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Electron Transfer Dissociation: Effects of Cation Charge State on Product Partitioning in Ion/Ion Electron Transfer to Multiply Protonated Polypeptides

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Abstract

The effect of cation charge state on product partitioning in the gas-phase ion/ion electron transfer reactions of multiply protonated tryptic peptides, model peptides, and relatively large peptides with singly charged radical anions has been examined. In particular, partitioning into various competing channels, such as proton transfer (PT) versus electron transfer (ET), electron transfer with subsequent dissociation (ETD) versus electron transfer with no dissociation (ET,noD), and fragmentation of backbone bonds versus fragmentation of side chains, was measured quantitatively as a function of peptide charge state to allow insights to be drawn about the fundamental aspects of ion/ion reactions that lead to ETD. The ET channel increases relative to the PT channel, ETD increases relative to ET,noD, and fragmentation at backbone bonds increases relative to side-chain cleavages as cation charge state increases. The increase in ET versus PT with charge state is consistent with a Landau-Zener based curve-crossing model. An optimum charge state for ET is predicted by the model for the ground state-to-ground state reaction. However, when the population of excited product ion states is considered, it is possible that a decrease in ET efficiency as charge state increases will not be observed due to the possibility of the population of excited electronic states of the products. Several factors can contribute to the increase in ETD versus ET,noD and backbone cleavage versus side-chain losses. These factors include an increase in reaction exothermicity and charge state dependent differences in precursor and product ion structures, stabilities, and sites of protonation.

Keywords

electron transfer dissociation; ion/ion reaction; multiply protonated peptide

1. Introduction

Tandem mass spectrometry is a core tool in protein identification and characterization, [1,2] which relies on the judicious selection of ion-type and dissociation method to maximize protein structure information.[3] Common dissociation methods [4] for protein/peptide ions include collision induced dissociation (CID) under a range of conditions, [5, 6, 7] surface-induced dissociation, [8] and infrared multi-photon dissociation (IRMPD).[9,10,11] These

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methods tend to give rise to cleavage at amide linkages, yielding b- and y- type sequence ions, and, if present, cleavages of labile side-chain modifications.[12,13] Ultraviolet photofragmentation of polypeptide ions [14,15] and collisions with metastable atoms [16,17,18] have been shown to have characteristics that distinguish them from the former methods, such as cleavages along other backbone bonds. Prominent among the methods that give rise to fragmentation that is distinct from CID of peptide and protein cations are electron capture dissociation (ECD),[19,20,21] and electron transfer dissociation (ETD), [22,23] which tend to induce backbone N-C α bond cleavages while retaining labile post-translational modifications and protein non-covalent interactions.

The significance of ECD/ETD in protein identification and characterization has prompted both experimental and theoretical studies to provide insights into the factors that underlie the overall processes. In the original ECD studies, free electrons were proposed to be captured initially into high lying Rydberg states and to localize eventually at the protonation sites of the peptide. Neutralization of the protonated site was proposed to generate a hydrogen atom that could subsequently be transferred to a nearby carbonyl group leading to cleavage of backbone N-C α bonds via an aminoketal radical intermediate.[24, 25] Subsequently, it was independently and contemporaneously proposed by Turecek et al. [26] and Simons et al. [27,28] that electrons can be directly captured by or transferred to amide antibonding orbitals, a process that is enabled by the positive coulomb field provided by the excess charges associated with a peptide ion. This idea followed the earlier suggestion that electrons could be captured in a coulomb stabilized σ^* orbital of a disulfide linkage, which can account for the selective cleavage of disulfide bonds in ECD/ETD.[29] This “coulomb assisted dissociation” model for backbone cleavage posits that the characteristic backbone N-C α bond cleavages occur from conformational changes in the excited electronic states of the incipient radical cations. [28] This model does not necessarily require the transfer of a mobile hydrogen atom to a nearby carbonyl group for bond dissociation and can therefore account for the production of c- and z- fragment ions from precursor ions in which excess positive charge is not due to protonation. [30] Simons et al. have also examined theoretically the process by which electrons can be transferred either through bond or through space to an anti-bonding amide bond orbital from relatively low-lying Rydberg orbitals of a charged site of peptide side chain.[31,32,33] The peptide charge state is relevant to the ECD/ETD mechanism in that it is associated with differences in the cation recombination energy, the electronic structures of precursor and product ions, the electrostatic field in the ions for the initially captured or transferred electrons, the higher order structure of peptide ions, the kinetic stabilities of the products, etc. For example, cation charge state for a given polypeptide ion is known to have significant effects on the dissociation of peptides ions in both ECD and ETD experiments in terms of dissociation patterns, sequence coverage, etc. [34,35,36]

