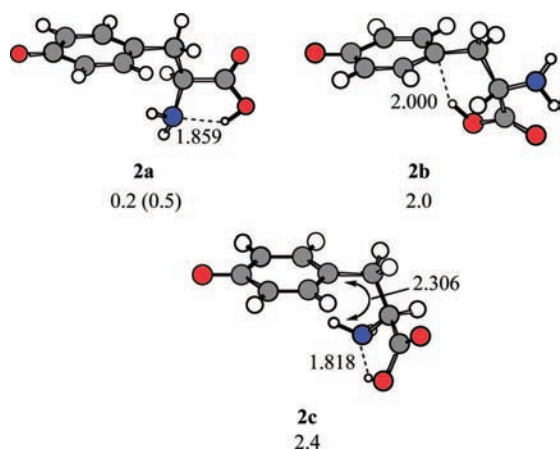
**Photoelectron Spectroscopy.** Photoelectron spectra of the deprotonated  $[M - H]^-$  ions of tyrosine, its methyl ester (TyrCO<sub>2</sub>CH<sub>3</sub>) and methyl ether (TyrOCH<sub>3</sub>), and *p*-hydroxybenzoic acid were obtained with a home-built apparatus that couples an ESI source and a temperature-controlled Paul trap with a magnetic-bottle photoelectron analyzer. Details of this instrument have been published recently,<sup>19</sup> and only a brief account is given here. The anions of interest were produced using ESI from 1 mM solutions, which were prepared by dissolving the corresponding acids in pure CH<sub>3</sub>CN or a 3:1 (v/v) CH<sub>3</sub>OH/H<sub>2</sub>O solution and adding a small amount of base. Anions from the ESI source were guided by a series of rf-only quadrupole devices and then bent 90° into the temperature-controlled Paul trap, where they were accumulated and cooled before being pulsed into the extraction zone of a time-of-flight mass spectrometer. The desired anions were mass-selected and decelerated and then were detached by a laser beam in the interaction zone of a magnetic-bottle photoelectron analyzer.

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**Figure 1.** B3LYP/aug-cc-pVDZ carboxylate structures **1a–d** for deprotonated tyrosine and their energies relative to **1a** (kcal mol<sup>-1</sup>) at 298 K.



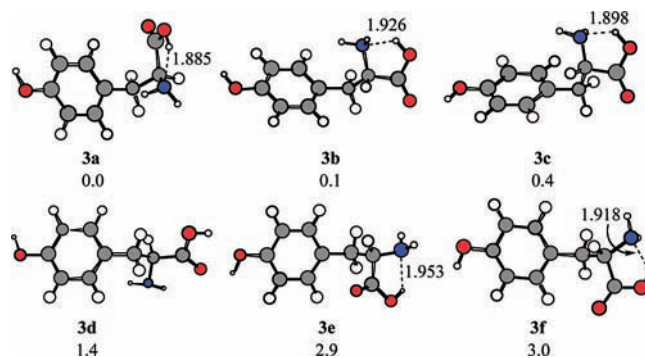
**Figure 2.** B3LYP/aug-cc-pVDZ phenoxide structures **2a–c** for deprotonated tyrosine and their energies relative to **1a** (kcal mol<sup>-1</sup>) at 298 K. The value in parentheses for **2a** is from G3B3 theory.

Two detachment photon energies were used in the current experiments: 193 nm (6.424 eV) from an ArF excimer laser and 266 nm (4.661 eV) from a Nd:YAG laser. Both lasers were operated at a 20 Hz repetition rate with the ion beam off on alternating laser shots for background subtraction. Photoelectrons were collected at nearly 100% efficiency by the magnetic bottle and analyzed in a 5.2 m long electron flight tube. Time-of-flight photoelectron spectra were collected and converted to kinetic energy spectra calibrated with the known spectra of I<sup>-</sup> and ClO<sub>2</sub><sup>-</sup>. The electron binding energy spectra reported were obtained by subtracting the kinetic energy spectra from the respective detachment photon energies. The energy resolution ( $\Delta E/E$ ) of the magnetic-bottle electron analyzer was  $\sim 2\%$  (i.e.,  $\sim 20$  meV for 1 eV electrons).

## Results and Discussion

**Theoretical Calculations.** Geometry optimizations were carried out on tyrosine and its conjugate bases. Both carboxylate and phenoxide ions were explored, and all three structures were subjected to Monte Carlo searches to try to locate the lowest-energy conformations. The 10 most stable structures obtained for each species were reoptimized at the B3LYP/aug-cc-pVDZ level, and their geometries and energies are given in the Supporting Information. The most stable deprotonated carboxylate (**1**) and phenoxide (**2**) anions, as well as several conformations of tyrosine (**3**), are illustrated in Figures 1, 2, and 3, respectively, but only the lowest-energy O–H rotamers are shown for **1** and **3**.

