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Using dissociation energies to predict observability of *b*– and *y* – peaks in mass spectra of short peptides

O. I. Obolensky^{a,1}, Wells W. Wu^b, R.-F. Shen^c, and Yi-Kuo Yu^a

^aNational Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD

^bNational Institute on Aging, National Institutes of Health, Baltimore, MD

^cCenter for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD

Abstract

RATIONALE—Peptide identification reliability can be improved by excluding from analysis those *m/z* peaks of candidate peptides, which can not be observed in practice due to various physical, chemical or thermodynamic considerations. We propose using dissociation energies (as opposed to proton affinities) as a predictor of observability of different *m/z* peaks in spectra of short peptides.

Experiment: Mass spectra of tetrapeptides AAAA, AAFA, AAVA, AFAA, AVAA, AFFA, AVVA have been measured in the CID activation mode on a grid of activation times 0.05 to 100 ms and normalized collision energy 10 to 35 %.

Computations: Lowest energy geometries and vibrational spectra were calculated for the precursor ions and their charged and neutral fragments using DFT at the TPSS/6-31G(d,p) level. Dissociation energies were calculated for all fragmentation channels leading to *b*– or *y*– fragments.

RESULTS—It is demonstrated that *m/z* peaks observed in the mass spectra correspond to the fragmentation channels with the lowest dissociation energies. Using 50 kcal/mol as the cut off value of dissociation energy, it was predicted that 28 out of 42 possible peaks in the *b*– and *y*– series of the seven tetrapeptides can be observed in mass spectra. In the experiments, 26 *b*– or *y*– peaks were observed, all of which are among the 28 predicted ones.

CONCLUSIONS—The use of dissociation energies generalizes the use of proton affinities for semi-quantitative predictions of relative intensities of different *m/z* peaks of short peptides. Further advances in this direction will pave the way for reliable quantitative predictions and, hence, for a significant improvement in robustness and accuracy of peptide and protein identification tools.

Keywords

short peptides; tandem mass spectrometry; protein identification; density functional theory; dissociation energy

obolensk@ncbi.nlm.nih.gov (O. I. Obolensky), yyu@ncbi.nlm.nih.gov (Yi-Kuo Yu).

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1. Introduction

Tandem mass spectrometry (MS/MS) techniques are routinely used for identification of proteins in biological samples. Most of MS/MS-based peptide identification methods operate by searching for similarities between the given experimental mass spectra and reference spectra. The reference spectra are either obtained from spectral libraries or theoretically generated from candidate peptides in a protein database. Methods relying on comparisons with spectral libraries suffer from the limited scope of existing libraries and, more generally, from various other restrictions inherent to spectral libraries (for example, the type of equipment or activation regimes used in the experiment can be different from those used for building the library). In the latter approach, the reference spectra are generated by assuming that the candidate peptides can dissociate at all possible locations, that is, all theoretical m/z peaks are equally weighted in scoring the similarity with the experimental mass spectra. In this case the reliability and sensitivity of the statistical analysis are greatly reduced because the valuable information about correlations in intensities of different m/z peaks (also called ion intensity relationships) is not utilized. It is expected that the ability to theoretically predict intensity profiles of mass spectra for arbitrary peptides will be useful in improving bioinformatics tools for protein and peptide identification.

Currently existing methods for predicting intensity profiles, see, e.g.,^{1,2}, employ statistical analysis of large MS spectra databases to deduce phenomenological rules of ion intensity relationships. Complementary to the this approach, another scheme can potentially be developed, in which physical and chemical properties of peptides are employed for predicting dissociation rates along different fragmentation pathways and hence the relative intensities of different m/z peaks in experimental mass spectra. The key parameters in the latter scheme are physical and thermodynamic quantities such as the energies and the entropies of the precursor ions, of the transition states and of the fragments. These quantities can, in principle, be extracted from experimental data³⁻⁵ with the use of methods of thermochemistry.⁶⁻¹³

Alternatively, these quantities can be calculated theoretically with sufficient degree of accuracy with the use of quantum chemistry methods, most notably density functional theory (DFT). Most of the *in silico* studies of the dissociation of short peptides have thus far focused on elucidating the fragmentation pathways and the geometries of the fragments and transition states. Significant progress was achieved in that direction. Knowledge of the energetics and the structural rearrangements taking place during the fragmentation process has proved very valuable in explaining various features of mass spectra of short peptides, e.g., sequence scrambling.¹⁴ A comprehensive review on the topic can be found in¹⁵⁻¹⁷ and more recent developments in¹⁸⁻²⁶.

