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Average Activation Energies of Low-energy Fragmentation Processes of Protonated Peptides Determined by a New Approach

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An attempt was made to estimate the average activation energies of low-energy fragmentation processes of protonated oligopeptides by combining RRKM theory and the results of electrospray ionization/surface induced dissociation (ESI/SID). The average internal energy was assumed to be deposited by three processes: thermal energy gained in the heated capillary of the electrospray source, energy gain in the capillary-skimmer region of the electrospray source, and energy deposition by collision with the surface. The latter fraction was calculated based on the position of the ESI/SID fragmentation of efficiency curves and the ratio of kinetic to internal energy conversion in SID. Using the average internal energy estimated from the experimental results, the average activation energies were evaluated by applying RRKM theory. The application of this approach for protonated leucine enkephalin resulted in an average activation energy of 36±5 kcal/mol for the lowest energy decompositions. The approach has also been applied to several other peptides in the mass range of 200–1200 Da, yielding average activation energies in the range of 35–47 kcal/mol.

Mass spectrometric fragmentation of protonated peptides gives important information on their structures. Tandem mass spectrometry has been widely used for sequence determination of peptides, and this has become one of the most important biochemical applications of mass spectrometry. There is, however, little known about the energetics of the fragmentation processes of protonated peptides, mainly because of a lack of suitable methods for determination of activation energy (E_a).

The classical term 'activation energy' is related to thermal equilibria, so, in principle, thermal decompositions of protonated peptides could provide activation energies of fragmentation. Thermal decompositions of non-covalent complexes and singly and multiply charged oligopeptides are of increasing importance,[1–4] in spite of the 'pitfalls' associated with these experiments.[2a] Smith and co-workers[2] have reported that the Arrhenius activation energies for the dissociation of mellitin decrease with increasing charge state within the range of 32–40 kcal/mol. They used a heated capillary with electrospray to dissociate the protonated melittin ions. Williams and co-workers[3] measured rate constants for the dissociation of protonated peptides promoted by black body radiation in a Fourier transform (FT) mass spectrometer at low pressure (less than 10^{-7} Torr). They reported activation energies of 1.3 eV and 0.6 eV for the loss of NH_3 and formation of the b_2/y_7 complementary pair from singly and doubly protonated bradykinin, respectively. The method applied by Williams and co-workers has the following advantages over thermal dissociation in the interface region:[3] precursor ions can be selected by the FT mass spectrometer before thermal dissociation, accurate measurement of the temperature of the ion environment and the elimination of interfering processes, such as droplet evaporation and ion desolvation. In addition, the ions can be stored in the cell for several seconds, decreasing the kinetic shift significantly.

Recently an attempt was also made to study thermal decomposition kinetics of protonated peptides in our laboratory.[4] Thermal dissociation of a protonated model peptide, leucine enkephalin and its dimer, was studied in a heated tube reactor which was attached to an electrospray (ESI) ion source.[4] (In this sense, our method is closely similar to that published by Smith and co-workers.[1]) Decomposition of the protonated leucine enkephalin was studied as a function of the temperature of the reactor, and the results were evaluated by using an Arrhenius plot. The Arrhenius activation energy of the decomposition of protonated leucine enkephalin so determined was 38 kcal/mol (1.65 eV) with an A factor given by $\log A = 15.7$.

Peptide fragmentations can also be studied by surface-induced disociation (SID), an ion-activation technique developed by Cooks and co-workers.[5,6] Recently it has been observed that the fragmentation efficiency (sum of fragment ion current divided by the total ion current) plotted against the laboratory SID collision energy can be used to characterize the ease of fragmentation of protonated peptides.[4,7–9] Such a fragmentation efficiency curve for leucine enkephalin, colliding on an octadecanethiolate self-assembled monolayer surface prepared on vapor deposited gold, is reproduced in Fig. 1. The laboratory collison energy corresponding to the inflection point of the logistic-type fragmentation efficiency curve, i.e., to *ca.* 50% fragmentation, is 33.2 eV in this case, and can be taken as the collision energy characterizing the fragmentation of the protonated leucine enkephalin (E_{chr}) within the allowed time frame of this mass spectrometer. Note that, in general, these inflection point values depend significantly on the peptide sequence and can be related to the ease of fragmentation.[7–9] It has been shown that peptides with basic residues (such as arginine or lysine) fragment at considerably higher SID energies than those containing no basic residues.

The distinct differences observed in the position of the

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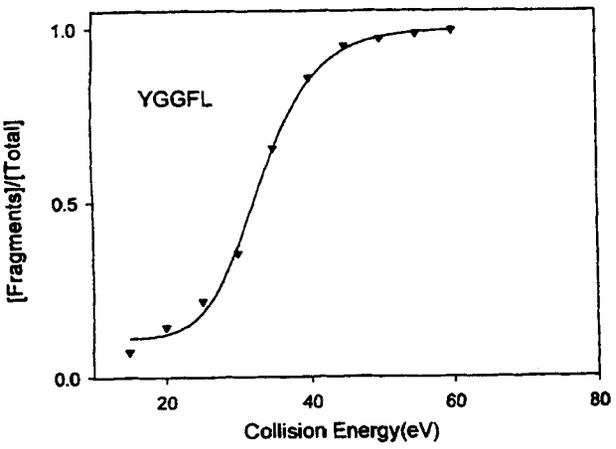

Figure 1. ESI/SID fragmentation efficiency curve of protonated leucine enkephalin on an octadecanoethiolate monolayer surface.