We have approached the study of factors that affect ETD by making systematic measurements of ion/ion product partitioning using model polypeptide systems. For example, we have used it to study the characteristics of the reagent anion in the competition between electron transfer and proton transfer [37], in the role of the protonation site within a given charge state, [38] and in the possible role of the amide hydrogen in the mechanism of ETD.[39] In this study, systematic product partitioning measurements were made to examine the effects of cation charge state on the competition between various channels in an ion/ion reaction that leads, at least in part, to ETD. These include proton transfer versus electron transfer (PT versus ET), electron transfer with subsequent dissociation (ETD) versus electron transfer with no dissociation (ET,noD), and fragmentation of backbone bonds (c,z-ETD) versus fragmentation of side chains (side-chainETD). Multiply protonated tryptic peptides were examined due to their relevance in widely used bottom-up proteomics. Model non-tryptic peptides and peptides of relatively large sizes were also employed to

reduce the complexity of the peptide composition and to investigate the effects of higher charge states, respectively.

2. Experimental Section

Materials

Model peptides, RGAGGRGAGGHL, RGAGGHGAGGRL, HGAGGRGAGGRL, RGAGGRGAGGRL, and RGAGGKGAGGRL were custom synthesized by CPC Scientific (Dublin, CA). Tryptic peptides were produced using digestion protocols reported elsewhere [40] from horse myoglobin, which was purchased from Sigma-Aldrich (St. Louis, MO) together with azobenzene and melittin from honey bee venom. Neuropeptide W-30 (human) and pTH-Related Protein (1–34) amide (human, rat) were obtained from Bachem Americas, Inc. (Torrance, CA). Methanol and glacial acetic acid were purchased from Mallinckrodt (Phillipsburg, NJ). All peptides were dissolved to a concentration of 10 μ M in 50/49/1 water/methanol/acetic acid solution for positive nanoelectrospray ionization.

Mass Spectrometer

All experiments were performed on a commercial quadrupole/time-of-flight (TOF) tandem mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, Concord, ON, Canada) modified for ion/ion reactions.[41] Coupled directly to the interface was a home-built dual ion source, [42] which consisted of a nanoelectrospray ion source to generate positive ions and an atmospheric pressure chemical ionization (APCI) source to produce reagent radical anions. All electronics were controlled by Daetalyt 3.14, a version of research software developed by AB Sciex. Electron transfer ion/ion reactions were implemented in Q2 linear ion trap in mutual storage mode, the procedure for which has been detailed elsewhere.[43] Briefly, the typical experiment consisted of four major steps: mass-resolved cation injection into Q2 (300 ms), mass-resolved anion injection into Q2 (300 ms), mutual storage of both polarities in Q2 (150 ms), and mass analysis of ion/ion reaction products by TOF (100 ms). Azobenzene was used to generate the electron transfer reagent as this is a convenient compound to use in our laboratory and provides moderately high electron transfer efficiencies. Other reagents, such as fluoranthene or azulene [22, 44], are commonly used in commercial systems and have been reported to give somewhat higher electron transfer efficiencies than azobenzene. However, under the APCI conditions used on our work, the radical anions of these compounds are rapidly and efficiently oxidized to species that do not undergo electron transfer with peptide cations. While the absolute partitioning can be expected to differ for different reagents, the general trends are expected to be consistent for all reagents, based on our experience with a variety of reagents [37]. Quantitative information about the two major components in the charge reduced species, i.e. proton transfer products and electron transfer products without subsequent dissociation, was obtained by manual deconvolution of the corresponding charge reduced precursor ion peaks with the assumption of Gaussian peak shape.

3. Results and Discussion

The effect of charge state on the dissociation of polypeptides upon electron transfer from a radical anion was investigated by reacting azobenzene anions with different charge states of peptides including tryptic peptides of myoglobin, model peptides, and peptides of relatively large size. The results from reaction of azobenzene anions with the triply and doubly protonated ions of the tryptic peptide of HLKTEAEMK are shown in Figures 1(a) and (b), respectively, which represent the typical dissociation behavior of peptides of different charge states investigated in this study. A range of reaction phenomena are noted in Figure 1. A prominent phenomenon is the dissociation of the polypeptide cation, which includes the