Each of the carboxylates has a CO<sub>2</sub><sup>-</sup>⋯H<sub>2</sub>N hydrogen bond, but there are no direct interactions with the hydroxyl group



**Figure 3.** B3LYP/aug-cc-pVDZ structures **3a–f** for tyrosine and their energies relative to **3a** (kcal mol<sup>-1</sup>) at 298 K.

because of the rigidity of the aromatic ring and the short tether in tyrosine. The same situation applies to the most stable phenoxide conformation and neutral tyrosine (i.e., there are CO<sub>2</sub>H⋯NH<sub>2</sub> interactions), but the former species does have slightly higher energy conformations with CO<sub>2</sub>H⋯C<sub>ipso</sub> (**2b**) and NH⋯C<sub>ipso</sub> (**2c**) hydrogen bonds. These arise because the excess charge on the phenoxide ring is partially delocalized to the para (ipso) carbon of the aromatic ring. Previous computations carried out by Bleiholder et al.<sup>20</sup> and Jones et al.<sup>21</sup> on tyrosine and its phenoxide conjugate base are in good accord with our findings, except that we also located two conformations having lower energies than previously reported for the carboxylate ion (i.e., **1a** and **1b** vs **1c**).<sup>22</sup>

The carboxylate **1a** is predicted to be only 0.2 kcal mol<sup>-1</sup> more stable than phenoxide **2a** at the B3LYP/aug-cc-pVDZ level. This difference is not particularly surprising given that acetic acid is only 0.6–1.9 kcal mol<sup>-1</sup> more acidic ( $\Delta G_{\text{acid}}^{\circ}$ ) than phenol in the gas phase,<sup>23</sup> but it is sufficiently small that the relative stabilities easily could be reversed. High-level G3B3 calculations were carried out to address this possibility, but the relative energy ordering was the same and the difference (0.5 kcal mol<sup>-1</sup>) nearly the same. The latter computations lead to a prediction based upon this energy difference that the deprotonated [M – H]<sup>-</sup> ion of tyrosine will consist of a 77% carboxylate/23% phenoxide mixture at equilibrium. An essentially identical 80/20 mixture is predicted if one does a population analysis using the DFT enthalpies and assumes a Maxwell–Boltzmann distribution, whereas this increases to 95% carboxylate/5% phenoxide if one uses free energies derived from the B3LYP entropies.

The computed acidities of tyrosine were obtained by using its lowest-energy neutral conformer (**3a**) and the best carboxylate (**1a**) and phenoxide (**2a**) anion structures. This led to  $\Delta H_{\text{acid}}^{\circ}$  values of 336.7 and 336.9 kcal mol<sup>-1</sup> for the carboxyl and phenolic positions of tyrosine, respectively, at the B3LYP/aug-cc-pVDZ level. If one corrects these values for the errors in

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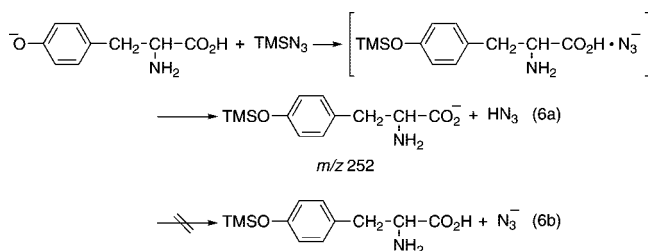
(22) Conformer **1a** was found to be lower in energy than **1c** regardless of whether a 6-31+G(d) or aug-cc-pVDZ basis set was used.

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This precluded us from transferring the  $[M - \text{TMS}]^-$  ion to the second reaction region (cell), but its reactivity could nevertheless be probed in the cell where it was formed. As expected on the basis of the reactivity of terephthalate, only the  $[\text{adduct} - \text{HN}_3]^-$  ion at  $m/z$  252 was observed (eq 6):

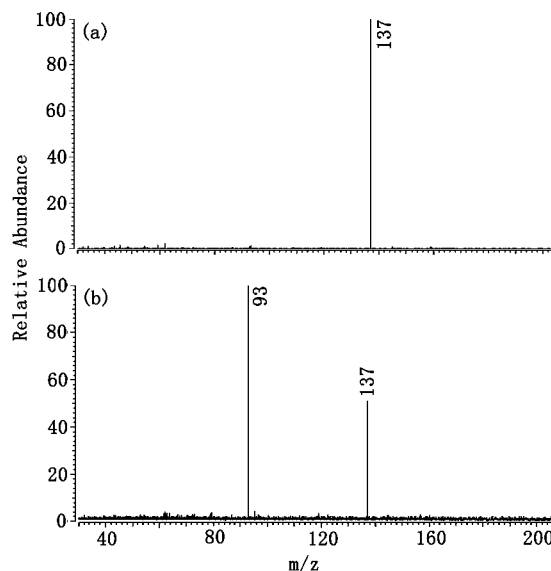


These results indicate that trimethylsilyl azide is a suitable reagent for differentiating the two  $[M - \text{H}]^-$  ions of tyrosine and can be used to determine their relative amounts.

