A Mass Spectrometric and Ab Initio Study of the Pathways for Dehydration of Simple Glycine and Cysteine-Containing Peptide $[M+H]^+$ Ions

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The gas phase fragmentation reactions of the $[M+H]^+$ and $[M+H-H_2O]^+$ ions of glycylglycine, glycylcysteine, *N*-acetylglycine, *N*-acetylcysteine, their corresponding methyl esters, as well as several other related model systems have been examined by electrospray ionization (ESI) tandem mass spectrometry (MS^n) using triple quadrupole and quadrupole ion trap mass spectrometers. Two discrete gas phase fragmentation pathways for the loss of water from glycine-containing peptides, corresponding to retro-Koch and retro-Ritter type reactions were observed. Two pathways were also observed for the loss of water from C-terminal cysteine-containing peptides: a retro-Koch type reaction and an intramolecular nucleophilic attack at the carbonyl of the amide bond by the cysteinyl side chain thiol. Various intermediates involved in these reactions, derived from the $[M+H-H_2O]^+$ ions of *N*-formylglycine and *N*-formylcysteine, were modeled using *ab initio* calculations at the MP2(FC)/6-31G*//HF/6-31G* level of theory. These calculations indicate that: (i) the retro-Koch reaction product is predicted to be more stable than the product from the retro-Ritter reaction for *N*-formylglycine, and (ii) the intramolecular nucleophilic attack product is preferred over the retro-Koch and retro-Ritter reaction products for *N*-formylcysteine. The results from these ab initio calculations are in good agreement with the experimentally determined ion abundances for these processes. (J Am Soc Mass Spectrom 1998, 9, 945–956) © 1998 American Society for Mass Spectrometry

As part of ongoing research into the gas phase ionic reactivities of biological model compounds, the reactions of the amino acids glycine and cysteine, with $(CH_3)_2Cl^+$ and $CH_3OCH_2^+$, were recently examined using chemical ionization tandem mass spectrometry [1,2]. During these studies we developed an interest in understanding the mechanisms of the fragmentation reactions of ionized (e.g., $[M+H]^+$ and $[M-H]^-$ ions) amino acids, peptides and their derivatives [3]. Such studies are of general interest since the determination of the amino acid sequence of a peptide by MS/MS of its $[M+H]^+$ or $[M-H]^-$ ions, requires cleavage of each peptide bond to yield "sequence" ions. Usually, those ions resulting from fragmentations not involving cleavage of the peptide backbone, designated "nonsequence" ions, are ignored and are generally regarded as a nuisance because they not only complicate the spectrum, but also deplete the overall ion current away from the desired "sequence" ion channels.

Although unified nomenclature schemes have been proposed for naming the resultant sequence ions for peptides [4] and oligonucleotide anions [5], the mechanisms and factors which influence nonsequence fragment ion production are not well understood. A more detailed understanding of the factors that govern the decomposition reactions of simple biomolecules in the gaseous phase, including the structure of the resultant fragment ions, the influence of potentially reactive amino acid side chains on peptide backbone fragmentation, and the mechanisms leading to the loss of small neutral molecules such as water, ammonia, and carbon monoxide, would greatly simplify the task of routine mass spectrometric based peptide sequencing and the

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Gas Phase Ion Chemistry of Biomolecules, Part 10. For part 9 see [3].

Dedicated to the memory of Professor B.S. Freiser, a true innovator in gas phase ion chemistry.

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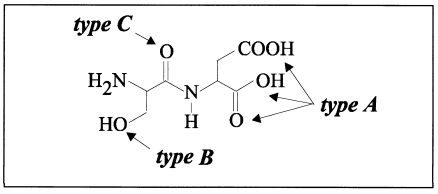
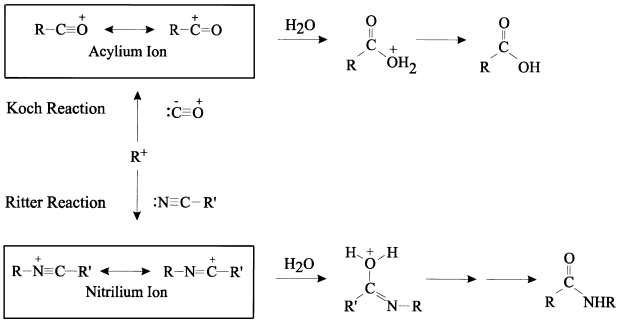


Scheme I

characterization of posttranslationally modified proteins [6]. A common nonsequence ion routinely observed in the MS/MS spectra of peptide $[M+H]^+$ ions arises from the loss of water (i.e., dehydration with concomitant $[M+H - H_2O]^+$ ion formation). Ballard and Gaskell's pioneering studies [7], which employed [^{18}O] labeling of the C-terminal carboxyl group, identified three possible sources for the loss of the oxygen atom, thereby suggesting three distinct types of dehydration pathway: the loss of H_2O from the C-terminal carboxylic acid or the side chains of aspartic or glutamic acid residues (***type A***); H_2O loss from the side chain hydroxyl of serine or threonine residues (***type B***); or the loss of H_2O from the amide carbonyl of the peptide backbone (***type C***) (Scheme I). Although convincing evidence for all three pathways was demonstrated, detailed mechanisms for each of these processes were not defined.

In recent years some progress has been made towards understanding the mechanisms of these reactions. For example, in earlier studies it was noted that the loss of water from the carboxylic acid group of glycine [1a] is the reverse process of the well known solution phase synthesis of carboxylic acids known as the Koch reaction [8a] (Scheme II). Thus H_2O losses of ***type A*** in Scheme I, involving the formation of an acylium ion, can be regarded as the retro-Koch reaction.

Recent work by Harrison [9a,b], Hunt [9c], and Gaskell [9d] indicates that the acyclic acylium ion for peptides is unstable and that the species formed is a stable cyclic oxazolone structure [designated as b_n ($n >$ 1) using the standard nomenclature] [4]. Several workers have previously noted that for amino acids, the b_1 ion formed initially fragments via loss of CO to give an immonium ion [10–12].



Scheme II

Dehydration pathways of ***type B*** (see Scheme I) have also received attention recently [11f, 13]. In particular, the fragment ion formed via the loss of H_2O from the $[M+H]^+$ ion of threonine has been studied using a combination of MS/MS techniques and ab initio calculations [13]. Experiments involving [^{18}O]-labeled threonine clearly indicate that the side-chain hydroxyl group is exclusively lost from the $[M+H]^+$ ion. Furthermore, ab initio calculations suggest that the structure of the resultant $[M+H - H_2O]^+$ ion is that of *N*-protonated dehydroamino-2-butyric acid.

Type C (Scheme I) water losses from peptides have not been further investigated since Gaskell's original report [7]. In keeping with analogies of solution phase chemistries, we note that the loss of water from an amide bond is the reverse of a well known solution phase process for the synthesis of amides, known as the Ritter reaction, with the formation of a stable nitrilium ion [8b] (Scheme II). Previous mass spectrometric experiments combined with ab initio calculations suggest that such a retro-Ritter process operates for protonated formamide in the gas phase [14]. (We agree with a reviewer's comment that the gas phase versus solution phase mechanisms are likely to be different for the retro-Koch and retro-Ritter reactions.)