cleavage of peptide backbone producing the c- and z- type fragment ions (the latter were detected as radical species) as well as reactions giving neutral losses, as indicated by the ammonia losses in this particular case. Charge reduction is another dominant channel as evident by the abundant charge reduced precursor ion species in both Figures 1(a) and (b). From the comparison of the theoretical isotope distribution of the doubly protonated peptide with the experimental isotope distribution of the charge reduced precursor ion of the same charge state, which is shown in the inset of Figure 1(a), the existence of intact products from both proton transfer and electron transfer products is evident. The intact electron transfer product ion is one mass unit higher than the proton transfer product and therefore the isotope envelopes from these two types of products overlap. The ion/ion reaction of the triply charged peptide with azobenzene anions shown in Figure 1(a) gives full sequence coverage for the peptide via production of 7 c-ions and 7 z-ions, which is in sharp contrast with the results from the doubly charged version, which shows only 37.5% sequence coverage from the formation of 2 c-ions and 1 z-ion. The production of higher sequence coverage from a higher peptide charge states has also been noted in previous studies [35,36] and is a common observation at high ratios of charge/residue. One systematic study, however, did not note this tendency [45], although post-activation of the charge reduced ion was employed, thereby precluding a direct comparison.

To clarify the effect of cation charge state on the dissociation of multiply protonated peptide ions in an electron transfer dissociation experiment, it is instructive to account for all of the competing channels into which the products can be partitioned. Proton transfer (PT) is always possible in an ion/ion reaction involving a multiply protonated polypeptide regardless of the identity of the reagent anion and, therefore, can compete with electron transfer (ET) when an ET reagent anion is used. Despite the high exothermicity associated with proton transfer from a multiply protonated polypeptide to a reagent anion, dissociation of the polypeptide is rarely observed as a result of ion/ion proton transfer.[46] Therefore, essentially all products from proton transfer are present in the post-ion/ion spectrum as the deprotonated peptide reactant species. On the other hand, dissociation of a multiply protonated polypeptide is commonly observed upon electron transfer. Within the dissociation channel, either a peptide backbone N-C α bond cleaves to produce c- and z- type sequence ions, an informative reaction channel referred here as c,z-ETD, or a side chain can be lost from some residues, which is denoted here as side-chainETD. The loss of a hydrogen atom upon electron capture is well-known [47]; however, hydrogen atom loss following electron transfer is indistinguishable from a proton transfer and, if it occurred, was treated here as proton transfer. Some peptide ions can survive the transfer of an electron, which leads to a hydrogen-rich charge reduced intact species, a reaction channel referred here as ET,noD. Charge reduction (i.e., the ET,noD channel) is undesirable when the objective is ETD and significant attention has been paid to minimizing the ET,noD products either by activating the peptide ions during the reaction, either thermally [40] or by photoactivation [48], or after formation, via collisional activation [49,50] or infra-red photodissociation [51]. In this work, we avoid such measures because we are interested in the contribution of charge reduction channel in the absence of any additional measures. The product partitioning examined here for a peptide cation/radical anion reaction is shown schematically in Figure 2.

Assuming no ion losses in the experiments, the sum of products from proton transfer, ET,noD, and ETD represents all the precursor ions that undergo an ion/ion reaction. The percentage partitioning among these three channels can be calculated using the equations below, respectively:

$$\%PT = \frac{\sum PT}{\sum PT + (ET, noD) + ETD} \times 100 \quad (1)$$

$$\%ET, noD = \frac{\sum ET, noD}{\sum PT + (ET, noD) + ETD} \times 100 \quad (2)$$

$$\%ETD = \frac{\sum ETD}{\sum PT + (ET, noD) + ETD} \times 100 \quad (3)$$

The total products from ETD and ET,noD channels account for the contribution from the ET channel, which is given in percentage by:

$$\%ET = \%ET, noD + \%ETD \quad (4)$$

The percentage partitioning from the two competing classes of dissociation reaction, c,z-ETD and side-chainETD, within the overall ETD channel is, respectively, represented by:

$$\%c, z - ETD = \frac{\sum c, z}{\sum ETD} \times 100 \quad (5)$$

$$\%side - chainETD = \frac{\sum side - chain}{\sum ETD} \times 100 \quad (6)$$

In this work, we have not examined in detail the specific partitioning among the various amino acid side-chain losses or among the competitive channels that give rise to N-C_α bond cleavage as much larger sets of peptide ions would be required to see statistically significant charge dependent patterns. Side-chain losses from ETD of a large set of peptides ions have been examined [52] and statistical analyses of the backbone cleavages channels from ETD have been reported [53,54], although precursor charge state was not the primary focus of these works and supplemental activation was used, at least in some instances.

The product partitioning into various reaction channels for the tryptic peptide shown in Figure 1 is summarized in Table 1 along with the results from other tryptic peptides of myoglobin. The extent of the ion/ion reactions for the product partitioning calculation was limited in all peptides studied to produce largely first generation products by using a short ion/ion reaction time. The minimal sequential reaction effected by a short ion/ion reaction time is reflected in Figure 1(a) by the low abundance of the second generation charge reduced precursor ions relative to that of the first generation charge reduced precursor ions. Product partitioning into the various channels shown in equations (1) – (6) was also determined for three model peptides and three peptides of relatively large size, which is summarized in Table 2 and 3, respectively. The comparison of product partitioning between various direct competing channels, i.e. PT versus ET, ETD versus ET,noD, and c,z-ETD versus side-chainETD, are discussed in the following sections.