ESI/SID fragmentation efficiency curves as a function of peptide sequence encouraged us to correlate these positions with the activation energy of peptide fragmentation. The work presented here is the first attempt to estimate the activation energy of peptide fragmentation by combining the ESI/SID experimental data and RRKM theory. Due to the approximations applied in the present work (see below), the present calculations cannot lead to 'precise' determination of activation energies. Nevertheless, this method can be considered as an alternative (and independent) way of estimating activation energies of peptide fragmentation.

The reliable estimation of the average internal energy of protonated peptides deposited during the ESI/SID process is of primary importance. As will be shown below, the main part of this internal energy is deposited by the collision with the surface. The conversion of the collision (kinetic) energy to the internal energy of the reactant ion in SID ($T \rightarrow V$ conversion) has been calculated by many authors.[10–20] The internal energy content of fragmenting ions can be measured by several different methods[10–20] including the 'thermometer molecule' method[10–14] and the 'deconvolution' method.[15–17] The various methods applied are based on different assumptions and approximations, and utilize different reactant ions, such as metal hexacarbonyls,[10,11] ferrocene,[12] and benzene.[17] By determining the internal energy of ions excited by collisions with a surface at various collision energies, the efficiency of kinetic-to-internal energy conversion can be determined.

The 'thermometer molecule' method and the 'deconvolution method' indicate that, on average, *ca.* 13–17% of the kinetic (collision) energy is converted to internal energy of the projectile ion using alkanethiolate (e.g., octadecanethiolate) monolayer surfaces. Taking into account the energy taken away by the neutral fragment, the original 'thermometer molecule' method can be modified. The use of this correction increases the internal energy of an ion calculated by the 'thermometer molecule' method. Preliminary calculations[20] suggest that this correction increases the conversion ratio from 13 to 15–17%. In the following analysis we shall use 17% kinetic-to-internal energy conversion on the hydrocarbon monolayer surface. It should be noted here that there is no direct evidence that the $T \rightarrow V$ conversion factors determined for metal carbonyls or small organic molecular ions (such as benzene) can be used for protonated peptides. Nevertheless, for alkanethiolate self-assempled monolayer surfaces, the *ca.* 15–17% energy conversion is a reasonable first approximation.

In spite of the differences in the particular $T \rightarrow V$ conversion values, all the methods applied so far for various reactant ions indicate that the kinetic energy→internal energy transfer in SID has the following common characteristics:[10–20]

(a) a significant percentage of the collision energy is converted into internal (vibrational) energy of the colliding ion;
(b) the amount of kinetic energy→internal energy conversion depends on the surface used; for example, fluorinated alkanethiolates always provide higher $T \rightarrow V$ conversion than alkanethiolate self-assembled monolayer surfaces;
(c) the internal energy deposited within the projectiles varies approximately linearly with the collision energy;
(d) product ions resulting from high energy processes, e.g., with average internal energy greater than 20 eV, may be formed;
(e) the internal energy distribution deposited by ion/surface collision is relatively narrow, especially in comparison with gas-phas collision-induced dissociation (CID).

Statistical rate theory, and its slightly modified version, the quasi-equilibrium theory (QET), have been used in mass spectrometry for many years. A good description can be found in the book of Robinson and Holbrook,[21] and a more qualitative discussion, giving a number of interesting semi-quantitative applications in mass spectrometry, can be found in the treatment on metastable ions.[22] Two interesting applications of the statistical rate theory for the study of high mass ions have appeared recently.[23,24]

The early version of the rate theory used the classical approximation (the oscillators are not quantized), the Rice–Rampsberger–Kassel (RRK) theory. This uses the simplified expression

$$k(E) = \nu(1 - E_0/E)^{s-1} \quad (1)$$

where $k(E)$ is the rate constant (given as a function of the internal energy, E), E_0 is the activation (critical) energy of fragmentation, E is the internal energy of the molecule and s is the number of oscillators in the molecule. To overcome the inaccuracy of the mathematical treatment (non-quantized oscillators) the concept of 'effective' oscillators has been introduced, decreasing the number of oscillators to *ca.* 20–30% of the true value. This form of the theory can be used only for qualitative or semi-quantitative purposes.

The Rice–Rampsberger–Kassel–Marcus (RRKM) theory uses a mathematical form taking into account the quantized nature of vibrations and rotations. In this formalism, there is no need to assume 'effective' oscillators, and the rate constant takes the following form:

$$k(E) = (\sigma/h)(G(E - E_0)/\rho(E)) \quad (2)$$

where σ is the reaction path degeneracy, h is Planck's constant, $G(E - E_0)$ is the number of states in the transition state with $(E - E_0)$ internal energy and $\rho(E)$ is the density of states in the reactant ion with E internal energy. $G(E - E_0)$ and $\rho(E)$ can be determined by a direct counting of states. It is important that state counting should be done accurately; the often-used Whitten–Rabinovich algorithm gives large errors.[24]

State counting requires, however, a knowledge of vibrational frequencies in the reactant (typically known) and in the transition state (typically unknown). The latter are, therefore, often estimated. Fortunately, many applications

are not sensitive to the choice of vibrational frequencies and, in such cases, quantitative studies can be performed even without a detailed knowledge of the transition state.