In this paper, using a combination of multistage tandem mass spectrometric (MS^n) methods and a series of ab initio calculations, the competition between ***type A*** (i.e., retro-Koch) and ***type C*** (i.e., retro-Ritter) reactions for the loss of water from the $[M+H]^+$ ions of simple glycine- and cysteine-containing dipeptides and model systems are examined. Additionally, a new mechanism for the loss of water from a cysteine-containing peptide involving an intramolecular nucleophilic side chain thiol attack on the carbonyl carbon of the amide bond is described.

Experimental

Materials

Glycine ($H_2NCH_2CO_2H$), glycine O-methyl ester ($H_2NCH_2CO_2CH_3$), sarcosine ($CH_3NHCH_2CO_2H$), cysteine [$H_2NCH(CH_2SH)CO_2H$], cysteine O-methyl ester [$H_2NCH(CH_2SH)CO_2CH_3$], S-methyl cysteine [$H_2NCH(CH_2SCH_3)CO_2H$], glycylglycine ($H_2NCH_2CONHCH_2CO_2H$), and glycylcysteine [$H_2NCH_2CONHCH(CH_2SH)CO_2H$] were obtained from Bachem (Bubendorf, Switzerland) and were used without further purification. N-methyl cysteine [$CH_3NHCH(CH_2SH)CO_2H$] was available from a previous study [1b]. Methanol (ChromAR grade) was purchased from Mallinkrodt (Melbourne, Australia). Acetyl chloride was purchased from Aldrich (Milwaukee, WI). Acetic anhydride was obtained from Fluka (Buchs, Switzerland). Acetic acid was obtained from Merck (Darmstadt, Germany).

General Procedure for the Methyl Esterification of Amino Acids and Dipepides

A solution of 2N HCl in methanol was prepared at 25°C by the dropwise addition of 800 μL acetyl chloride to 5 mL of anhydrous methanol with stirring. After 5 min, a 1 mL aliquot of this reagent was added to 10 mg of lyophilized amino acid or dipeptide. The reaction was allowed to proceed for 2 h at 25°C then the sample was dried by lyophilization.

General Procedure for N-Acetylation of Amino Acid Methyl Esters

The lyophilized amino acid methyl esters (10 mg) were dissolved in 100 μL of 50-mM ammonium bicarbonate pH 7.8. To this solution 1 mL of the acetylation reagent (prepared by the addition of 250 μL of acetic anhydride to 750 μL methanol) was added and the reaction allowed to proceed for 2 h at 25°C. The sample was then dried by lyophilization. The resulting crude N-acetyl (NAc-) amino acid O-methyl ester was then used without further purification.

Mass Spectrometry

The experiments described were carried out using either (i) a Finnigan LCQ [15] quadrupole ion trap mass spectrometer or (ii) a Finnigan model TSQ-700 [15] triple quadrupole mass spectrometer. Both instruments were equipped with a Finnigan electrospray ionization (ESI) source. $[M+H]^+$ ions were formed by ESI using the following typical conditions. The sample (approximately 100 pmol/μL dissolved in 50% MeOH containing 1% (aq) acetic acid) was introduced to the ESI source via a length of 50 μm i.d. × 190 μm o.d. fused-silica tubing at a flow rate of 3 μL/min using a Harvard syringe drive. A potential of −5.0 kV and a sheath gas of nitrogen (ultrahigh purity) at a pressure of 35 psi were employed. The heated capillary was maintained at 200°C. The $[M+H]^+$ ion intensity was optimized by adjustment of the tube lens voltage, taking care to minimize "in-source" fragmentation.

MS^n experiments were performed on mass selected ions in the LCQ using standard isolation and excitation procedures. MS/MS experiments in the triple quadrupole mass spectrometer were performed by collision-induced dissociation (CID) of mass selected ions in the second quadrupole using argon as the collision gas at a pressure of 2–2.5 mtorr. Energy-resolved experiments were performed by stepping, in increments of 2.5 V, the collision offset voltage of the second quadrupole. To maintain efficient ion transmission through the third quadrupole, the offset voltage was set at a value 2.5 V above that of the collision cell. Data were acquired at a rate of 100 Th/s. All data points collected were the average of ten scans.

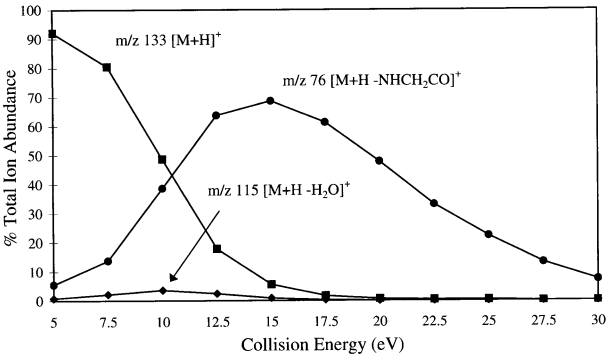


Figure 1. Triple quadrupole energy resolved MS/MS breakdown graph of the $[M+H]^+$ ion of glycylglycine: (filled square) $[M+H]^+$ (m/z 133), (filled circle) $[M+H - NHCH_2CO]^+$ (m/z 76), (filled diamond) $[M+H - H_2O]^+$ (m/z 115).

Computational Methods

The structures of ions and neutral peptides were optimized using standard ab initio gradient techniques at the Hartree–Fock (HF) level of theory, performed on the SPARTAN [16] molecular modeling package (Version 4.1.1) with the standard 6-31G* basis set [17]. All optimized structures were subjected to vibrational analysis to determine the nature of the localized stationary point, followed by calculation of the single point energy at the MP2(FC)/6-31G* level of theory. Energies were corrected for zero-point vibrations scaled by 0.9 [18]. In each case, only a limited set of possible rotamers was explored [19]. Complete structural details and lists of vibrational frequencies for each HF/6-31G* optimized structure are available from the authors upon request.

Results and Discussion

Tandem-Mass Spectrometric Studies of the Loss of Water from the $[M+H]^+$ Ions of Simple Glycine-Containing Dipeptides and Model Systems

Tandem-mass spectrometry of the $[M+H]^+$ ion of glycylglycine. The fragment ion yields and the relative appearance energies for the CID fragmentation of glycylglycine were examined by an energy-resolved experiment using a triple quadrupole mass spectrometer (Figure 1).