Effects of Cation Charge States on PT versus ET

When ETD is the objective, PT is a deleterious side reaction that is absent in the ECD process. The competition between proton transfer and electron transfer can be affected by several factors including both the identities of electron transfer reagent anions and the identities and locations of the charge carrying sites, which have been studied previously. [37,38,55] The cation charge state is also expected to play a significant role in the competition between the two reaction channels, since the number of charges on a multiply protonated polypeptide cation strongly affects energy surfaces over which ion/ion reactions proceed, which influences the product partitioning into the two channels. Both proton transfer and electron transfer are highly exothermic reactions, with the former being thermodynamically favored over the latter for most cation/anion combinations. The lower exothermicity associated with electron transfer leads to the crossing of the reactant and product states at a larger distance for electron transfer than for proton transfer. If the probability for a net crossing is high at this point, electron transfer can be kinetically favored over proton transfer despite its being thermodynamically disfavored. We have modeled the fraction of ion/ion reactions that occur via electron transfer by estimating the cross-section for electron transfer using a curve-crossing picture and by modeling proton transfer as proceeding via a long-lived complex (see below). The assumption is that any ion/ion encounter that does not lead to electron transfer at a crossing-point will result in proton transfer. We note that there may also be proton transfer at a crossing point and that this cross-section may be higher than the cross-section for formation of a long-lived complex at high charge states. Hence, by modeling proton transfer as proceeding exclusively via a long-lived complex, we may underestimate the total PT cross-section. However, the location of the crossing point for proton transfer is within that for the crossing point for electron transfer. Hence, the qualitative dependence of the fractional contribution from ET as a function of peptide charge will be reflected in this model.

The cross-section (σ) for each of the two processes, PT or ET, is given by

$$\sigma_{rxn} = P_{rxn} \pi b_{rxn}^2 \quad (7)$$

where P_{rxn} is the probability of reaction at classical impact parameter, b_{rxn} . The square of the impact parameter (in atomic units) for each of the interactions involving a multiply charged cation and a singly charged reagent anion takes the form:

$$b_{rxn}^2 = r_{rxn}^2 \left[1 + \frac{2Z_{cat}}{r_{rxn}\mu v_{rel}^2} \right] \quad (8)$$

where Z_{cat} is the unit charge of the analyte cation; μ is the reduced mass; v is the relative velocity of the two collision partners, and the r_{rxn} is the reaction distance. In the case of proton transfer reaction via complex formation, which can be considered as a hard sphere collision, the reaction distance can be estimated as the sum of the radii of the two collision partners. In the case of electron transfer, an estimate of the reaction distance r_{ET} can be taken as the distance at which the ground state curves of the entrance channels cross with those of the exit channels, which is given (in atomic units) for a multiply charged cation and a singly charged anion as

$$r_{ET} \approx \frac{(-Z_{cat})}{\Delta H_{ET}} \quad (9)$$

where ΔH_{ET} is defined by

$$\Delta H_{ET} = EA(A) - RE(MH_n^{n+}) \quad (10)$$

where the EA(A) is the electron affinity of the reagent A and the RE is the recombination energy of the analyte cation. The cation recombination energy is assumed here to take the form:

$$RE(Z_{cat})(eV) = 5.4 + 1.1^*(Z_{cat} - 1) \quad (11)$$

The assumed linear relationship between cation RE and its charge state is based on a recent study showing that for polypeptide cations, there is an average increase of 1.1 eV/charge in ionization energy, which is expected to be mirrored in the ion recombination energy. [56] This relationship is assumed to be most applicable to cases in which single excess charges are dispersed to different sites within the molecule, as is the case with multiple protonation of polypeptides.

