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Electron Capture Dissociation of Gaseous Multiply-Charged Proteins Is Favored at Disulfide Bonds and Other Sites of High Hydrogen Atom Affinity

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Abstract: Disulfide bonds in gaseous multiply-protonated proteins are preferentially cleaved in the mass spectrometer by low-energy electrons, in sharp contrast to excitation of the ions by photons or low-energy collisions. For S−S cyclized proteins, capture of one electron can break both an S−S bond and a backbone bond in the same ring, or even both disulfide bonds holding two peptide chains together (e.g., insulin), enhancing the sequence information obtainable by tandem mass spectrometry on proteins in trace amounts. Electron capture at uncharged S−S is unlikely; cleavage appears to be due to the high S−S affinity for H• atoms, consistent with a similar favorability found for tryptophan residues. RRKM calculations indicate that H• capture dissociation of backbone bonds in multiply-charged proteins represents nonergodic behavior, as proposed for the original direct mechanism of electron capture dissociation.

Introduction

Electrospray ionization[1] has extended the unique capabilities of tandem mass spectrometry (MS/MS) to the structural characterization of large molecules,[2] even in 10^{-17} mol amounts.[3] MS/MS has been especially applicable to *linear* biomolecules, such as proteins[2−4] and nucleotides,[2,5] because backbone cleavage of the mass-selected multiply protonated molecule yields products whose masses are directly indicative of the sequence. For proteins, adding energy by methods such as collisionally activated dissociation (CAD)[6] causes specific cleavage at amide bonds to yield *b*- and *y*-type (N- and C-terminal) fragment ions (eq 1). These data have proved to be especially useful in identifying proteins and locating errors in the DNA derived sequence, post-translational modifications, and derivatized active sites.[2−4] However, critical stabilization in many extracellular proteins is due to the branching or cyclization of internal

$$R-\overset{\overset{\text{O}}{\|}}{C}\text{-}\underset{b}{\overset{}{\vdots}}\text{-}\underset{y}{\overset{\overset{\text{H}^+}{|}}{N}H}\text{-}CHR'\text{-} \tag{1}$$

disulfide bonds. The multiple N- and C-termini of a branched protein produce overlapping *b* and *y* ions, and a cyclic ion yields structurally ambiguous products that arise from cleavage of any two of its ring bonds. Low-energy CAD yields little S−S bond dissociation.[7] It is customary before MS/MS measurement to reduce S−S bonds (usually followed by −SH alkylation), greatly increasing analysis time and sample requirements. Disulfide linked polypeptide ions formed by matrix-assisted laser desorption ionization do exhibit S−S cleavages for spontaneous (lowest energy) dissociations; with proteolytic digestion, this can provide effective mapping of the disulfide bond positions.[8] We report here that S−S bonds in gaseous multiply-charged protein ions are preferentially cleaved by the new technique of electron capture dissociation (ECD)[9] to provide key sequence information in addition to that from its unique *c*, *z*• (eq 2) cleavages and *b*,

$$R-\overset{\overset{\text{O}}{\|}}{C}\text{-}NH\text{-}CHR'\text{-} \xrightleftharpoons{\cdot H\text{----}} R-\overset{\overset{\text{OH}}{|}}{\underset{\cdot}{C}}\text{-}NH\text{-}CHR'\text{-} \longrightarrow R-\underset{c}{\overset{\overset{\text{OH}}{|}}{C}}=NH + \underset{z\cdot}{\cdot CHR'\text{-}} \tag{2}$$

y fragment ions from energetic dissociation.[10] However, this S−S bond preference is in conflict with our original assumption[9] that *c*, *z*• ions are produced by electron capture at a proton on

