Effects of Matrix Structure/Acidity on Ion Formation in Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

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Abstract: The involvement of ground and excited state proton transfer reactions in matrix-assisted laser desorption ionization (MALDI) of bradykinin and bovine insulin is examined using a series of *p*-substituted aniline compounds as matrices. Semiempirical calculations of ground and excited state acidity of the *p*-substituted aniline and anilinium ions are presented. A linear correlation between log (analyte $[A + H]^+$ ion yield) and matrix acidity is obtained. The behavior of the seven *p*-substituted anilines is discussed in terms of the relationship between matrix compound structure, reactivity, and ability to act as a MALDI matrix.

Introduction

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry has rapidly developed as an important technique for the analysis of peptides and proteins.[1,2] With the introduction of new matrices[3] and a better understanding of the role of sample preparation (*e.g.*, the role of sample morphology[4,5] and solvent effects[6]), substantial progress has also been made in the use of MALDI for the analysis of other important classes of biological molecules.[7−9] Although general criteria for selecting good MALDI matrices have been presented[10] and energy deposition and desorption dynamics have been extensively studied,[11] a detailed mechanism(s) for MALDI analyte ion formation has not been formulated.

Ehring et al.[12] proposed that MALDI analyte ion formation is initiated by the formation of a matrix radical cation $MH^{+\bullet}$ (positive ion mode) which then reacts with the matrix and/or the analyte by proton transfer. The proposed radical cation mechanism is based on the observed ionization behavior of a large variety of substituted aromatic compounds following laser desorption and ionization at 337 or 266 nm. Those substituted aromatic molecules that worked best as MALDI matrices form both radical matrix $MH^{+\bullet}$ and protonated matrix $[MH + H]^+$ ions. If the matrix $MH^{+\bullet}$ ion initiates analyte ionization, then the compounds that act as MALDI matrices at 337 nm should work better at 266 nm where the two-photon energy (9.32 eV) is more than sufficient to photoionize most substituted aromatic compounds. However, 266 nm excitation of indole-2-carboxylic acid decreases the effectiveness of this compound as a MALDI matrix relative to that observed at 337 nm. The authors decided that the ionic species ultimately responsible for the ionization of the analyte must be the matrix $[MH + H]^+$ ion and not the matrix $MH^{+\bullet}$.

In an earlier paper,[13] we rationalized the utility of several substituted phenol (*e.g.*, 3,5-dimethoxy-4-hydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid) and nitrogen bases (*e.g.*, 4-nitroaniline and 2,4-dinitroaniline,[13] thymine,[14] pyrimidines, pyridines, and basic benzene derivatives[15]) as MALDI matrices on the basis of the increased acidity of the low-lying electronically excited states.[16] That is, absorption of a photon by the matrix produces a strongly acidic species that can transfer a proton to the analyte yielding the observed protonated analyte $[A + H]^+$ ion. On the basis of our hypothesis that excited states are involved in the ionization mechanism of MALDI, we showed that the reactivity of a matrix compound could be influenced by heavy atom substitution.[17] Heavy atom substituents such as Cl, Br, and I increase the probability for intersystem crossing

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from the excited singlet to the triplet state and thus extend the lifetime of the photoexcited species.[18] Recent studies by Liao and Allison[19] lend additional support to the involvement of excited states in the ionization mechanism of MALDI, and recent work by Wu et al.[20] with the matrix 3-hydroxypicolinic acid also suggest that excited state acidity plays a key role in MALDI. On the basis of these fundamental studies, we would argue that an overriding criteria for selecting a matrix compound is the relative acidity (or basicity for negative ion MALDI) as compared to the analyte basicity (or acidity).

To probe further the chemical processes responsible for the formation of the analyte $[A + H]^+$ ions, we examined the behavior of seven *p*-substituted anilines at 337 nm and 266 nm excitation. We determine the acidity of the ground and first excited singlet states of the *p*-substituted aniline and anilinium ions using semiempirical calculations. The seven *p*-substituted anilines are then discussed in terms of the relationship between matrix compound structure and its ability to act as a MALDI matrix.

Experimental Section

Mass Spectrometry. MALDI experiments were performed using a linear time-of-flight (TOF) mass spectrometer that has been described previously.[17] Either a N_2 laser (337 nm, Laser Photonics LN300) or frequency-quadrupled Nd:YAG laser (266 nm, Spectra Physics Quanta Ray GCR Series) provided irradiation for ionization. Both the N_2 (5 ns pulse width) and Nd:YAG laser (8 ns pulse width) were operated at a repetition rate of 5 Hz. Laser power was controlled with a rotatable variable neutral density filter wheel. The average laser power density was measured using an energy meter (Scientech, Vector D200). Laser spot size was held constant at approximately 150 μm $\times$ 150 μm at each wavelength. Accelerating potentials used for the studies reported herein were +15 kV. Ions were detected using a standard dual microchannel plate detector biased at −2.20 kV. The transient ion signal was collected by a 300 MHz digital oscilloscope (LeCroy 9450A) and transferred to a personal computer for spectrum analysis (GRAMS386 version 1.0, Galactic Corp.) and storage. The solution phase UV absorption spectrum of each *p*-substituted aniline was taken using a Carey spectrophotometer.