The relative intensities of the product ions formed from the gas phase fragmentation of the $[M+H]^+$ ion of glycylglycine (m/z 133) are plotted with respect to increasing collision energy (laboratory frame of reference). An examination of the breakdown graph indicated that glycylglycine fragmented primarily via cleavage of the amide bond to yield a "sequence ion" (designated as a y_1 ion using the standard nomenclature), as evidenced by the appearance of an ion at m/z 76. In addition, a small but detectable "nonsequence ion" type fragmentation leading to the loss of H_2O (m/z 115) was observed. The collision energy at which the maximum yield of this $[M+H - H_2O]^+$ ion at m/z 115

Table 1. LCQ CID MS/MS of the $[M+H]^+$ ions of dipeptides and N-acetyl glycine and cysteine model systems

Neutral species	$[M+H]^+$ ion[a]	Daughter ions: *m/z*, (loss), abundance %[b]
$H_2NCH_2C(O)NHCH_2CO_2H$	133	115(H_2O)18, 105(CO)26, 88(CO, NH_3)19, 87(HCO_2H)1, 76($HNCH_2CO$)100
$H_2NCH_2C(O)NHCH_2CO_2CH_3$	147	129(H_2O)1, 119(CO)2, 115(CH_3OH)93, 102(CO, NH_3)35, 90($HNCH_2CO$)100, 87(HCO_2CH_3)1
$H_2NCH_2C(O)NHCH(CH_2SH)CO_2H$	179	161(H_2O)100, 122($HNCH_2CO$)2
$H_2NCH_2C(O)NHCH(CH_2SH)CO_2CH_3$	193	175(H_2O)100, 161(CH_3OH)1, 136($HNCH_2CO$)1
$CH_3C(O)NHCH_2CO_2H$	118	100(H_2O)13, 76(CH_2CO)100
$CH_3C(O)NHCH_2CO_2CH_3$	132	114(H_2O)2, 100(CH_3OH)77, 90(CH_2CO)100
$CH_3C(O)N(CH_3)CH_2CO_2H$	132	114(H_2O)22, 90(CH_2CO)100
$CH_3C(O)N(CH_3)CH_2CO_2CH_3$	146	114(CH_3OH)100, 104(CH_2CO)26
$CH_3C(O)NHCH(CH_2SH)CO_2H$	164	146(H_2O)100, 122(CH_2CO)94, 118(HCO_2H)3
$CH_3C(O)NHCH(CH_2SH)CO_2CH_3$	178	160(H_2O)100, 146(CH_3OH)68, 136(CH_2CO)50
$CH_3C(O)NHCH(CH_2SCH_3)CO_2H$	178	160(H_2O)45, 136(CH_2CO)100, 132(HCO_2H)5, 130($HSCH_3$)47, 90(CH_2CO, HCO_2H)6
$CH_3C(O)NHCH(CH_2SCH_3)CO_2CH_3$	192	See Figure 6B
$CH_3C(O)N(CH_3)CH(CH_2SH)CO_2CH_3$	192	See Figure 6A

[a]Formed via ESI.
[b]Only those ions greater than 1% relative abundance are shown.

was observed (~10 eV) was significantly lower than that of the *m/z* 76 sequence ion (~15 eV). Several ions at *m/z* 30, 48, 88, and 105 were also observed at higher fragmentation energies (~25 eV, data not shown). The ion at *m/z* 30, corresponding to formation of the immonium ion (designated as a_1 using the standard nomenclature) could be formed either directly, by loss of CO from the unstable b_1 ion, or by further fragmentation of the y_1 ion. Similar results for the fragmentation of protonated glycylglycine have been reported recently by Kerbarle [11h].

To examine the structural basis by which the $[M+H]^+$ ion of glycylglycine loses H_2O, the fragmentation of glycylglycine was examined using multistage tandem mass spectrometry (MS^3) on an ion trap mass spectrometer. The MS/MS product ion spectrum of glycylglycine (*m/z* 133) is listed in Table 1.

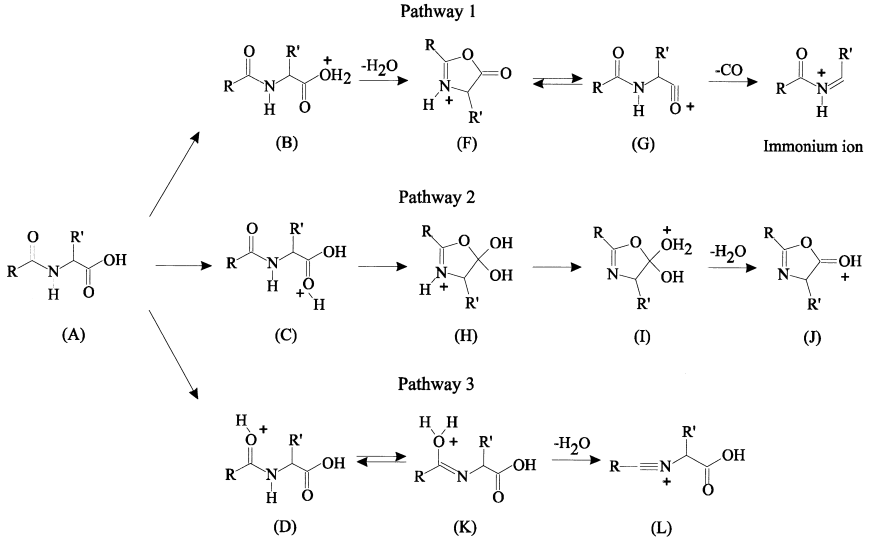
The major fragment ion observed at *m/z* 76 corresponds to the sequence ion fragmentation of the amide bond with loss of the N-terminal glycine amino acid residue. Additionally, ions at *m/z* 115, 105, and 88 corresponding to the loss of H_2O, CO, and both CO and NH_3 respectively, were also observed. The ion corresponding to the loss of H_2O at *m/z* 115 was of particular interest given the lack of knowledge regarding the mechanisms behind the ***type A*** (i.e., retro-Koch) and ***type C*** (i.e., retro-Ritter) reactions for the loss of water from protonated peptides in the gas phase.

Two structures have been previously proposed for the product ion formed following the ***type A*** (retro-Koch) loss of water from the carboxylic acid of a peptide. Harrison and co-workers [9a,b] have shown convincing evidence for an N-protonated cyclic oxazolone structure (F), resulting from protonation at the OH of the carboxylic acid (B) (Scheme III, pathway 1). Hunt and co-workers [9c] have proposed a CO protonated oxazolone structure (J), resulting from the loss of water following protonation of the carbonyl oxygen of the carboxylic acid (C) (Scheme III, pathway 2).

The mechanism proposed by Harrison predicts that the fragmentation of the N-protonated oxazolone involves ring opening to an unstable acyclic acylium ion (G), followed by loss of CO to form a stable immonium ion product (Scheme III, pathway 1). This is demonstrated for the $[M+H - H_2O]^+$ ion (*m/z* 115) of glycylglycine that, when subjected to MS^3, results in the sole formation of the immonium ion at *m/z* 87 via the loss of CO (Table 2).

Tandem-mass spectrometry of the $[M+H]^+$ ion of glycylglycine O-methyl ester. As described above, an alternate pathway for the loss of water from the backbone of a protonated peptide involves loss of water from the amide bond via a retro-Ritter type reaction (Scheme III, pathway 3). This process involves protonation of the carbonyl oxygen of the amide bond (D), transfer of the hydrogen atom from the amide nitrogen to the protonated carbonyl (K), and subsequent loss of water to form the nitrilium ion product (L). To probe the structure of the $[M+H - H_2O]^+$ ion of glycylglycine further and to obtain additional experimental evidence to confirm the site of H_2O loss, the methyl ester derivative of glycylglycine (*m/z* 147) was prepared and the $[M+H]^+$ ion subjected to MS/MS. The observed fragmentation pattern was similar to that of the underivatized peptide (Table 1) except that the loss of CH_3OH instead of H_2O was observed as the major fragmentation product, as expected for the ***type A*** retro-Koch reaction.

