Chapter 3. Mapping Disulfide Bonds in Insulin with the Route 66 Method: Selective Cleavage of S-C Bonds using Alkali and Alkaline Earth Metal Enolate Complexes

3.1. Abstract

Simple and fast identification of disulfide linkages in insulin is demonstrated with a peptic digest using the Route 66 method. This is accomplished by collisional activation of singly and doubly charged cationic Na^+ and Ca^{2+} complexes generated using electrospray ionization mass spectrometry (ESI-MS). Collisional activation of doubly charged metal complexes of peptides with intermolecular disulfide linkages yields two sets of singly charged paired products separated by 66 mass units resulting from selective S-C bond cleavages. Highly selective elimination of 66 mass units, which corresponds to the molecular weight of hydrogen disulfide (H_2S_2), is observed from singly charged metal complexes of peptides with disulfide linkages. The mechanism proposed for these processes involves a process initiated by formation of a metal stabilized enolate at Cys, followed by cleavage of the S-C bond. Further activation of the products yields sequence information that facilitates locating the position of the disulfide linkages in the peptic digest fragments. For example, doubly charged Ca^{2+} complex of the peptic digest product GIVEQCCASVCSL/FVNQHLCGSHL yields paired products separated by 66 mass units resulting from selective S-C bond cleavages at an intermolecular disulfide linkage under low energy CID. Further activation of the product comprising the first of the two chains reveals the presence of a second disulfide bridge, an intramolecular linkage.

Experimental and theoretical model peptide studies provide mechanistic details for the selective cleavage of the S-C bond.

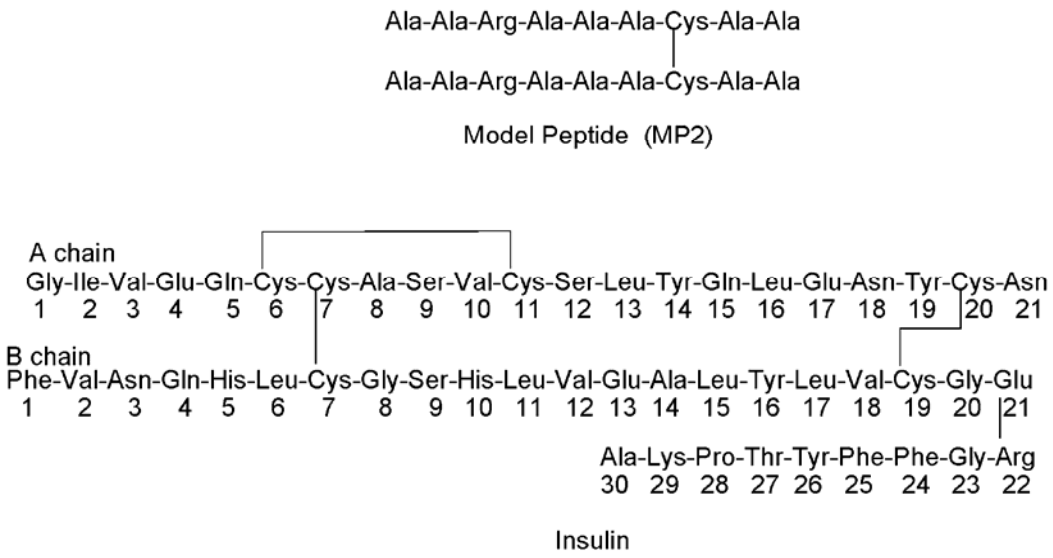
3.2. Introduction

Disulfide bonds are one of the most important post-translational modification (PTM) processes due to their unique role in determining the three-dimensional structures and stabilities of proteins.[1-3] Although various PTM sites in peptides have been identified using tandem mass spectrometry (MS^n),[4-11] disulfide bonds are not readily characterized by MS^n studies of protonated peptides.[2] Several studies achieved selective cleavages of the S-S and S-C bond at disulfide linkages using metal complexes of peptides.[12-16] Transition metal (Ni^{2+}, Co^{2+}, and Zn^{2+}) complexes of oxytocin exhibit dissociation pathways related to S-S and S-C bond cleavages under ECD conditions[14] and under sustained off-resonance irradiation collision induced dissociation (SORI-CID) conditions.[15] Selective S-S bond cleavages in cationic gold(I) complexes of peptides under low energy CID conditions were also reported.[13,16] We have recently reported what we call the Route 66 method for locating disulfide bonds in peptides. This methodology is based on the highly selective elimination of H_2S_2 (66 mass units) from singly charged sodiated and alkaline earth metal (Mg^{2+} and Ca^{2+}) bound peptide cations with disulfide linkages under CID conditions.[12] The process is initiated starting with a metal stabilized enolate anion at Cys, followed by cleavage of the S-C bond. Further MS^n spectra reveal additional details of the peptide structure in the region between the newly formed dehydroalanine residues.

In this paper, we report application of the Route 66 method for locating the position of disulfide linkages in insulin. This dipeptide hormone, widely used as a model system for

the identification of disulfide bonds along with sequence information, offers the challenge of three closely spaced disulfide bonds, including both intramolecular and intermolecular linkages.[17-19] A number of studies have reported sequence analysis of insulin using mass spectrometry.[19-23] Complete sequence analysis of insulin was demonstrated using the reduced protein with low energy CID.[22] ECD of the oxidized B-chain of insulin yields almost complete sequence information.[23] The CID of singly to triply charged insulin yields fragments resulting from intermolecular disulfide bond cleavages.[19] Although these top-down methods yield sequence information of insulin including the position of the Cys residues, locating the position of the original disulfide linkages in the protein remains a challenge. Analysis of a peptic digest is preferred for the investigation of this protein, since the acidic pH preserves the original disulfide bonds.[2] Furthermore, pepsin attacks a wide range of amide linkages yielding product peptides involving cleavage between most Cys residues.[18] Our previous study reported that ESI mass spectrum from the pepsin digest of insulin shows eight major ion peaks comprise five singly charged and three doubly charged ion peaks.[12] As the preliminary investigation, we examine doubly charged Na^+ and Ca^{2+} complexes of disulfide bridge linked model peptide $(AARAAACAA)_2$ (MP2). Then, we demonstrate that collisional activation of cationic Na^+ and Ca^{2+} complexes of insulin peptic digest fragment allows for straightforward analysis of the disulfide linkages in the parent molecules. The mechanisms and energetics of the observed reactions further examined by means of computational modeling. Structures of MP2 and insulin examined in this study are shown in Scheme 1.

Scheme 1. Structures of model peptide and insulin examined in this study.



3.3. Experimental

Calcium dichloride ($CaCl_2$), insulin from bovine pancreas, iodine (I_2), pepsin from porcine stomach mucosa, and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO). The model peptide AARAAACAA was purchased from BiomerTechnology (Concord, CA). All solvents (water, methanol, and benzene) were purchased from EMD Chemicals Inc. (Gibbstown, NJ). For the formation of an intermolecular disulfide bond joining a dimer of the model peptide AARAAACAA, 10 mM of the peptide in 300 μL of water was mixed with 10 mM of I_2 in 300 μL of benzene. The solution mixture was stirred vigorously for 30 minutes at room temperature. The aqueous solution was extracted after centrifugation. The sample solution was diluted to an appropriate concentration for ESI with 50:50 water/methanol solvent. The product corresponding to the dimeric peptide MP2 linked via a disulfide bond was confirmed by ESI mass spectrometric analysis. All metal complex samples were prepared by dissolving stoichiometric amounts of metal chloride and the analyte sample in the solvent. Total concentration of the sample solution was varied from 100 to 200 μM. Pepsin digests of insulin were prepared by incubating 0.1 mg of insulin from bovine pancreas with 0.025 mg of pepsin from porcine stomach mucosa in water containing 1% acetic acid by volume at 37 °C for 6 hours. Then pepsin was removed out using a Millipore Microcon centrifugal filter fitted with an Ultracel YM-10 membrane. The sample solution was diluted to an appropriate concentration for ESI. Metal complex samples were prepared by dissolving 40 μM of metal chloride in the diluted peptic digest solution.

