Mapping Disulfide Bonds in Insulin with the Route 66 Method: Selective Cleavage of S—C Bonds Using Alkali and Alkaline Earth Metal Enolate Complexes

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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 is 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, the 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 collision-induced dissociation. Further activation of the product comprising the A chain reveals the presence of a second disulfide bridge, an intramolecular linkage. Experimental and theoretical studies of the disulfide linked model peptides provide mechanistic details for the selective cleavage of the S—C bond. (J Am Soc Mass Spectrom 2009, 20, 157–166) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Disulfide bonds are one of the most important post-translational modification (PTM) processes because of 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 electron-capture dissociation (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 (dA) residues.

In this study, 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

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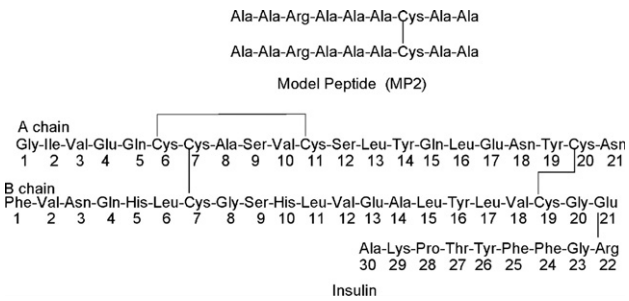
sequence information [23]. The CID of singly to triply charged insulin yields fragments resulting from intermolecular disulfide bond cleavages [19]. Although these top-down methods yield sequence information of insulin including the position of the Cys residues, locating the position of the original disulfide linkages in the protein remains a challenge. Analysis of a peptic digest is preferred for the investigation of this protein because 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 the electrospray ionization (ESI) mass spectrum from the pepsin digest of insulin shows eight major ion peaks, comprising five singly charged and three doubly charged ion peaks [12]. In the present investigation, we examine doubly charged Na^+ and Ca^{2+} complexes of the disulfide linked model peptide $(AARAAACAA)_2$ (MP2). Then, we demonstrate that collisional activation of cationic Na^+ and Ca^{2+} complexes of insulin peptic digest fragments allows for straightforward analysis of the disulfide linkages in the parent molecule. The mechanisms and energetics of the observed reactions are further examined by means of computational modeling. Structures of MP2 and insulin examined in this study are shown in Scheme **1**.

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, USA). The model peptide AARAAACAA was purchased from BiomerTechnology (Concord, CA, USA). All solvents (water, methanol, and benzene) were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). 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 I_2 in 300 μL of benzene. The solution mixture was stirred vigorously for 30 min 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 h. Then pepsin was removed using a Microcon centrifugal filter (Millipore, Billerica, MA, USA) 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 metal chloride in the diluted peptic digest solution.



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

Experiments were performed on an LCQ Deca ion trap mass spectrometer (ITMS; ThermoFinnigan LLC, San Jose, CA, USA) in positive mode. Electrospray voltage parameters of 4.5 kV, capillary voltage of 8 V, and capillary temperature 275 °C were set for ESI. The temperature of the MS analyzer was about 23 °C before the experiment and about 24 °C during the experiment. The pressure was estimated to be approximately 10^{-3} torr He inside the trap. Metal-complexed peptides of interest were isolated and fragmented via low-energy CID. Continuous isolation of selected 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 30 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 (Assisted Model Building and Energy Refinement) 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, USA). 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, Inc., Portland, OR, USA) 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 dehydroalanine and Δ of parent ion (M) refer to peptide ions containing a disulfide bond and

dA, respectively. The numerical subscript refers to the number of units in the parent ion when more than one repeated monomeric unit exists in the ion. For example, a parent ion (M) with two peptides, which are X and Y, linked via two disulfide bonds is referred to as ds_2M_{XY}. 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 Ca^{2+} is referred to as y_n^{Ca}. The numerical subscript refers to the number of equivalent amino acid units in the fragment ions of metal complexes of the peptic peptides.