There have been, however, few attempts to predict relative intensities of different m/z peaks. These attempts were limited to comparing the probabilities of retaining the charge on one or the other fragments. A natural quantity for making such comparisons is the proton affinities to the neutral fragments.²⁷ For example, in the $b_x - y_z$ fragmentation pathway,^{16,28} the ratio of abundances (i.e., intensities of the corresponding m/z peaks in mass spectra) of complementary b_x and y_z fragments, when entropy effects are neglected, is approximated as^{29,30}

$$\ln \left(\frac{b_x^+}{y_z^+} \right) \approx \left(\frac{PA_{N-term} - PA_{C-term}}{kT_{eff}} \right) \quad (1)$$

where T_{eff} is the effective temperature, $\text{PA}_{\text{N-term}}$ and $\text{PA}_{\text{C-term}}$ are the proton affinities of the neutral fragments corresponding to the b_x and y_z ions. We will denote such neutral fragments as b_x^0 and y_z^0 . From now on, to eliminate any chance of confusion, we will use a superscript plus sign for ions, b_x^+ and y_z^+ .

Proton affinities were successfully utilized in analysis of evolution of mass spectra in series such as H-Val-Xxx-OH,³¹ H-Gly-Gly-Xxx-OH,³² H-Xxx-Gly-Gly-OH,³³ and H-Gly-Xxx-Phe-OH,³⁴ where the Xxx amino acid was varied. Analysis based on proton affinities, however, did not lead to meaningful results for a very similar series H-Gly-Xxx-Gly-OH.³³

It is our current proposal to compare dissociation energies of different fragmentation channels in order to predict observability and eventually relative intensities of the corresponding m/z peaks in mass spectra. The dissociation energies (or reaction enthalpies in thermochemistry) are widely and successfully used for determining the relative rates of fragmentation in various fields of chemistry and physics, e.g., in atomic cluster fission.^{35,36}

The dissociation energy is defined as the energy difference between the final state of the system (two or more neutral and charged fragments) and the initial state (protonated precursor ion). The energy of the initial state is simply the energy of the precursor peptide E_{MH^+} . The energy of the final state is the sum of the energies of the charged and neutral fragments. For example, if a precursor peptide consists of k residues, there will be $2(k-1)$ fragmentation channels along the $b_x - y_z$ pathway with the final energies $E(b_n^+) + E(y_{k-n}^0)$ and $E(y_n^+) + E(b_{k-n}^0)$, where $n = 1, 2, \dots, (k-1)$. Correspondingly, there will be $2(k-1)$ dissociation energies

$$E_{b_n} \equiv (E(b_n^+) + E(y_{k-n}^0)) - E_{\text{MH}^+} \quad (2)$$

$$E_{y_n} \equiv (E(b_{k-n}^0) + E(y_n^+)) - E_{\text{MH}^+} \quad (3)$$

related to the intensity ratio for any pair of fragmentation channels by

$$\ln\left(\frac{I_1}{I_2}\right) \approx \left(\frac{E_2 - E_1}{kT_{\text{eff}}}\right). \quad (4)$$

Here I_1 and I_2 are the intensities and E_1 and E_2 are the dissociation energies.

The use of dissociation energies generalizes the use of proton affinities. Indeed, the proton affinity to the N-terminus fragment is defined as $\text{PA}(b_n) = E(b_n^0) - E(b_n^+)$, while the proton affinity to the corresponding C-terminus fragment is $\text{PA}(y_{k-n}) = E(y_{k-n}^0) - E(y_{k-n}^+)$. Thus, for a special case of the complementary fragmentation channels $\text{MH}^+ \rightarrow b_n^+ + y_{k-n}^0$ and $\text{MH}^+ \rightarrow y_{k-n}^+ + b_n^0$, Equation (4) reduces to Equation (1)

$$\begin{aligned} \text{PA}(b_n) - \text{PA}(y_{k-n}) &= (E(b_n^0) - E(b_n^+)) - (E(y_{k-n}^0) - E(y_{k-n}^+)) \\ &= E_{y_{k-n}} - E_{b_n}. \end{aligned} \quad (5)$$

The key difference between using proton affinities and using dissociation energies is that proton affinities cannot be used for making predictions regarding the relative intensities of m/z peaks which originate in cleavage of different bonds, e.g., b_2^+ and b_3^+ peaks.