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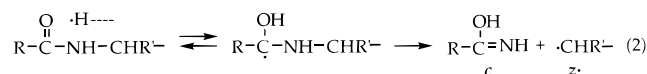
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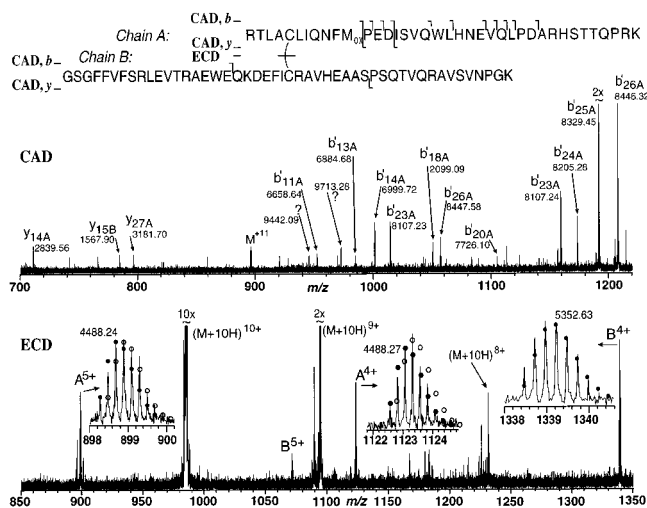(10) The omitted protons in these equations are discussed in the text.

a carbonyl group; the proton affinity of S—S is low relative to many other protein functionalities.

Experimental Section

Peptides were from Ted Thannhauser of the Cornell Biotechnology Facility, the 10 and 49 kDa samples are from the IgE protein from Hannah Gould, the ribonuclease is from Harold Scheraga, and the porcine insulin is from Sigma.

MS/MS spectra were obtained from ESI-produced ions trapped in a modified Finnigan 6T Fourier transform (FT) instrument.[9,11–13] The precursor ions were isolated by SWIFT[14] and interacted with low-energy (<0.2 eV) electrons from an external electrically heated filament, cooled simultaneously with a 10^{-5} Torr Ar pulse. An extensive description of the instrumentation and operating parameters is in preparation.[13] Peaks were not recorded below m/z 500, so that additional terminal fragment ions of $m < 500$ could have been formed.

Results and Discussion

Capture of a low-energy (<0.2 eV) electron by an $(M + nH)^{n+}$ protein ion yields the odd-electron reduced ion $(M + nH)^{(n-1)+\bullet}$. Some of these dissociate rapidly to produce c and $z^{\bullet}$ product ions (eq 2), plus minor amounts of $a^{\bullet}$ and y ions (eq 3).[9,12,13] These products (except for y) contrast sharply with the

$$\underset{R-C-NH-CHR'^{\llcorner}}{\overset{O}{\underset{\|}{}}\overset{\cdot H----}{}} \longrightarrow \underset{R-C-NH_2-CHR'^{\llcorner}}{\overset{O}{\underset{\|}{}}\overset{\bullet}{}} \longrightarrow \underset{a\cdot}{R\cdot} + \underset{y}{\overset{O}{\underset{\|\|}{C}} + NH_2CHR'^{\llcorner}} \quad (3)$$

b, y products (eq 1) from CAD[6] or infrared multiphoton dissociation (IRMPD).[15,16] Equally unusual and useful, ubiquitin with 75 backbone bonds showed[9] ECD cleavages at 50 (now 66)[13] of these, triple those by CAD or IRMPD (formation of c, $z^{\bullet}$ on the N-terminal side of Pro requires cleavage of two bonds, so these products of the three Pro residues are not observed). Thus ECD provides far more sequence information, presumably because specific dissociations are influenced far less by the structural characteristics of neighboring side chains. It was postulated[9] that the protons on protein side chains of highest proton affinity (Lys, Arg, His) were solvated to the amide backbone sites of somewhat lower proton affinity, so that electron capture at the shared proton appeared to be the cause of the dominant, but relatively nonselective backbone cleavage of eq 2. Thus it was a surprise to find that the most intense peak in the ESI spectrum of a 34-residue peptide containing one disulfide bond was a fragment ion from S—S cleavage (Figure 1); protonation at S—S is improbable because the proton affinity [B3LYP/6-31G(d)][17] of CH_3SSCH_3 is calculated to be 23.6 kcal/mol *less* than that of $CH_3CONHCH_3$.