Deprotonation of tyrosine with a  $\text{OH}^-/\text{NH}_2^-$  mixture was carried out under equilibrating conditions where the  $[M - \text{H}]^-$  ion was allowed to interact with neutral tyrosine. It was subsequently transferred to the second reaction region, where only  $\text{TMSN}_3$  was present. Upon their reaction, product ions at  $m/z$  42 and 252 in a 30:70 ratio were produced (Figure 4); the increase in the former ion and the decrease in the latter species at longer times are due to the secondary reaction of the carboxylate ion ( $m/z$  252) with  $\text{TMSN}_3$  to afford  $\text{N}_3^-$  ( $m/z$  42). This result indicates that the equilibrium composition of deprotonated tyrosine is 70% phenoxide and 30% carboxylate, which is in accord with computations indicating that these two species are similar in energy. The direct B3LYP and G3B3 calculations lead to a reversed ion ratio, but the use of isodesmic reactions in which one corrects for the errors in the computed acidities of acetic acid and phenol leads to the observed relative stability order.

Since the phenoxide ion is found to be more stable than the carboxylate anion in the gas phase whereas the order is reversed in solution, an interesting question arises: what will the ion composition be when ESI is employed? To address this situation, tyrosine was sprayed from a 3:1 (v/v)  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  solution, and the  $[M - \text{H}]^-$  ion was reacted with  $\text{TMSN}_3$ . The same 30:70 product ratio as before was observed, indicating that during the electrospray process and the subsequent isolation of the  $[M - \text{H}]^-$  ion, its isomeric structure is altered. In contrast, when tyrosine was sprayed from  $\text{CH}_3\text{CN}$  or  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  mixtures with 99:1 to 50:50 ratios, the  $[M - \text{H}]^-$  ion was found to be almost entirely ( $\sim 95\%$ ) the carboxylate anion. Small amounts of methanol in the acetonitrile (1–4%), however, led back to the formation of the phenoxide ion. These results indicate that changes in the solvent composition can alter the identity of an anion.<sup>6</sup> This may be due to the formation of different ions at the exit of the ESI source or a subsequent ion/molecule reaction with  $\text{CH}_3\text{OH}$ .

To explore the generality of this finding, *p*-hydroxybenzoic acid was examined, because its conjugate base has been reported to be a phenoxide ion in the gas phase and a carboxylate anion in solution.<sup>24</sup> We confirmed this by generating the  $[M - \text{H}]^-$  ion via electron ionization and allowing it to equilibrate with the neutral acid for varying amounts of time before transferring the ions to the second reaction region and fragmenting them by collision-induced dissociation (CID). At short time intervals, an  $[M - \text{CO}_2]^-$  ion due to the fragmentation of the carboxylate ion was observed as the most abundant ion in the spectrum. At



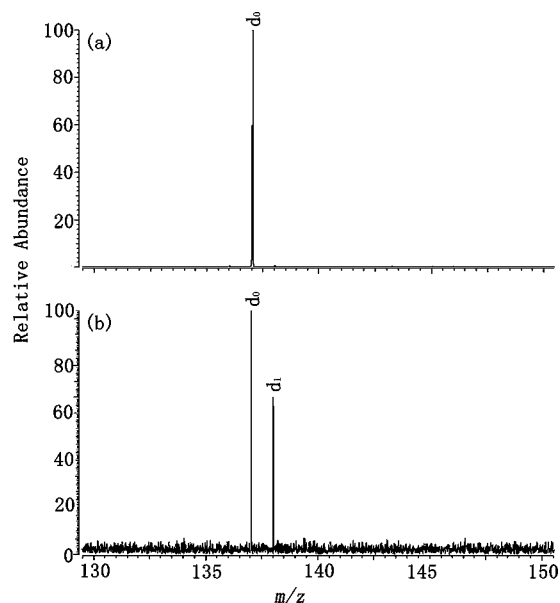
**Figure 5.** On-resonance CID of deprotonated *p*-hydroxybenzoic acid ions sprayed from two different solvent systems: (a) 3:1 (v/v)  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ ; (b)  $\text{CH}_3\text{CN}$ .

longer times, the  $[M - \text{CO}_2]^-$  ion disappeared because an acid-catalyzed isomerization to the more stable phenoxide ion occurred. Computations also indicate that the phenoxide ion is more stable than the carboxylate anion, and at the B3LYP/au-gc-pVDZ and G3B3 levels, the energy differences are 8.2 and 5.5  $\text{kcal mol}^{-1}$ , respectively; the latter method also provides an acidity at the phenolic position that is in excellent accord with experiment (335.5 vs  $335.9 \pm 2.1$   $\text{kcal mol}^{-1}$ , respectively), whereas the B3LYP value is too acidic by 4.1  $\text{kcal mol}^{-1}$ .