In the case of hard sphere collisions, we assume that proton transfer occurs when the two partners come into physical contact (i.e., $P_{hs} = 1$) such that the cross-section for complex formation can be estimated as πb_{hs}^2 . However, the probabilities of electron transfer at the crossing point, at which the energies for the entrance and exit channels are equal, are usually treated using a model based on Landau-Zener theory. [57,58] The net probability for electron transfer can be expressed as

$$P_{ET} = 2P_{LZ}(1 - P_{LZ}) \quad (12)$$

where P_{LZ} represents the so-called Landau-Zener probability and is given by

$$P_{LZ} = e^{-\frac{2\pi H_{12}^2 \langle \chi_{1v'} | \chi_{2v''} \rangle^2}{\frac{dr}{dt} \left(\frac{Z_{cat}}{r_{ET}^2} \right)}} \quad (13)$$

where H_{12}^2 is the coupling matrix element at the point of the closest approach, $\langle \chi_{1v'} | \chi_{2v''} \rangle^2$ represents the Franck-Condon overlap between reactant and product vibrational wave functions associated with the transition from $1v'$ to $2v''$, dr/dt is the radial velocity of the reactants at the crossing point, and the remainder of the denominator gives the difference in slopes of the entrance and exit channels at the crossing point. The slope of the exit channel is very small when one of the reactants is singly charged because the corresponding product is a neutral species, which makes the exit channel that of an ion/molecule interaction. We therefore assume the slope of the exit channel to be zero in this work. The radial velocity at the crossing point can be estimated as:

$$\frac{dr}{dt} \approx \left[\frac{2Z_{cat}}{r_{ET}\mu} \right]^{0.5} \quad (14)$$

The coupling matrix element in equation (13), H_{12}^2 , which indicates the strength of the electronic coupling between states, is a very difficult term to evaluate *a priori* for the large biomolecule systems investigated in this study. However, a parameterized expression for H_{12}^2 has been reported by Olson and co-workers [59,60], which is based on data collected from reactions of a number of relatively small singly charged ions and was used recently in the investigation of parameters that affect the proton transfer and electron transfer in ion/ion reactions of polypeptide cations. [37]

$$H_{12} = \left(1.044 \sqrt{EA} \sqrt{RE} \left(\frac{\sqrt{2EA} + \sqrt{2RE}}{2} \right) r_{ET} \right) \times \exp \left(-0.857 \left(\frac{\sqrt{EA} + \sqrt{2RE}}{2} \right) r_{ET} \right) \quad (15)$$

With all of the relationships and parameters just described, it is possible to estimate qualitatively the fraction of products directed into the electron transfer channel with the masses of the reactants, the EA of the reagent, and the RE and charge state of the analyte cation, and the Franck-Condon factors for the anion, using the following relationship:

$$\frac{\sigma_{et}}{\sigma_{tot}} = \frac{P_{et} \pi b_{et}^2}{P_{et} \pi b_{et}^2 + (1 - P_{et}) \pi b_{hs}^2} \quad (16)$$

Figure 3 shows the predicted fraction of electron transfer as a function of polypeptide cation charge state in an ion/ion reaction using the radical anion of azobenzene as the reagent. The input data to the calculation include a 300 K cation with mass of 1000 Da, a 300 K anion with mass of 180 Da, electron affinity of 0.56 eV, and Franck-Condon overlap of 0.18 [37]. The solid curve corresponds to a protein cation with charges sequestered on the surface of the ion, while the dashed line corresponds to a situation where charges are taken as a point charge in the core of the protein cation. The solid and dashed curves demonstrate qualitatively similar effects of cation charge state on the percentage of products directed into the electron transfer channel, with the former predicting a consistently higher contribution from electron transfer than the latter, which is largely due to the increased impact parameter when charges are located on the cation surface. Both curves predict an increased fraction of electron transfer with charge state at low cation charge states, which is consistent with the experimental data collected from doubly and triply charged tryptic peptides and model peptides shown in Tables 1 and 2 (columns 2 and 3) as well as the data collected for the triply and quadruply charged melittin cations (Table 3, Rows 3, 4 and columns 2, 3).

Both cases also predict a decrease in the relative contribution of electron transfer beyond the maximum predicted for $Z_{CAT}=4+$. The model, however, is predictive only for the ground states of the entrance and exit channels. When this crossing point occurs at large distances where P_{ET} for the ground states is low (see Figure 4(a)), there are no other states that can be engaged in electron transfer. This situation prevails on the low side of the maximum of Figure 3. On the other hand, when the crossing point is on the other side of the P_{ET} curve (see Figure 4(b)), crossings that populate excited electronic states of the products can be probable. Hence, a decrease in the overall contribution from electron transfer may not be observed experimentally at high charge states due to the contributions from excited electronic states. Indeed, no clear decrease in the contribution of electron transfer is noted in these experiments.