Noncyclic S—S Bonds. Electrospray ionization of the Figure 1 peptide, an S—S bonded dimer of a Cys-terminal peptide, gave isotopic peaks corresponding to $(RSSR + nH)^{n+}$ ions. CAD (SORI)[6b,c] dissociation of the $(M + 5H)^{5+}$ ions gave 6 b and 4 y ions (with others of $m < 500$ possible); these are indicated by the longer vertical bars in the linear sequence (Figure 1, top). This spectrum shows little S—S cleavage, with the abundance of $(RSH + 2H)^{2+}$ only 3% of that of the y_{10} ion. Interacting

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Figure 1. Top: Schematic description of cleavages (vertical bars) in SWIFT-isolated 5+ ions from electrospray ionization of this symmetrical R—S—S—R peptide (R is the 17-mer shown) caused by CAD (SORI)[6b,c] and ECD; numerical values refer to product charge states. CAD gives b, y ions and ECD gives c, $z^{\bullet}$ ions, except for the cleavages at sulfur (see text). Bottom: ECD spectrum.

the $(M + 5H)^{5+}$ ions with minimum energy electrons produced the ECD spectrum of Figure 1, bottom. The most abundant fragment ion corresponds to S—S bond cleavage with retention of the neutralized H$^{\bullet}$ to yield $(RSH + 2H)^{2+}$ (eq 4).[10] This

$$R-S-S-R' + \cdot H \longrightarrow R-\overset{\overset{\displaystyle H}{|}}{S} \cdot S-R \longrightarrow RSH + \cdot SR' \quad (4)$$

spectrum shows far more extensive dissociation after e^- capture than does ECD of non-S—S proteins,[9] with little of the reduced $(M + 5H)^{4+\bullet}$ remaining. The ECD spectrum of $(M + 3H)^{3+}$ from the corresponding 17-mer monomer without an S—S bond (N-terminal Cys) shows $(M + 3H)^{2+\bullet}$ as one of the larger product ions,[12] and loss of —SH yields only a minor peak. In the Figure 1 spectrum the complementary $(RS^{\bullet} + 2H)^{2+\bullet}$ ion of eq 3 is negligible, but this odd-electron product should be less stable; further losses from it of S, CH_2S, and NH_3 account for other peaks. Again,[9,12] ECD also produces far more cleavages (14 c, 10 $z^{\bullet}$ ions, $m > 500$) than CAD (Figure 1, top). Thus this ECD spectrum clearly delineates the S—S bond position as well as providing nearly complete sequence information (31 backbone bonds cleaved of 37 possible; CAD cleaved four of the remaining six).

A larger LysC digest peptide, $M_r = 9839.9$, represents residues 135–172 (here termed AS$^{\bullet}$) and 175–222 (BS$^{\bullet}$) of Fcϵ3-4 of IgE joined by an S—S bond between Cys-139 and Cys-199 (Figure 2, top).[18] No CAD fragments result from S—S bond cleavage; only a b_{19} B-chain fragment and y_{24}, y_{27} A-chain fragments contain a single terminus, while eight b fragments of the A chain still contain the B chain. However, the ECD spectrum of $(M + 10H)^{10+}$ was dominated by $(M + 10H)^{9+\bullet}$,

(16) Matrix-assisted laser desorption ionization at high photon intensity can also give c, $z^{\bullet}$ products (Brown, R. S.; Lennon, J. J. *Anal. Chem.* **1995**, *67*, 3990–3999) so that ECD offers an explanation for these unexpected MALDI observations.

(17) Without zero-point energy corrections: Gaussian 94, Revision E.2, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. Gaussian, Inc.: Pittsburgh, PA, 1995. Beck, A. D. *J. Chem. Phys.* **1993**, *98*, 5648. Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev.* **1988**, *37*, 785.

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Figure 2. ESI of a 9839.9 Da peptide from a LysC digest of Fcε3-4 of human IgE, corresponding to residues 135−172 (AS•) and 175−222 (BS•) and joined by a disulfide bond, gave a $(M+10H)^{10+}$ ion that was SWIFT isolated and subjected to CAD (upper spectrum) and ECD (lower spectrum). Dots on inserts in the lower spectrum show calculated isotopic distributions: for A^{5+}, the filled circles correspond to $(AS• + 5H)^{5+•}$ and the open circles to $(ASH + 5H)^{5+}$; for A^{4+}, $(AS• + 4H)^{4+}$ and $(ASH + 4H)^{4+}$; for B^{4+}, only $(BSH + 4H)^{4+}$ is shown.
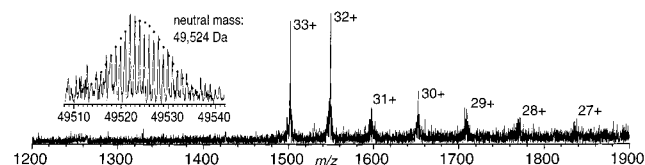
Figure 3. From the ESI spectrum of a deglycosylated Fcε3−4 construct (a disulfide bonded homodimer) from human IgE (insert, a high-resolution expansion of a single charge state, converted to neutral mass), the $(M + 33)^{33+}$ was isolated by SWIFT to yield the ECD spectrum shown.