ESI of *p*-hydroxybenzoic acid from a 3:1  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  mixture affords an  $[M - \text{H}]^-$  ion that neither fragments upon CID nor undergoes hydrogen–deuterium exchange upon exposure to  $2.9 \times 10^{-7}$  Torr of *tert*-BuOD for 300 s. In contrast, when the ion is generated from  $\text{CH}_3\text{CN}$  under the same experimental conditions, it readily loses  $\text{CO}_2$  upon CID and incorporates 1 deuterium upon reaction with the deuterated reagent (Figures 5 and 6). The latter transformation undoubtedly occurs via a relay mechanism in which the carboxylate is isomerized to the more stable phenoxide (Scheme 1). The reverse process is energetically unfavorable; consequently, the phenoxide ion does not undergo H/D exchange with *tert*-BuOD. These findings are analogous to those for tyrosine in that the gas-phase structure (i.e., the phenoxide ion) is produced from  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  whereas the liquid-phase species (i.e., the carboxylate anion) is formed from  $\text{CH}_3\text{CN}$ .

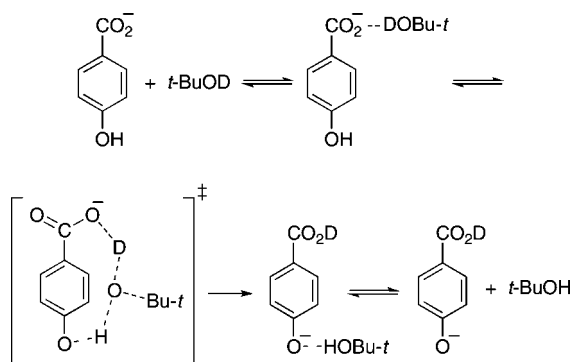
**Photoelectron Spectroscopy.** Tyrosine, its methyl ester ( $\text{TyrCO}_2\text{CH}_3$ ), and its methyl ether ( $\text{TyrOCH}_3$ ) were sprayed from 3:1  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  solutions, and the photoelectron spectra of the  $[M - \text{H}]^-$  ions were obtained, as shown in Figure 7. The two methylated control compounds contain only one deprotonation site and exclusively produce phenoxide ions (Figure 7b) and carboxylate ions (Figure 7c), respectively. The adiabatic electron detachment energy (ADE) of the phenoxide ( $2.40 \pm 0.10$  eV) is quite different from that of the carboxylate ( $3.55 \pm 0.10$  eV), in accord with previously reported values for  $\text{C}_6\text{H}_5\text{O}^-$  ( $2.253 \pm 0.006$  eV)<sup>25</sup> and  $\text{C}_6\text{H}_5\text{CO}_2^-$  ( $3.59 \pm 0.05$

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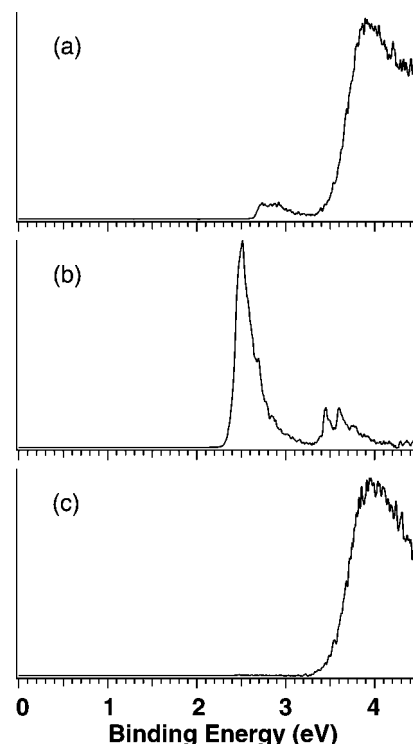


**Figure 6.** Comparison of H/D exchange with *tert*-BuOD for 300 s at  $2.9 \times 10^{-7}$  Torr of deprotonated *p*-hydroxybenzoic acid ions sprayed from two different solvent systems: (a) 3:1 (v/v)  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ ; (b)  $\text{CH}_3\text{CN}$ .

**Scheme 1.** Proposed H/D Exchange Mechanism for Deprotonated *p*-Hydroxybenzoic Acid



eV).<sup>26</sup> The band shapes also are distinct in that the former is narrower than the latter. These observations enabled us to assign the weak, low-energy feature in the tyrosine spectrum to the phenoxide ion and the more intense, higher-energy band to the carboxylate anion (Figure 7a). The computational results are in good accord with these assignments (Table 1), and the observation of both isomers (**1** and **2**) is consistent with the reactivity studies. While it is difficult to determine the relative amounts of the two ions by PES, it appears that there is considerably more of the carboxylate ion on the basis of the spectrum in Figure 7a. The spectrum did not change noticeably when  $\text{CH}_3\text{CN}$  was used as the solvent, which contrasts with the findings noted above. We attribute this difference to the different ESI sources used in the two experiments. In the PES experiment, a home-built ESI source with an ion guide was used,<sup>19</sup> whereas a Micromass Z-Spray source outfitted with a hexapole trap was employed in the FTMS studies. These differences result in significantly shorter ion trapping times in the home-built device (i.e., 100 ms vs 1–5 s). As a result, it tends to form solution-phase structures, in contrast to the Z-Spray source.