Results and Discussion

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 product peptides. Both paired protonated and sodiated products from MP2 are separated by 66 mass units, corresponding 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 calcium 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 Figure 1c and d, respectively. The MS^3 spectrum of the Ca^{2+} bound product at *m/z* 779.4 exhibits the dominant product resulting from

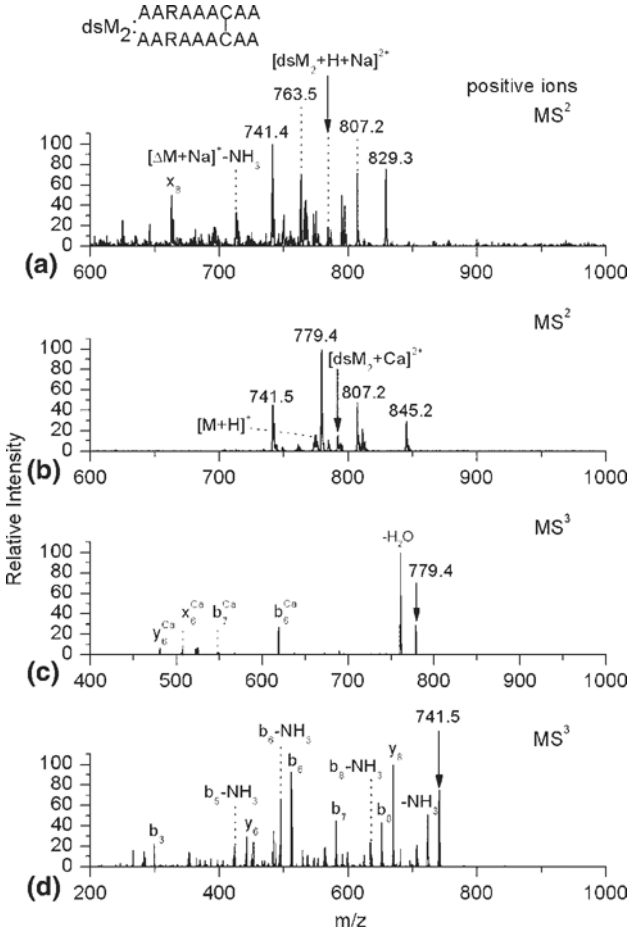


Figure 1. CID spectra of doubly charged (**a**) monosodiated MP2 and (**b**) monocalcium bound MP2 showing dominant product pairs separated by 66 mass units. (**c**) MS^3 spectrum of the MS^2 product at *m/z* 779.4 from (**b**). (**d**) MS^3 spectrum of the MS^2 product at *m/z* 741.5 from (**b**). Arrows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

dehydration (Figure 1c). Other than dehydration, the products comprise b-type fragments (b_6 and b_7) formed by cleavages on both sides of the 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 on both sides of the dA residue are observed in the MS^3 spectrum of the product at *m/z* 741.5 (Figure 1d).

Low-Energy CID of Metal Complexes of the Pepsin Digest of Insulin

The ESI mass spectrum of pepsin digest of insulin exhibits eight major ion peaks that show good agreement with earlier published analyses (Table 1) [17, 18]. The masses and segments of observed metallated peptic digest ions of insulin with added sodium and calcium are included in Table 1.

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

Segment[a,b]	Sequence[b]	Charge state	Protonated	*m/z* Sodiated	Calcium
B26–30	YTPKA	+1	579.3	601.4[c]	617.3[e]
				623.5[d]	
B25–30	FYTPKA	+1	726.4	748.5[c]	—[g]
				770.4[d]	
B23–30	GFFYTPKA	+1	931.2	953.3[c]	—[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]	1300.5[e]
				1303.6[d]	1319.6[f]
A1–13/B1–13	GIVEQCCASVCSL/FVNQHLCGSHLVE	+2	1395.5	1406.5[c]	1415.1[e]
				1417.5[d]	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 Scheme **1**.
[b]Peptide pair with disulfide linkage is indicated with "/".
[c]Monosodiated ion.
[d]Disodiated ion.
[e]Monocalcium bound ion.
[f]Dicalcium bound ion.
[g]Because of the low intensity of this fragment, the metal complex species was not observed or not sufficiently abundant for MS^2 analysis.

Singly Charged Sodiated Peptides from Pepsin Digest of Insulin

As summarized in Table 1, abundant singly charged sodiated peptide ions are observed for three peptic digest products from insulin, which correspond to YTPKA, FYTPKA, and NYCN/LVCGERGFF. By scanning MS^2 of singly charged sodiated peptic digest 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, were presented in our earlier study [12]. The CID of sodiated NYCN/LVCGERGFF yields a major product from H_2S_2 elimination. The observed elimination of H_2S_2 indicates the presence of a disulfide linkage in the peptide.