In the present paper, we combine the characteristic collision energy (E_{chr}) determined from ESI/SID fragmentation efficiency curves with the energy conversion factor in SID and evaluate the results in terms of the RRKM theory. From these calculations, with clearly stated approximations, the activation energy for the fragmentation of protonated peptides can be determined.

The term, 'activation energy' for fragmentation of a protonated peptide used here and also in the paper on thermal kinetics,[4] has to be clarified. First, the activation energy calculated below must be distinguished from the appearance energy: the latter involves the kinetic shift that is dependent on the method of analysis and instrument type. Secondly, the term "critical" energy is also used in mass spectrometry,[22] and we use 'activation energy' in the same sense (neglecting that we may not have 'classical' thermal equilibrium). Finally, a given protonated peptide has a large number of possible fragmentation channels, each with a given activation (critical) energy. The activation energy measured here (and also by thermal kinetics[4]) will be an *average* value of those processes which give abundant ions at the laboratory collision energy studied. The selection of an appropriate collision energy requires careful consideration. At first sight, any collision energy, i.e. any point of the ESI/SID fragmentation efficiency curve, could be used to estimate the average internal energy. However, at very low collision energies it is much more likely that one observes fragmentation processes resulting from parent ions at the high energy tail of the internal energy distribution, so this point is not as informative for the *average* internal energy distribution. The selection of a very high collision energy can also be questioned, because at this energy, higher energy processes such as side-chain cleavages, may also occur. It seems, therefore, reasonable to select an intermediate point at which the avarage internal energy can be estimated and at which the lower energy processes are still dominant. In the present paper, we therefore use the collision energy that corresponds to the inflection point of the ESI/SID fragmentation efficiency curves. At this SID energy, several fragmentation channels are open (that are similar to those of low energy CID), so it is more correct to use the term 'average activation energy'. (For example, the activation energy for the formation of each individual b_n ion is different but the appearance of b_n ions with similar intensities in the ESI/SID spectra of several peptides and the results of *ab initio* bond-order calculations[25,26] indicate that the activation energies for the b_n ion formation are expected to be close to each other.)

EXPERIMENTAL AND COMPUTATIONAL DETAILS

SID experiments were performed by using the tandem quadrupole mass spectrometer described earlier.[11] Two Extrel quadrupoles are positioned at right angles to each other and the target surface intersects the ion optical path at 45°. An octadecanethiolate self-assembled monolayer prepared by the spontaneous assembly of octadecanethiol on vapor-deposited, plasma-cleaned gold was used as a surface. The peptides were ionized by an electrospray source designed according to Reference 27. The singly charged protonated peptides were selected by the first quadrupole analyzer, and collided with the surface target at a given SID collision energy.

The average lifetime of the selected ions following the collisions can be assumed to be related to the average rate constant. As was indicated above, this assumption is presumably valid at the collision energy of the inflection point of the ESI/SID fragmentation efficiency curves. The ions, following collision with the surface, are accelerated to *ca.* −30 eV potential, relative to the surface, over a distance of 12 mm; then by a lens system to the entrance of the second quadrupole analyser (a further 5 mm distant). The average potential within this lens system was *ca.* 85 V. The flight time of an ion can be determined with the following expression:

$$t = cl(m/(E_1 + E_2))^{1/2} \quad (3)$$

where t is the flight time, l is the length of the flight path, m the mass of the ion, and E_1 and E_2 are the kinetic energies of the ion at the beginning and at the end of the flight region, respectively, while c is a conversion factor (1.018×10^{-4}, if second, meter, eV and dalton units are used). (Note that the ions leaving the surface do have an initial kinetic energy, of a few eV.[5b,6] This shortens the flight time between the collision and the entrance to the quadrupole analyser, and, consequently, the liftime of the decomposing ions will be smaller by about 2–10%. It has been shown by model calculations that this effect would correspond to a negligible difference in the calculated activation energies and so has been neglected.

In the present paper, fragmentation efficiency curves measured previously using an octadecanethiolate monolayer surface[7,8] are discussed for the following peptides: leucine enkephalin, YGGFL (inflection point, or characteristic collision energy, E_{chr}: 33.2 eV); trialanine, A_3 (20.1 eV); pentaalanine, A_5 (254 eV); prolyl-tetraalanine, PAAAA (32.8 eV); lysyl-tetraalanine, KAAAA (38.8 eV); AAKAA (36.2 eV); AAAAK (35.9 eV); arginyl-tetraalanine, RAAAA (46.8 eV); des-Arg1-bradykinin, PPGFSPFR (78.8 eV); des-Arg9-bradykinin, RPPGFSPF (78.3 eV); substance P frag. 4–11, PQQFFGLM-NH_2 (58.5 eV); substance P frag. 2–11, PKPQQFFGLM-NH_2 (79.1 eV). The peptide sequences here are given in one-letter codes, the characteristic points of the fragmentation efficiency curves (E_{chr}, inflection points, close to 50% fragmentation probability) are given in parenthesis.

The temperature of the ESI capillary was kept at 400 K. This temperature was measured at the outer wall in such a way that the thermocouple did not touch the metal wall. In our more recent setup we directly measured the wall temperatures. Based on these measurements and the assumption that the temperature inside the capillary is slightly less than the wall temperature, it can be predicted that the temperature inside the capillary was in the range of 400 to 450 K in our present experiments.