**Figure 7.** Photoelectron spectra at 266 nm for deprotonated  $[\text{M} - \text{H}]^-$  ions from (a) tyrosine (Tyr), (b)  $\text{TyrCO}_2\text{CH}_3$ , and (c)  $\text{TyrOCH}_3$ .

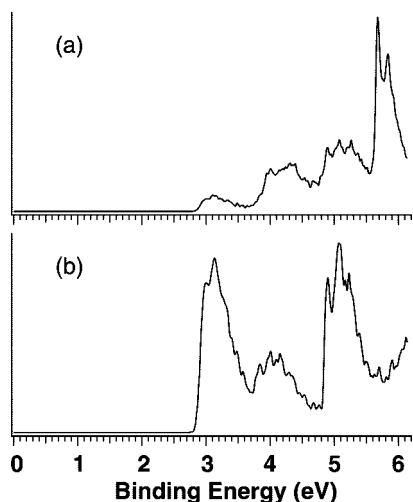
**Table 1.** Calculated (B3LYP/aug-cc-pVDZ and G3B3) and Experimental Adiabatic and Vertical Electron Detachment Energies of Deprotonated Tyrosine (**1a** and **2a**), Benzoate Ion, Phenoxide Ion, and the Conjugate Bases of *p*-Hydroxybenzoic Acid at 298 K<sup>a</sup>

compound	computations		experiment	
	ADE	VDE	ADE	VDE
$\text{TyrCO}_2^-$ ( <b>1a</b> )	— <sup>b</sup>	3.65	$3.55 \pm 0.10$	$3.90 \pm 0.10$
$\text{TyrO}^-$ ( <b>2a</b> )	2.64	2.72	$2.70 \pm 0.05$	$2.74 \pm 0.10$
$\text{C}_6\text{H}_5\text{CO}_2^-$	3.42 (3.63) <sup>c</sup>	3.79	$3.59 \pm 0.05$	$3.95 \pm 0.10$
$\text{C}_6\text{H}_5\text{O}^-$	2.21 (2.34) <sup>c</sup>	2.25	$2.253 \pm 0.006$	—
$\text{HOC}_6\text{H}_4\text{CO}_2^-$	3.36	3.91	$3.75 \pm 0.10$	$4.0 \pm 0.1$
$\text{HO}_2\text{CC}_6\text{H}_4\text{O}^-$	2.97	3.08	$2.85 \pm 0.10$	$3.0 \pm 0.1$

<sup>a</sup> All values are in eV. <sup>b</sup> Carbon dioxide was lost upon optimization of the radical, and a stable carboxyl radical could not be located. <sup>c</sup> G3B3 energies are given in parentheses.

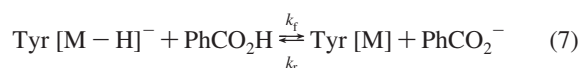
To explore this further, *p*-hydroxybenzoic acid was examined. The photoelectron spectrum of the  $[\text{M} - \text{H}]^-$  ion produced from either a 3:1  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  or  $\text{CH}_3\text{CN}$  solution displays four groups of bands (Figure 8), and the two lowest-energy ones are attributed to the phenoxide and carboxylate anions, respectively. This assignment is based upon analogy to the tyrosine spectra and is in good accord with the computational results (Table 1). The relative proportions of the phenoxide and carboxylate ions appear to strongly depend on the ESI solutions with the phenoxide ion dominating in the  $\text{CH}_3\text{CN}$  ESI solution, which is not surprising since there is a greater energetic difference between the isomers derived from *p*-hydroxybenzoic acid. B3LYP/aug-cc-pVDZ and G3B3 computations indicate that the phenoxide ion is the more stable one by 8.2 (B3LYP) and 5.5 (G3B3) kcal mol<sup>-1</sup>. It is unclear why the two ESI sources behave differently and why tyrosine and *p*-hydroxybenzoic acid do not seem to respond the same way with regard to the solvent from which they are sprayed in the PES instrument, but these observations are worthy of future exploration.

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**Figure 8.** Photoelectron spectra at 193 nm for deprotonated *p*-hydroxybenzoic acid  $[M - H]^-$  ions sprayed from (a) 3:1 (v/v)  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  and (b)  $\text{CH}_3\text{CN}$  solutions.