$(BSH + 4H)^{4+}$ (5352.6 Da), and $(AS• + 5H)^{5+•}$ (4488.2 Da) ions, uniquely defining the length of each of the component peptides.

This tendency for the even-electron RSH product (eq 3) to originate from the R group of higher charge is observed in other spectra and appears to arise from S−S bond polarization. Calculations with H• added to CH_3SSCH_3 assumed symmetrical charge planes differing by a 1 e$^-$ charge 15 Å apart along the molecular axis. The formation of CH_3SH (vs $CH_3S•$) at the positively charged end was found to be favored by 4.9 kcal/mol.

ECD of $(M + 33H)^{33+}$ of a symmetrical S−S bound dimer from deglycosylated Fcε3-4 protein from IgE, $M_r = 49524$ (Figure 3), gave the reduced ions $(M + 33H)^{(26−32)+}$, but any $(0.5M + 16H)^{16+}$ ions from S−S cleavage were of too low abundance to be clearly identified beneath the $(M + 32H)^{32+•}$ isotopic peaks.[18] For proteins larger than 20 kDa without disulfide bonds, ECD similarly gave only reduction, with no bond cleavages.[9]

Cyclic S−S Bonds. The peptides [Lys-8]-vasopressin, H-CYFQNCPKG-NH2, and Val-Asp-[Arg-8]-vasopressin, H-VD-CYFQNCPRG-NH2, gave similar MS/MS spectra. CAD of $(M + H)^{1+}$ and $(M + 2H)^{2+}$ from the latter yielded mainly b_8, b_9, b_{10}, and H_2O-loss ions, but none from cleavages within the cyclic structure. Its ECD spectrum of $(M + 2H)^{2+}$ has a dominant $(M − HS• + 2H)^{1+}$, with smaller amounts of $(M + 2H)^{1+•}$ and $(M − CH_2S + 2H)^{1+•}$. Its z_9, z_{10}, and c_{10} ions indicate
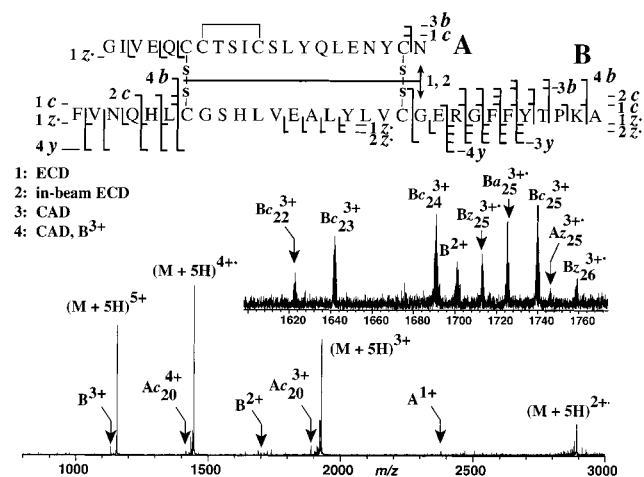
Figure 4. As in Figure 1, for 5+ ions from the electrospray spectrum of insulin subjected to (1) ECD (bottom spectrum), (2) IR excitation during ECD ("in beam"), (3) CAD, and (4) CAD of the B^{3+} ions from ECD. Cleavages in the B chain shown with arrows yield even-electron z ions that contain the C-terminus of the B-chain after A-chain loss (eq 5).