Experiments were performed on a Thermo Finnigan LCQ Deca ion trap mass spectrometer (ITMS) in positive mode. Electrospray voltage of 5 kV, capillary voltage of 9 V, and capillary temperature 275 °C were set as parameters for ESI. The temperature of

the MS analyzer was ~23 °C before the experiment and ~24 °C during the experiment. The pressure is estimated to be ~10^{-3} torr He inside the trap. Metal complex ions of interest were isolated and fragmented via low energy CID. Continuous isolation of the cluster ions followed by CID (MS^n) was performed until the track of the isolated ion was lost. The ESI mass spectra reported in this study were obtained by averaging thirty scanned spectra.

The mechanisms and energetics of the S-C bond and S-S bond cleavage reactions were evaluated using density functional theory (DFT) calculations. Candidate structures for the Ca^{2+} complex of the dipeptide $(ACA)_2$ linked by a disulfide bond were generated using the AMBER force field by subjecting a starting conformation to 200 ps of dynamics at 500 K, then cooling it to 50 K over a variable period of time using HyperChem 7.52 (Hypercube, Gainesville, FL). For intermediates, 200 ps of dynamic simulation was executed at 300 K followed by cooling to 50 K over a variable period of time. The charge distribution of the complex was assigned at the PM3 level in each annealing sequence. Over 100 structures were generated and typically 20 candidate structures were determined. The lowest-energy structures were determined using DFT with the candidate structures. The DFT calculations were performed using Jaguar 6.0 (Schrödinger, Portland, OR) utilizing the Becke three-parameter functional (B3)[24] combined with the correlation functional of Lee, Yang, and Parr (LYP),[25] using the LACVP basis set. Thermodynamic properties were calculated assuming ideal gas at 298.15 K.

The nomenclature proposed by Roepstorff and Fohlman[26] was used for the parent and fragment ions. The prefixes “ds” and “Δ” refer to peptide ions containing a disulfide bond and dehydroalanine, respectively. The dehydroalanine residue is referred to as “dA” in displayed peptide sequences. The element symbol superscript for the fragment ion

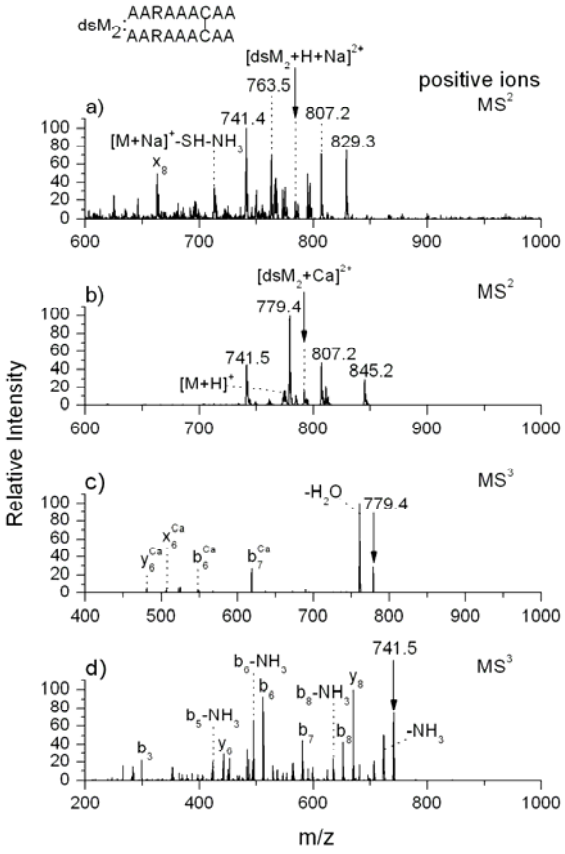
refers to the metal cation in the singly charged metal complex of the fragment. For example, a y_n fragment ion complex with a Ca^{2+} is referred to as "y_n^{Ca}." The numerical subscript refers to the number of equivalent amino acid units in the molecular ion when more than one repeated unit is present.

3.4. Results and Disucssion

3.4.1. Low Energy CID of Metal Complexes of MP2. We have recently demonstrated the low energy CID of singly charged cationic Na^+ and Ca^{2+} complexes of MP2, which is the dimeric model peptide $(AARAAACAA)_2$ linked by an intermolecular disulfide bond.[12] The CID of the monosodiated MP2 yields the dominant product resulting from the elimination of H_2S_2 (-66 mass units) with monomeric products from the cleavages of the S-S and S-C bonds. A dramatic increase of the relative abundance of the product from the elimination of H_2S_2 is observed from singly charged alkaline earth metal (Ca^{2+} and Mg^{2+}) complexes of MP2. In the present work we extend these earlier studies to include doubly charged cationic complexes of MP2 anticipating that the results can be useful in interpreting CID studies of the abundant doubly charged Na^+ and Ca^{2+} complexes observed in the ESI mass spectrum of the peptic digest of insulin.

Figure 1 shows MS^n spectra of doubly charged cationic complexes of MP2 with a Na^+ ion and a Ca^{2+} ion. The low energy CID spectrum of the monosodiated peptide is shown in Figure 1a. The CID of the doubly charged sodiated peptide yields four distinct singly charged products. The products at m/z 741.4 and m/z 807.2 are protonated product peptides and at m/z 763.5 and m/z 829.3 are monosodiated prouct peptides. Both paired protonated and sodiated products from MP2 are separated by 66 mass units, which

Figure 1. CID spectra of doubly charged (a) monosodiated MP2, and (b) monocalcium bound MP2 showing dominant product pairs separated by 66 mass units. (c) MS^3 spectrum of the MS^2 product at m/z 779.4 from (b). (d) MS^3 spectrum of the MS^2 product at m/z 741.5 from (b). Arrows indicate the ion peaks being isolated and collisionally activated.


dsM2: AARAAACAA
AARAAACAA
a)
[dsM2+H+Na]2+
763.5
741.4
807.2
829.3
[M+Na]+-SH-NH3
x8
positive ions
MS2
b)
[dsM2+Ca]2+
779.4
741.5
807.2
845.2
[M+H]+
MS2
c)
-H2O
779.4
x6Ca
y6Ca
b6Ca
b7Ca
MS3
d)
741.5
b6-NH3
b6
y8
b8-NH3
b5-NH3
b7
b8
-NH3
y6
b3
MS3
Relative Intensity
m/z

corresponds to the molecular weight of H_2S_2. This results from the S-C bond cleavage at the intermolecular disulfide linkage. The low energy CID of doubly charged Ca^{2+} bound peptide yields highly selective cleavages of S-C bonds. Two singly charged calicium bound products resulting from the S-C bond cleavages are observed at m/z 779.4 and m/z 845.2 as well as two singly charged protonated products at m/z 741.5 and m/z 807.2 (Figure 1b). It is notable that only a minor product from the S-S bond cleavage is observed at m/z 775.4 in the CID spectrum (Figure 1b). The MS^3 spectra of the products at m/z 779.4 and m/z 741.5 are shown in Figures 1c and 1d, respectively. The MS^3 spectrum of the Ca2+ bound product at m/z 779.4 exhibits the dominant product resulting from dehydration (Figure 1c). Other than dehydration, the products comprise b-type fragments (b_6 and b_7) formed by cleavages between the dehydroalanine (dA) residue. The collisionally activated protonated product at m/z 741.5 dissociates to yield a broad range of b- and y-type fragments. Similar to the MS^3 of the product at m/z 779.4, b-type fragments (b_6 and b_7) formed by cleavages between the dehydroalanine (dA) residue are observed in the MS^3 spectrum of the product at m/z 741.5 (Figure 1d).

3.4.2. Low Energy CID of Metal Complexes of the Pepsin Digest of Insulin. The ESI mass spectrum of pepsin digest of insulin shows 8 major ion peaks, which show good agreement with earlier published analyses (Figure 2a).[17,18] The ESI mass spectra of the pepsin digest with sodium and calcium are shown in Figures 2b and 2c. The masses and segments of observed peptic digest ions of insulin are summarized in Table 1.