**Equilibrium Acidity.** In 1992, O'Hair et al.<sup>1</sup> used the Cooks kinetic acidity method to determine that  $\Delta H_{\text{acid}}^\circ(\text{tyrosine}) = 336.4 \pm 3.1 \text{ kcal mol}^{-1}$ . More recently, Poutsma and co-workers<sup>21</sup> remeasured this quantity using the extended kinetic method, which includes entropy effects, and obtained a value of  $337.7 \pm 2.6 \text{ kcal mol}^{-1}$ . These two results are in good accord with each other and computational predictions for this value, but the formation of a mixture of phenoxide and carboxylate ions could lead to systematic errors. There have also been few amino acid equilibrium determinations in the gas phase, and only one involving a polar side chain.<sup>3</sup> Consequently, the equilibrium constants for the acid–base reactions of tyrosine with benzoic acid and fluoroacetic acid were determined. Since  $K = k_f/k_r$ , this was accomplished by measuring the forward and reverse rate constants for the proton-transfer reactions, as illustrated in eq 7 for the former case:



Three independent measurements for each quantity afforded  $k_f = (4.01 \pm 0.14) \times 10^{-10} [(6.55 \pm 0.16) \times 10^{-10}] \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ ,  $k_r = (3.93 \pm 0.27) \times 10^{-10} [(4.26 \pm 0.42) \times 10^{-10}] \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ , and  $K = 1.02 \pm 0.08 [1.54 \pm 0.15]$ , where in each case the first value is for the benzoic acid determination and the following one in brackets is for the fluoroacetic acid measurement. These results lead to  $\Delta\Delta G_{\text{acid}}^\circ = 0.0 \pm 0.6$  and  $-0.3 \pm 0.6 \text{ kcal mol}^{-1}$ , respectively, if one adopts a conservative uncertainty of  $\pm 100\%$  for  $K$  in the data analysis. By combining these values with  $\Delta G_{\text{acid}}^\circ(\text{PhCO}_2\text{H}) = 333.0 \pm 2.0 \text{ kcal mol}^{-1}$  and  $\Delta G_{\text{acid}}^\circ(\text{FCH}_2\text{CO}_2\text{H}) = 331.6 \pm 2.0 \text{ kcal mol}^{-1}$ ,<sup>1</sup> one obtains  $\Delta G_{\text{acid}}^\circ(\text{tyrosine}) = 333.0 \pm 2.1$  and  $331.9 \pm 2.1 \text{ kcal mol}^{-1}$ , respectively. These two independent determinations of  $\Delta G_{\text{acid}}^\circ(\text{tyrosine})$  were averaged, yielding a value of  $332.5 \pm 1.5 \text{ kcal mol}^{-1}$  for this quantity.

To obtain  $\Delta H_{\text{acid}}^\circ(\text{tyrosine})$ , the entropies for tyrosine and its conjugate base are needed. The latter quantities (109.8 and 111.1 e.u., respectively) were computed by using unscaled B3LYP/aug-cc-pVDZ vibrational frequencies and weighting the entropies of all of the low-energy conformers that were located by assuming Maxwell–Boltzmann energy distributions. This leads to  $\Delta S_{\text{acid}}^\circ(\text{tyrosine}) = 27.3 \text{ cal mol}^{-1} \text{ K}^{-1}$ , and adopting an uncertainty of  $\pm 2$  e.u. then yields  $\Delta H_{\text{acid}}^\circ(\text{tyrosine}) = 340.7 \pm$

**Table 2.** Apparent Rate Constants for the H/D Exchange Reactions of the Tyrosine  $[M - H]^-$  Ion with Deuterated Alcohols

ROD	$\Delta H_{\text{acid}}^\circ$ <sup>a</sup>	$k \times 10^{13} (\text{cm}^3 \text{ molecule}^{-1} \text{ s}^{-1})$ <sup>b</sup>		
		$d_0 \rightarrow d_1$	$d_1 \rightarrow d_2$ <sup>c</sup>	$d_2 \rightarrow d_3$
$\text{CH}_3\text{CH}_2\text{OD}$	$378.3 \pm 1.0$	$3.3 \pm 0.1$	$2.2 \pm 0.7 (3.3 \pm 1.0)$	<sup>d</sup>
$\text{C}_6\text{H}_5\text{CH}_2\text{OD}$	$370.0 \pm 2.1$	$58 \pm 17$	$36 \pm 11 (54 \pm 16)$	$14 \pm 4$
$\text{CF}_3\text{CH}_2\text{OD}$	$361.7 \pm 2.2$	$930 \pm 280$	$570 \pm 170 (860 \pm 260)$	$69 \pm 21$