Sample Preparation. 4-Nitroaniline, 4-aminophenol, 4-aminotoluidine, 4-aminobenzoic acid, ethyl 4-aminobenzoate, and 4-chloroaniline were purchased from Sigma Chemical Company (St. Louis, MO). 4-Aminobenzonitrile was purchased from Fluka (Switzerland). These compounds were used as MALDI matrices without further purification. Bradykinin (M_r 1059.6 Da) and bovine insulin (M_r 5733 Da) were chosen as test analytes for MALDI analysis. Bradykinin and bovine insulin were purchased from Sigma Chemical Company and used without further purification.

Bradykinin and bovine insulin solutions were prepared at concentrations of 0.93 and 0.18 mM in methanol, respectively. All matrix solutions were prepared in methanol, each at a concentration of 0.11 M. MALDI samples were prepared by codepositing the matrix and analyte solutions on the probe tip and allowing them to crystallize. Matrix-to-analyte molar ratios used in the experiments were 120:1 for bradykinin and 600:1 for bovine insulin. Each MALDI mass spectrum consisted of the average ion signal acquired from 100 laser shots. Seven replicate experimental runs for bradykinin and eight replicate experimental runs for bovine insulin using each of the *p*-substituted aniline compounds as matrix were performed to allow estimation of standard deviation of MALDI analyte $[A + H]^+$ ion yields.

Semiempirical Calculations. The semiempirical calculations were performed using MOPAC version 6.0[21a] running on a MacIntosh Quadra 900. The starting geometries were obtained using the Molecule Editor program.[21b] The calculations were performed by the standard PM3[22] program based on the restricted Hartree−Fock method. Ground state geometries were optimized using analytical gradients (STO-6G basis set), and the search was terminated when the change in energy upon successive interations was less than 0.000 01 kcal/mol. The heats of formation (ΔH_f) of the *p*-substituted aniline compounds and corresponding conjugate bases were calculated for the ground and first excited singlet states. Two forms of these compounds were considered as possible acids: the *p*-substituted aniline neutral (acidic center -NH_2) and the anilinium ion (acidic center -NH_3^+). All calculations were performed with full optimization of all geometrical parameters (bond lengths, bond angles, and dihedral angles) without any symmetry constraint. The conformations with the lowest energy were selected for correlation analysis.

Table 1. Solution Phase Absorption Spectra of *p*-Substituted Aniline Compounds used as MALDI Matrices

	S_1	S_2	absorption coefficient (M^{-1} cm^{-1})	
p-substituent	λ_{max} (nm)	λ_{max} (nm)	266 nm	337nm
-OH	297	241	440	91
-CH_3	285	244	680	36
-Cl	295	247	730	30
-$CO_2C_2H_5$	277	220	1800	55
-COOH	275	225	4700	110
-CN	275	215	3200	150
-NO_2	370	235	1900	7200

Results

The present study compares the MALDI ion yields for seven *p*-substituted anilines (substituents -OH, -CH_3, -Cl, -COOH, -$CO_2C_2H_5$, -CN, and -NO_2). The selection of these particular compounds for these studies is based on the wide variance in electron-releasing and electron-withdrawing properties of the substituents. For example, the -OH group is a strong electron-releasing substituent, whereas the -NO_2 group is a strong electron-withdrawing substituent. These groups affect the acid/base properties of the *p*-substituted anilines by destabilizing/stabilizing the conjugate base (or stabilizing/destabilizing the acid), depending on the relative amount of electron-releasing/electron-withdrawing character of the substituent.

Comparisons of Matrix $[MH + H]^+$ and $MH^{+\bullet}$ Ion Yields at 377 and 266 nm. The solution phase absorbance of the *p*-substituted anilines was determined at 266 and 337 nm. The positions of the first and second excited electronic state maxima and molar absorptivities of each *p*-substituted aniline are shown in Table 1. The thin film absorbance is expected to be shifted to longer wavelengths due to the crystalline structure of the film. Positive ion mass spectra of the seven *p*-substituted anilines were then obtained at 337 and 266 nm. The ratios of $[MH + H]^+/MH^{+\bullet}$ ion abundances are shown in Table 2. Note that the matrix $MH^{+\bullet}$ is more abundant than the $[MH + H]^+$ at 337 nm excitation for all *p*-substituents with the exception of the -NO_2 substituent. When the excitation laser wavelength is changed to 266 nm, the matrix $MH^{+\bullet}$ is more abundant than the $[MH + H]^+$ for all seven *p*-substituted anilines.

The seven *p*-substituted aniline compounds were tested as MALDI matrices using bradykinin and bovine insulin as analytes. Figure 1 contains the 337 nm MALDI mass spectra of bovine insulin obtained using 4-aminophenol and 4-nitroaniline as matrices. The laser energy was measured to be 120 μJ/pulse (100 MW/cm^2) and 60 μJ/pulse (55 MW/cm^2) for 4-aminophenol and 4-nitroaniline, respectively. The compounds

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Table 2. Relative Abundance of $[M + H]^+/MH^{+\bullet}$ Ions Observed in the TOF Mass Spectrum Near Threshold for Desorption and Ionization of the *p*-Substituted Aniline Compounds as a Function of Incident Laser Wavelength

	$[MH + H]^+/MH^{+\bullet}$	
p-substituent	337 nm	266 nm
-OH	0.20 (±0.01)	0.16 (±0.03)
$-CH_3$	0.21 (±0.05)	0.21 (±0.04)
-Cl	0.34 (±0.1)	0.21 (±0.01)
$-CO_2C_2H_5$	0[a]	0.16 (±0.04)
-COOH	0.42 (±0.04)	0.09 (±0.03)
-CN	0[b]	0.07 (±0.04)
$-NO_2$	3.5 (±0.4)	0.4 (±0.1)

[a] $[MH + H]^+$ and $MH^{+\bullet}$ ion signals not observed; only fragments. [b] $[MH + H]^+$ ion signal not observed; $MH^{+\bullet}$ ion signal observed.