The thermal energy of a harmonic oscillator (divided by kT) is

$$\langle E \rangle / kT = (h\nu/kT)(1/\exp[h\nu/kT] - 1) \quad (4)$$

where $\langle E \rangle$ is the mean energy of an oscillator, h is Planck's constant, ν is the vibrational frequency, k is the Boltzmann constant and T is the absolute temperature. The value $h\nu/kT$ is a dimensionless quantity, and is equal to $1.4388\bar{\nu}/T$ (where $\bar{\nu}$ is the more-usually used wave number, measured in cm^{-1} units, and the temperature is measured in K). If the thermal energy is to be related to a mol sample (instead of an oscillator) $\langle E \rangle / RT$ should be used instead of $\langle E \rangle / kT$ in the left-hand side of Eqn. (4), R being the universal gas

constant, 8.62×10^{-5} eV/molK.

RRKM calculations were performed by the 'RRKM large' program written by Christie, and described recently.[24] The algorithm is capable of calculating reaction rates for large molecules with large excess energies. Internal rotations are treated as low frequency vibrations. The program calculates the number of states by a direct state count, using no approximations.

In the calculations, two different frequency schemes have been used for the ground state of the reactant ion of the protonated peptides: the frequency model given by Christie[24] and the frequencies listed in Table 2 of Reference 23 (Griffin and McAdoo). In both cases, the frequencies were scaled to the size of the peptide studied. For the transition state, four different models have been used: a 'loose' and a 'tight' frequency model given by Christie,[24] the model given by Griffin,[23] and a simple frequency scheme modeling a direct bond cleavage at the amide bond (modeling for example, charge-directed **b** ion formation). Because *ab initio* and MNDO bond-order calculations show significant weakening of the amide bond in the amide nitrogen protonated forms, which can be regarded as fragmenting structures for charge-directed simple bond cleavage of the amide bond leading to **b** ions,[26,27] the simple frequency model was specified as follows: the reaction coordinate is 1200 cm^{-1}; five (bending and torsional) frequencies become smaller in the transition state by a factor of 2:800 change to 400 cm^{-1}; 600 change to 300 cm^{-1}; 400 change to 200 cm^{-1}; 200 change to 100 cm^{-1} and 100 change to 50 cm^{-1}. Although the division of the original frequencies by the factor of 2 is arbitrary, the modified frequencies may reflect a loose transition state model, which will, therefore, be referred to as a 'simple loose transition state' model.

RESULTS AND DISCUSSION

Leucine enkephalin

The first model compound is protonated leucine enkephalin, whose ESI/SID fragmentation[7,8] and thermal kinetics[1] have been studied recently. It has a mass of 556 Da, and has 228 internal degrees-of-freedom (DOF).

The internal energy of decomposing ions is due to three main contributions: i, internal energy of the neutral before ionization (thermal energy); ii, increase of the internal energy resulting from electrospray ionization (ESI); and iii, increase of the internal energy due to the ion-activation step, SID. As was mentioned above, the temperature representing the internal energy of the sample molecules is estimated to be between 400 and 450 K. Substituting peptide ground-state vibrational frequencies into Eqn. (4), the thermal energy of the molecule can be determined at 400 K, as it is 0.19 kT (or 6.6 meV) per oscillator, while at 450 K this value is 0.22 kT (or 8.5 meV). The difference due to different frequency schemes is minor, within *ca.* 1%. Using these values, the average thermal energy in leucine enkephalin is 1.72 ± 0.20 eV; the major part of the ambiguity originates from determination of the temperature of the sample molecules.

Ionization is usually accompanied by excitation. In electrospray, this is mainly due to collisional activation in the capillary/skimmer region. In our recent experiments,[7,8] the voltage between the capillary and the skimmer was kept at 30 V. This is sufficient to prevent significant cluster formation, but does not increase the internal energy of the samples to a significant degree.[7,8] Another study on the ESI fragmentation of the tetramethyl ammonium ion also suggests that the increase of internal energy due to ionization and possible low-energy collisional activation in the flight region is small, estimated to be between 0.5 and 1.0 eV, i.e. 0.75 ± 0.25 eV.[28]

The increase of internal energy due to the SID activation step is the largest contribution to the internal energy of protonated leucine enkephalin. The characteristic (inflection) point of the fragmentation efficiency curve of protonated leucine enkephalin using an octadecanethiolate monolayer surface is 33.2 eV[7,8] (Fig. 1). The efficiency of the conversion of kinetic into internal energy on this surface is, on average, 17%, as discussed in the introduction. SID at 33.2 eV collision energy, therefore, increases the internal energy of protonated leucine enkephalin by 5.64 eV. The estimated accuracy, including various possible sources of errors (e.g. the reproduibility of the position of the ESI/SID fragmentation efficiency curves[7,8] and the validity of the 17% $T \rightarrow V$ factor), is *ca.* $\pm 10\%$ (*ca.* ± 0.56 eV).

Combining the three main contributions discussed above, the average internal energy of protonated leucine enkephalin fragmenting with *ca.* 50% probability between the ion/surface collision and mass analysis is 8.1 eV. The estimated uncertainties for the three sources of internal energy discussed above are ± 0.20, ± 0.25, and ± 0.56 eV. Calculating the mean square of these errors, the overall error in the internal energy is estimated to be *ca.* ± 0.65 eV or $\pm 8\%$.