Effects of Cation Charge State on ETD versus ET,noD

The %ETD and %ET,noD for different charge states of the three categories of polypeptides are listed in columns 4 and 5 in Tables 1–3. The ratio of the ETD/ET,noD channels (i.e., the ratio of columns 4 and 5) is shown in column 6. A consistent observation of increased ETD relative to ET,noD with cation charge state is noted for almost all peptides investigated, which has also been observed in most previous studies [35,36]. An exception [53] employed post-activation of the ET,noD products, which precludes a direct comparison with this work. The increase in the ratio of ETD/ET,noD is most striking for the ions going from $Z_{CAT}=2+$ to $Z_{CAT}=3+$. Several factors likely contribute to this observation. This includes the fact that the electron transfer product from the $Z_{CAT}=2+$ reaction is singly charged and therefore experiences no internal electrostatic repulsion that might facilitate the separation of

fragments of like charge generated by backbone cleavages. A second effect may arise from differences in the extent of stabilizing hydrogen bonding and dipole-dipole interactions in the precursor ions due to the differences in the internal electrostatic repulsion in the precursor ion. The tendency to minimize electrostatic repulsion, for example, can disrupt some non-covalent intramolecular interactions that otherwise might prevent fragments from separating. Evidence for these effects is apparent from the effectiveness associated with the simultaneous and post-activation methods for increasing ETD for doubly protonated peptides. [40,48,49,50,51] The cation recombination energy also increases with cation charge state resulting in an increase in the reaction enthalpy. Furthermore, a mechanistic effect associated with differences in the electronic state structures associated with cations of different net charge cannot be precluded.

Despite the general trend for increasing contribution of ETD relative to ET,noD with cation charge there is considerable variation in the differences seen from a given charge state to the next for different peptide cations. This is clearly seen for the model peptides of Table 2, for example. The differences noted in the comparisons of the $Z_{CAT}=2+$ and $Z_{CAT}=3+$ charge states are consistent with previous systematic studies designed to examine the roles of the sites of protonation.[38] For example, ET,noD has been noted to be maximized when protonation takes place at a histidine residue, which has been attributed to the exothermic isomerization of the His (3H)-imidazoline radical to the more stable (2H)-imidazoline isomer under sterically favorable conditions.[61,62] For a given charge state, the histidine containing model peptides all show higher contributions from ET,noD than the peptide ions without histidine. Lysine was noted as giving rise to the least ET,noD and this is consistent with the near absence of the charge reduced electron transfer product for the triply protonated RGAGGKGAGGRL peptide. When doubly protonated, however, this peptide, which is likely to be protonated at the arginine residues, shows a relatively large ET,noD contribution. Site of protonation effects are less readily apparent in the data of Tables 1 and 3 due to the more random nature of the sequences in these systems. However, Table 2 clearly shows that site of protonation modulates the effect of total charge on the relative contributions of ETD and ET,noD.

Effects of Cation Charge States on c,z-ETD versus side-chainETD

The value of ETD as a structural probe lies in the generation of sequence informative c- and z-ions, although side-chain losses can provide composition information that can be of use in peptide identification.[52] Within the overall ETD channel, there is a clear peptide charge state dependence associated with the appearance of backbone cleavage fragments versus side-chain fragments. The effects of cation charge states on the product partitioning between the backbone cleavage and neutral losses for the three categories of polypeptides are illustrated in columns 7 and 8 in Tables 1–3. A consistent trend is apparent for all peptides studied that shows increased contributions from backbone cleavages as the charge state of a given peptide increases. There are, however, relatively large variations in the product partitioning into either c,z-ETD or side-chainETD channels for different polypeptides of same charge states. The identities of the protonation sites are likely factors in this variation, as is apparent from the results for the model peptides of Table 2. Of the three common basic amino acid residues (i.e., lysine, histine, and arginine), arginine is the most prone to side-chain loss upon electron capture and histidine is the least. [38] All of the peptides in Table 2 have at least two arginine residues in the sequence and all of the ions showed significant side-chain loss, which was dominated by losses associated with the arginine side-chain (data not shown). The triply protonated peptide that showed the largest contribution from side-chain loss (53%) was that of RGAGGRGAGGRL whereas the doubly protonated peptide that showed the least side-chain loss (62%) was that of HGAGGRGAGGRL. These and

other observations indicate that, in addition to charge state, the identities of the protonation sites affect the extent of side-chain loss.

As with the competition between ETD and ET,noD, the Coulomb fields in the precursor and product ions are expected to play significant roles in affecting the product partitioning between c,z-ETD and side-chainETD channels. The Coulomb field in the precursor, for example, plays roles in determining the sites of protonation, the three dimensional structure of the ion (including the extent of intramolecular hydrogen bonding), and the electronic state structure of the ion, which can influence the evolution and eventual localization of the captured electron. In the case of the electron transfer peptide product ion, electrostatic repulsion, or lack thereof, can influence the overall stability of the electron transfer product as well as the relative propensities for the competitive cleavage channels. For example, side-chains are lost essentially exclusively as neutral species in ETD whereas backbone cleavages are usually accompanied by charge separation when the product ion is multiply charged. The side-chain loss channels can be expected to be relatively more prominent at low charge states because they are not strongly affected by electrostatic repulsion and the neutral side-chains are generally less likely to be engaged in stabilizing intramolecular interactions that might otherwise prevent fragments from separating from one another. The success of simultaneous activation and post-activation methods in enhancing the production of c- and z-ions from electron transfer to doubly protonated peptides is strong evidence that stabilizing interactions in the precursor and product ions play a role in the 'spontaneous' production of peptide backbone fragments.