the terminal residues (c_7 and c_8 from the Lys-8 variant); the lack of ECD cleavage at the N-terminal side of the Pro tertiary nitrogen (favored for CAD) was noted earlier.[9] Contrary to eq 2, the z_5, z_7, and z_8 ions (z_7 and z_8 from the Lys-8 variant) are even-electron species, consistent with ring cleavages at both a backbone position and S−S (eq 5). The S−S bond should have

$$(5)$$
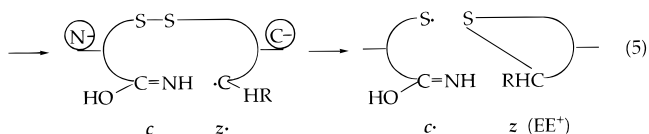
a high affinity for an initially formed $z•$ radical, as well as for H•, to induce its low-energy cleavage (eq 4). Initial formation of an RS• fragment is possible, but this would have marginal kinetic energy for initiation of the higher energy eq 2 cleavage. No $c•$ or c ions consistent with initial eq 2 cleavage within the ring were found. The even-electron $(M − HS• + 2H)^{1+}$ must also arise from ECD cleavage of two bonds; CAD of this ion also gave no products that arise from cleavage of the original ring, suggesting formation of the recyclized monosulfide ion.[12]

Porcine insulin ($M_r = 5776.6$) contains two S−S bonds connecting its A and B chains (Figure 4, top) designated here as •SAS• (2381.0 Da) and •SBS• (3395.6 Da). CAD (designated as "3b" and "3y") of its $(M + 5H)^{5+}$ ions yields eight b and six y product ions, all corresponding to exocyclic cleavages in the two C-termini; formation of none requires cleavage of the interchain ring. From eq 1, the fragment ion corresponding to the loss of 132.10 Da indicates the C-terminal amino acid N (Asn, 114.04 Da + 18.01, H_2O). Of the b, y fragments, five are complementary pairs whose masses sum to that of $(M + 5H)^{5+}$. Four pairs define the sequence -GFF-, while these and the other b, y ions are consistent with the terminal sequence (314.19 Da) TYFFGR(EG). The terminal 314.19 Da cannot contain the C-terminal N assigned above. Although it does indicate, correctly, the C-terminal −PKA, it also corresponds to possible N-terminal sequences such as (VTN).

ECD of $(M + 5H)^{5+}$ forms 10 c and 12 $z•$ product ions (designated as "1c" and "1z", Figure 4) representing exocyclic cleavages in both the N- and the C-termini. These ions indicate (eq 2) at least two C-termini, one with the residue −N and the other with the sequence −RGFFY(TP)KA. For the correspond-

ing N-termini, the data are consistent with F(VN)QHL for one and (170.11)(228.11)Q- for the other, with 170.11 as a terminal (GL) or (AV) and the mass difference 228.11 as the next (VE) or (LD) (these L assignments could also be the isomeric I residues). Thus the ECD spectrum suggests that the protein contains two covalently-linked peptide chains. Dominant fragment ions (B^{3+}, B^{2+}, A^{1+}, Figure 4) of complementary masses [those summing to the mass of $(M + nH)^{n+}$] correspond to the A + B chains; ECD has preferentially cleaved both S−S interchain bonds. Deconvolution of the overlapping isotopic peaks indicates that the major products (although variable run-to-run) of the more highly charged B-chain are the even-electron ions $(HSBSH + 3H)^{3+}$ (most abundant) and $(SBS + 3H)^{3+}$ (possibly cyclic) and the corresponding 2+ ions; the much less abundant A-chain ions are mainly the odd-electron $[S(A − H)-S^{\bullet} + H]^{1+\bullet}$ and $(HSAS^{\bullet} + H)^{1+\bullet}$ (minor species representing the gain, or loss, of S from these ions are also present). Note that both S−S bonds cleaved in the formation of the most abundant $(HSBSH + 3H)^{3+}$ ion have involved transfer of an extra $H^{\bullet}$ to the most highly charged species, possibly as indicated in eq 6.