Doubly Charged Sodiated Peptides from Pepsin Digest of Insulin

Abundant doubly charged sodiated peptide ions are observed for three peptic digest products YQLENYCN/LVCGERGFF, GIVEQCCASVCSL/FVNQHLCGSHL, and GIVEQCCASVCSL/FVNQHLCGSHLVE (Table 1). Figure 2 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 2a). 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 singly charged, protonated B-chain peptide, with —SSH replacing —SH at Cys.

Figure 2b shows the CID spectrum of the 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-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 dA (*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 dA 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 2c. 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

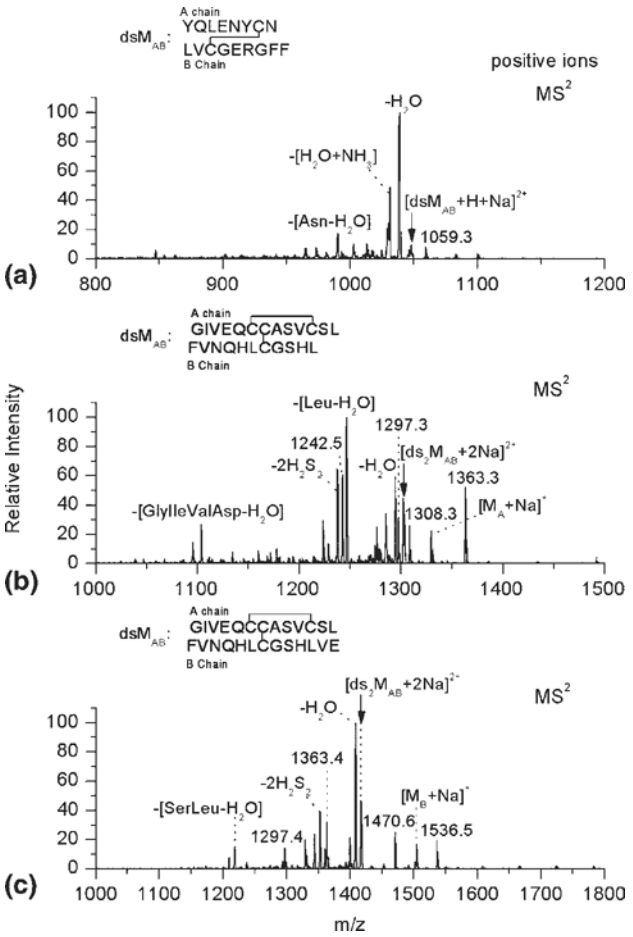


Figure 2. (**a**) MS^2 spectrum of doubly charged monosodiated peptic digest fragment from insulin YQLENYCN/LVCGERGFF. (**b**) MS^2 spectrum of doubly charged disodiated peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHL, showing major products involving disulfide bond cleavages. (**c**) MS^2 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.

observed at *m/z* 1470.6 and *m/z* 1536.5 from the A-chain peptide.

Doubly Charged Calcium Complexes of Peptides from Pepsin Digest of Insulin

The observed calcium bound pepsin digest of insulin from the ESI mass spectrum is summarized in Table 1. Abundant doubly charged calcium complexes are observed for three peptic digest products YQLENYCN/LVCGERGFF, GIVEQCCASVCSL/FVNQHLCGSHL, and GIVEQCCASVCSL/FVNQHLCGSHLVE. Figures 3–5 show low-energy CID spectra of doubly charged Ca^{2+} bound peptides YQLENYCN/LVCGERGFF, GIVEQCCASVCSL/FVNQHLCGSHL, and GIVEQCCASVCSL/FVNQHLCGSHLVE.

Doubly Charged Dicalcium Complex of YQLENYCN/LVCGERGFF

As seen in Figure 3a, 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 a singly charged monocalcium bound B-chain peptide with a disulfide and a dehydroalanine, respectively. Further MS^n analysis was performed to locate the position of the disulfide linkage in the dipeptide.