Dissociation energies determine the fragmentation reaction rate and therefore the observability of different m/z peaks in mass spectra in situations when the energies of the final states are much higher than the energies of the transition states. Computations suggest that this is the case for fragmentation of pentaalanine¹⁶, for fragmentation of all seven tetrapeptides considered in this work (see Table 1 in Section 3), and, generally, for fragmentation of short peptides with non-polar side chains.³⁷ We note that this is a very fortunate circumstance because energies of the fragments are much easier to calculate than to find transition state geometries and their energies. However, for longer peptides or peptides with acidic and/or basic side chains, transition states may turn out to be higher than the final states and our method would not be applicable. In this case in order to find the fragmentation propensity at each amide bond, one may analyse the stability of the corresponding N-protonated isomer, as has been recently proposed.²²

Finally, we stress that the usage of the dissociation energies (or, more generally, enthalpies and free energies of dissociation processes) is not at all a new concept in physics, thermodynamics or thermochemistry.^{6-8,29,38} However, this approach has been applied to dissociation of peptides only in the “reverse” direction: to deduce energies, enthalpies and free energies from experimental data with the help of the kinetic method and its modifications. In this work we show that these quantities can be found *in silico* and employed in the “forward” direction, for making predictions regarding fragment observability in experimental mass spectra.

2. Methods

2.1. Experimental

Experiments were performed on a commercial Finnigan LTQ XL mass spectrometer in the collision-induced dissociation (CID) activation mode. The mass spectra were acquired in centroid mode on a grid of activation times t_a varying from 0.05 to 100 ms and normalized collision energy (NCE) varying from 10 to 35 per cent. Multiple mass spectra were measured and averaged for each t_a / NCE grid point to improve signal to noise ratio. 10 pmol/ul solutions of AAAA (Bachem) and AAFA, AAVA, AFAA, AVAA, AFFA, AVVA (Princeton Biomolecules Corp., Langhorne, PA) were infused into an ESI source using a syringe pump at a flow rate of 3 microliter per minute. The spray voltage and ion transfer tube temperature were set at 4.0 kV and 275°C, respectively. We observed the same dominant m/z peaks as in the previous experiments,^{4,15,39} except for very short activation times during which sequential emergence of various m/z peaks was observed as t_a was increased.

2.2. Computational

Numerical calculations were carried out with the use of Gaussian 09 software package⁴⁰ on the Biowulf computer cluster at the National Institutes of Health. We have tested performance of several density functionals (B3LYP, PBE, TPSS), as well as basis sets (6-31G(d), 6-31G(d,p), 6-31G(2df,2dp)). We found that TPSS/6-31G(d,p) is the optimum model chemistry with respect to the balance of speed, stability and accuracy. Zero point energies were taken into account, while more subtle effects, such as counterpoise corrections, were neglected based on relatively large uncertainty introduced by the choice of the functional.^{41,42}

The configuration space of the peptides and their fragments was searched extensively and the geometries with the lowest energies were identified for each protonation site of the precursor ions and for all charged and neutral fragments. Normal modes and vibrational spectra were calculated for each geometry to determine the zero point energy correction and

to permit further RRKM (Rice–Ramsperger–Kassel–Marcus⁴³) analysis. Transition states were validated by the IRC method.

The lowest energy geometries of the precursor ions for all studied peptides are head-to-tail configurations in which the peptide forms some kind of cyclic structure, with a hydrogen bond between the added proton (attached to the nitrogen at the N-terminus) and the amide oxygen at the C-terminus. Recent infrared multiphoton dissociation (IRMPD) data⁴⁴ provided experimental evidence supporting such cyclic structures in polyalanines. In y_3^+ fragments the head-to-tail and extended configurations compete with some prevalence of extended geometries, and for even shorter fragments y_2^+ extended configurations prevail. Neutral y_n^0 fragments ($n = 1 \dots 3$) are C-terminal peptides.