The Coulomb field in the precursor ion, however, may also play a role via Coulomb stabilization of some electronic states that can lead to ETD. According to the direct attachment version of the "Coulomb assisted direct electron attachment" model [26–28] put forward by Turecek et al. and Simons et al., electrons can be directly captured by the amide π^* antibonding orbital if Coulombic stabilization is sufficiently high. Alternatively, electrons evolve from higher to lower Rydberg orbitals with eventual intramolecular electron transfer to amide antibonding orbitals, which leads to the backbone dissociation of the ion. [32,33] The probability for either electron capture or transfer to amide antibonding orbitals is therefore expected to be dependent upon charge state for a given polypeptide ion. If the antibonding orbitals are less likely to be populated due to a low Coulomb field, the electrons will more likely remain localized at the protonation sites leading to greater side-chain loss.

4. Conclusions

This study is the first to compare simultaneously the charge state dependencies of the competitions between proton transfer versus electron transfer (PT vs. ET), electron transfer with dissociation versus without dissociation (ETD vs. ET,noD), and side-chain cleavage versus backbone cleavage (side-chainETD vs. c,z-ETD) for an ion/ion reaction involving multiply protonated peptides and an electron transfer reagent anion. For the systems examined here, ET increase relative to PT as the peptide charge state increases. This observation is consistent with a model based on Landau-Zener theory, which predicts an optimum charge state for a given state-to-state transition. However, due to the large number of accessible excited states associated with a large polypeptide ion, charge states higher than the optimum for a ground state-to-ground state transition may show high electron transfer efficiencies because excited states with high transition probabilities are accessible. This situation does not prevail for charge states below the optimum value. In other words, a peptide can carry too little charge for efficient electron transfer but probably not too much, at least for the charge range accessible by electrospray.

The study of charge state effects on the fate of the electron transfer products (i.e., ETD versus ET,noD and c,z-ETD versus side-chainETD) is complicated by multiple effects that changing the charge state can have on a peptide ion. These effects include, for example, changes in precursor conformation, sites of protonation, alteration of the electronic state structure, etc. as well as changes in product ion conformations and stabilities, relative propensities for competitive cleavage reactions, etc. It is difficult to separate the possible contributions from each of these factors. Nevertheless, some general conclusions can be drawn. Electron transfer with subsequent dissociation (ETD) increases with polypeptide charge relative to charge reduction without dissociation (ET,noD). This effect is clearly noted in comparing the $Z_{CAT}=2+$ and the $Z_{CAT}=3+$ charge states and is likely amplified by the fact that the charge reduced peptide is singly charged in the case of the $Z_{CAT}=2+$ precursor and lacks internal electrostatic repulsion. Major changes in ETD versus ET,noD are also noted for the larger polypeptides at higher charge states, which is also consistent with the importance of electrostatic repulsion in the products and the importance of the charge/residue [34] and its effect on the internal Coulomb field, in addition to total absolute charge.

Among the ETD channels, charge state also plays a significant role in the competition between backbone cleavage and side-chain loss with the former increasingly prominent as the charge state increases. The Coulomb field of the electron transfer product also likely plays a role here, in addition to other factors, such as the identity of the protonation site. However, this observation may also reflect the role of the Coulomb field in determining which electronic states are populated either initially or upon subsequent electron transfer.

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Highlights

- The z-state dependencies of the competitions between PT vs. ET, ETD vs. ETnoD, and side-chain cleavage vs. backbone cleavage in ETD are described.
- ET increase relative to PT as peptide z-state increases.
- ETD increases relative to ET,noD as peptide z-state increases.
- Back-bone cleavage increases relative to side-chain loss as z-state increases.
- Factors that underlie these trends are discussed.

Figure 1. Spectra derived from ETD reactions of azobenzene radical anion with (a) triply and (b) doubly protonated tryptic peptide, HLKTEAEMK, from myoglobin.

Figure 2. Schematic diagram of the product partitioning in a reaction between a multiply protonated peptide and a radical anion.

Figure 3. Predicted ratio of electron transfer in an ETD process as a function of cation charge state, Z_{cat} . The solid curve corresponds to charges on the surface of the protein cation, while the dashed curve corresponds to charges in the core of the cation.