$$(6)$$
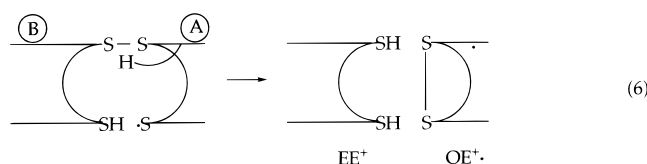
ECD also yields Bz_{15}, an even-electron ion whose formation is consistent with initial intra-ring c, $z^{\bullet}$ cleavage followed by $z^{\bullet}$ attack on the adjacent S−S bond (eq 5). Unexpectedly, the relative proportion of these double cleavage products could be greatly increased by collisions or IR irradiation[15a] of the ions during their exposure to electrons (designated as "2-z", Figure 4, top). Thus ECD during ("in beam")[13] collisional trapping of the electrosprayed ion beam in the FTMS cell ($\sim10^{-5}$ Torr Ar) yields B^{3+} in an abundance comparable to that of $(M + 5H)^{4+\bullet}$ and produces the new double-cleavage even-electron products $Bz_{16}−z_{18}$. As found for vasopressin, no $Bc^{\bullet}$ products from intrachain cleavage were formed. This spectrum also has Bc_4, Bc_5, $Bz_{11}^{\bullet}$ (a new B-chain cleavage) peaks not shown in the normal ECD spectrum. The B chain 3+ ion mixtures from ECD were subjected to CAD to yield b, y products (designated as "4b" and "4y" in Figure 4, top), as expected from the dissociation of even-electron ions. These represent seven B-chain cleavages not seen in the CAD spectrum of $(M + 5H)^{5+}$, but none between Cys-7 and Cys-19; that of $V^2−N^3$ is not represented in any other spectrum.

In the ECD or CAD spectra, cleavages have occurred on the terminal side of each of the four cysteines, defining the sequences of the 23 amino acids of these termini except for the pairs $I^{2A}−V^{3A}$ and $E^{4A}−Q^{5A}$. The only cleavages within the ring are those of ECD yielding *even-electron z* ions of eq 5, not the $z^{\bullet}$ ions of eq 2. To the extent this behavior is true for other S−S cyclic proteins (as it is, above, for the vasopressins), the combined data from ECD and CAD spectra appear useful for sequence characterization, including the intrachain bond positions, for such cross-linked S−S structures.

Ribonuclease A, 13.7 kDa, is a single chain protein with four intrachain S−S bonds. ECD of $(M + 9H)^{9+}$ gave $(M + 9H)^{(6−8)+}$ but no fragment ions. However, a 30 min exposure of these gaseous ions to 10^{-7} Torr of D_2O^{19} exchanged ~32 D atoms (vs 29 D measured earlier)[19a] for $(M + 9H)^{9+}$, 38 D for

$(M + 9H)^{8+\bullet}$, 48 D for $(M + 9H)^{7+}$, and a higher value for $(M + 9H)^{6+\bullet}$. These values are consistent with selective ECD cleavage of S−S bonds and ring opening to a more exposed conformation; if recyclization of the new radical site occurs, apparently this still leaves a more open structure.

Mechanisms of ECD: H$^{\bullet}$ Atom Capture. ECD cleavage of the S−S bond is highly favored over that producing c, $z^{\bullet}$ or the minor $c^{\bullet}$, z or $a^{\bullet}$, y ions.[9,12,13] However, B3LYP/6-31+G(d) calculations[17] indicate that the electron affinity of CH_3SSCH_3 is 93 kcal/mol less than that of the charged species CH_3C^+-$(OH)NHCH_3$. The protonated side chains should be preferentially solvated to the backbone carbonyl groups[20] (the proton affinity of CH_3SSCH_3 is calculated[17] to be 23.6 kcal/mol less than that of $CH_3CONHCH_3$). Thus, as previously postulated,[9] most protons when neutralized by e^- should be nearer to backbone carbonyl groups (eq 2) than to S−S bonds. Although facile $H^{\bullet}$ loss should result from e^- neutralization of the protonated side chains of His,[21] Arg, and Lys, $H^{\bullet}$ loss peaks are nearly absent in protein ECD spectra.[9,13] As a mechanistic alternative, the $H^{\bullet}$ atom affinity of CH_3SSCH_3 is calculated[17] to be 23.9 kcal/mol *greater* than that of $CH_3CONHCH_3$, with $H^{\bullet}$ addition yielding (eq 4) the hypervalent intermediate that should spontaneously cleave to CH_3SH and $^{\bullet}SCH_3$.