The $[M+H - CH_3OH]^+$ ion (*m/z* 115), was subjected to MS^3 and the resulting spectrum (Table 2), as expected, showed the loss of CO to form the immonium ion product at *m/z* 87. Interestingly, a low abundance ion (*m/z* 129) corresponding to the loss of H_2O was also observed in the MS/MS spectrum of glyclylglycine-OMe (Table 1). Unfortunately, the yield of this ion was not sufficient to perform subsequent MS^3. As this loss of water must occur remote to the C-terminus, it



Scheme III

appeared likely that the mechanism of water loss was of ***type C*** (i.e., the retro-Ritter reaction) as described above.

Tandem-mass spectrometry of the $[M+H]^+$ ion of N-acetyl glycine O-methyl ester. To gain further insights into the formation of the retro-Koch and retro-Ritter products, a simple model peptide system using the N-acetyl (NAc-), and O-methyl (OMe-) amino acid derivative of glycine was prepared. Previously, model peptide systems have been used successfully to probe the reactivities of peptides within the gas phase [1c]. The LCQ MS/MS spectrum of the $[M+H]^+$ ion of NAc-glycine-OMe (m/z 132) is given in Table 1. The types of observed fragment ions corresponded closely to those seen in the MS/MS spectrum of the $[M+H]^+$ ion of glycylglycine-OMe. The intensity of the fragment ion at m/z 114 corresponding to the loss of water from the $[M+H]^+$ ion of NAc-glycine-OMe was not sufficient to allow MS^3.

Tandem-mass spectrometry of the $[M+H]^+$ ion of N-acetyl sarcosine O-methyl ester. As transfer of the hydrogen atom from the amide nitrogen to the protonated carbonyl of the amide bond is required for the retro-Ritter reaction to proceed (Scheme III, pathway 3), the NAc-, OMe- derivative of sarcosine [NMe-glycine, $CH_3CON(CH_3)CH_2CO_2CH_3$] was synthesized to determine if the $[M+H - H_2O]^+$ ion of NAc-glycine-OMe was formed via the retro-Ritter pathway. The MS/MS spectrum of NAc-sarcosine-OMe (m/z 146) is listed in Table 1. An ion at m/z 128, corresponding to the loss of water, was not observed for the NMe-model peptide, suggesting that the retro-Ritter pathway for the loss of water does operate for the simple glycine-containing dipeptide and model peptide systems examined.

Although the mass spectrometric data provided convincing evidence for the retro-Koch and retro-Ritter reactions in the formation of the $[M+H - H_2O]$ ions of

Table 2. LCQ CID MS^3 of the $[M+H - H_2O]^+$ ions of dipeptides and N-acetyl glycine and cysteine model systems.

Neutral species	$[M+H - H_2O]^+$ ion[a]	Daughter ions: *m/z*, (loss), abundance %[b]
$H_2NCH_2C(O)NHCH_2CO_2H$	115	87(CO)100
$H_2NCH_2C(O)NHCH_2CO_2CH_3$	129	MS/MS ion too small to obtain MS^3[c]
$H_2NCH_2C(O)NHCH(CH_2SH)CO_2H$	161	144(NH_3)2, 133(CO)1, 132($HNCH_2$)100, 115(HCO_2H)25, 100( )1, 89( )50, 86($HNCH_2$, HCO_2H)1, 69( )8, 62( )1
$H_2NCH_2C(O)NHCH(CH_2SH)CO_2CH_3$	175	158(NH_3)9, 146($HNCH_2$)41, 119(H_2NCH_2CN)1, 115(HCO_2CH_3)12, 89( )26, 62( )1
$CH_3C(O)NHCH_2CO_2H$	100	72(CO)100
$CH_3C(O)NHCH_2CO_2CH_3$	114	MS/MS ion too small to obtain MS^3[c]
$CH_3C(O)N(CH_3)CH_2CO_2H$	114	86(CO)100
$CH_3C(O)NHCH(CH_2SH)CO_2H$	146	118(CO)70, 105(CH_3CN)41, 100(HCO_2H)100, 76(CO, CH_2CO)3
$CH_3C(O)NHCH(CH_2SH)CO_2CH_3$	160	See Figure 5A
$CH_3C(O)NHCH(CH_2SCH_3)CO_2H$	160	132(CO)100, 119(CH_3CN)8
$CH_3C(O)N(CH_3)CH(CH_2SH)CO_2H$	160	132(CO)43, 114(HCO_2H)2, 90(CO, CH_2CO)2
$CH_3C(O)N(CH_3)CH(CH_2SH)CO_2CH_3$	174	resistant to fragmentation

[a]Formed via ESI-MS/MS of the respective $[M+H]^+$ ions shown in Table 1.
[b]Only those ions greater than 1% relative abundance are shown.
[c]MS/MS of the $[M+H - CH_3OH]^+$ ion yielded CO loss.

Table 3. Energies of species involved in the dehydration of N-formylglycine and N-formylcysteine

N-formylglycine derived species		Total energies (Hartree) HF/6-31G*	MP2(fc)/6-31G*	ZPE[a]	Relative energies (kcal/mol) HF[b]	MP2[c]
$HC(O)NHCH_2COOH$	(A)	−395.58067	−396.64070	0.08785	+206.2	+202.1
$[HC(O)NHCH_2COOH_2]^+$	(B)	−395.86644	−396.92429	0.09457	+31.1	+28.4
$[HC(O)NHCH_2C(OH)_2]^+$	(C)	−395.89195	−396.94531	0.09972	+18.3	+18.4
$[HC(OH)NHCH_2COOH]^+$	(D)	−395.92241	−396.97589	0.10097	0	0
$[HC(O)NH_2CH_2COOH]^+$	(E)	−395.89348	−396.95518	0.10059	+17.9	+12.8
$[HC(O)NHCH_2CO]^+$	(F)	−319.86029	−320.73056	0.07440	+28.5[d]	+27.3[d]
$[HC(O)NHCH_2CO]^+$	(G)	−319.86029	−320.73053	0.07439	+28.5[d]	+27.3[d]
$[HC(O)NHCH_2C(OH)_2]^+$	(H)	−395.90353	−396.95917	0.10082	+11.8	+10.4
$[HC(O)NCH_2C(OH)OH_2]^+$	(I)	−395.86366	−396.91962	0.09667	+34.2	+32.6
$[HC(O)NCH_2COH]^+$	(J)	−319.82806	−320.69592	0.07324	+48.0[d]	+48.3[d]
$[HC(OH_2)NCH_2COOH]^+$	(K)	−395.87126	−396.92959	0.09370	+27.5	+24.5
$[HCNCH_2COOH]^+$	(L)	−319.82875	−320.69875	0.07079	+46.1[d]	+45.0[d]
N-formylcysteine derived species						
$[HC(OH)NHCH(CH_2SH)COOH]^+$	(M)	−832.42168	−833.74363	0.12838	+32.4	+32.5
$[HCNH(OH_2)CH(CH_2S)COOH]^+$	(N)	−832.47264	−833.79466	0.12767	0	0
$[HCNHCH(CH_2S)COOH]^+$	(O)	−756.43445	−757.56609	0.10437	+15.6[d]	+18.8[d]
$[HCONHCH(CH_2SH)CO]^+$	(P)	−756.40587	−757.52921	0.10144	+31.7[d]	+40.1[d]
$[HCNCH(CH_2SH)COOH]^+$	(Q)	−756.36622	−757.49163	0.09802	+54.4[d]	+61.6[d]
H_2O	—	− 76.01075	− 76.19595	0.02069		

[a]ZPE = zero point energy, which has been corrected by a scaling factor of 0.9.
[b]At the HF/6-31G*//HF/6-31G*+0.9 ZPE level of theory.
[c]At the MP2(fc)/6-31G*//HF/6-31G*+0.9 ZPE level of theory.
[d]The energy of the whole system (peptide+H_2O) is reported.

glycylglycine, the distinction between the Harrison and Hunt pathways for formation of the retro-Koch product and the effect of the site of protonation on the observed fragmentation pathway is difficult to address experimentally. Therefore, we have used ab initio calculations to address the above questions, and to determine the relative stabilities of several of the intermediates proposed in Scheme III for both the retro-Koch and retro-Ritter reactions for the dehydration of glycine containing peptides.