Figure 4. Schematic energy diagrams showing the entrance and exit channels for an ion/ion reaction involving a multiply protonated molecule, MH_n^{n+} , and a singly-charged reagent anion, $A^{\bullet}$. On the left of each diagram is a schematic plot of P_{ET} for an electron transfer involving ground states of the entrance and exit channels. (a) A depiction of the situation that prevails when the crossing point is located at the low energy side of the P_{ET} curve. (b) A depiction of the situation that prevails when the crossing point is located on the high energy side of the P_{ET} curve.

Table 1
Ion/Ion Reaction Product Partitioning for Triply and Doubly Charged Tryptic Peptide Cations in Reaction with Azobenzene Radical Anions

Peptide (charge state)	% PT	%ET	%ETD	%ET.noD	ETD/ ETnoD	%c,z-ETD	%side-chainETD
HLKTEAEMK (3+)	33±1	67±1	47±2	20±1	2.4	87±1	13±1
HLKTEAEMK (2+)	65±2	35±2	22±3	14±1	1.6	69±3	31±3
HPGDFGADAQGAMTK (3+)	35±1	65±1	48±3	17±1	2.8	83±2	17±2
HPGDFGADAQGAMTK (2+)	74±1	26±1	6±1	20±1	0.3	3±1	97±1
VEADIAGHGQEVLR (3+)	49±1	51±1	33±2	17±1	1.9	55±1	45±1
VEADIAGHGQEVLR (2+)	77±1	23±1	7±1	15±1	0.5	8±1	92±1
KHGTVVLTALGGILK (3+)	32±1	68±1	48±3	20±2	2.4	86±1	14±1
KHGTVVLTALGGILK (2+)	89±1	11±1	1±1	10±1	0.1	<1	~100
HGTVVLTALGGILK (3+)	38±1	62±1	48±1	13±1	3.7	60±1	40±1
HGTVVLTALGGILK (2+)	61±1	39±1	15±2	24±2	0.6	56±1	44±1

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Ion/Ion Reaction Product Partitioning for Triply and Doubly Charged Model Peptide Cations in Reaction with Azobenzene Radical Anions

Table 2

Peptide (charge state)	% PT	%ET	%ETD	%ET,noD	ETD/ ETnoD	%c,z-ETD	%side- chainETD
RGAGRGAGGHL (3+)	24±1	76±1	63±1	13±1	4.8	65±2	35±2
RGAGRGAGGHL (2+)	52±1	48±1	18±1	31±1	0.6	19±1	81±1
RGAGGHCAGGRL (3+)	25±1	75±1	59±1	15±1	3.9	66±1	34±1
RGAGGHCAGGRL (2+)	50±1	50±1	14±1	36±1	0.4	24±2	76±2
HGAGRGAGGRL (3+)	28±1	72±1	53±1	19±1	2.8	65±1	35±1
HGAGRGAGGRL (2+)	53±1	47±1	21±1	26±1	0.8	38±2	62±2
RGAGRGAGGRL (3+)	35±1	65±1	63±1	2±1	32	47±1	53±1
RGAGRGAGGRL (2+)	51±1	49±1	24±1	25±1	1.0	10±1	90±1
RGAGGKGAGGRL (3+)	27±1	73±1	73±1	<1	>73	63±1	37±1
RGAGGKGAGGRL (2+)	54±1	46±1	12±1	34±1	0.4	9±1	91±1

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Table 3
Ion/Ion Reaction Product Partitioning for Multiply Charged Larger Peptide Cations in Reaction with the Azobenzene Radical Anions

Peptide (charge state)	% PT	%ET	%ETD	%ET,noD	ETD/ ETnoD	%c,z-ETD	%side- chainETD
Melittin (5+)	34±1	66±2	48±1	18±1	2.7	64±1	36±1
Melittin (4+)	56±1	44±3	24±2	19±2	1.3	16±3	84±3
Melittin (3+)	88±1	12±3	9±1	3±1	3.0	7±2	93±2
Neuropeptide W-30 (6+)	42±3	58±3	45±4	14±2	3.2	72±5	28±5
Neuropeptide W-30 (5+)	50±1	50±1	38±1	12±1	3.3	67±3	33±3
Neuropeptide W-30 (4+)	68±1	32±1	10±1	22±1	0.5	26±2	74±2
pTH related protein (7+)	44±4	56±4	40±6	16±2	2.5	84±1	16±1
pTH related protein (6+)	54±1	46±1	24±1	22±1	1.1	79±1	21±1
pTH related protein (5+)	65±2	35±2	16±1	19±1	0.8	52±3	48±3
pTH related protein (4+)	76±3	24±3	11±1	13±2	0.8	2±1	98±1