For confirmation of this basic mechanism, $H^{\bullet}$ affinities were calculated for other protein functionalities. The $H^{\bullet}$ affinity of the 3-methylindole functionality of tryptophan was found to 4.3 kcal/mol *more* favorable than that of S−S. There are two Trp residues in myoglobin and one in ubiquitin; the four c and $z^{\bullet}$ ions[9] resulting from cleavage on the C-terminal side of these Trp residues (possibly as in eq 7) are five times more abundant

$$(7)$$
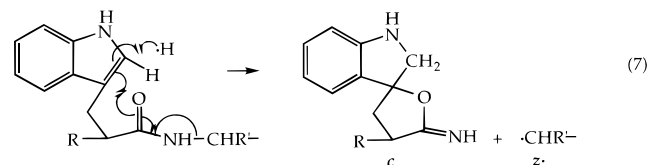
than the average of c, $z^{\bullet}$ ions for 514 ECD fragments from proteins containing 451 amino acids.[22] In the same way, in the ECD spectrum of a 15-mer peptide (N-terminal Cys) the Trp (ninth residue) C-side products exhibit abundances 2.5× those of any other residue.[12] However, for the corresponding symmetrical 30-mer dimer, RSSR, the ECD spectrum of the combined 7+ and 5+ ions, like Figure 1, again showed dominant monomer peaks $(RSH + 3H)^{3+}$ and $(RSH + 2H)^{2+}$, and cleavages at the C-side of Trp gave ions seventh in abundance vs those of the other residues. If $H^{\bullet}$ capture at Trp is actually more favorable than that at S−S, the subsequent eq 4 cleavage at S−S apparently is more favorable than that of eq 7.[23]

Nonergodic Dissociation. The electron affinity of a quaternary ammonium ion (e.g., Lys, RNH_3^+) is ~90 kcal/mol,[25] while that for such a species in a multiply protonated protein should

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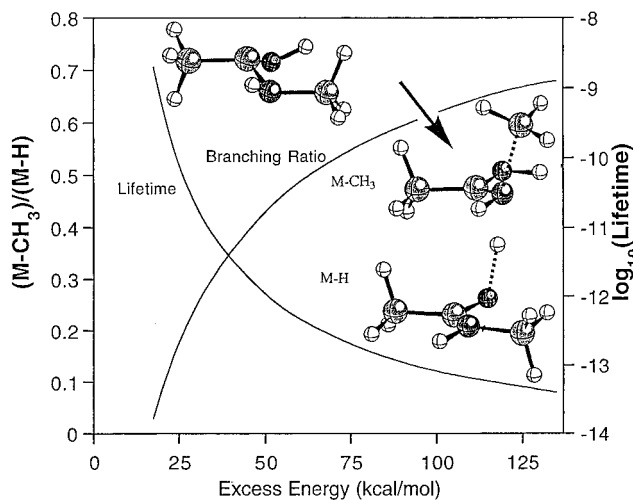
Figure 5. Branching ratios for the competitive losses of hydroxyl H[•] and N-substituted CH_3 from $CH_3\dot{C}(OH)NH$ from RRKM calculations using B3LYP/6-31G(d) geometries and frequencies.[17]

be even higher. Thus neutralization should form an excited species (e.g., the hypervalent $R\dot{N}H_3$) that immediately ejects H[•] with high kinetic energy.[25] Capture of this H[•] at the amide carbonyl (eq 2) could thus supply substantial excitation energy to the odd-electron product that is basically unstable; for the reverse reaction of the model system $CH_3\dot{C}(OH)NHCH_3 \rightarrow$ H[•] + $CH_3CONHCH_3$ $\Delta G°_{B3LYP} = -2.9$ kcal/mol.[17] RRKM calculations (Figure 5) indicate that the competitive c, $z^{•}$ cleavage $CH_3\dot{C}(OH)NH-CH_3$ is more favorable at high excitation energies, consistent with the increased dissociation with "in beam" excitation. These $\sim 10^{-12}$ s lifetimes should be almost as short for the corresponding protein backbone cleavage $-CHR\dot{C}(OH)NH-CHR'-$, consistent with nonergodic dissociation[26] as postulated previously for the mechanism in which the neutralized proton was already bonded to the amide carbonyl.[9] Further, randomization of the >90 kcal/mol neutralization energy over the whole protein would have a negligible effect on the rate of the eq 2 reaction. However, if the c, $z^{•}$ cleavage is nonergodic, why does the presence of the S—S bond greatly reduce the probability of this backbone cleavage? A possible explanation is that S—S can capture more energetic H[•] atoms than can the amide carbonyl; these H[•] atoms otherwise would be collisionally cooled for carbonyl capture. Further, the latter is reversible (first step of eq 2) so that a re-released H[•] atom would have an additional opportunity for nonreversible S—S capture.