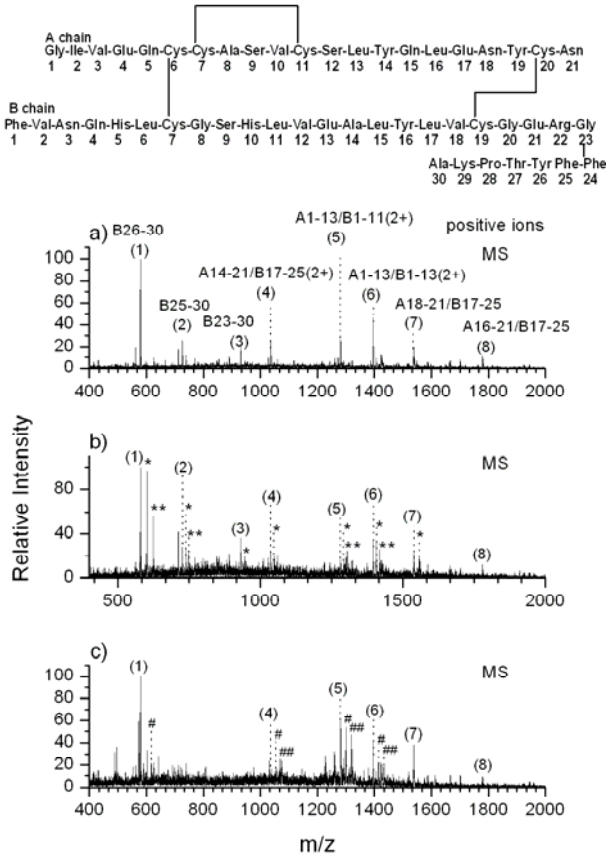
3.4.2.1. Singly Charged Sodiated Peptides from Pepsin Digest of Insulin. As seen in Figure 2b, abundant singly charged sodiated peptide ions are observed for three peptic digest products (1), (2), and (7) from insulin, which correspond to YTPKA, FYTPKA, and NYCN/LVCGERGFF. By scanning MS^2 of singly charged sodiated peptic digest

Table 1. Major peptide ions in the mass spectrum of the 6 hours pepsin digest of insulin

Segment[a,b]	Sequence[b]	Charge state	m/z (protonated)	m/z (sodiated)	m/z (calcium)
B26-30	YTPKA	+1	579.3	601.4[c] 623.5[d]	617.3[e]
B25-30	FYTPKA	+1	726.4	748.5[c] 770.4[d]	-[g]
B23-30	GFFYTPKA	+1	931.2	953.3[c,g]	-[g]
A14-21/ B17-25	YQLENYCN/ LVCGERGFF	+2	1036.4	1047.4[c]	1055.5[e] 1074.0[f]
A1-13/ B1-11	GIVEQCCASVCSL/ FVNQHLCGSHL	+2	1281.6	1292.5[c] 1303.6[d]	1300.5[e] 1319.6[f]
A1-13/ B1-13	GIVEQCCASVCSL/ FVNQHLCGSHLVE	+2	1395.5	1406.5[c] 1417.5[d]	1415.1[e] 1434.1[f]
A18-21/ B17-25	NYCN/ LVCGERGFF	+1	1537.6	1559.5[c]	-[g]
A16-21/ B17-25	LENYCN/ LVCGERGFF	+1	1779.5	1801.5[c,g]	-[g]

[a]Amino acid numbering for the two chains is shown in Figure 8. [b]Peptide pair with disulfide linkage is indicated with "/." [c]Monosodiated ion. [d]Disodiated ion. [e]Monocalcium bound ion. [f]Dicalcium bound ion. [g]Due to low intensity of this fragment the metal complex species was not observed or not sufficiently abundant for MS^2 analysis.

Figure 2. (a) ESI-MS of pepsin digest of insulin in positive mode. (b) ESI-MS of pepsin digest of insulin with NaCl in positive mode. Monosodiated and disodiated complexes are indicated as single asterisk and double asterisks, respectively, next to the numbered protonated segments. (c) ESI-MS of pepsin digest of insulin with $CaCl_2$ in positive mode. Monocalcium and dicalcium complexes are indicated as single sharp and double sharps, respectively, next to the numbered protonated segments. Structure of insulin is shown with amino acid numbering of the two chains.

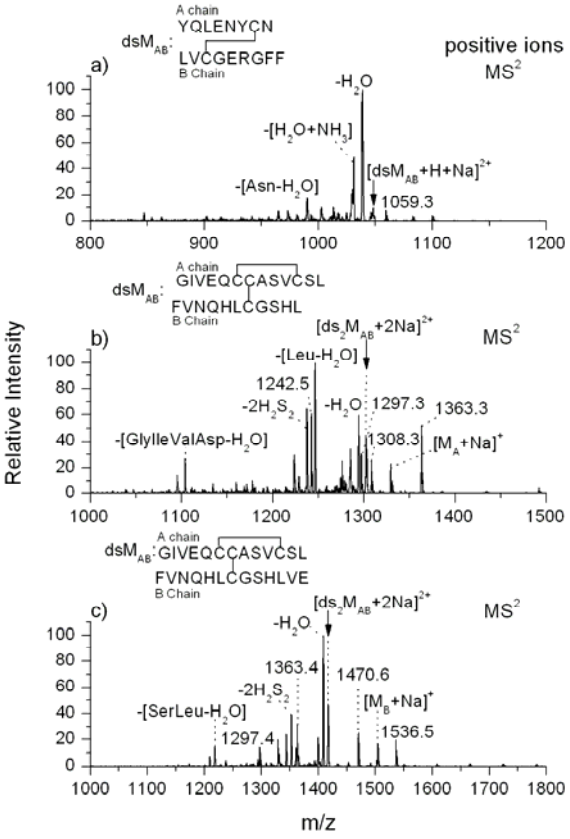

A chain
Gly-Ile-Val-Glu-Gln-Cys-Cys-Ala-Ser-Val-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
B chain
Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Ala-Lys-Pro-Thr-Tyr Phe-Phe
30 29 28 27 26 25 24
a) B26-30
(1)
A1-13/B1-11(2+)
(5)
positive ions
MS
A14-21/B17-25(2+)
(4)
A1-13/B1-13(2+)
(6)
B25-30
(2)
B23-30
(3)
A18-21/B17-25
(7)
A16-21/B17-25
(8)
b)
c)
Relative Intensity
m/z

components for loss of 66 mass units (H_2S_2) it is possible to identify peptide fragments with disulfide linkages. CID of sodiated peptides YTPKA at m/z 601.2 and FYTPKA at m/z 748.5 yield major products resulting commonly from the elimination of C-terminal Ala (- 89 mass units). No product is found from the elimination of H_2S_2 (-66 mass units). Results for CID of monosodiated dipeptide NYCN/LVCGERGFF at m/z 1559.5, linked via an intermolecular disulfide bond, has been presented in our earlier study.[12] The CID of sodiated NYCN/LVCGERGFF yields a major product from the H_2S_2 elimination. The observed elimination of H_2S_2 indicates the existence of a disulfide linkage in the peptide.

3.4.2.2. Doubly Charged Sodiated Peptides from Pepsin Digest of Insulin. Abundant doubly charged sodiated peptide ions are observed for three peptic digest products (4), (5), and (6), which correspond to YQLENYCN/LVCGERGFF, GIVEQCCASVCSL/FVNQHLCGSHL, and GIVEQCCASVCSL/-FVNQHLCGSHLVE (Figure 2b). Figure 3 shows CID spectra of the major doubly charged sodiated peptic digest fragments (Table 1) of insulin. The CID of doubly charged monosodiated dipeptide YQLENYCN/LVCGERGFF yields abundant products resulting from dehydration and combined dehydration and ammonia elimination (Figure 3a). In addition, a minor product from the elimination of A chain C-terminal Asn is observed in the spectrum. The product at m/z 1059.3 is the protonated B-chain peptide with –SSH replacing –SH at Cys.