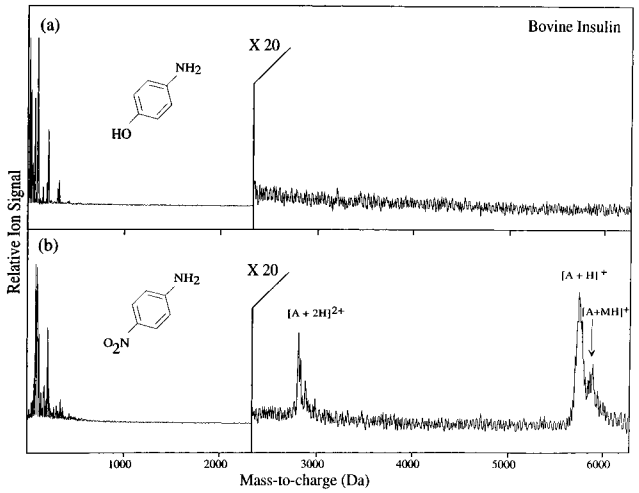

Figure 1. MALDI TOF mass spectra (337 nm) of bovine insulin using the *p*-substituted aniline matrices (a) 4-aminophenol (120 μJ/pulse) and (b) 4-nitroaniline (62 μJ/pulse).

4-aminophenol and 4-nitroaniline represent the two extremes in reactivity observed across the series of *p*-substituted anilines. 4-Aminophenol produced no $[A + H]^+$ ions regardless of laser power (Figure 1a), compared to the large abundance of $[A + H]^+$ ions produced by 4-nitroaniline (Figure 1b) under fairly standard MALDI conditions.[1] In addition to the singly charged bovine insulin $[A + H]^+$ ions observed for 4-nitroaniline, matrix−analyte adduct $[A + MH + H]^+$ ions and the doubly charged $[A + 2H]^{2+}$ ions are present in the mass spectrum. Figure 2 compares the 266 nm MALDI mass spectra of bovine insulin obtained using 4-aminophenol and 4-nitroaniline as matrices. At 266 nm, the laser energy was measured to be 400 μJ/pulse (222 MW/cm^2) and 140 μJ/pulse (78 MW/cm^2) for 4-aminophenol and 4-nitroaniline, respectively, which is roughly a factor of 1.5−2.5 higher than that used at 337 nm. No $[A + H]^+$ ion signal is observed with 4-aminophenol matrix (Figure 2a). 4-Nitroaniline produced weak $[A + H]^+$ ion signal (Figure 2b), compared to the strong $[A + H]^+$ ion signal obtained for 337 nm MALDI. Similar results (not shown) were obtained for MALDI of bradykinin; 4-aminophenol produced weak $[A + H]^+$ ion signal, and 4-nitroaniline produced the most $[A + H]^+$ ion signal at an incident laser wavelength of 337 nm. All seven *p*-substituted anilines produced less bradykinin $[A + H]^+$ ion signal at 266 nm relative to 337 nm.

Analysis of Calculated Gas-Phase Acidities. Semiempirical and *ab initio* calculations have been used previously to determine substituent effects on the ground state acidity of aniline[24a] and anilinium ions.[24b] Tables 3 and 4 list the results of the

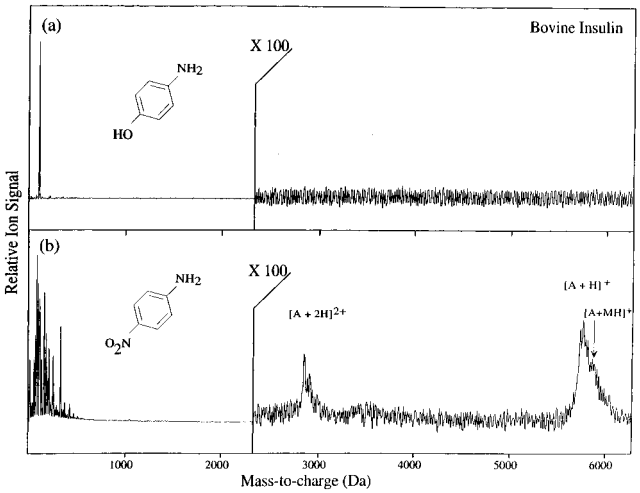

Figure 2. MALDI TOF mass spectra (266 nm) of bovine insulin using the *p*-substituted aniline matrices (a) 4-aminophenol (400 μJ/pulse) and (b) 4-nitroaniline (140 μJ/pulse).