There is much less clarity regarding the structures of both charged and neutral b^- fragments despite the large number of experimental and theoretical studies.⁴⁵ While shorter b^+ fragments are believed to be oxazolone derivatives,⁴⁵⁻⁵¹ the structure of b_4^+ ion is the subject of an active debate. There is some theoretical and experimental evidence pointing to linear oxazolone and cyclic structures.^{45,52-54} However, recent detailed computational, MS³ and IRMPD analysis has suggested existence of non-canonical structures of b_4^+ ions which are formed from tetrapeptides by loss of water.^{19,55}

Neutral b^0 fragments can be diketopiperazine derivatives or oxazolone derivatives. In the former case the dissociation energies are significantly lower, but the transition states are prohibitively high and fragmentation paths involve *cis-trans* isomerization.^{17,19,56} Based on the conclusions of previous studies we did not investigate further these diketopiperazine fragmentation pathways. Instead, we assumed that cleavage of amide bonds and formation of b^- and y^- ions proceeds along the $b_x - y_z$ fragmentation pathway^{17,45} which results in oxazolone derivative structures for the neutral b^0 fragments. Similarly, we chose not to use the structures of b_4^+ ions suggested in⁵⁵ because they do not form along the $b_x - y_z$ fragmentation pathway and because our preliminary computations indicated a high transition barrier (48.3 kcal/mol for GGGG and 51.0 kcal/mol for AAAA) to the geometry preceding the water loss. This precursor structure contains a 5-membered imidazol ring

$C'_1 - N_2 - C_2^\alpha - C'_2 - N_3$ with the OH group attached to C'_1 . A possible for the transition state being so high is that it involves a very unfavorable bending of the peptide's backbone⁵⁷ due to twisting of the dihedral angle ω_1 . The transition state on the second stage of the fragmentation process (detachment of OH group and capture of an extra hydrogen to form a water molecule) is much lower (30.5 kcal/mol for GGGG and 31.9 kcal/mol for AAAA) and is comparable to that for formation of the more standard oxazolone-based b_4^+ ion.

Geometries and energies of the precursor ions and of positive and neutral fragments used for determining the dissociation energies for all seven tetrapeptides are provided in the supporting materials. We also provide energies and geometries for transition states leading to formation of the non-canonical structures of b_4^+ ions.⁵⁵

3. Results and discussion

In Table 1 we summarize results of our DFT calculations of dissociation energies for all fragmentation channels in b^- and y^- series. also provide dissociation energies for We fragmentation resulting in formation of $m/z = MH^+ - 18$ ion (loss of water) which is essentially b_4^+ ion in the case of tetrapeptides. Even though this fragmentation channel can not be used for peptide identification purposes, it has the lowest dissociation and transition

state energies and does appear in mass spectra for all seven peptides. Note that b_1^+ fragment ions are not stable and therefore there are no corresponding dissociation energies.

One of the most striking features of the data presented in Table 1 is that the dissociation energies depend very little on side chain composition and sequence. This suggests that at least for these neutral, nonpolar side chains the fragmentation process is governed mainly by backbone reconfigurations. For all seven peptides, the $MH^+ \rightarrow b_4^+ + H_2O$ fragmentation channel has the lowest dissociation energy, followed by the $MH^+ \rightarrow b_3^+ + y_0^1$, $MH^+ \rightarrow y_2^+ + b_2^0$, and $MH^+ \rightarrow b_2^+ + y_2^0$ processes.

In Table 1 we also present energies of transition states for amide bond cleavage, the essential step along the $b_x - y_z$ fragmentation pathway, as well as the transition state energies for the water loss reaction. For each fragmentation channel we found many different transition states with similar energies. There might exist transition states with even lower energies, so the values presented in Table 1 should be considered as upper bounds on the transition barriers. Nevertheless, even these transition barriers are lower than the energies of the final (fragmented) states.

The fact that the energies of the transition states are lower than the dissociation energies suggests that, at least for short non-polar peptides, the dissociation energy is the most important physical quantity governing the fragmentation process and determining its rate. Threshold measurements (Table 2) support this conclusion. Indeed b_4^+ is the first fragment which appears in mass spectra already at 0.05 ms of activation time t_a , followed by b_3^+ (at $t_a = 0.4$ ms), y_2^+ (0.8 ms) and b_2^+ (1.2 ms). An experiment-based RRKM modeling of tetraalanine fragmentation⁴ also resulted in the smallest activation energy for the b_4^+ fragmentation channel. These results are in agreement with the data on tetraglycine, showing the dominance of the b_4^+ fragment.^{19,55,58}

As the activation time increases, the intensity of b_4^+ peak increases very slowly, while intensities of b_3^+ and y_2^+ peaks rise at a much faster pace. By $t_a = 10$ ms, when most of precursor ions are fragmented, in five of the seven peptides the y_2^+ peak dominates and in the other two (AAVA and AFAA) the b_3^+ peak is the most intense one (Table 3). Out of 42 possible m/z peaks in the b - and y - series of the seven peptides, 26 peaks are clearly present in the mass spectra. The remaining 16 peaks have relative intensities smaller than 1% of the total intensity and thus counted as “unobserved”.