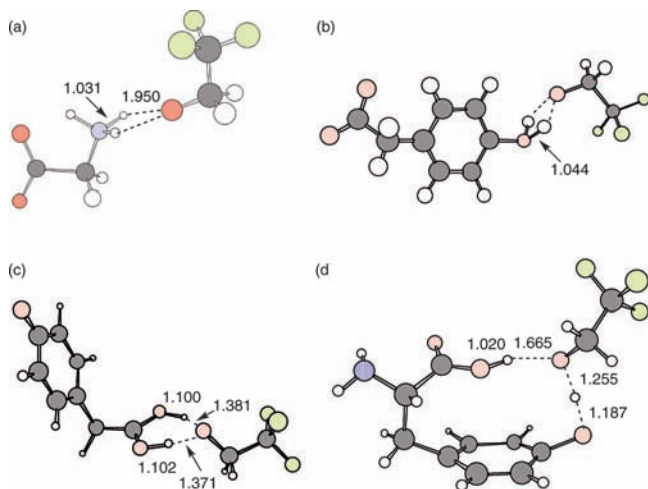
<sup>a</sup> All values are for the protic acids and come from ref 1. <sup>b</sup> The uncertainties are assumed to be  $\pm 30\%$ . <sup>c</sup> Values in parentheses are statistically corrected for the two kinetically equivalent amine hydrogens; that is, the value for  $k(d_1 \rightarrow d_2)$  was multiplied by 1.5. <sup>d</sup> This reaction was too slow for its rate to be accurately measured.

$1.6 \text{ kcal mol}^{-1}$ . This value is a phenomenological acidity because it represents a composite of the two acidic sites in tyrosine. The individual acidities of the carboxylic and phenolic positions could not be reliably determined, but a 70:30 phenoxide/carboxylate equilibrium mixture corresponds to a free-energy difference of only  $0.5 \text{ kcal mol}^{-1}$  and an enthalpy difference of  $1.0 \text{ kcal mol}^{-1}$  in this case. As a result, the acidities for the two different sites must be very similar and are within the reported uncertainty of the composite value. Our experimental determination also is in good accord with computed B3LYP/aug-cc-pVDZ and G3B3 predictions based upon isodesmic reactions. That is, when the calculations are corrected for the errors in the computed acidities of benzoic acid and phenol, values of  $338.3$  (B3LYP) and  $339.0$  (G3B3)  $\text{kcal mol}^{-1}$  for the phenolic position and  $339.5$  (B3LYP) and  $339.9$  (G3B3)  $\text{kcal mol}^{-1}$  for the carboxylic site are obtained. Our equilibrium determination also agrees with the earlier kinetic method measurements within the combined experimental uncertainties but suggests that the latter results are too small by  $3\text{--}4 \text{ kcal mol}^{-1}$ .

**Hydrogen–Deuterium Exchange.** Upon exposure to ethanol-OD, benzyl alcohol-OD, and 2,2,2-trifluoroethanol-OD, the  $[M - H]^-$  ion of tyrosine undergoes three H/D exchanges with apparent rate constants spanning the range from  $10^{-10}$  to  $10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$  (Table 2). If these rate constants are statistically corrected to account for the two equivalent amino hydrogens, then the first two rate constants are found to be the same within experimental error whereas the third is smaller. This indicates that the amino hydrogens undergo H/D exchange more rapidly than the hydroxyl hydrogen on the OH or  $\text{CO}_2\text{H}$  group and that two processes are involved in the incorporation of the three deuteriums. The first of these accounts for the reactivity of the  $\alpha$ -amino group in tyrosine and presumably occurs via the same pathway by which glycine undergoes exchange. Molecular modeling indicates that this is a four-centered flip-flop process that occurs via a zwitterionic transition state (Figure 9a); there is no energy minimum corresponding to the zwitterion on the potential surface. Several processes for the exchange of the remaining hydrogen were considered. First, a four-centered flip-flop pathway involving the phenol was computed (Figure 9b).<sup>27</sup> Second, a six-centered flip-flop mechanism involving the carboxyl group was calculated (Figure 9c). Finally, a relay process interchanging the phenoxide and carboxylate anion centers was modeled (Figure 9d).<sup>28</sup> Of these possibilities, the first has a prohibitively large barrier of  $28.4 \text{ kcal mol}^{-1}$  relative to the separated reactants; the second is

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**Figure 9.** B3LYP/aug-cc-pVDZ-modeled H/D exchange pathways. (a) Four-centered flip-flop transition state (TS) between deprotonated glycine and 2,2,2-trifluoroethanol (TFE); the TS is zwitterionic. (b) Four-centered flip-flop TS between *p*-hydroxyphenyl acetate ( $\text{HOC}_6\text{H}_4\text{CH}_2\text{CO}_2^-$ ) and TFE; the TS is zwitterionic. (c) Six-centered flip-flop TS between *p*-carboxymethylphenoxide ( $-\text{OC}_6\text{H}_4\text{CH}_2\text{CO}_2\text{H}$ ) and TFE; the TS is zwitterionic. (d) Relay TS between deprotonated tyrosine and TFE.

feasible and cannot be ruled out because it has a barrier of  $-5.9$  kcal mol $^{-1}$ , but the third pathway is the most likely exchange route since it has a computed barrier of  $-12.8$  kcal mol $^{-1}$ . Consequently, we envision that the exchange of the carboxyl hydrogen involves the isomerization of the phenoxide to the carboxylate ion by a relay process that is mediated by the exchange reagent. A second molecule of the deuterated alcohol could isomerize the carboxylate anion back to the phenoxide ion via the same relay pathway, but the resulting product would then contain the deuterium at the carboxyl position.