Ab Initio Studies of Retro-Koch versus Retro-Ritter Reactions for the Loss of Water from the $[M+H]^+$ ion of N-Formylglycine

The simplest model to study the loss of water from the $[M+H]^+$ ion of peptides by the retro-Koch and retro-Ritter reactions is N-formylglycine [9a] (Scheme III, where R = H and R′ = H). We have performed ab initio calculations to determine the stable conformers of neutral N-formylglycine, the various protonated forms, and several intermediates on both the retro-Koch and retro-Ritter pathways for the loss of water. The results of the calculations for each of the species (Species A–L) are summarized in Table 3.

Determination of the preferred site of protonation for N-formylglycine. Neutral N-formylglycine (Species A, Figure 2) (see Table 3 for energies), can be protonated at four different sites; the OH and CO of the carboxylic acid and the carbonyl oxygen and nitrogen of the amide bond. Each of these structures (Species B–E, Figure 2) (see also Table 3) were derived from the optimized structure of neutral N-formylglycine and then subjected to energy minimization at the HF/6-31G* level of theory. Clearly, the thermodynamically preferred site of protonation was the carbonyl oxygen of the amide bond, which was found to be 12.8 kcal/mol more stable than protonation at the amide nitrogen and 18.4 and 28.4 kcal/mol more stable than protonation at the CO and OH of the carboxylic acid, respectively (at the MP2(FC)/6-31G*//HF/6-31G*+ZPE correction level of theory). This result is consistent with previous ab initio calculations on simple amide systems [14a] and the simple dipeptide glycylglycine [14b]. It was observed that protonation at the carboxyl OH (Figure 2B) leads to significant lengthening of the C–OH bond, and loss of water consistent with previous results for protonated glycine [11d]. Protonation at the NH of the amide bond (Figure 2E) leads to weakening of the proximal C–N bond, whereas protonation at the amide carbonyl (Figure 2D) leads to significant strengthening of the C–N bond as indicated by the shorter bond length observed. These last two results are consistent with previous reports by other workers [20].

Given that the thermodynamically preferred site of protonation was the carbonyl oxygen of the amide bond, how does this result relate to the observed fragmentation reactions of $[M+H]^+$ ions, particularly those of the simple glycine-containing peptides where amide bond cleavage is the major fragmentation process? The mechanism for "indirect" amide bond cleav-

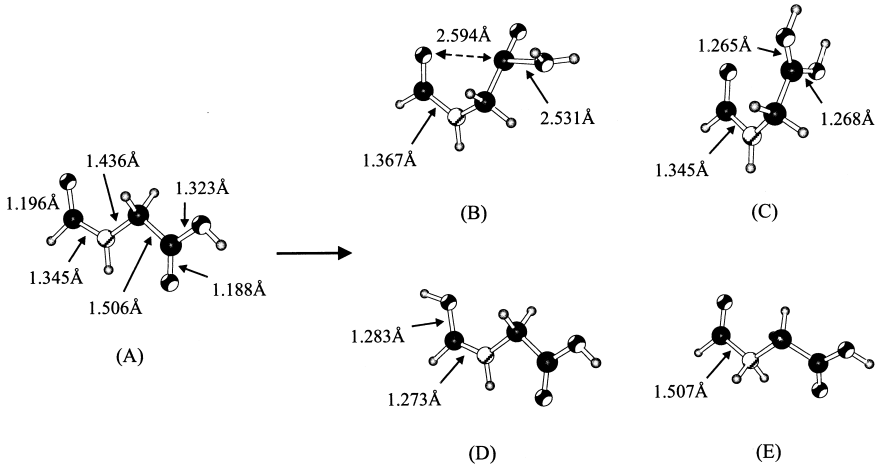


Figure 2. HF/6-31G* optimized structures of (**A**) neutral N-formylglycine, (**B**) carboxylic OH-protonated N-formylglycine, (**C**) carboxylic CO-protonated N-formylglycine, (**D**) amide carbonyl CO-protonated N-formylglycine, and (**E**) amide NH-protonated N-formylglycine.

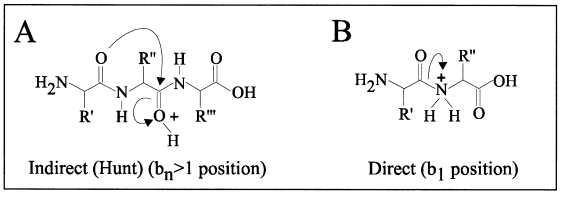
age proposed by Hunt [9c], suggests that protonation at the carbonyl oxygen of the amide bond is followed by cyclization to the oxazolone and, following proton transfer, cleavage of the amide bond (Scheme IVA).

However, this proposal does not explain the amide bond fragmentation observed for the above mentioned examples for glycylglycine (i.e., at the b_1 position) as Hunt's mechanism can only account for fragmentation of the amide bond where $b_n > 1$ (note that n is equal to the number amide bonds from the N-terminus).

Direct protonation at the amide nitrogen (Scheme IV) is required in order for cleavage at the b_1 position (i.e., at the first amide bond from the N-terminus) to occur. The energy required to overcome the barrier to proton transfer from the initially preferred carbonyl oxygen to the amide nitrogen, to effect amide bond cleavage, is most likely supplied by collisional "heating" employed during the fragmentation process. This notion of intramolecular proton transfer in peptide ions has been called the "mobile proton model" and has been used to rationalize unimolecular fragmentation reactions [21] as well as bimolecular reactions [22].

Ab initio studies of species involved in the dehydration of the $[M+H]^+$ ion of N-formylglycine by the retro-Koch and retro-Ritter reactions. As noted previously, there are three possible processes for the loss of water from the backbone of a protonated peptide (Scheme III). To gain insights into the relative energetics for each of the proposed intermediates in the pathways for the loss of water, the structures of the species shown in pathway 1 (the retro-Koch, Harrison), pathway 2 (the retro-Koch, Hunt) and pathway 3 (the retro-Ritter) of Scheme III were calculated. The thermodynamically preferred product following dehydration by the Harrison scheme (Scheme III, pathway 1) was shown to be the N-protonated oxazolone structure (F) where the highest energy intermediate along the pathway was the initially protonated species (B) (Figure 3). The acyclic acylium ion (G), upon energy minimization, was found to spontaneously cyclize to species (F). Our results are consistent with previous calculations at the HF/6-31G** level of theory by Harrison [9a], that indicated that for both the N-protonated oxazolone and acylium ion dehydration products of N-formylglycine, the oxazolone is stable, whereas the acylium ion lies at a saddle point or transition state on the potential energy surface.