A comparison of Tables 1 and 3 shows that dissociation energy is not the only quantity needed for predicting the ion intensity relationships between different m/z peaks in mass spectra of short peptides. This conclusion is in agreement with the results of the previous studies of collision-induced and surface-induced dissociation of short peptides⁴ in which RRKM analysis of experimental data was employed. The non-negligible sequential fragmentation and the non-uniformity of pre-exponential (entropic) factors evidently play an important role in emergence of the observed intensity profiles in mass spectra.

Let us point out, however, that all 26 observed b - and y - peaks have dissociation energies below 50 kcal/mol. Only two out of 16 unobserved fragmentation channels have dissociation energies below 50 kcal/mol. Therefore, it appears that dissociation energies can serve as a very good indicator of whether a particular m/z peak can appear in a mass spectrum or not. Further studies on the cut off value of dissociation energy (and its dependence on the type of mass spectrometer and the precursor ions activation regime) are apparently necessary. Nevertheless our data demonstrate that dissociation energies may serve as a reliable criterion

for eliminating m/z peaks which cannot be observed in practical mass spectra from peptide identification scoring functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Dissociation energies (kcal/mol) for fragmentation channels resulting in formation of *b*– or *y*– series fragments. Energies of the transition states for cleaving the corresponding amide bonds are given in parentheses. TPSS/TPSS/6-31G(d,p) model chemistry is used for geometry optimization and energy calculations.

Peptide	Fragments					
	b_2^+	b_3^+	b_4^+	y_1^+	y_2^+	y_3^+
AAAA	45.4 (34.6)	39.0 (28.8)	32.5 (30.5)	52.4 (28.8)	42.2 (34.6)	53.6
AAVA	46.0 (34.0)	37.9 (28.3)	32.3 (29.9)	52.4 (28.3)	41.1 (34.0)	53.8
AVAA	43.4 (31.0)	36.9 (28.4)	32.9 (30.4)	53.0 (28.4)	42.2 (31.0)	53.6
AVVA	43.8 (27.5)	36.7 (23.7)	32.9 (30.0)	52.9 (23.7)	40.9 (27.5)	56.7
AAFA	44.8 (32.5)	37.8 (27.9)	30.7 (28.5)	53.0 (27.9)	38.0 (32.5)	52.3
AFAA	43.7 (33.5)	38.4 (29.5)	32.7 (30.2)	54.0 (29.5)	43.7 (33.5)	53.7
AFFA	43.0 (31.2)	36.0 (28.4)	31.3 (30.8)	54.5 (28.4)	39.3 (31.2)	52.4

Table 2

Intensities of various m/z peaks (per cent of the total intensity) in mass spectra of AVVA. Normalized collision energy is 12.5%. Intensities of all observed fragments are tabulated, a_3^* denotes $a_3^+ - 17$ fragments.

t_a (ms)	Fragments							
	b_2^+	b_3^+	b_4^+	y_2^+	a_2^+	$(a_3^*)^+$	a_4^+	other
0.05		0.01	0.11					99.88
0.4		0.02	0.11					99.86
0.8		0.13	0.11	0.18				99.58
1.2	0.05	1.29	0.25	1.75		0.05		96.59
1.6	0.25	3.84	0.57	5.38	0.04	0.36	0.03	89.49
2.0	0.63	7.54	0.94	10.39	0.13	0.96	0.10	79.21
								0.10

Table 3

Intensities of various m/z peaks (per cent of the total intensity). Activation time is 10 ms. Normalized collision energy is 12.5%. In column “other” the sum of intensities of all non b - and non y - series fragments are given.

Peptide	Fragments							
	b_2^+	b_3^+	b_4^+	y_1^+	y_2^+	y_3^+	MH ⁺	other
AAAA	2.6	29.8	9.3	0.2	39.6	0.5	10.9	7.1
AAVA	0.8	46.0	5.1	0.0	31.2	0.3	1.9	14.7
AVAA	14.8	24.3	7.1	0.3	39.7	0.2	4.1	9.5
AVVA	3.7	34.8	3.7	0.0	48.2	0.1	0.9	8.6
AAFA	0.1	21.8	10.0	0.0	53.3	0.4	1.8	12.6
AFAA	22.8	40.3	8.0	0.1	13.8	0.5	2.5	12.0
AFFA	2.7	25.9	9.0	0.0	47.9	0.3	0.4	13.8