Figure 3b shows the 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

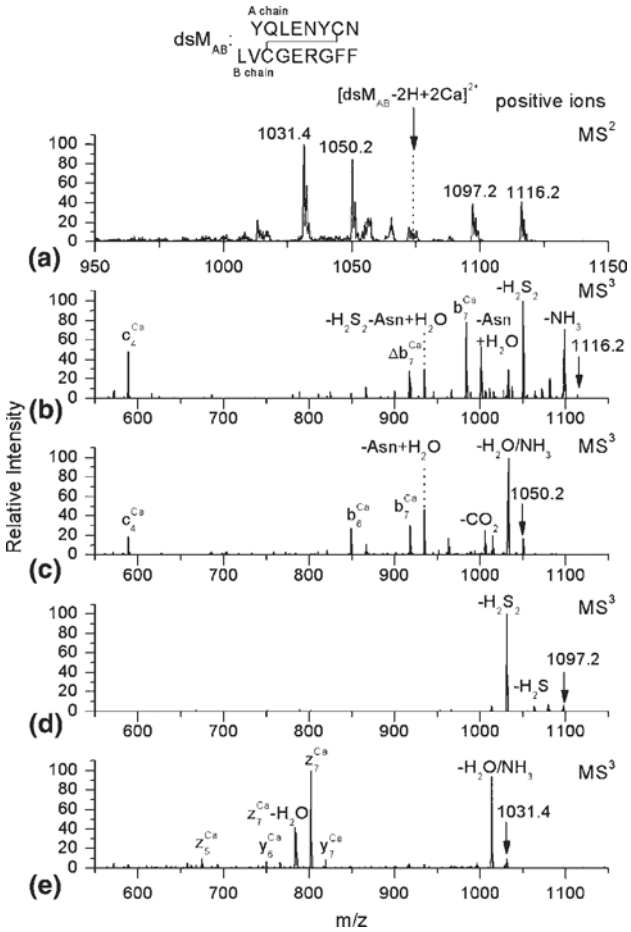


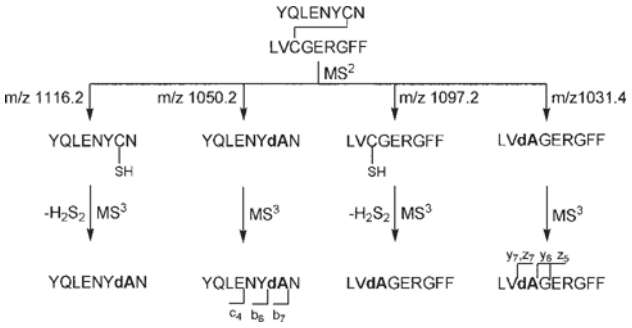
Figure 3. (**a**) CID spectrum of doubly charged dicalcium bound peptic digest fragment from insulin YQLENYCN/LVCGERGFF. (**b**) MS^3 of product at 1116.2 from (**a**). (**c**) MS^3 of product at 1050.2 from (**a**). (**d**) MS^3 of product at 1097.2 from (**a**). (**e**) MS^3 of product at 1031.4 from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

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 3c. 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 3d and e 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 the doubly charged dicalcium bound YQLENYCN/LVCGERGFF cation are summarized in Scheme **2**.

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 4a). 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 a singly charged monocalcium bound peptide from the A-chain peptide with Cys converted to a disulfide and dehydroalanine, respectively. A product separated