semiempirical molecular orbital theory calculations for the *p*-substituted aniline compounds in the ground and first excited singlet states, respectively. The first column of Table 3 refers to Scheme 1 which contains the *isodesmic*[23] proton transfer reactions involving the *p*-substituted aniline neutrals (reaction 1a) and the *p*-substituted anilinium ions (reaction 1b). First, the ground state heats of formation ΔH_f are calculated for the acid and the corresponding conjugate base of each *p*-substituted aniline species. Then, the energy difference $\delta(\Delta H_f)$ is calculated using the *isodesmic* proton transfer reaction (Scheme 1, reaction 1a or 1b). This $\delta(\Delta H_f)$ gives the calculated effect of the substituent on acidity relative to that of unsubstituted aniline or anilinium ion (aniline was not used as a matrix in this study). Our calculated $\delta(\Delta H_f)$ values agree reasonably well with the STO-3G *ab initio* calculated energy differences $\delta(\Delta E)$[24] and experimentally determined free energy differences $\delta(\Delta G)$[24a] shown in columns 6 and 7. The average deviation of the semiempirical calculated $\delta(\Delta H_f)$ values compared to *ab initio* $\delta(\Delta E)$ values is 14% of the total range of the substituent effects (2.5 to −27.8). Table 4 contains the calculated $\delta(\Delta H^*_f)$ values for the *p*-substituted aniline species in the zeroth vibrational level of their first excited singlet states. Experimental and/or calculated values are unavailable for comparison. However, we can calculate the energy difference of a vertical excitation (*i.e.*, geometries are optimized for the corresponding ground states and used to determine properties of the excited singlets in higher vibrationally states) and compare the results to experimental values derived from the λ_{max} of bands in the corresponding absorption spectra. The solution phase absorption maxima are observed at 280 and 285 nm for aniline[25] and 4-aminotoluidine, respectively. We would expect the λ_{max} to blue shift in the gas phase. In fact, our calculated energy differences corresponding to λ_{max} values of 261 nm for aniline and 264 nm for 4-aminotoluidine (results not shown) are blue shifted.

Table 4 also lists the difference in acidity between the ground states and the excited states of the *p*-substituted aniline species, as formally defined using the Förster cycle[26] (Scheme 2). Förster's excited state acidities of the *p*-substituted aniline species are scaled relative to the unsubstituted aniline and

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Table 3. Computed Ground State Energies (kcal/mol) for *p*-Substituted Aniline Species Using the PM3 Semiempirical Method

reaction	*p*-substituent	$(\Delta H_f)_{acid}$	$(\Delta H_f)_{conj.\ base}$	$\delta(\Delta H_f)^a$	$\delta(\Delta E)^b$	$\delta(\Delta G)^c$
1a (aniline neutral)	-OH	−23.1	−30.6	−0.5		
	$-CH_3$	12.1	4.7	−0.4	1.0	1.2
	-Cl	14.5	1.3	−6.2		
	-H	21.4	14.4	0	0	0
	$-CO_2C_2H_5$	−65.6	−87.2	−14.6		
	-COOH	−69.0	−92.0	−16.0		
	-CN	55.8	32.3	−16.5	−20.2	
	$-NO_2$	10.8	−24.0	−27.8	−27.7	
1b (anilinium ion)	-OH	128.1	−23.1	2.5		
	$-CH_3$	164.2	12.1	1.8	2.4	3.2
	-Cl	169.9	14.5	−1.6		−1.8
	-H	175.2	21.4	0	0	0
	$-CH_2C_2H_5$	92.7	−65.6	−4.5		
	-COOH	91.0	−69.0	−6.2		
	-CN	217.3	55.8	−7.7	−10.4	
	$-NO_2$	179.2	10.8	−14.6	−14.8	

[a] $\delta(\Delta H_f) = (\Delta H_f)_{X\ conj.\ base} - (\Delta H_f)_{X\ acid} - (\Delta H_f)_{H\ conj.\ base} + (\Delta H_f)_{H\ acid}$. [b] STO-3G *ab initio* results for *p*-substituted anilinium ions taken from ref 24. [c] Reference 24a.

Table 4. Computed Excited State Energies (kcal/mol) and Förster's Excited State Acidities for *p*-Substituted Aniline Species Using the PM3 Semiempirical Method

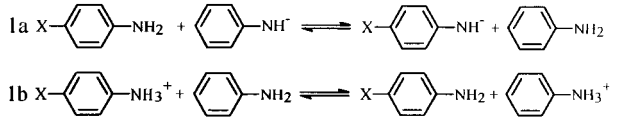
reaction	*p*-substituent	$(\Delta H^*_f)_{acid}$	$(\delta H^*_f)_{conj.\ base}$	$\delta(\Delta H^*_f)^a$	Förster's excited state acidity[b]
1a (aniline neutral)	-OH	69.80	30.56	−10.22	−0.44
	$-CH_3$	97.81	68.40	−0.39	0
	-Cl	101.77	64.58	−8.16	−0.09
	-H	106.37	77.35	0	0
	$-CO_2C_2H_5$	15.96	−13.59	−0.52	0.64
	-COOH	12.19	−18.48	−1.65	0.65
	-CN	137.56	99.53	−9.01	0.34
	$-NO_2$	88.64	59.26	−.36	1.25
1b (anilinium ion)	-OH	229.28	69.80	15.86	0.62
	$-CH_3$	264.04	97.81	9.10	0.34
	-Cl	277.92	101.77	−0.82	0.04
	-H	281.70	106.37	0	0
	$-CO_2C_2H_5$	192.92	15.96	−1.63	0.13
	-COOH	195.55	12.19	−8.03	−0.08
	-CN	322.30	137.56	−9.41	−0.08
	$-NO_2$	295.73	88.64	−31.76	−0.80

[a] Geometries optimized for the S_1 state yielding excited singlets in their ground vibronic states with $\delta(\Delta H^*_f)$ corresponding to a (0−0) transition. [b] Excited state acidity = $[\delta(\Delta H^*_f)_X - \delta(\Delta H_f)_X]/[\delta(\Delta H^*_f)_H - \delta(\Delta H_f)_H] - 1$ (see text for further discussion).