Figure 3b shows the CID spectrum of doubly charged disodiated dipeptide GIVEQCCASVCSL/FVNQHLCGSHL. This dipeptide is linked by one intermolecular disulfide bond between $^{A}Cys_7$ and $^{B}Cys_7$. In addition, the A chain peptide contains one intramolecular linkage between $^{A}Cys_6$ and $^{A}Cys_{11}$. Various products, resulting from dehydrations (m/z 1285.5 and m/z 1276.5), the S-S bond cleavage (m/z 1329.3), the C-

Figure 3. (a) MS^2 spectrum of doubly charged monosodiated peptic digest fragment from insulin YQLENYCN/LVCGERGFF. (b) MS^2 spectrum of doubly charged disodiated peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHL showing major products involving disulfide bond cleavages. (c) MS^2 spectrum of doubly charged disodiated peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHLVE showing major products involving disulfide bond cleavages. Arrows indicate the ion peaks being isolated and collisionally activated.


A chain
YQLENYCN
dsM_AB:
LVCGERGFF
B Chain
a)
positive ions
MS2
-H2O
-[H2O+NH3]
[dsMAB+H+Na]2+
-[Asn-H2O]
1059.3
A chain
GIVEQCCASVCSL
dsMAB:
FVNQHLCGSHL
B Chain
b)
[ds2MAB+2Na]2+
MS2
-[Leu-H2O]
1242.5
-2H2S2
-H2O
1297.3
1363.3
-[GlyIleValAsp-H2O]
1308.3
[MA+Na]+
A chain
dsMAB: GIVEQCCASVCSL
FVNQHLCGSHLVE
B Chain
c)
[ds2MAB+2Na]2+
MS2
-H2O
1363.4
1470.6
-2H2S2
[MB+Na]+
-[SerLeu-H2O]
1536.5
1297.4
Relative Intensity
m/z

terminal residue elimination (m/z 1246.5), and cleavages at Asp residue (m/z 1104 and m/z 1095.3), are observed in the spectrum. In addition, CID of the doubly charged disodiated dipeptide yields four distinct singly charged monosodiated products at m/z 1242.5, m/z 1297.3, m/z 1308.3, and m/z 1363.3. The products at m/z 1242.5 and m/z 1308.3 are separated by 66 mass units. The products result from the conversion of Cys into dehydroalanine (m/z 1242.5) and disulfide group (m/z 1308.3) from A chain peptide via S-C bond cleavage. The products at m/z 1297.3 and m/z 1363.3 result from conversion of Cys into either a dehydroalanine or a disulfide group on the B chain peptide. The product resulting from the eliminations of two H_2S_2 indicates two disulfide linkages in the dipeptide.

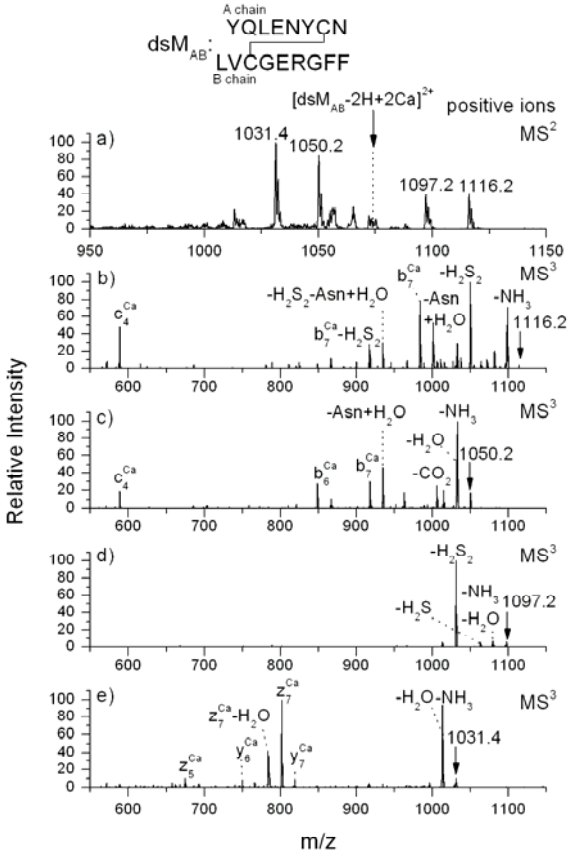
The dipeptide GIVEQCCASVCSL/FVNQHLCGSHLVE contains one intermolecular linkage between $^{A}Cys_7$ and $^{B}Cys_7$ along with intramolecular linkage between $^{A}Cys_6$ and $^{A}Cys_{11}$. The CID spectrum of doubly charged disodiated GIVEQCCASVCSL/-FVNQHLCGSHLVE is shown in Figure 3c. The product resulting from the eliminations of two H_2S_2 is observed at m/z 1352.5. Two singly charged monosodiated products separated by 66 mass units are observed at m/z 1297.4 and m/z 1363.4 from the B chain peptide. The other two singly charged monosodiated products separated by 66 mass units are observed at m/z 1470.6 and m/z 1536.5 from the A chain peptide.

3.4.2.3. Doubly Charged Calcium Complexes of Peptides from Pepsin Digest of Insulin. The ESI mass spectrum of calcium bound pepsin digest of insulin is shown in Figure 2c. Abundant doubly charged calcium complexes are observed for three peptic digest products (4), (5), and (6). Figures 3-5 show low energy CID spectra of doubly charged Ca^{2+} bound peptides YQLENYCN/LVCGERGFF, GIVEQCCASVCSL/ FVNQHLCGSHL, and GIVEQCCASVCSL/FVNQHLCGSHLVE.

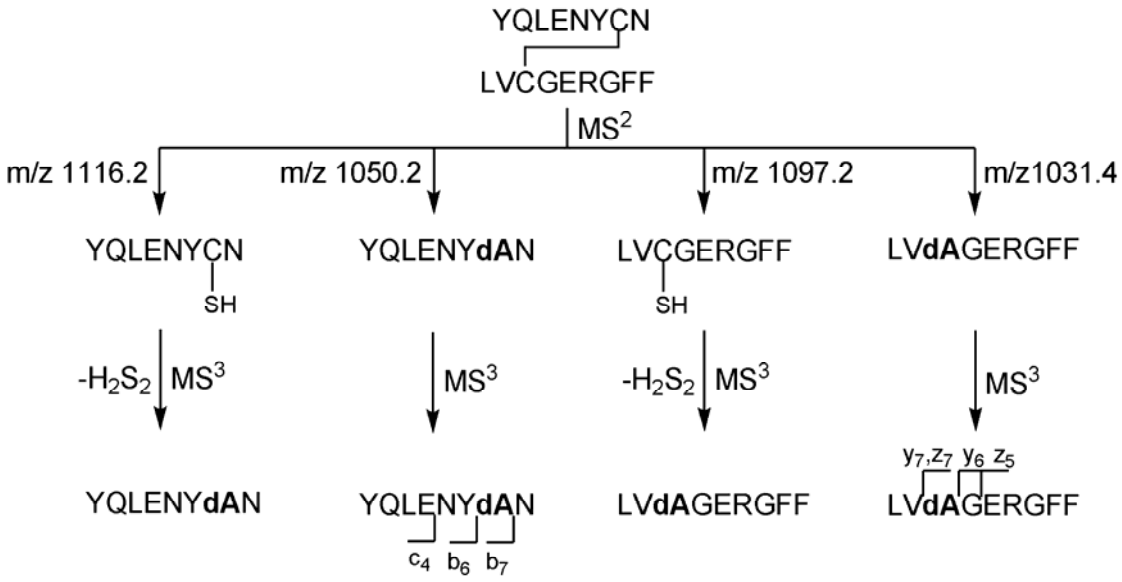
3.4.2.4. Doubly Charged Dicalcium Complex of YQLENYCN/LVCGERGFF. As seen in Figure 4a, CID of doubly charged dicalcium bound dipeptide YQLENYCN/LVCGERGFF yields four distinct products. The products at m/z 1116.2 and m/z 1050.2 and the products at m/z 1097.2 and m/z 1031.4 are separated by 66 mass units. The two sets of products indicate the presence of an intermolecular disulfide linkage in the peptide. The products at m/z 1116.2 and m/z 1050.2 are singly charged monocalcium bound A chain peptides with Cys converted to a disulfide and a dehydroalanine, respectively, via S-C bond cleavages. The products at m/z 1097.2 and m/z 1031.4 are singly charged monocalcium bound B chain peptide with a disulfide and a dehydroalanine, respectively. Further MS^n analysis has been performed in order to locate the position of the disulfide linkage in the dipeptide.