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

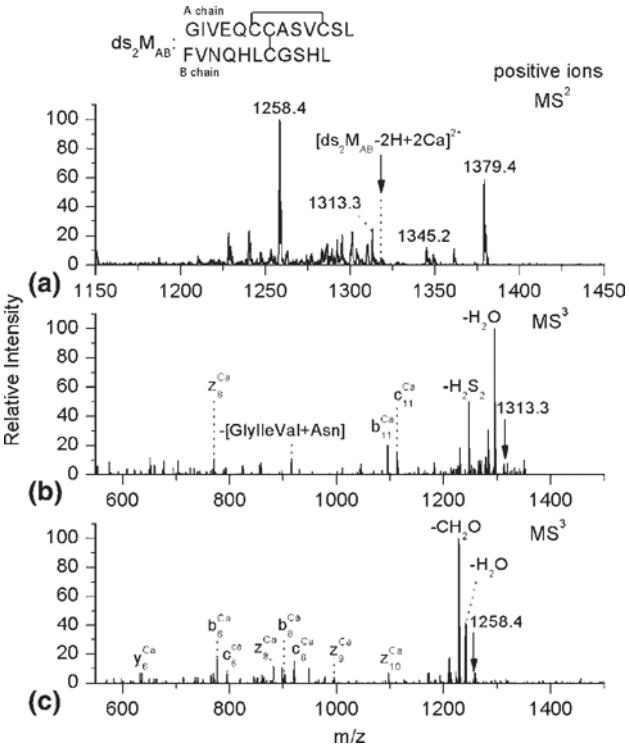


Figure 4. (**a**) CID spectrum of doubly charged dicalcium bound peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHL. (**b**) MS^3 of product at 1313.3 from (**a**). (**c**) MS^3 of product at 1258.4 from (**a**). Arrows indicate the ion peaks being isolated and collisionally activated.

by 66 mass units from the product at *m/z* 1258.4 is not observed in the spectrum. The product at *m/z* 1258.4 is the singly charged monocalcium bound peptide with dehydroalanine from the B-chain peptide.

Figure 4b shows the MS^3 spectrum of the MS^2 product at *m/z* 1313.3. Other than a 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 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 4c 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 intramolecular disulfide linkage. Other products from z-type fragments are also observed. All observed CID pathways of the doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHL cation are summarized in Scheme **3**.

Doubly Charged Dicalcium Complex of GIVEQCCASVCSL/FVNQHLCGSHLVE

Figure 5a shows the CID spectrum of the doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHLVE cation. In analogy to other doubly charged metal complex dipeptides with intermolecular disulfide bonds, four distinct products are observed via CID. The abundant products at *m/z* 1486.6 and *m/z* 1552.6 originate from the B chain and are separated by 66 mass units. The A-chain peptide is also observed at *m/z* 1151. The two products at *m/z* 1379.3 and *m/z* 1313.5 from the A-chain peptide were discussed in the previous section.

The MS^3 spectrum of the product at *m/z* 1486.6 yields a major product resulting from dehydration (Figure 5b). 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 the 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 the doubly charged dicalcium bound GIVEQCCASVCSL/FVNQHLCGSHLVE cation are summarized in Scheme **4**.

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 the 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 Figure 1c and d, 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.

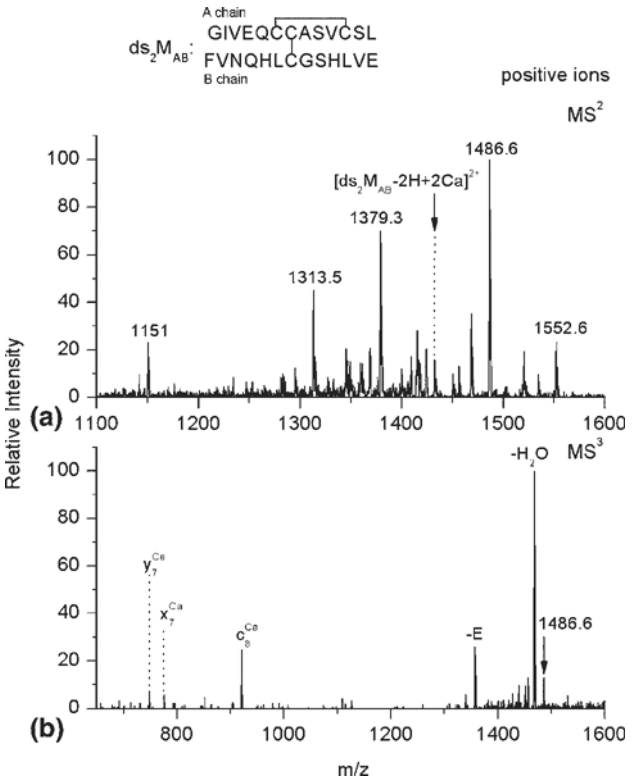
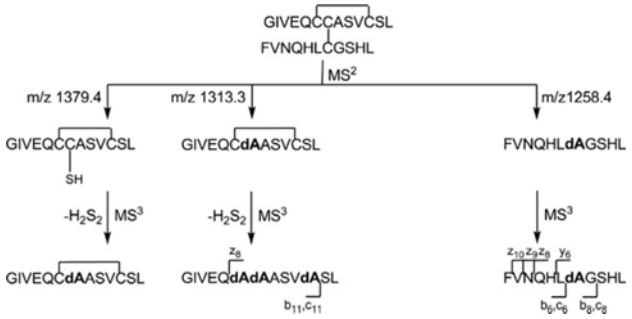
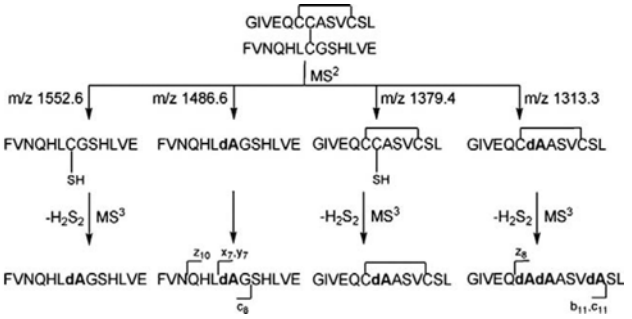