Table 5. Regression Outputs for Semiempirically Calculated Properties of *p*-Substituted Aniline Species versus Hammett σ_p^- Values ($\delta(\Delta H_f)$ or Excited State Acidity = ρ (σ_p^-) + C)

reaction		ρ	C	r^2
1a (aniline neutral)	$\delta(\Delta H_f)$ Förster's	−17.23	−3.28	0.95
	excited state acidity	0.86	−.06	0.86
1b (anilinium ion)	$\delta(\Delta H_f)$ Förster's	−9.74	0.15	0.95
	excited state acidity	−0.61	0.27	0.73

Scheme 1



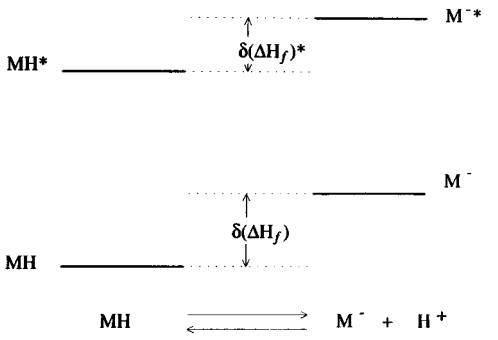
X = -OH, $-CH_3$, -Cl, -COOH, $-CO_2C_2H_5$, -CN, $-NO_2$

determined using eq 1 where $\delta(\Delta H^*_f)_X$ and $\delta(\Delta H_f)_X$ are the

$$\text{Förster's excited state acidity} = [\delta(\Delta H^*_f)_X - \delta(\Delta H_f)_X]/[\delta(\Delta H^*_f)_H - \delta(\Delta H_f)_H] - 1 \quad (1)$$

calculated energy differences for proton transfer from the *p*-substituted aniline species (X = -OH, $-CH_3$, *etc.*) and $\delta(\Delta H^*_f)_H$ and $\delta(\Delta H_f)_H$ are the calculated energy differences for proton transfer from the unsubstituted species. If the *p*-substituted species become less acidic in the excited state than the unsubstituted aniline neutral or anilinium ion, then the calculation of Förster's excited state acidity yields a positive value. If the *p*-substituted species become more acidic in the excited state than the unsubstituted aniline neutral or anilinium ion, the calculated Förster's excited state acidity is negative.

Scheme 2



A further check of the validity of the calculated Förster's excited state acidities is obtained by plotting these values against the Hammett[27−30] substituent σ_p^- values. The σ_p^- values are −0.37, −0.15, 0.19, 0.68, 0.73, 0.89, and 1.27[29] for *p*-

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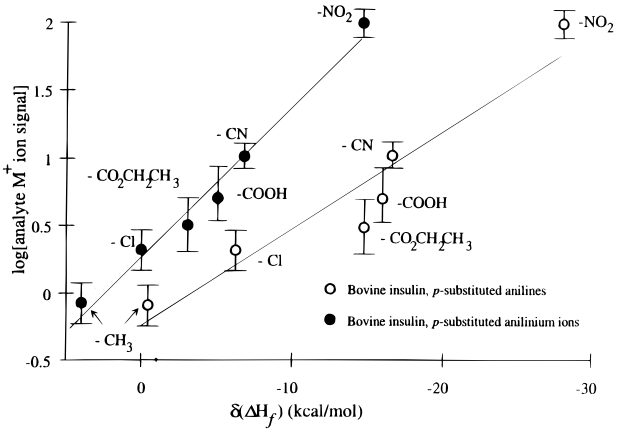

Figure 3. MALDI bovine insulin $[A + H]^+$ ion yield plotted as a function of the calculated effect of the *p*-substituents on the ground state acidity of aniline and anilinium ions. The correlation coefficient for $[A + H]^+$ ion yield as a function of *p*-substituted aniline acidities is 0.91 and as a function of *p*-substituted anilinium ion acidities is 0.98. The stand deviation of the $[A + H]^+$ ion signal is given by the error bars.

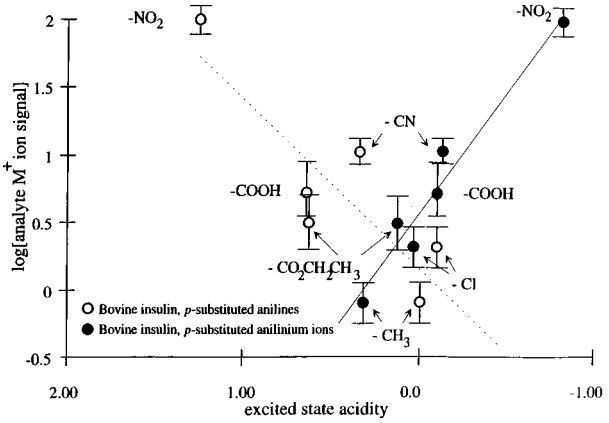

Figure 4. MALDI bovine insulin $[A + H]^+$ ion yield plotted as a function of the calculated difference between ground and excited state acidity of the *p*-substituted aniline and anilinium ions. The correlation coefficient for $[A + H]^+$ ion yield as a function of *p*-substituted aniline excited state acidities is 0.73 and as a function of *p*-substituted anilinium ion acidities is 0.94. The standard deviation of the $[A + H]^+$ ion signal is given by the error bars.

substituents -OH, $-CH_3$, -Cl, $-CO_2C_2H_5$, -COOH, -CN, and $-NO_2$, respectively. These "exalted" substituent values were originally determined for proton transfer reactions using the *p*-substituted aniline series and take into account the enhanced interaction between the *p*-substituent and the $-NH_2$[28] (or $-NH_3^+$[31]) reaction center. The $\delta(\Delta H_f)$ values calculated from ground state properties for both Scheme 1 reactions 1a and 1b have satisfactory correlations with experimentally determined Hammett σ_p^- values ($r^2 = 0.95$ in both cases). The correlation of Förster's excited state acidities with σ_p^- values is found to be significantly smaller ($r^2 = 0.86$ and 0.73, respectively) for excited state reactions 1a and 1b. The fact that the Förster's excited state acidities have lower correlations is not surprising; it is possible that the *p*-substituents affect the excited state reaction chemistry in a manner that is different than the ground state reaction chemistry.