Figure 4b shows MS^3 spectrum of the product at m/z 1116.2. The major product observed at m/z 1050.4 results from elimination of H_2S_2. The MS^3 product at m/z 1050.4 confirms that the MS^2 product at m/z 1116.2 contains a disulfide group via the S-C bond cleavage. Products resulting from C-terminal Asn elimination are observed at m/z 935.2 ($-H_2S_2$-Asn+H_2O) and m/z 1001.5 (-Asn+H_2O). The products comprise b-type fragments (b_7 and b_7-H_2S_2) formed by cleavage at the disulfide site. A significant fragment c_4 is also observed at m/z 589.2. The MS^3 spectrum of the product at m/z 1050.2 is shown in Figure 4c. Similar to the MS^3 spectrum of the peptide at m/z 1116.2, the spectrum exhibits products resulting from C-terminal Asn elimination and the c_4 fragment. No product resulting from the elimination of H_2S_2 is observed, indicating the absence of a disulfide group in the peptide. Two b-type fragments (b_6 and b_7) at dehydroalanine residues locate the position of the intermolecular disulfide linkage in the A chain peptide.

Figure 4. (a) CID spectrum of doubly charged dicalcium bound peptic digest fragment from insulin YQLENYCN/LVCGERGFF. (b) MS^3 of product at 1116.2 from (a). (c) MS^3 of product at 1050.2 from (a). (d) MS^3 of product at 1097.2 from (a). (e) MS^3 of product at 1031.4 from (a). Arrows indicate the ion peaks being isolated and collisionally activated.


A chain
YQLENYCN
dsM_AB:
LVCGERGFF
B chain
[dsM_AB-2H+2Ca]2+
positive ions
a)
MS2
1031.4
1050.2
1097.2
1116.2
b)
MS3
c)
MS3
d)
MS3
e)
MS3
Relative Intensity
m/z

Scheme 2. Dissociation pathways of doubly charged dicalcium bound YQLENYCN/LVCGERGFF inferred from CID. Indicated product probed by MS^3.

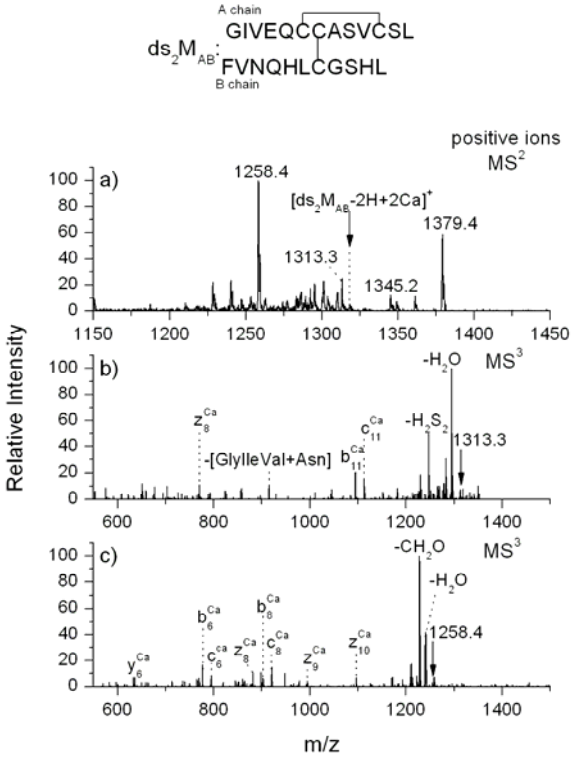


Figures 4d and 4e show MS^3 spectra of the MS^2 products at m/z 1097.2 and m/z 1031.4 from the B chain peptide. The MS^3 of the product at m/z 1097.2 yields the exclusive product at m/z 1031.3 resulting from elimination of H_2S_2. The MS^3 spectrum of the MS^2 product at m/z 1031.4 yields z- and y-type fragments. Formation of y_6 and y_7 fragments at the dehydroalanine residue locates the position of the disulfide linkage in the B chain peptide. In addition, products comprising z-type fragments (z_5 and z_7) are observed in the spectrum. CID pathways of doubly charged dicalcium bound YQLENYCN/-LVCGERGFF cation are summarized in Scheme 2.

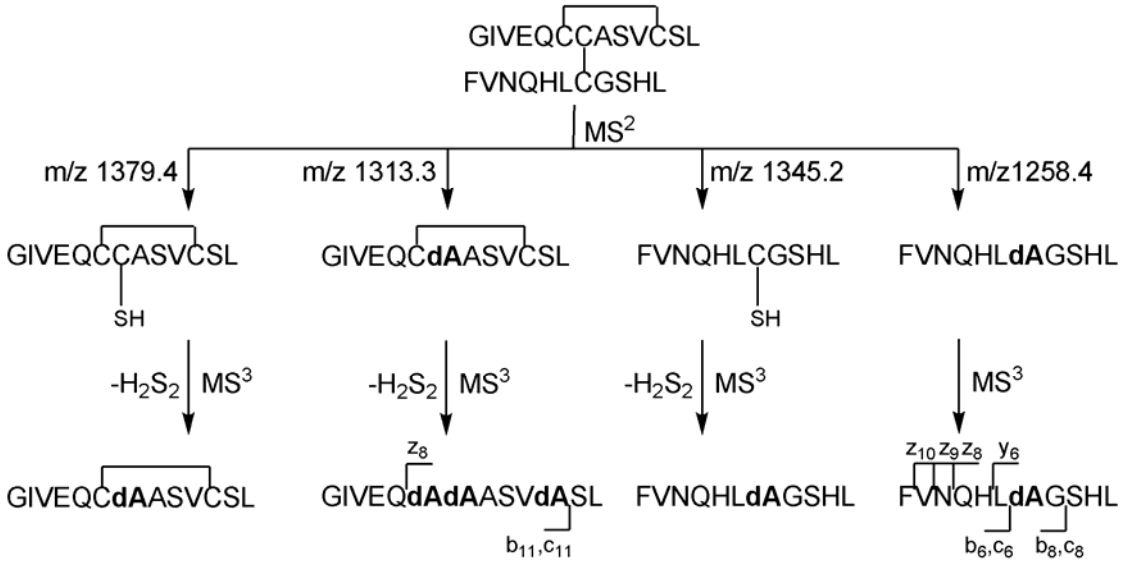
3.4.2.5. Doubly Charged Dicalcium Complex of GIVEQCCASVCSL/ FVNQHLCGSHL. The MS^2 spectrum of the doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHL peptide shows two distinct products at m/z 1379.4 and m/z 1258.4 (Figure 5a). A product separated by 66 mass units from the product at m/z 1379.4 is observed at m/z 1313.3. The MS^2 products at m/z 1379.4 and m/z 1313.3 are singly charged monocalcium bound peptide from A chain peptide with Cys converted to a disulfide and dehydroalanine, respectively. A product separated by 66 mass units from the product at m/z 1258.6 is not observed in the spectrum. The product at m/z 1258.6 is the singly charged monocalcium bound peptide with dehydroalanine from B chain peptide.

Figure 5b shows the MS^3 spectrum of the MS^2 product at m/z 1313.3. Other than dehydration product, an ion resulting from the elimination of H_2S_2 is observed as a major product in the MS^3 spectrum. Of note, the MS^2 product at m/z 1313.3 contains a dehydroalanine residue converted from Cys via the S-C bond cleavage. The dipeptide GIVEQCCASVCSL/FVNQHLCGSHL contains one intermolecular linkage and one intramolecular disulfide linkage. From this it is inferred that the observed selective

Figure 5. (a) CID spectrum of doubly charged dicalcium bound peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHL. (b) MS^3 of product at 1313.3 from (a). (c) MS^3 of product at 1258.4 from (a). Arrows indicate the ion peaks being isolated and collisionally activated.