Figure 5. (**a**) CID spectrum of doubly charged dicalcium bound peptic digest fragment from insulin GIVEQCCASVCSL/FVNQHLCGSHLVE. (**b**) MS^3 of product at 1486.6 from (**a**). Arrows indicate the ion peaks being isolated and collisionally activated.

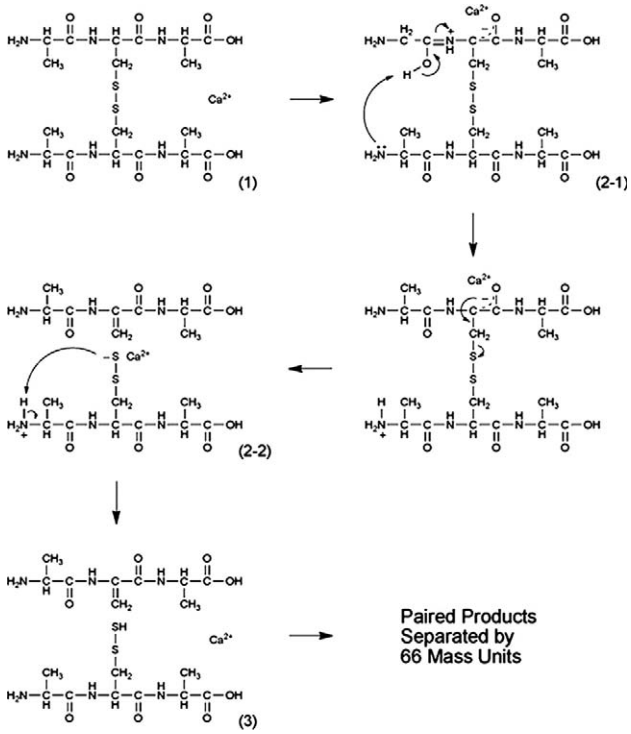
Figure 6 shows the DFT calculated changes of electronic energy (ΔE) associated with cleavages involving S—S and S—C bonds in the Ca^{2+} bound dimeric peptide, $(ACA)_2$, linked by an intermolecular disulfide bond. The optimized geometries of the corresponding intermediates are shown below the diagram. The S—S bond cleavage process of calcium bound $(ACA)_2$ is endothermic by approximately 113 kJ/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 about



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



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



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 6.

210 kJ/mol compared with 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 the Ca^{2+} ion to interact mainly with the amide/carboxylic acid oxygen atoms. Once the proton is transferred to the disulfide group, immediate separation of the two fragments occurs as the result of a charge repulsion and both are observed in the MS^2 spectrum (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, the Ca^{2+} ion interacts with the sulfur atom and the amide oxygen atoms after the S—S bond cleavage. We suggest that the interaction between hard acid Ca^{2+} with the relatively soft base sulfur atom causes the process of S—S bond cleavage to be less favorable 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 bonds.

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 conditions 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 products evidencing the presence of disulfide linkages via CID of doubly charged Na^+ complexes (Figure 2b and c). The CID of the doubly charged disodiated peptides yields the products resulting from two H_2S_2 eliminations. Each singly charged Na^+ cation may interact with each disulfide linkage to effect cleavage to yield two H_2S_2 eliminations when the peptide complexes are collisionally activated.