The results of our ab initio calculations on the intermediates involved in the retro-Koch (Hunt) mechanism (Scheme III, pathway 2) indicated that the final product (J) resides at the highest energy minima coordinate along the reaction pathway. Although cyclization of (C) to (H) results in an energy decrease, proton transfer across the ring from (H) to (I) would be



Scheme IV

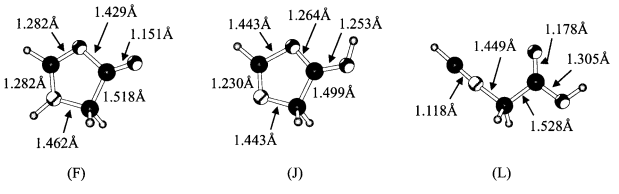


Figure 3. HF/6-31G* optimized structures of (**F**) the retro-Koch-(Harrison) N-protonated oxazolone, (**J**) the retro-Koch(Hunt) CO-protonated oxazolone, and (**L**) the retro-Ritter nitrilium ion product of N-formylglycine.

Table 4. Relative energies [at the MP2(FC)/6-31G*//HF/6-31G*+0.9 ZPE level of theory] of the stable product ions resulting from the dehydration of N-formylglycine and N-formylcysteine

	Relative energies (kcal/mol)			
	N-formylglycine		N-formylcysteine	
Retro-Koch(Harrison)	(F)	0	(P)	0
Retro-Koch(Hunt)	(J)	+19.5		—
Retro-Ritter	(L)	+17.5	(Q)	+21.4
Intramolecular nucleophilic attack		—	(O)	−21.3

expected to be energetically demanding. Although we have not calculated the transition state to this proton transfer, it is likely to be a high energy process because of the ring strain required. An alternative pathway for loss of H_2O from (H) may involve a 1,1 elimination to give species (F), the thermodynamically preferred product from the retro-Koch (Harrison) mechanism.

The intermediates involved in the retro-Ritter mechanism for water loss from protonated N-formylglycine have also been examined using ab initio calculations. It can be seen from the energetically "uphill" reaction profile that formation of the nitrilium ion retro-Ritter product would not be favored thermodynamically where the difference in energies between species (D) and (K) following proton transfer is 24.5 kcal/mol and a further difference of 20.5 kcal/mol between (K) and (L) for the loss of water was calculated.

A comparison of the relative energies for each of the structures (F, J, and L) (Table 4) indicated an overall preference for the retro-Koch(Harrison) reaction pathway. Thus the ab initio results indicate the following order for water loss based upon the stabilities of the final products: retro-Koch(Harrison) > retro-Ritter > retro-Koch(Hunt).

The relative order of stabilities of the dehydration products (F), (J), and (L) from Figure 3 compare favorably with the experimentally observed product ions from the MS/MS of the [M+H]+ ion of glycylglycine-OMe and NAc-glycine-OMe (Table 1). Thus, the relative ion abundances for the loss of CH_3OH (m/z 115 and 100, respectively) corresponding to the retro-Koch(Harrison) reaction product (F), and loss of H_2O (m/z 129 and 114, respectively) corresponding to the retro-Ritter reaction product (L), compare favorably with the theoretically determined values for the simple N-formylglycine model peptide system. As the experimentally observed relative ion abundances appear to follow the thermodynamically preferred fragmentation pathways determined by ab initio calculations, it would also appear that the kinetic product is also the thermodynamically favored product in the various reaction pathways. We recognize that a proper theoretical analysis of these fragmentation pathways would require an examination of the transition states. This is beyond the scope of this study because there are essentially no experimental or theoretical benchmarks to test the appropriate level of theory for transition state calculations on such molecules.

Tandem-Mass Spectrometric Studies of the Loss of Water from the $[M+H]^+$ Ions of Simple Cysteine-Containing Dipeptides and Model Systems

Having obtained both experimental and theoretical evidence to support the proposed mechanisms for loss of water from the backbone of a protonated peptide, we were interested in the influence of potentially reactive side chains on the dehydration pathways. The side chain thiol of cysteine was chosen for evaluation as previous studies on the gas phase ion–molecule reactions of cysteine have indicated that the thiol side chain can act as an intermolecular nucleophile [1b]. Furthermore, the identification and characterization of post-translationally modified cysteine containing proteins and the arrangement of cysteine connectivities in proteins is important because of the vital role that cysteine plays both functionally (for example, in the catalytic site of the cysteine proteases), and in stabilizing the overall structure of a protein by disulfide bond formation. Failure to reduce and S-alkylate proteins prior to proteolytic digestion may result in the generation of extremely complex peptide maps that complicate subsequent analysis strategies. In most cases, this is because of disulfide bond formation following digestion. Therefore, the ability to identify and sequence modified cysteine residues is crucial to protein sequencing strategies [23].

Tandem-mass spectrometry of the $[M+H]^+$ ion of glycylcysteine. For the above reasons, we were interested in the effects that cysteine may have on dehydration of the peptide backbone. The fragment ion yield and relative appearance energies of the product ions for the energy resolved CID fragmentation of the $[M+H]^+$ ion of glycylcysteine are shown in Figure 4. Compared to glycylglycine, the breakdown graph of glycylcysteine exhibited a significant increase in the abundance of the $[M+H-H_2O]^+$ ion (m/z 161). [In contrast to glycylcysteine, the MS/MS of the $[M+H]^+$ ion of cysteinylglycine fragmented almost exclusively to form an ion at m/z 162, corresponding to the loss of NH_3. The related $[M+H-NH_3]^+$ ion of protonated cysteine has recently been shown, by MS^n tandem mass spectrometric and ab initio techniques, to be an episulfonium ion, formed via intramolecular nucleophilic attack by the thiol side chain at the alpha carbon with subsequent elimination

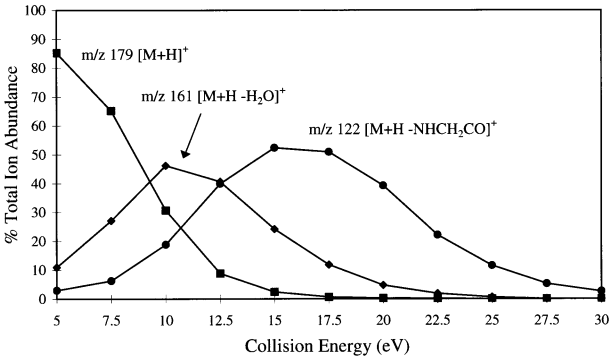


Figure 4. Triple quadrupole energy resolved MS/MS breakdown graph of the [M+H]+ ion of glycylcysteine: (filled square) $[M+H]^+$ (m/z 179), (filled circle) $[M+H - NHCH_2CO]^+$ (m/z 122), (filled diamond) $[M+H - H_2O]^+$ (m/z 161).

of NH_3 (O'Hair, R. A. J.; Styles, M.; Reid, G. E., manuscript submitted).]