## Conclusions

Deprotonated tyrosine was found to consist of a 70:30 mixture of phenoxide and carboxylate anions at equilibrium. This was determined using trimethylsilyl azide as a chemical probe for the composition of the deprotonated tyrosine  $[\text{M} - \text{H}]^-$  ion and is consistent with photoelectron spectra that clearly reveal the presence of both anions. These results are also in accord with B3LYP/aug-cc-pVDZ and G3B3 calculations based upon isodesmic reactions, which indicate that the phenoxide ion is more stable than the carboxylate ion by 1.2 and 0.9 kcal mol $^{-1}$ , respectively.

Equilibrium acidity measurements were carried out, and this work represents only the second such determination for an amino acid with a polar side chain. Our values for tyrosine ( $\Delta G_{\text{acid}}^\circ = 332.5 \pm 1.5$  kcal mol $^{-1}$  and  $\Delta H_{\text{acid}}^\circ = 340.7 \pm 1.6$  kcal mol $^{-1}$ ) are in good accord with the results of computations and kinetic acidity measurements, but the latter results are 3–4 kcal mol $^{-1}$  smaller than the two equilibrium acidity determinations carried out to date. The adiabatic electron detachment energy for the tyrosine  $[\text{M} - \text{H}]^-$  phenoxide ion is  $2.70 \pm 0.05$  eV, which is  $10.3 \pm 1.2$  kcal mol $^{-1}$  larger than that for the bare  $\text{C}_6\text{H}_5\text{O}^-$  phenoxide. This is not surprising given that the difference in

the proton affinities is  $7.6 \pm 1.7$  kcal mol $^{-1}$  and that the carboxyl group in tyrosine stabilizes the phenoxide anion center. Similarly, the ADE of *p*- $\text{HO}_2\text{CC}_6\text{H}_4\text{O}^-$  is  $2.85 \pm 0.10$  eV, which is  $13.7 \pm 2.3$  kcal mol $^{-1}$  larger than that for  $\text{C}_6\text{H}_5\text{O}^-$ , in accord with  $\Delta\text{PA} = 12.4 \pm 2.3$  kcal mol $^{-1}$  computed at the G3B3 level; the calculated proton affinities (PAs) are 349.5 and 335.5 kcal mol $^{-1}$  for  $\text{C}_6\text{H}_5\text{O}^-$  and *p*- $\text{HO}_2\text{CC}_6\text{H}_4\text{O}^-$ , respectively, both of which are in excellent agreement with the corresponding measured values of  $348.3 \pm 0.7$  and  $335.9 \pm 2.1$  kcal mol $^{-1}$ . The latter differences are greater than those for tyrosine, but this is expected because the two functional groups are directly conjugated to each other in *p*-hydroxybenzoic acid.

Hydrogen–deuterium exchange experiments were also carried out on the tyrosine  $[\text{M} - \text{H}]^-$  ion. Three deuterium atoms were incorporated into the anion, but the amino hydrogens were replaced more rapidly than the carboxyl hydrogen. Two different pathways must be involved, and we suggest that the  $\text{NH}_2$  group reacts via a four-centered flip-flop process involving a zwitterionic transition structure. The carboxyl group most likely transfers a proton to the deuterated alcohol as it simultaneously transfers a deuteron to the phenoxide anion center. An isomerized ion containing one deuterium results from this relay pathway, and this does not change if a subsequent isomerization back to the phenoxide ion takes place in the presence of the deuterated reagent. The location of the label, however, would then be at the carboxyl position.

Finally, it is interesting to note that deprotonated tyrosine is a carboxylate anion in aqueous solution but primarily exists as a phenoxide in the gas phase. These two isomeric species can be differentiated, and the identity of the  $[\text{M} - \text{H}]^-$  ion generated by electrospray was identified. It was found that the resulting anion has the gas-phase composition rather than the solution structure when the sample is sprayed from  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  using a commercial source but that the liquid-phase ion dominates when  $\text{CH}_3\text{CN}$  is used as the solvent. Similar results were found for *p*-hydroxybenzoic acid, while a home-built ESI source gave different ion compositions. This may be due to the formation of different isomeric ratios at the exits of the ESI sources and/or subsequent ion/molecule reactions. In any case, it is clear that the isomeric identity of an anion can be altered by the experimental conditions.

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**Supporting Information Available:** Computed B3LYP/aug-cc-pVDZ geometries (as xyz coordinates) and energies for the computed structures and complete ref 15. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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