The flight time between the surface (collision target) and the entrance into the quadrupole analyser, which is the average lifetime of the decomposition ions, is 6.2 μs, as determined based on the values given in the experimental section. This can be converted to an average rate constant of fragmentation of 1.61×10^5 s^{-1}. Knowing the internal energy and the activation energy of an ion, its rate of fragmentation can be calculated using RRKM theory. Conversely, from the internal energy and the rate constant the activation energy may be determined. Application of RRKM theory, however, requires knowledge of the ground- and transition-state frequencies of the fragmenting ion. These are not known for protonated leucine enkephalin, so approximations are needed. There are various frequency scheme in the literature applicable for peptide fragmentations.[23,24] We evaluate these models below and estimate the errors they may introduce in the determination of the activation energy.

RRKM calculations on protonated leucine enkephalin have been performed using the frequency model of Christie[24] described for the ground state and the 'loose' transition state of peptides. The dependence of the rate constant upon the internal energy of the ion, using the arbitrarily chosen activation energy of 1.50 eV (*ca.* 35 kcal/mol), is shown in Fig. 2 (solid line). Using the same transition-state model, but peptide ground-state frequencies given by Griffin,[23] a very similar curve is calculated (Fig. 2, broken line). The difference between the two curves can also be expressed in terms of activation energy. The second model, using 1.48 eV activation energy (Fig. 2, dotted line), gives practically identical rate constants (especially around 10^5 s^{-1} value) to that obtained by the first model with 1.50 eV activation energy (solid curve). Based on this comparison it is concluded that the choice of estimated ground-state frequencies of Christie vs. Griffin does not affect the results of RRKM rate calculations to a significant degree: the error in the activation energy connected with the estimation of peptide ground-state vibrational frequencies is

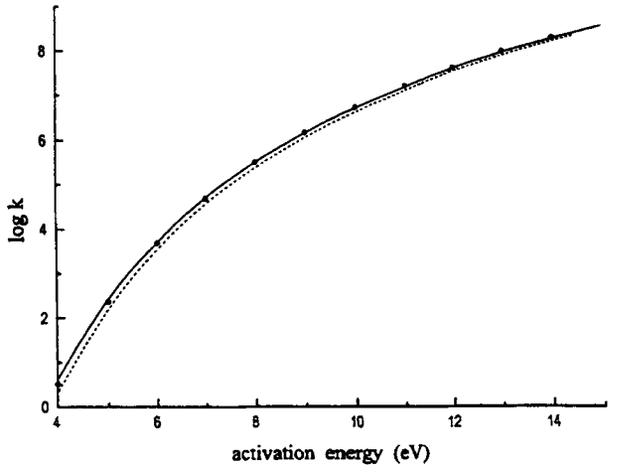

Figure 2. Dependence of rate constant on internal energy according to the frequency model of (a) Christie's 'loose' transition state model[24] (solid line) and (b) Griffin's model[23] (dotted line). In both cases, the activation energy was arbitrarily set to $E_a = 1.50$ eV.

ca. 0.02 eV (1–2%) in this case. In the following, the ground-state frequency model of Christie[23] will be used.

Various transition-state frequency models have also been tested. The rate constant vs. internal energy curves (again using 1.50 eV activation energy) are shown in Fig. 3. These curves differ from each other more than do those shown in Fig. 2, indicating that the choice of the transition state model has a larger effect on the RRKM calculations. The highest rate constants are obtained using the 'simple loose transition state' model, developed to model the direct cleavage of a peptide band yielding **b** ions (curve (a) in Fig. 3). This choice is supported by energy-resolved mass spectra of small peptides, indicating that, at low internal energy, **b** ions are most usually formed. (The thermal dissociation of leucine enkephalin leads to a very intense $\mathbf{a}_4^+$ ion,[4] which can be formed from $\mathbf{b}_4^+$ by CO loss. At the inflection point of the ESI/SID curve of leucine enkephalin, the **a** and **b** ions represent *ca.* 50% of the total fragment ions.[4]) The Arrhenius pre-exponential factor corresponding to this transition state model is $10^{15.5}$ (calculated by the 'RRKM large' program). This agrees very well with that obtained experimentally ($10^{15.7}$) using thermal kinetics.[4] It should be mentioned that no attempts were made to 'optimize' this transition state model to give a 'correct' pre-exponentional factor.

The 'loose' tansition state models of Christie[24] (curve (b)) and Griffin[23] (curve (c)) result in lower pre-exponential factors ($10^{14.7}$ and $10^{13.8}$, respectively) and lower rate constants, when the same activation energy (1.50 eV) is used (Fig. 3). The 'tight' transition state of Christie[24] (curve (d)), modeling a rearrangement reaction, gives the lowest reaction rate and pre-exponential factor ($10^{12.6}$), again using the same activation energy of 1.50 eV. Differences in reaction rates (and pre-exponential factors) using various transition state models can be converted to differences in activation energies. Using Christie's 'loose' transition state model with 1.50 eV activation energy, the rate constant will be $1.61 \times 10^5\ s^{-1}$ at 8.1 eV internal energy (which is the characteristic internal energy estimated above). To reach the same rate constant at the same internal energy the 'simple loose transition state' model indicates 1.54 eV, that of Griffin 1.40 eV activation energy.