As was observed for the MS/MS of glycylglycine, the collision energy at which the maximum yield of the $[M+H - H_2O]^+$ ion (m/z 161) was seen (~10 eV, laboratory frame of reference) was significantly lower than that for the m/z 122 sequence ion (~15 eV) suggesting a lower energy requirement for its formation. Once again, as discussed earlier for the MS/MS of glycylglycine, several ions (m/z 133, 132, 105, 87, 76, 59, 30, and 18) were also observed as the products formed from higher energy fragmentation processes. These ions were attributed, using MS/MS and MS^3, to the loss of H_2O and CO (m/z 133) from the $[M+H]^+$ ion, loss of $NHCH_2$ (m/z 132) from the $[M+H - H_2O]^+$ (m/z 161) ion and losses of NH_3 (m/z 105), NH_3 and H_2O (m/z 87), H_2O and CO (m/z 76) and NH_3, H_2O, and CO (m/z 59) from the $[M+H - NHCH_2CO]^+$ (m/z 122) ion. In addition the loss of CO from the unstable b_1 ion (m/z 30) was observed, as was an ion corresponding to the ammonium ion, NH_4^+ (m/z 18) (data not shown).

Tandem-mass spectrometry of the $[M+H]^+$ ion of glyclycysteine O-methyl ester. To probe the structure of the $[M+H - H_2O]^+$ ion of glycylcysteine (m/z 161), the methyl ester derivative of glycylcysteine (m/z 193) was prepared. The LCQ MS/MS product ion spectrum of the $[M+H]^+$ ion of glycylcysteine-OMe is listed in Table 1. Compared to the MS/MS spectrum of the $[M+H]^+$ ion of glycylglycine-OMe, where the major product ion resulted from fragmentation of the amide bond, the major ion (m/z 175) observed from the MS/MS of glycylcysteine-OMe corresponded to the loss of water. Furthermore, the m/z 175 ion corresponding to the loss of H_2O is more pronounced than that corresponding to the loss of CH_3OH (m/z 161), which is the reverse of that observed for glycylglycine-OMe. The MS^3 spectrum of the $[M+H - H_2O]^+$ ion (m/z 175) of glycylcysteine-OMe (Table 2) revealed major product ions at m/z 158, 146, 115, and 89 and is dramatically different

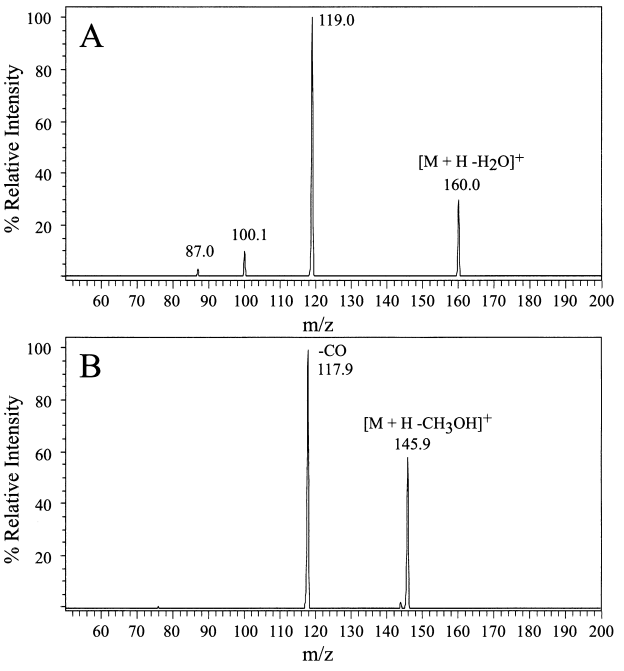


Figure 5. (**A**) MS^3 spectrum of the $[M+H - H_2O]^+$ ion of NAc-cysteine-OMe and (**B**) the $[M+H - CH_3OH]^+$ ion of NAc-cysteine-OMe.

from the MS^3 spectrum of the $[M+H - CH_3OH]^+$ ion of glycylglycine-OMe.

Tandem-mass spectrometry of the $[M+H]^+$ ions of N-acetyl cysteine O-methyl ester, N-acetyl cysteine N, O-methyl ester and N-acetyl cysteine S, O-methyl ester. To explain the significantly higher abundance of the $[M+H - H_2O]^+$ ion in the MS/MS spectra of glycylcysteine-OMe compared to that of glycylglycine-OMe, the NAc-OMe-amino acid derivative of cysteine was prepared. Additionally, the N-acetyl, O-methyl derivative of NMe-cysteine $[CH_3CON(CH_3)CH(CH_2SH)CO_2CH_3]$ was synthesized because transfer of the acidic proton from the amide nitrogen to the carbonyl is required for the retro-Ritter reaction to proceed. The LCQ MS/MS spectra of the $[M+H]^+$ ion is listed in Table 1, whereas the MS^3 of the $[M+H - H_2O]^+$ and $[M+H - CH_3OH]^+$ ions of NAc-cysteine-OMe are shown in Figure 5A, B, respectively. Note that (i) the MS^3 spectrum of the $[M+H - H_2O]^+$ and $[M+H - CH_3OH]^+$ ions of NAc-cysteine-OMe are quite different, suggesting that they have different structures and (ii) the exclusive loss of CO from the $[M+H - CH_3OH]^+$ ion of NAc-cysteine-OMe is the same as that observed for both the $[M+H - H_2O]^+$ ion of glycylglycine and the $[M+H - CH_3OH]^+$ ions of glycylglycine-OMe and NAc-Gly-OMe and is consistent with formation of an oxazolone structure [9a,b].

In contrast to NAc-sarcosine-OMe, where H_2O loss was not observed, the formation of an ion at m/z 174 from the MS/MS spectrum of NAc-cysteine-N-OMe (m/z 192), consistent with the loss of water, suggests

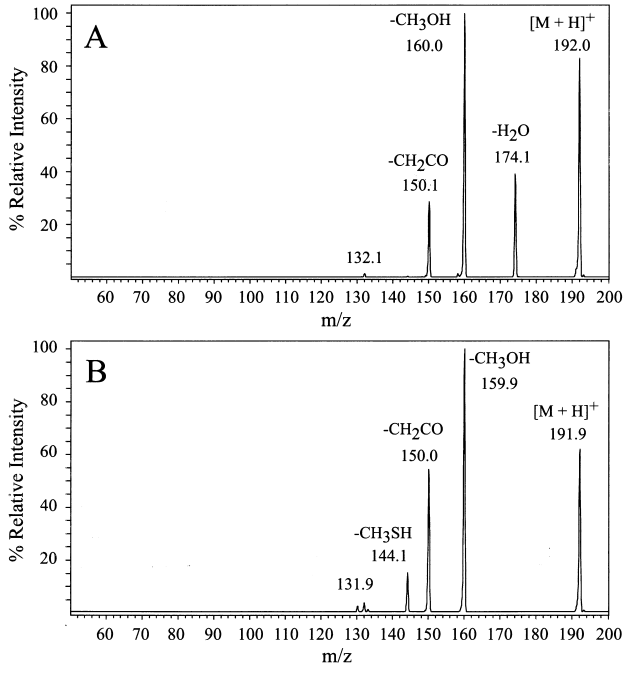


Figure 6. LCQ MS/MS spectra of the $[M+H]^+$ ions of (**A**) NAc-cysteine-N,OMe and (**B**) NAc-cysteine-S,OMe.

that the retro-Ritter reaction is not the major contributing process for water loss in this system (Figure 6A). As the only other possible source for the hydrogen atom required for water loss from the amide bond is the side chain thiol proton of cysteine, the NAc-cysteine-S-OMe derivative, $[CH_3CONHCH(CH_2SCH_3)CO_2CH_3]$ was synthesized. The MS/MS spectrum of the $[M+H]^+$ ion at (m/z 192) is shown in Figure 6B.