As seen in Figures 3–5, higher selectivity for cleavage of S—C bonds is observed for the Ca^{2+} bound complex compared with the sodiated complex via CID. The strong interaction between the amide oxygen atom at

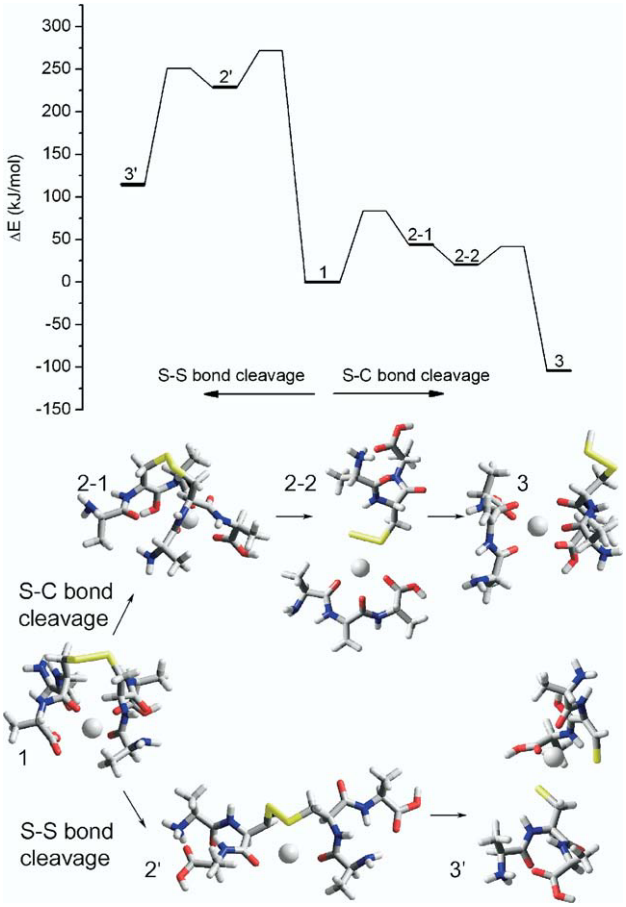
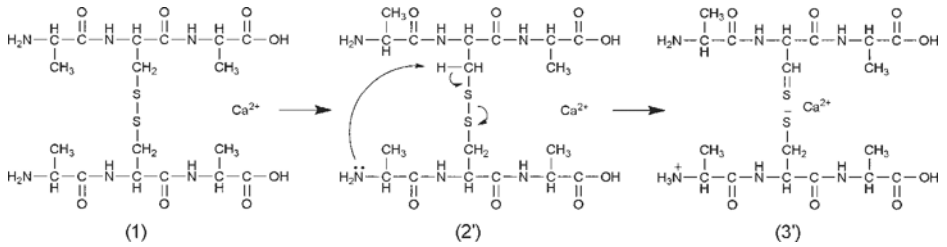


Figure 6. Reaction coordinate diagrams showing relative energies in kJ/mol for S—S bond cleavage (left side) and S—C bond cleavage (right side) of doubly charged calcium bound dimeric tripeptide, $(ACA)_2$, linked by intermolecular disulfide bond at the B3LYP/LACVP level, including zero-point correction 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 Schemes **5** and **6**.



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 6.

Cys and divalent Ca^{2+} appears to facilitate enolate formation of Cys, leading in turn to highly selective S—C bond cleavage. The MS^3 spectra of products with dA residues allow us to identify peptides with intramolecular disulfide linkages (Figure 4b; Schemes **3** and **4**). Otherwise, MS^3 spectra provide sequence information of the product peptide, revealing the location of the Cys residue that 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 conditions, the process being assisted by charge repulsion between monomeric product peptides.

Conclusion

The three disulfide linkages in insulin were characterized using mass spectrometry. The peptic digest fragments containing intermolecular disulfide linkages were 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. More detailed structural information for the peptide, including the location of the disulfide linkages and the presence of additional intramolecular disulfide linkages, was achieved by further activation of the product peptides to identify the position of dehydraoalanine residues. The mechanism proposed for these processes, supported by theoretical studies of model systems, involves formation of a metal-stabilized enolate at Cys, followed by cleavage of the S—C bond. We believe that the approach described in the present and previous [12] studies, which we refer to as the Route 66 method, is a promising methodology to determine the presence and location of both inter- and intramolecular disulfide linkages in peptides and proteins.

Acknowledgments

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