The 'tight' transition state model of Christie results in a much lower activation energy (1.24 eV). The Arrhenius pre-exponential factor using this latter model is, however, in clear disagreement with that obtained from thermal kinetics[4] ($10^{12.6}$ vs. $10^{15.7}$). For this reason it was concluded that the 'tight' transition state model is not suitable to describe the typical fragmentation of a small protonated peptide, leucine enkephalin, and this model is discarded in the calculations of the present paper. Note, however, that the application of this 'tight' transition state model would be desirable to make predictions on rearrangement processes of peptide fragmentation but such a study is beyond the scope of the present work.

The activation energies determined by the 'simple loose transition state' model, by Christie's 'loose' transition state model, and Griffin's transition state model are fairly similar. From these, Christie's 'loose' transition state model, which gives an intermediate activation energy, will be used in the following calculations.

By running a series of RRKM calculations, the amount of internal energy necessary to drive the reaction with a given rate can be determined as a function of the activation energy. Such a correlation is shown in Fig. 4, calculated for protonated leucine enkephalin at a fragmentation rate of $1.61 \times 10^5\ s^{-1}$. By assuming that the internal energy of the

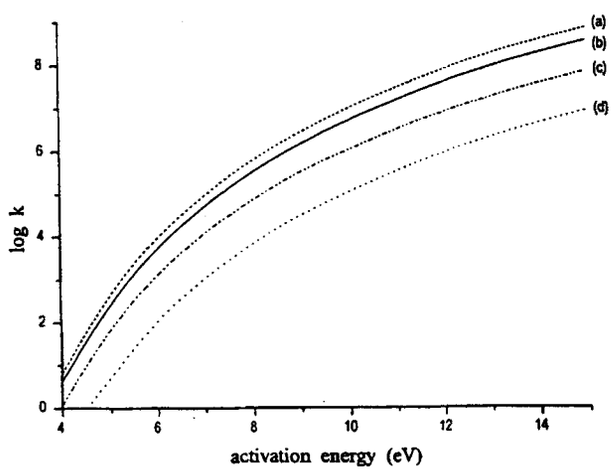

Figure 3. Dependence of the rate constant upon the internal energy of the ion using (a) Christie's 'simple loose transition state' model (see text for definition), (b) Christie's 'tight transition state' model.[24] (c) Griffin's transition state model,[23] and (d) Christie's 'tight transition state' model.[24] In all cases, the activation energy was arbitrarily chosen as $E_a = 1.50$ eV.

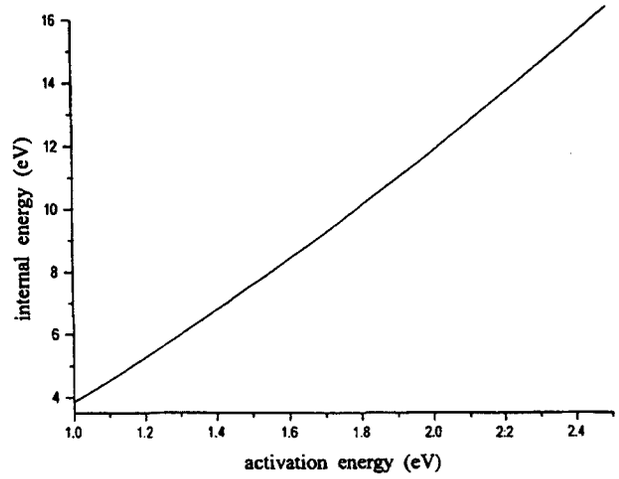

Figure 4. Correlation between the internal energy and the activation energy. In the calculations Christie's ground-state peptide frequencies and his loose transition state model were used (see Ref. 24). The rate constant is $1.61 \times 10^5\ s^{-1}$ calculated from the flight time of protonated leucine enkephalin (see text for details).

decomposing ions (with this reaction rate) is 8.1 eV (see above), the activation energy of fragmentation can be determined as 1.56 eV (36 kcal/mol). This value is in good agreement with that determined recently from thermal kinetics (38 kcal/mol).[4] Although the average internal energy for leucine enkephalin cannot be compared directly with those of bradykinin, it is worth noting that the above values (36–38 kcal/mol) are slightly higher than that reported by Williams ad co-workers for the lowest energy fragmentation (NH_3 loss) of singly charged bradykinin (1.3 eV or *ca.* 30 kcal/mol).[3] This is, however, not a surprising result because in our ESI/SID experiments the internal energy content is much higher than that provided by black body radiation in the FTMS instrument. In our ESI/SID experiments, it is very difficult to detect specifically the lowest energy process: other processes with slightly higher activation energies are dominant. (For a comparison of the energetics of **y**, **b**, and **a** ion formation, based on ion kinetic energy loss measurements in high energy CID, see the very recent publication by Glish and co-workers.[29])