A chain
GIVEQCCASVCSL
ds2MAB:
FVNQHLCGSHL
B chain
positive ions
MS2
a)
1258.4
[ds2MAB-2H+2Ca]+
1379.4
1313.3
1345.2
b)
-H2O
MS3
z8Ca
c11Ca
-H2S2
1313.3
-[GlyIleVal+Asn]
b11Ca
c)
-CH2O
MS3
-H2O
b6Ca
b8Ca
1258.4
c6Ca
z8Ca
c8Ca
z10Ca
y6Ca
z9Ca
Relative Intensity
m/z

Scheme 3. Dissociation pathways of doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHL inferred from CID. Indicated product probed by MS^3.

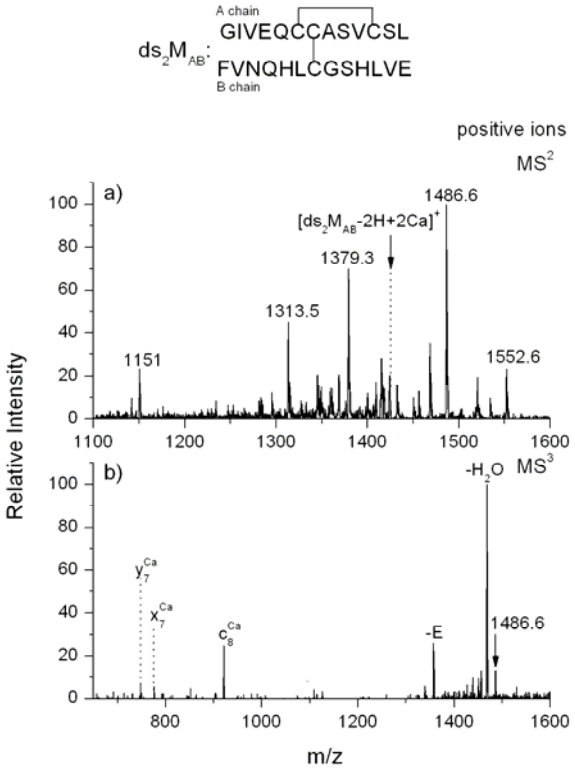


elimination of H_2S_2 is from the intramolecular disulfide linkage in the A chain peptide. The observation of b_{11} and c_{11} fragments at $^{A}Cys_{11}$ and z_8 fragment at $^{A}Cys_6$ locate the position of the intramolecular disulfide linkage. Figure 5c shows the MS^3 spectrum of the MS^2 product at m/z 1258.4. The major product at m/z 1228.3 results from the elimination of CH_2O (-30 mass units) from $^{B}Ser_9$. The products comprising b-type (b_6 and b_8) and c-type (c_6 and c_8) fragments locate the position of the intramoelcular disulfide linkage. Other products from z-type fragments are also observed. All observed CID pathways of doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHL cation are summarized in Scheme 3.

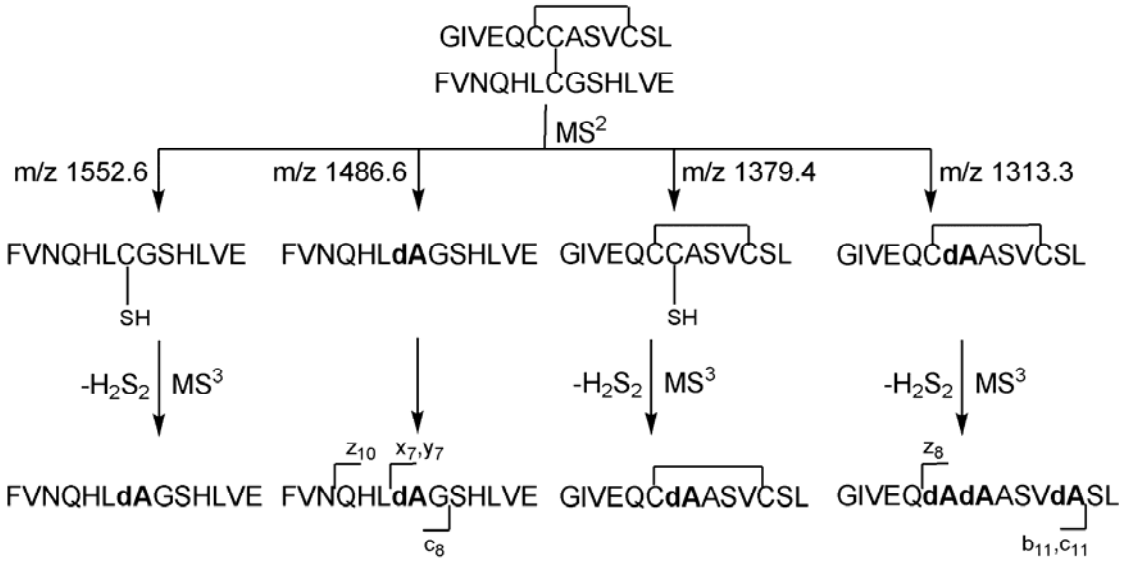
3.4.2.6. Doubly Charged Dicalcium Complex of GIVEQCCASVCSL/ FVNQHLCGSHLVE. Figure 6a shows the CID spectrum of doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHLVE cation. In analogy to other doubly charged metal complexes dipeptides with intermolecular disulfide bonds, four distinct products are observed via CID. The abundant products at m/z 1486.6 and m/z1552.6 originate from the B chain and are separated by 66 mass units. The formation of doubly charged dicalcium bound dipeptide comprising the c_8 fragment in the B chain. The A chain peptide is also observed at m/z 1151. Two products at m/z 1379.3 and m/z 1313.5 from the A chain peptide are already discussed in the previous section.

The MS^3 spectrum of the product at m/z 1486.6 yields a dominating product resulting from dehydration (Figure 6b). The product from the elimination of C-terminal Glu is observed at m/z 1357.6. The products comprising x_7 and y_7 fragments at dehydroalanine residue are also observed. Combined with the c_8 fragment, the x_7 and y_7 fragments locate the position of the disulfide linkage in the B chain peptide. The CID pathways of doubly

Figure 6. (a) CID spectrum of doubly charged dicalcium bound peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHLVE. (b) MS^3 of product at 1486.6 from (a). Arrows indicate the ion peaks being isolated and collisionally activated.


A chain
GIVEQCCASVCSL
ds2MAB:
FVNQHLCGSHLVE
B chain
positive ions
MS2
a)
1486.6
[ds2MAB-2H+2Ca]+
1379.3
1313.5
1151
1552.6
Relative Intensity
b)
-H2O
MS3
y7Ca
X7Ca
c8Ca
-E
1486.6
m/z

Scheme 4. Dissociation pathways of doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHLVE inferred from CID. Indicated product probed by MS^3.



charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHLVE cation are summarized in Scheme 4.

3.4.3. Selective S-C Bond Cleavage Processes Involving Enolate at Cys. This study demonstrates selective S-C bond cleavage of doubly charged sodiated and calcium bound disulfide linked dipeptide cations under low energy CID conditions. As seen in Figure 1, only a minor product is yielded by S-S bond cleavage process. As we proposed, the formation of an enolate anion at the Cys residue initiates the selective elimination of H_2S_2 from singly charged metal complex peptides under low energy CID.[12] The selective S-C bond cleavage from doubly charged collisionally activated metal complex peptides is considered to be initiated from an enolate structure at the Cys residue (Scheme 5). As seen in Figures 1c-1d, the MS^3 products comprise b-type fragments (b_6 and b_7) formed by cleavage between the dehydroalanine (dA) residue from MP2. This suggests that the hydrogen atoms attached to the sulfur atoms originated from the α-carbon of Cys as a result of the enolation process.

Figure 7 shows the DFT calculated changes of electronic energy (ΔE) associated with cleavages involving S-S and S-C bonds in the Ca^{2+} bound dimeric peptide, $(ACA)_2$, linked by a intermolecular disulfide bond. The optimized geometries of the corresponding intermediates are shown below the diagram. The S-S bond cleavage process of calcium bound $(ACA)_2$ is endothermic by ~27 kcal/mol. However, the process is exothermic overall for the S-C bond cleavage. The intermediate species associated with the S-C bond cleavage are energetically favored by ~50 kcal/mol when compared to intermediates involved in the cleavage of the S-S bond. The S-C bond cleavage is initiated by enolate at Cys and stabilized by interaction of a Ca^{2+} ion with the disulfide group. The proton transfer from the N-terminal amine to the disulfide group allows Ca^{2+}

Scheme 5. Proposed S-C bond cleavage mechanisms of doubly charged cationic Ca^{2+} bound dipeptide $(ACA)_2$ linked by intermolecular disulfide bond. Optimized geometries and energy changes for corresponding numbered states of Ca^{2+} bound $(ACA)_2$ are shown in Figure 7.