Correlation of MALDI Analyte $[A + H]^+$ Ion Yield and Calculated Gas Phase Acidities. The 337 nm MALDI analyte $[A + H]^+$ ion yield was measured for bradykinin and bovine insulin using each *p*-substituted aniline compound as matrix. Figures 3 and 4 show plots of the MALDI $[A + H]^+$ ion yields for bovine insulin plotted against the calculated ground state acidities and Förster's excited state acidities for the series of *p*-substituted aniline matrices. Note that the bovine insulin $[A + H]^+$ ion signal was not observed when 4-aminophenol was used as matrix. Recall that the more negative the $\delta(\Delta H_f)$ (or $\delta(\Delta H^*_f)$) value, the more acidic the *p*-substituted aniline matrix. Linear regression of the data assuming ground state reactivity yields r^2 values of 0.91 for *p*-substituted aniline species and 0.98 for the *p*-substituted anilinium ion species acting as the acid. Plots of the results of the MALDI ion yields for bradykinin (not shown) yield r^2 values of 0.77 and 0.86, respectively. Linear regression assuming excited electronic state reactivity of the data contained in Figure 4 yields r^2 values of 0.73 and 0.94 for the p-substituted aniline and anilinium species, respectively; the r^2 values for bradykinin data are 0.65 and 0.86 (graphs not shown).

Discussion

Previous fundamental studies of desorption ionization, including MALDI, focus primarily on issues related to energy deposition and desorption dynamics[11] (*i.e.*, the physics of the desorption ionization process). However, few studies actually examine the reaction chemistry that gives rise to the protonated analyte molecule $[A + H]^+$.[12,13,19,32−34] In most desorption ionization experiments, it is difficult to identify the processes that initiate the ion-forming reactions. For example, fast atom bombardment (FAB) ionization involves impacting a kiloelectronvolt energy particle (Ar^0, Xe^0, Cs^+) onto a liquid matrix (typically glycerol, G). As a result of the particle bombardment, material is ejected into the vacuum and clusters of the solvent G_nH^+ are formed as are protonated $[A + H]^+$ ions. It is unclear whether gas phase $[A + H]^+$ ions are produced simply because they exist as such in solution or are the result of gas phase or condensed phase proton transfer reactions.[33] The major objective of this study is to critically examine the role of proton transfer reactions in the MALDI process. Our basic premise is that photon absorption by the matrix initiates two separate events: (i) energy deposition into the solid lattice that ultimately results in the ejection of material into the gas phase[11b] and (ii) formation of reactive matrix species that yield an ionized form of the analyte. Recent studies by Yeung and co-workers[5] and Ehring and Sundqvist[35] show that some of the laser energy deposited into the matrix results in the formation of species that also decay by radiative processes. Although radiative and nonradiative decay processes affect the total yield of ionic species, we cannot measure this parameter in the mass spectrometric experiment. We will focus the remainder of this discussion on processes involving proton transfer.

Plausible mechanisms for ion formation in MALDI are summarized in Scheme 3. Radical cations of the matrix can be formed directly by multiphoton ionization (MPI) (reaction 1). In addition, the absorption of photons by the matrix can yield an excited state matrix species (MH*), which can react with other matrix molecules to form the radical anion $MH^{-\bullet}$, and the radical cation, $MH^{+\bullet}$ (reaction 2).[36] Under conditions where separated radical cations are formed, they could react by

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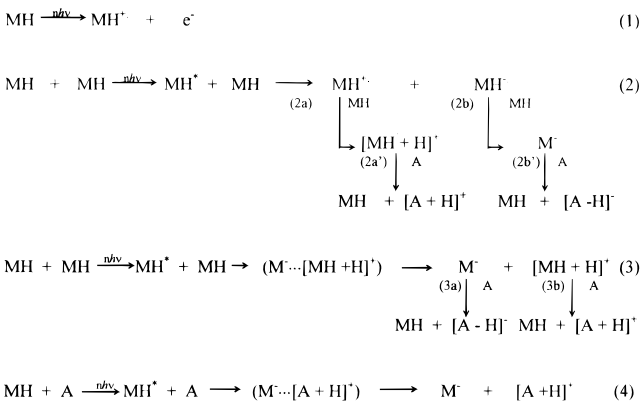
Scheme 3



proton transfer with other matrix molecules to produce $[MH + H]^+$ and M^- ions (reactions 2a and 2b, respectively). Protonation of the analyte A could occur *via* reaction with $MH^{+\bullet}$ and/or $[MH + H]^+$. Note, radical ion−molecule reactions of this type are discussed within the ionization model proposed by Ehring et al.[12] Similarly deprotonation of the analyte can occur by reaction of $MH^{-\bullet}$ and/or M^- with the analyte. Reactions of this type could be responsible for the formation of $[A - H]^-$ ions in negative ion mode MALDI. If the laser fluence is sufficiently high that MPI of the matrix results in formation of fragment ions of the matrix, it is possible that even- and odd-electron fragments of the matrix are formed, and these species may also react with matrix and analyte. Alternatively, if MH* is acidic, it can react with matrix or analyte molecules to donate a proton to form $[MH + H]^+$ or $[A + H]^+$ ions (reactions 3 and 4). Reactions 3 and 4 correspond to excited state proton transfer (ESPT), and formation of $[MH + H]^+$ or $[A + H]^+$ as gas phase ions requires the partitioning of the excess energy of proton transfer into internal energy as well as energy to affect ion pair separation ($M^-\cdots[MH + H]^+$ or $M^-\cdots[A + H]^+$). The energy requirements for ion pair separation of an isolated molecule can be quite high, as much as 15 eV,[37] but this energy is greatly reduced in a "solvated" system[38] as might be found in the crystalline solid or the dense phase as the material is desorbed from the sample surface.