Uncertainties in the activation energy, so determined, come from two main sources: the internal energy determination and the rate calculation. As discussed, the internal energy is determined with an accuracy of *ca.* ±8%. Using Fig. 4, this corresponds to an error of *ca.* ±0.08 eV in the activation energy determined. The errors in the RRKM calculation also come from various independent sources, discussed above individually. The average rate constant is supposed to be determined with an accuracy of 10% based on the flight time of the ions between the surface and the second quadrupole; this converts to an error of less than 0.01 eV in the activation energy (calculated using Figs. 2 and 4). The selection of the ground-state frequency model introduces *ca.* 0.02 eV error in the activation energy. The main errors of RRKM calculation come from the estimation of transition-state frequencies which causes *ca.* ±0.10 eV error in the activation energy determined. The main sources of errors are independent of each other; therefore, they can be treated statistically (the overall error is the square room of the sum of error squares). The overall errors in the determination of activation energy by the present method is therefore estimated to be about ±0.13 eV. As the errors come from various independent sources, none has a particularly large individual effect o the overall reliability of the calculations. If, for example, the error in conversion of SID collision energy to internal energy were twice as large as estimated (20% instead of 10%), the overall error in the determination of activation energy would be increased only from 0.13 to 0.17 eV. To be on the same side, we estimate the overall accuracy of our method, including sources of systematic errors, to be better than ±0.20 eV (5 kcal/mol).

The main sources of error described above are primarily systematic errors. In any comparison of similar or related molecules (e.g. protonated peptides) most of the errors associated with experimental uncertainties will cancel if the same experimental setup is used. The experimental uncertainties include, for example, the determination of the effective temperature of the ions and $T \rightarrow V$ energy converson in SID. The 'characteristic' collision energy of an SID collision efficiency curve can be determined with a reproducibility of *ca.* ±1 eV, which translates to an uncertainty of 0.02 eV in activation energy. The errors of RRKM calculations due to arbitrary transition-state frequency models are not necessarily similar for various peptides because the relative abundance of **a**/**b** ions in the ESI/SID spectra obtained at the inflection-point energy varies with the sequence of the peptide. Nevertheless, it is worth comparing the activation energies obtained by the above method for a few small peptides and investigating how these values are related to intuitive expectations (e.g., the presence or absence of residues containing basic side chains, such as arginine or lysine).

Other protonated peptides

The method described above was applied to the determination of the activation energies of other small peptides. The activation energies corresponding to the main low-energy fragmentation channels are shown in Table 1. Based on the activation energies of peptides listed in Table 1, several general trends can be observed:

1. The activation energies of fragmentation of various peptides fall between 1.5 and 2.0 eV. (Taking into account possible systematic errors (±0.2 eV, see above), this range could shift to 1.3–1.8 or to 1.7–2.2 eV.) This is a relatively small range, reflecting the similarities of peptides and of their fragmentations. As might be expected, the small variation of the E_0 of peptide fragmentations may be one of the underlying factors making peptide sequencing possible by mass spectrometry.
2. The activation energy of non-basic peptides is smaller

Table 1. Average activation energies (E_a) for fragmentation reactions of protonated peptides (eV). The number of degrees of freedom (DOF) of the protonated ion, the characteristic collision energy (E_{chr} in eV) of SID fragmentation-efficiency curves obtained on the octadecanethiolate surface and the E_{chr}/DOF ratio are also shown.

Peptide	Sequence	E_{chr}	DOF	E_{chr}/DOF	E_a
Leucine Enkephalin	YGGFL	33.2	228	0.146	1.56
Trialanine	AAA	20.1	96	0.209	1.75
Pentaalanine	AAAAA	25.4	156	0.163	1.61
Prolyl-tetraalanine	PAAAA	32.8	168	0.195	1.76
Lys^1-tetraalanine	KAAAA	38.8	189	0.205	1.80
Lys^3-tetraalanine	AAKAA	36.2	189	0.191	1.74
Lys^5-tetraalanine	AAAAK	35.9	189	0.190	1.73
Arginyl-tetraalanine	RAAAA	46.8	195	0.240	2.03
des-Arg^1-Bradykinin	PPGFSPFR	78.8	375	0.210	1.92
des-Arg^9-Bradykinin	RPPGFSPF	78.3	375	0.209	1.91
Substance P frag. 4–11	PQQFFGLM-NH_2	58.5	402	0.145	1.60
Substance P frag. 2–11	PKPQQFFGLM-NH_2	79.1	507	0.156	1.67

than that of basic peptides of similar size. As a rough guide, three categories may be constructed: i, non-basic pepteides; ii, basic peptides (containing proline, lysine or, possibly, histidine); and iii, very basic peptides (containing arginine). The activation energy of fragmentation increases by *ca.* 0.2 eV between these three groups of peptides. As examples, comparisons include pentaalanine and leucine enkephalin vs. prolyl-tetraalanine and lysyl-tetraalanine vs. arginyl-tetraalanine, which are small peptides of similar size. Among larger peptides, substance P fragment 4–11 may be compared with des-Arg-bradykinin (Table 1).

This difference qualitatively agrees well with the mobile proton model of peptide fragmentation:[7–9] (a) protonation occurs at the most basic site; (b) the proton migrates to a less basic amide nitrogen on the peptide backbone; and (c) the peptide fragments by cleavage of the amide bond at the protonated amide nitrogen. Migration of the proton from the basic site to the backbone is endothermic; and this is the reason for the higher activation energy of fragmentation of basics vs. non-basic peptides. (For further experimental and theoretical details regarding the 'mobile proton model', see e.g. Refs 7–9.)