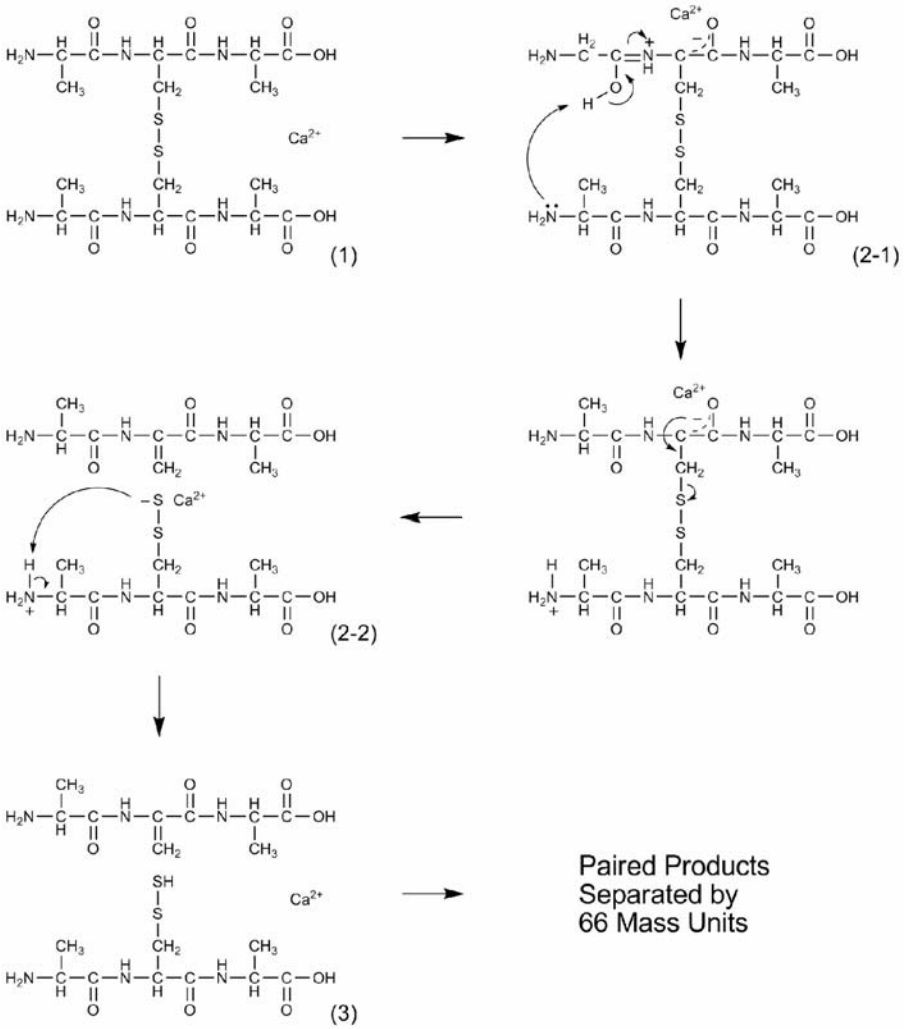
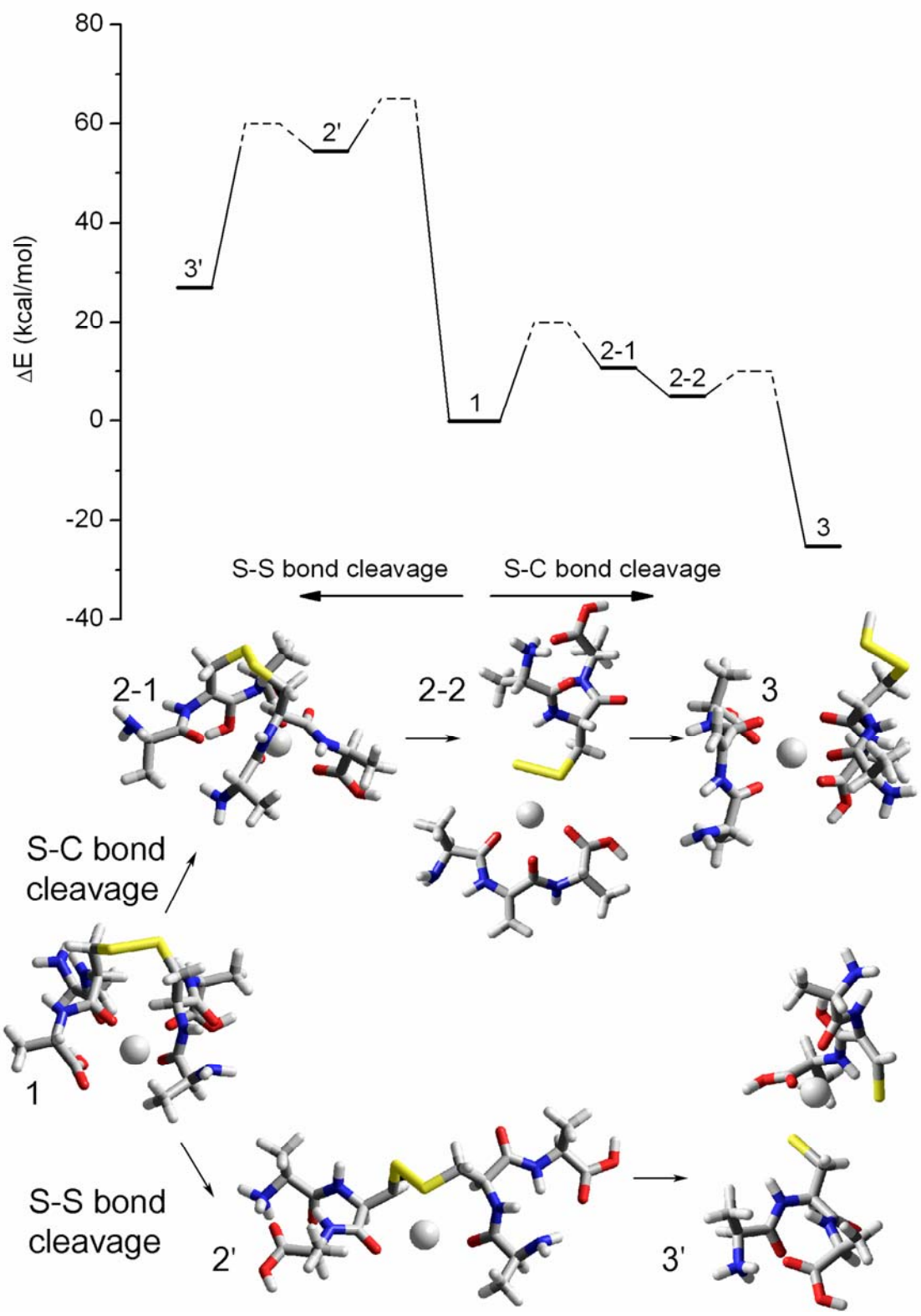


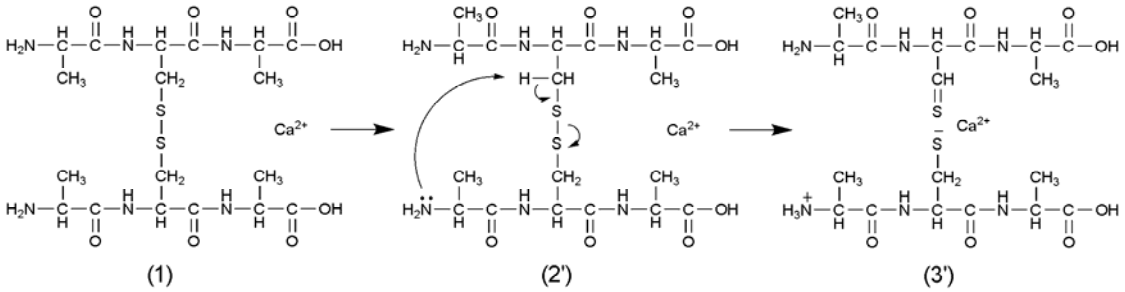
Figure 7. Reaction coordinate diagrams showing relative energies in kcal/mol for S-S bond cleavage (left side) and S-C bond cleavage (right side) of doubly charged calcium bound dimeric tripeptide, $(ACA)_2$, linked by intermolecular disulfide bond at the B3LYP/LACVP level, including zero-point correction obtained at the same scaled level. Barrier heights are not known. Optimized geometries for corresponding states are obtained at the same scaled level. The reaction mechanism of each numbered step is shown in Scheme 5.