The discussion of our results begins with an examination of the wavelength dependent mass spectra of each of the *p*-substituted anilines. Table 2 shows the effect of 337 and 266 nm excitation on the relative abundance of $[MH + H]^+/MH^{+\bullet}$ ion signals. Note that the radical cation $MH^{+\bullet}$ is more abundant than the $[MH + H]^+$ in the 337 nm mass spectra of all *p*-substituents with the exception of $-NO_2$. The $MH^{+\bullet}$ ion is the dominant ionic species in the 266 nm mass spectra of all of the *p*-substituted anilines. The most significant increases in $MH^{+\bullet}$ relative to $[MH + H]^+$ ions are observed in the 266 nm mass spectra of 4-nitroaniline. The 266 and 337 nm MALDI studies show that matrices that work well at 337 nm (*i.e.*, yield a large abundance of analyte $[A + H]^+$ ions) do not work as well at 266 nm. There does not appear to be a direct correlation with the presence or absence of a particular matrix species. Despite no change in the relative $[MH + H]^+/MH^{+\bullet}$ ion signal, 4-aminotoluidine produces bovine insulin $[A + H]^+$ ion signal when excited at 337 nm but not at 266 nm. On the other hand, the most significant change in $[MH + H]^+/MH^{+\bullet}$ ion signal occurs for 4-nitroaniline. 4-Nitroaniline produces a much weaker bovine insulin $[A + H]^+$ ion signal at 266 nm where there is a corresponding lower $[MH + H]^+/MH^{+\bullet}$ ion signal. We interpret these results as evidence that the ionization mechanism of MALDI is probably influenced by the particular excited state accessed via the excitation photon wavelength. If the proton transfer reaction depended solely on the relative abundance of $MH^{+\bullet}$ ions formed, then the *p*-substituted anilines would generally act as better MALDI matrices at 266 nm where the dominant ion is $MH^{+\bullet}$. The wavelength dependent behavior of these MALDI matrices is not unique to the *p*-substituted anilines examined in this study. For example, we observed that α-cyano-4-hydroxycinnamic acid[39] works better as a MALDI matrix at 337 nm than at 266 nm. As mentioned previously, similar observations were reported for indole-2-carboxylic acid.[12] In terms of understanding MALDI, the studies at 337 and 266 nm underscores the point that the photoinduced proton transfer reaction(s) must be driven at the excitation wavelength. That is, proton transfer must be an available nonradiative decay channel of the photoexcited matrix molecule. Steadman and Syage[40] and Robinson and coworkers[41] have demonstrated the influence of excitation wavelength in determining the photoionization versus ESPT reaction chemistry of aromatic acids, such as phenol,[40] 1-naphthol, and 2-naphthol,[41] in clusters of ammonia and mixtures of ammonia and methanol.

We now turn the discussion to the ionization reactions illustrated in Scheme 3. Reaction 1 clearly takes place as evidenced by the presence of matrix radical cations $MH^{+\bullet}$ for all *p*-substituted aniline compounds studied. However, the extent to which analyte ionization reactions involving the $MH^{+\bullet}$ species (reaction 2) occur cannot be determined by our 266 and 337 nm results. Clearly, simply increasing the relative abundance of the $MH^{+\bullet}$ ion does not result in a corresponding increase in analyte $[A + H]^+$ ion signal.

The $[A + H]^+$ ion formation illustrated in reaction 3 involves proton transfer from the protonated matrix molecule (anilinium ion) which is generated *via* an ESPT from a neutral matrix molecule. Our semiempirical calculations demonstrate that changes in acidity of the *p*-substituted anilinium ions correlate with the Hammett substituent σ_p^- values; that is, electron-releasing substituents are acid-weakening, whereas electron-withdrawing substituents are acid-strengthening. Figures 3 and 4 show a correlation between the relative abundance of the bovine insulin $[A + H]^+$ ion signal and the calculated effect of the *p*-substituents on the aniline matrix acidities. As indicated in Figure 3, *p*-substituents that increase acidity lead to increased analyte $[A + H]^+$ ion formation and *vice versa*. The MALDI bovine insulin $[A + H]^+$ ion signal shows a strong relationship with the calculated ground state acidity of the *p*-substituted anilinium ions ($r^2 = 0.98$). This result is consistent with behavior that would be expected for an ionization mechanism proceeding by Scheme 3, reaction 2 and/or reaction 3, in which the reacting *p*-substituted anilinium ion is in the ground electronic state. We also find that a correlation exists ($r^2 = 0.94$) between the excited state acidities of *p*-substituted anilinium ions and MALDI bovine insulin $[A + H]^+$ ion yield (Figure 4). This correlation suggests that if *p*-substituted

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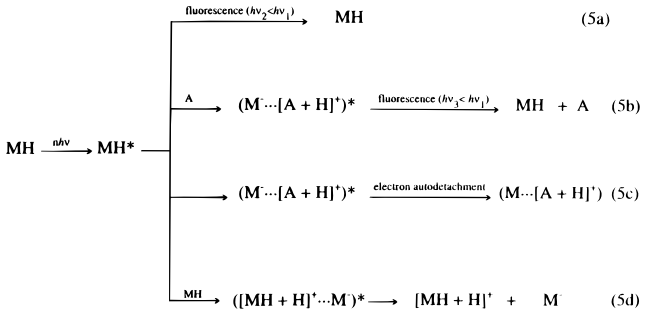
Scheme 4



anilinium ions are present in the desorption, these matrix ions should be considered as possible proton sources.