Quantitatively, however, this simple explanation is in some disagreement with the activation energies determined. The difference in gas-phase basicity between a 'non-basic' and a 'moderately basic' amino acid (i.e. proline, lysine, or histidine) is *ca.* 0.4 eV; that between a moderately basic amino acid and the bery basic arginine is *ca.* 0.6 eV. These energy differences are much larger than the differences in determined activation energies. A possible explanation for this discrepancy might be that peptides may be protonated at various positions (i.e. not exclusively at the most-basic site), and that these forms are not in thermal equilibrium with each other. Basic sites may be protonated with larger probability than non-basic sites, but not as much as would be required by thermodynamic control. Another possibility is that the RRKM model calculations used in this study provide less accurate activation energies with increasing size.

3. Peptide size also influences the energy activation measured. In groups of similar preptides (e.g. basic or non-basic) smaller peptides require somewhat larger activation energies for fragmentation (Table 1). Comparisons include trialanine vs. pentaalanine or leucin-enkephalin; arginyl-tetraalanine vs. des-Arg-bradykinins; and prolyl- and lysyl-tetraalanine vs. substance P fragments. In the molecular mass range studied (from *ca.* 200 to 1200 Da), the activation energy of fragmentation of a peptide doubled in size is reduced by *ca.* 0.10 eV. A plausible explanation for this dependence is that larger precursors can form larger fragments, which are usually more stable, due to the possibility of internal hydrogen bonds, etc., than smaller ones. As the product ions become more stable, the activation energy decreases. It is also possible that the secondary structure plays a more important role, with increasing size, in proton transfer and/or fragmentation.
4. Based on the small number of systems investigated here, variations in the sequence, but not in amino acid composition, of a peptide appear to affect the activation energy only to a small degree (within the margin of experimental error. This is shown, for example, for lysyl-tetraalanine and des-Arg-bradykinin isomers. Further experimental and theoretical work is necessary to reveal whether these small changes can be attributed to the differences in secondary structure.
5. The characteristic SID collision energies (E_{chr}) of the fragmentation efficiency curves indicate the ease of fragmentation of a peptide. This has an approximately linear correlation with the internal energy of the molecule, as discussed above and also in a very recent publication by Glish and co-workers.[29] An important effect determining E_{chr} is the size of the peptide. If E_{chr} is scaled linearly with the degrees of freedom of the decomposing ion (E_{chr}/DOF constant), a measure of excitation (to a first order of approximation) which is independent of molecular size is obtained. This 'scaled' E_{chr}/DOF value is, furthermore, in good linear correlation with the activation energy, as shown in fig. 5.

$$E_a = 0.92 + 4.44[E_{chr}/\mathrm{DOF}] \quad (5)$$

If, therefore, the E_{chr}/DOF value is scaled to the value of E_a in the case of, e.g., leucine enkephalin, it is then easy to get a measure of approximate activation energy from the ESI/SID fragmentation efficiency curves.

CONCLUSIONS

Evaluation of ESI/SID data using the RRKM rate theory provided information on the average activation energy of low-energy peptide fragmentations. In spite of the early stage of development of this method, the activation energies presented here are in remarkably good agreement with recently reported experimental values determined by thermal decompositions.[1,3,4] Results on various small and medium sized peptides indicate that activation energies of peptide fragmentation fall within a narrow range (*ca.* 35–45 kcal/mol). Differences in activation energy due to amino acid composition (basicity) are qualitatively similar, but much smaller than differences in the gas-phase basicities of the individual amino acid residues. The reason for this is unclear; it may have implications for understanding the precise mechanism of peptide fragmentations. Similarly, better understanding of the effect of peptide size on the average activation energies and internal energy distribution requires further experimental and theoretical investigation.

The method developed here is based on several assumptions and could be refined in future. An enhanced accuracy of temperature measurements and a better knowledge of

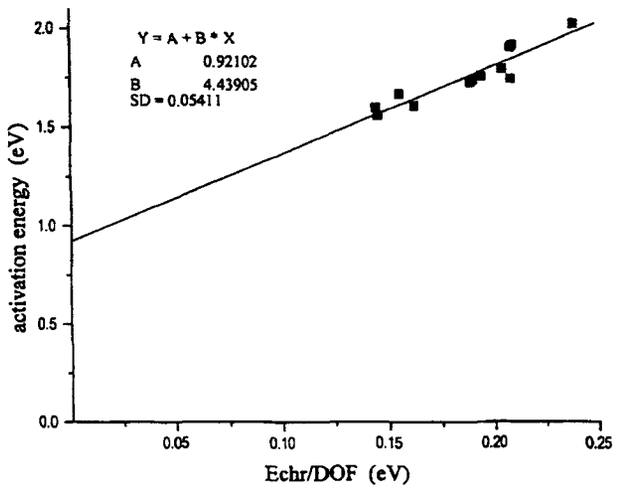

Figure 5. Correlation between activation energy (E_a) and linearly scaled SID characteristic collision energy (E_{chr}/DOF). The line shown satisfies the relationship $Y = 0.92102 + 4.43905X$ (standard deviation 0.05411).

energy transfer both in the capillary/skimmer region and at the surface will obviously make the method more sophisticated. The extension of the present approach to higher energy processes and to rearrangements with low frequency factors is also desirable. Obviously, the method presented in this paper should not be considered as an exclusive way of determining activation energies for peptide fragmentation. Nevertheless, the present approach, together with the results of alternative experimental measurements and theoretical approaches, can help in a better understanding of the energetics and mechanism of fragmentation of protonated peptides.

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