80
60
40
20
0
-20
-40
ΔE (kcal/mol)
3'
2'
1
2-1
2-2
3
S-S bond cleavage
S-C bond cleavage
2-1
2-2
3
S-C bond
cleavage
1
S-S bond
cleavage
2'
3'

ion to interact mainly with the amide/carboxylic acid oxygen atoms. Once the proton is transferred to the disulfide group, immediate monomeric dissociation occurs due to charge repulsion as observed from the MS^2 spectra (Figure 1b). The S-S bond cleavage process requires unfavorable proton transfer from the β-carbon at Cys to the N-terminal amine (Scheme 6). In addition, Ca^{2+} ion interacts with the sulfur atom and the amide oxygen atoms after the S-S bond cleavage. We infer that the interaction between hard acid Ca^{2+} with relatively soft base sulfur atom causes the process of S-S bond cleavage to be less stable than the S-C bond cleavage.[27] As a result, highly selective S-C bond cleavage is achieved from alkali and alkaline earth metal complexes of peptides with intermolecular disulfide bond.

3.4.4. The Route 66 Method for Locating Disulfide Linkages in Peptides. Simple and fast identification of peptides with disulfide linkages in the peptic digest of insulin is demonstrated under low energy CID condition using Na^+ and Ca^{2+} complexes. Disulfide linkages in insulin are able to be located via further activation of the CID products. Two dipeptides, GIVEQCCASVCSL/FVNQHLCGSHL and GIVEQCCASVCSL/-FVNQHLCGSHLVE, which possess one intermolecular disulfide linkage and one intramolecular disulfide linkage each, yield evident products of the presence of disulfide linkages via CID of doubly charged Na^+ complexes (Figures 2b and 2c). The CID of the doubly charged disodiated peptides yield the products resulting from two H_2S_2 eliminations. Each singly charged Na^+ cation may interact with each disulfide linkage to stabilize enolate process at Cys to yield two H_2S_2 eliminations when the peptide complexes are collisionally activated.

Scheme 6. The S-S bond cleavage reaction of doubly charged cationic Ca^{2+} bound dipeptide $(ACA)_2$ linked by intermolecular disulfide bond requires a proton transfer from β-carbon at Cys. Optimized geometries and energy changes for corresponding numbered states of Ca^{2+} bound $(ACA)_2$ are shown in Figure 7.



As seen in Figures 3-5, higher selectivity on the cleavages of S-C bonds is observed from Ca^{2+} bound complex compared to sodiated complex via CID. Doubly charged calcium cation interacts more strongly with amide oxygen atom at Cys than singly charged Na^+ cation. As a result, more stable enolate processes at Cys yield higher selective S-C bond cleavages via CID. The MS^3 spectra of the products with dA residue allow us to identify the peptide with the intramolecular disulfide linkage (Figure 5b; Schemes 3 and 4). Otherwise, MS^3 spectra provide sequence information of the product peptide revealing the location of the Cys residue, which forms the disulfide bond in the peptide (Schemes 2-4). For the doubly charged metal complexes with both intermolecular and intramolecular disulfide linkages, the S-C bond cleavage occurs preferentially at the intermolecular disulfide linkage under low energy CID condition due to charge repulsion between monomeric product peptides.

3.5. Conclusion

Positions of disulfide linkages in insulin are located from peptic digest using mass spectrometry under low energy CID condition. The peptides containing disulfide linkages are easily identified from MS^2 of doubly charged Na^+ and Ca^{2+} complexes by a high abundance of paired products separated by 66 mass units. The structural information of the peptide including the location of the disulfide linkages and the presence of intramolecular disulfide linkages is achieved by further activation of the products. Similar to the elimination of H_2S_2 from singly charged alkali and alkaline earth metal complexes of peptides, the selective S-C bond cleavages of the doubly charged metal complexes are initiated from a metal stabilized enolate at Cys, with the intermediate products being further stabilized by the cation.

3.6. Acknowledgment

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3.7. References

(1) Thornton, J. M. *J. Mol. Biol.* 1981, *151*, 261-287.

(2) Gorman, J. J.; Wallis, T. P.; Pitt, J. J. *Mass Spectrom. Rev.* **2002**, *21*, 183-216.

(3) Chelius, D.; Wimer, M. E. H. *J. Am. Soc. Mass. Spectrom* **2006**, *17*, 1590-1598.

(4) Biemann, K.; Scoble, H. A. *Science* **1987**, *237*, 992-998.

(5) Mann, M.; Jensen, O. N. *Nat. Biotechnol.* **2003**, *21*, 255-261.

(6) Resing, K. A.; Johnson, R. S.; Walsh, K. A. *Biochemistry* **1995**, *34*, 9477-9487.

(7) Gibson, B. W.; Cohen, P. *Method Enzymol.* **1990**, *193*, 480-501.

(8) Huddleston, M. J.; Annan, R. S.; Bean, M. F.; Carr, S. A. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 710-717.

(9) Clauser, K. R.; Hall, S. C.; Smith, D. M.; Webb, J. W.; Andrews, L. E.; Tran, H. M.; Epstein, L. B.; Burlingame, A. L. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 5072-5076.

(10) Qin, J.; Chait, B. T. *Anal. Chem.* **1997**, *69*, 4002-4009.

(11) Reid, G. E.; Roberts, K. D.; Kapp, E. A.; Simpson, R. J. *J. Proteome Res.* **2004**, *3*, 751-759.

(12) Kim, H. I.; Beauchamp, J. L. *J. Am. Chem. Soc.* **2008**, *130*, 1245-1257.

(13) Gunawardena, H. P.; O'Hair, R. A. J.; McLuckey, S. A. *J. Proteome Res.* **2006**, *5*, 2087-2092.

(14) Kleinnijenhuis, A. J.; Mihalca, R.; Heeren, R. M. A.; Heck, A. J. R. *Int. J. Mass Spectrom.* **2006**, *253*, 217-224.

(15) Mihalca, R.; van der Burgt, Y. E. M.; Heck, A. J. R.; Heeren, R. M. A. *J. Mass Spectrom.* **2007**, *42*, 450-458.

(16) Lioe, H.; Duan, M.; O'Hair, R. A. J. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2727-2733.

(17) Morris, H. R.; Pucci, P. *Biochem. Biophys. Res. Commun.* **1985**, *126*, 1122-1128.

(18) Sun, Y. P.; Smith, D. L. *Anal. Biochem.* **1988**, *172*, 130-138.

(19) Wells, J. M.; Stephenson, J. L.; McLuckey, S. A. *Int. J. Mass Spectrom.* **2000**, *203*, A1-A9.

(20) Zubarev, R. A.; Kruger, N. A.; Fridriksson, E. K.; Lewis, M. A.; Horn, D. M.; Carpenter, B. K.; McLafferty, F. W. *J. Am. Chem. Soc.* **1999**, *121*, 2857-2862.

(21) Brown, R. S.; Lennon, J. J. *Anal. Chem.* **1995**, *67*, 3990-3999.

(22) Stephenson, J. L.; Cargile, B. J.; McLuckey, S. A. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2040-2048.

(23) Polfer, N. C.; Haselmann, K. F.; Zubarev, R. A.; Langridge-Smith, P. R. R. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 936-943.

(24) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648-5652.

(25) Lee, C. T.; Yang, W. T.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785-789.

(26) Roepstorff, P.; Fohlman, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601-601.

(27) Pearson, R. G. *J. Am. Chem. Soc.* **1963**, *85*, 3533-&.