In Scheme 3 reaction 4, proton transfer to ionize the analyte occurs directly from the photoexcited neutral matrix species. The MALDI analyte ion yields do not correlate ($r^2 = 0.73$ and 0.65 for bovine insulin and bradykinin, respectively) to the calculated Förster's excited state acidities of the *p*-substituted aniline neutrals. The *p*-substituted aniline neutrals appear to become weaker acids in the excited state as opposed to the *p*-substituted anilinium ions which become stronger acids in the excited state. To the best of our knowledge, no studies have been performed to probe the changes in acid/base chemistry for deprotonation of excited state *p*-substituted anilines. The MALDI analyte ion yields do correlate with the calculated ground state acidities of the *p*-substituted aniline neutrals ($r^2 = 0.91$ and 0.77 for bovine insulin and bradykinin, respectively) and suggest that a proton transfer reaction analogous to reaction 4 that does not involve photoexcitation should be added to Scheme 3.

Other routes for deactivation and/or reaction of the photoexcited matrix that have not been considered thus far and could be probed experimentally are illustrated in Scheme 4. The deactivation of the photoexcited matrix neutral (reaction 5a) and/or conjugate base in the ion pair (reaction 5b) by emission of a photon could be determined by monitoring the matrix luminescence. Autodetachment of an electron from the conjugate base of the matrix could also occur if the anion is formed with internal energy above the electron affinity of the anion. In order to evaluate the role of electron autodetachment, electron affinity values for the anilide ions are needed, and we are currently in the process of determining these values. Yet another alternative, as suggested by our semiempirical calculations, is the photoexcited *p*-substituted anilines become weaker acids (*i.e.*, stronger bases) in the excited state. Photoexcitation of the matrix may drive proton abstraction (reaction 5d) from surrounding matrix molecules and/or analytes to form $[MH + H]^+$ ions which can go on to act as proton sources. A preliminary examination of the negative ion mode MALDI mass spectra of these *p*-substituted anilines lends support to proton abstraction occurring between matrix and analyte.[44]

Whether the proton transfer occurs in the solid state, in the dense phase as the material is desorbed from the sample surface, or in the gas phase cannot be determined from these studies. While the excited state $[MH + H]^+$ ion could theoretically donate a proton in the solid state or in the dense phase, the relatively short lifetime (fluorescent radiative lifetime on the order of 1−10 ns) of the excited singlet state may preclude the direct involvement of excited singlet state species in the gas phase production of analyte ions; however, the studies on heavy-atom effects[17] implicate involvement of triplet excited states, and these long-lived species would contribute to a gas phase ionization process. Measurements of the ion initial kinetic energy distributions of MALDI-formed ions suggest that some analyte $[A + H]^+$ ion formation occurs in the gas phase.[42,43] All of the anilinium ions with electron-withdrawing *p*-substituents in this study can act as gas phase acids either in their excited or ground states.

Conclusions

In this paper we present results showing that changes in matrix structure which increase matrix acidity increase the MALDI analyte $[A + H]^+$ ion yield and *vice versa*. Semiempirical calculations were performed assuming thermodynamic control of the proton transfer reaction. This trend should also be observed if kinetics control the chemistry and the activation barrier for proton transfer is small. To understand more fully the influence of thermodynamics versus kinetics in the MALDI experiment, it would be useful to examine the ion−molecule reaction rates involving these *p*-substituted aniline compounds. Furthermore, the results of the studies conducted at 266 and 337 nm suggest that the matrix excited electronic state is important to the observation of the MALDI effect. The *p*-substituted anilinium ion appears to act as proton donor to the analyte as opposed to the aniline $MH^{+\bullet}$ ion. We have not been able to determine unambiguously whether the ground or excited state *p*-substituted anilinium ion is responsible for the proton donation. The mechanism of analyte $[A + H]^+$ ion formation is complex, involving both condensed phase and gas phase reactions, and likely to involve both $[MH + H]^+$ and $MH^{+\bullet}$ species. A detailed examination of the behavior of the *p*-substituted aniline compounds in negative mode MALDI is currently in progress and should yield additional insight into the role of the matrix in the analyte ionization mechanism.

Acknowledgment. Preliminary results were presented at the 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL (1994). This work was supported by grants from the National Science Foundation (CHE-8821780) and the US Department of Energy, Office of Basic Energy Science, Division of Chemical Science (DE-FG05-85-ER13434). The TOF mass spectrometer was constructed with funds provided by the Center for Macromolecular Design, the College of Science Research Enhancement Program, and the Department of Chemistry at Texas A&M University. M.G.K. also thanks Dow Chemical USA for its financial support through an ACS Analytical Division Graduate Summer Fellowship and W. R. Grace & Company for its financial support through a 1994−1995 College of Science Graduate Fellowship.

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