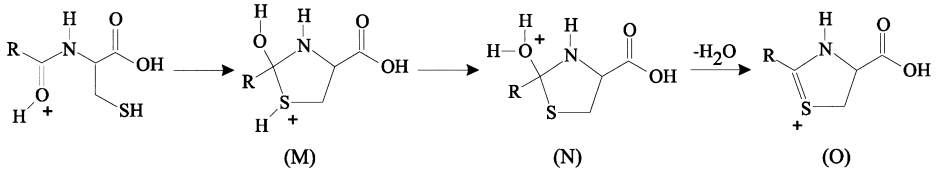
Loss of water from the S-methyl derivative was not observed indicating that the pathway for water loss is "switched off" following removal of the thiol proton. This result clearly demonstrates involvement of the nucleophilic thiol side chain in the pathway for water loss from the amide bond in this model peptide. Thus, we propose a mechanism whereby intramolecular nucleophilic attack by the thiol side chain occurs at the protonated carbonyl of the amide bond, followed by proton transfer from the side chain thiol to induce water loss (Scheme V). (Interestingly, MS/MS of the $[M+H]^+$ ion of NAc-cysteine-S,OMe produced an ion at m/z 144 that was not seen with either the NAc-cysteine-OMe or NAc-cysteine-N-OMe derivatives. This fragment ion may result from protonation at an additional site not previously discussed, the thiol side chain of cysteine, followed by nucleophilic attack by the preceding carbonyl oxygen to eliminate CH_3SH. The formation of product ions resulting from the thiol side chain cleavage of modified cysteine and cysteine-containing peptides has been recognized previously [24, 25].) To further examine this novel pathway for water loss, ab initio calculations were performed on the intermediates involved in this mechanism.

Ab Initio Studies of the Retro-Koch, Retro-Ritter, and Intramolecular Thiol Attack Reactions for the Loss of Water from the $[M+H]^+$ Ion of N-Formylcysteine

Our past experience with simple amino acids and their derivatives indicate that cysteine, due to both its larger size (more electrons) and larger number of conformers, is a considerably more demanding system than glycine on which to perform ab initio calculations [1, 17]. Based upon the results above, which indicate that the stabilities of the final products in the fragmentation pathways appear to be more important than the initial site of protonation, we have limited our ab initio calculations to the products formed by the loss of water from the $[M+H]^+$ ion of N-formylcysteine (Scheme III, where R = H and R′ = CH_2SH) (Table 3) via the intramolecular nucleophilic attack process and retro-Koch and retro-Ritter reactions. The optimized structures of the intramolecular nucleophilic attack product (O), the retro-Koch(Harrison) N-protonated oxazolone product (P), and the retro-Ritter product (Q) of N-formylcysteine are shown in Figure 7. The ab initio calculations on N-formylcysteine indicate that the stabilities of the products (Table 4) following dehydration are in the order of intramolecular nucleophilic attack (0 kcal/mol) > retro-Koch(Harrison) (+21.3 kcal/mol) > retro-Ritter (+42.7 kcal/mol).

When the relative energies of both the glycine- and cysteine-containing model systems are compared (Table 4), it is apparent that the retro-Koch reaction is favored in both cases by approximately 20 kcal/mol over the retro-Ritter reaction product.

However, for cysteine-containing peptides, where an alternate pathway for water loss involving a stronger nucleophile is introduced (i.e., the intramolecular nucleophilic attack pathway), even more favorable characteristics for the formation of the dehydration product are observed. Hence, the intramolecular thiol attack



Scheme V

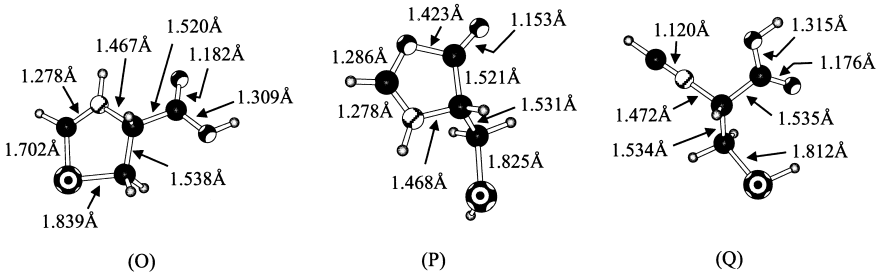


Figure 7. HF/6-31G* optimized structures of (**O**) the side chain thiol intramolecular nucleophilic attack at the carbonyl of the amide bond product, (**P**) the retro-Koch(Harrison) N-protonated oxazolone product, and (**Q**) the retro-Ritter nitrilium ion product of N-formylcysteine.

product for cysteine is preferred over the retro-Koch product by approximately a further 20 kcal/mol. Once again, there is a good correlation between the favored pathway determined from the ab initio calculations on the N-formyl model system and the experimentally observed MS/MS product ion abundances from the protonated N-acetyl peptide model system.

Conclusions

The gas phase fragmentation channels for the loss of water from the $[M+H]^+$ ion of some simple protonated dipeptides and peptide models has been examined. Three clearly distinct fragmentation pathways for the loss of water from the peptide backbone were observed. Two of these pathways correspond to the reverse reactions of analogous solution phase processes known as the Koch and Ritter reactions and have therefore been termed retro-Koch and retro-Ritter reactions. The third process operates for simple peptide and peptide model systems, XaaCys (where Xaa = glycine or CH_3CO), and involves intramolecular nucleophilic attack by the thiol side chain of the cysteine amino acid to eliminate water. Ab initio calculations on N-formylglycine, the simplest model system for the loss of water from the $[M+H]^+$ ion of peptides, indicated relative stabilities following dehydration of N-formylglycine in the order of the retro-Koch(Harrison) (0 kcal/mol), retro-Ritter (+17.5 kcal/mol), and retro-Koch(Hunt) (+19.5 kcal/mol) products. Ab initio calculations on N-formylcysteine reveal an order of stabilities following dehydration of N-formylcysteine of the intramolecular nucleophilic attack product (0 kcal/mol) followed by the retro-Koch-(Harrison) (+21.3 kcal/mol) and retro-Ritter (+42.7 kcal/mol) products.

Further work is required to assess the role of other side chains on the fragmentation reactions of the peptide backbone. A comprehensive study on the fragmentation of other simple model peptide systems (i.e., NAc-Xaa-OMe, where Xaa = any amino acid) is currently underway and should provide valuable insights towards the goal of routine characterization of novel and posttranslationally modified proteins by de novo mass spectrometric-based sequencing methodologies.

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