Extending this postulate, larger protein ions will have more extensive tertiary structures (also more stable conformers than those in solution),[19c] providing more nonreactive collisions of the released H[•] atoms as well as more stabilization of the backbone bonds. From Figure 5, H[•] captures by larger protein ions would have a lower probability of causing backbone

cleavage, instead producing the reduced protonated molecule, as found for ECD of proteins >20 kDa.

Two related methods lower the ion charge without causing such dissociations. In Beynon's "electron capture induced decomposition" method, the multiply charged ion (e. g., $C_6H_6^{2+}$) captures an electron from a collision gas,[27] so that the energy deposited is less than that from a free electron by the difference in ionization energies;[28] IE(e^-) = 0. Interaction of $(M + nH)^{n+}$ with negative ions gives mainly proton abstraction and association, with multiply protonated proteins yielding no c, $z^{•}$ products.[29]

The reaction of H[•] atoms (2% H[•] in H_2 generated in an exterior microwave discharge) with small gaseous odd-electron molecular ions abstracts a counterpart H[•] to form the even-electron $(M - H)^+ + H_2$.[30] However, $CH_3S^{+•}CH_3 + H^{•}$ gave $CH_3SH^{+•} + {}^{•}CH_3$ ($\Delta H = +3.6$ kcal/mol) instead of the favored $CH_3=SH^+ + CH_4$ ($\Delta H = -46$ kcal/mol), consistent with H[•] capture at the S atom followed by "kinetically controlled cleavage".[30] Here electron pairing of H[•] with the radical site on sulfur apparently provides the excess energy necessary for the thermodynamically unfavored reaction, similar to that of Figure 4. The reaction of H[•] with $CH_3CON(CH_3)_2^{+•}$ yielded $HN^{+•}(CH_3)_2$ and $H_2N^+(CH_3)_2$; this corresponds to H[•] attack on $N^{+•}$,[30] not on the carbonyl as in eq 2. Application of this novel H[•] reaction scheme to even-electron protein ions could test this ECD mechanism further.

Other H[•] Reactions. Intramolecular hydrogen transfer reactions (i.e., rearrangements) have been studied extensively. Thus the favorable H[•] affinity shown here for the amide carbonyl parallels the characteristic hydrogen rearrangement of carbonyl compounds in photochemistry (Norrish Type II reaction) or in electron ionization mass spectra. The H[•] rearrangement $RCH_2S-R'^{+•} \rightarrow RCH=S^+H + {}^{•}R'$ in the latter spectra of sulfides is not observed for the corresponding oxygen-containing species.[32]

Conclusions

Electron capture by a multiply-protonated peptide or protein is a high-energy process that can release an energetic hydrogen atom. Its subsequent chemistry, with a transition state that appears to involve an intramolecularly mobile H[•] atom, has little precedent in solution reactions. Instead of escaping the gaseous protein, the H[•] apparently is collisionally de-excited until its kinetic energy is favorable for capture by a functional group of the gaseous protein ion. The capture tendency appears to be relatively independent of the intramolecular distance to the functionality, and mainly dependent on its H[•] atom affinity. For eq 2 cleavage, the kinetic energy of the H[•] at capture is also a factor; this must supply sufficient excess energy for near-instantaneous (nonergodic) dissociation (Figure 3) despite conformational protein stabilization that generally increases with increasing molecular weight. As predicted by theoretical H[•] affinity calculations,[17] capture and cleavage at the disulfide bond

(23) John Brauman has made a novel suggestion as an alternative to our proposal for H[•] atom capture at the disulfide bond. The original e^- capture could form a high-n Rydberg state, expected to be long-lived,[24a] that would have a favorable intersystem crossing to an S—S dissociative state. Long-lived ion−electron complexes are postulated as intermediates in the ion−electron recombination process.[24b] These proposals are under further investigation.

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(eq 4) is favored; if S—S is not present, capture and cleavage adjacent to a tryptophan residue (eq 7) is somewhat favored, with a general tendency otherwise for capture at backbone amide carbonyl groups followed by c, $z^{\bullet}$ cleavage (eq 2). Thus ECD should be a valuable complement to the well-established methods (e.g., CAD, IRMPD) for dissociation of multiprotonated proteins, including those with S—S bonds. The extent to which these combined MS/MS methods can provide *de novo* sequencing of proteins is under investigation.