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Effects of disulfide linkages on gas-phase reactions of small multiply charged peptide ions

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Abstract

Multiply protonated ions of disulfide-intact and -reduced peptides were generated by electrospray ionization and studied by Fourier transform ion cyclotron resonance mass spectrometry. The effects of disulfide bonds on gas-phase deprotonation reactions and hydrogen/deuterium (H/D) exchange were investigated. Insight into conformations was gained from molecular dynamics calculations. For ions from three small peptides containing 9–14 amino acid residues, H/D exchange is more sensitive to changes in conformation than deprotonation. However, with both gas-phase reactions the more diffuse forms of the peptides (as determined by molecular modeling) react more readily. The effects of disulfide cleavage on the conformations and on the reactions were found to depend upon the sequence of the peptide. For $[M + 3H]^{3+}$ of TGF-α (34–43), reduction of the disulfide linkage leads to a greatly extended structure and a dramatic increase in the rate and extent of H/D exchange. In contrast, $[M + 2H]^{2+}$ of Arg^8 -vasopressin becomes slightly more compact upon cleavage of the disulfide bond; these reduced ions are slower to react. For $[M + 3H]^{3+}$ of somatostatin-14, reduction of the disulfide bond has little effect on conformation or gas-phase reactivity. Overall, these results indicate that there is no general rule on how cleavage of a disulfide bond will effect a peptide ion's gas-phase reactivity. (Int J Mass Spectrom 182/183 (1999) 233–241) © 1999 Elsevier Science B.V.

Keywords: Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; Disulfide bond; Peptide conformation; Hydrogen/deuterium exchange; Deprotonation

1. Introduction

Physical and chemical properties and biological functions of proteins strongly depend upon their conformations. Methods such as optical rotation, spectrophotometry, viscometry, fluorescence, and circular dichroism have been used to characterize protein conformations. In addition, solution-phase hydrogen/deuterium (H/D) exchange, often employing nuclear magnetic resonance (NMR) analysis, has been widely utilized in confrontational studies [1,2]. The maximum number of exchanges is compared to the total number of labile hydrogens in the protein. It is generally accepted that there are more exchanges for a protein in its unfolded form than in its compact state. Recently, mass spectrometry has also become an important method for probing protein conforma-

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Dedicated to the memory of Ben Freiser to commemorate his many seminal contributions to mass spectrometry and gas phase ion chemistry.

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tions and folding processes [3–25]. Mass spectral analysis can give accurate information on the number of solution-phase exchanges [5–11]. In addition, H/D exchange can be performed in the mass spectrometer to study gas-phase conformations [20–26].

Disulfide bonds play an important role in three-dimensional structure. Differences in conformation are expected for disulfide-intact ("native") and -reduced ("denatured") proteins and peptides. Several mass spectral studies have explored the effects of disulfide cleavage on electrospray ionization (ESI) charge state distributions, proton transfer and H/D exchange reactions, and ion collision cross sections. For example, Katta and Chait [5] showed that higher charge state ions were obtained by ESI from a reduced lysozyme solution than from a native solution. The implication is that reduced lysozyme, which undergoes more H/D exchanges in solution, is more diffuse and thus more readily protonated than the disulfide-intact protein. For nine proteins, Loo and co-workers [4] also observed that cleavage of disulfide bonds produced ions of higher charge state by ESI. In gas-phase H/D exchange experiments performed with a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, Suckau et al. [21] found that disulfide-reduced RNase A ions underwent more exchanges than ions from the intact form. Winger and co-workers studied gas-phase H/D exchange of native and reduced bovine proinsulin and α-lactalbumin in an "inlet/reaction" capillary interface prior to a triple quadrupole mass spectrometer [20]. Interestingly, they reported that the disulfide-intact ions underwent more H/D exchanges than the reduced ions. They suggested that exchange was enhanced by Coulomb repulsion arising from a more compact conformation, with the assumption being that the intact forms were more compact. In a later report, Winger and co-workers found no significant differences in gas-phase deprotonation rates for the disulfide-intact and -reduced forms of these peptides [13]. In another proton transfer study, Gross et al. [14] investigated the reactions of disulfide-reduced and -intact lysozyme $[M + nH]^{n+}$. For $n = 9–11$, the intact form was more reactive, which was attributed to higher Coulomb repulsion driving proton removal in compact conformations. However, for ions with charge states of $n \leq 8$ both forms exhibited similar reactivities. Clemmer and co-workers [19] studied the confrontations of gaseous lysozyme ions, $n = 5–18$, using ion mobility mass spectrometry techniques. Reduced ions had collision cross sections much larger than those of the disulfide-intact ions, indicating that the reduced ions were largely unfolded. In ion mobility studies by Douglas and co-workers [15], reduced bovine pancreatic trypsin inhibitor (BPTI) ions also had larger cross sections than their disulfide-containing counterparts; however, the difference in size was small ($\sim$17%) and both forms were relatively compact.

These previous studies suggest that cleavage of a peptide or protein's disulfide bond can either enhance or reduce gas-phase reactivity. In the present work, this effect is investigated by deprotonation and H/D exchange reactions on three small peptides in their disulfide-intact and -reduced forms. In order to correlate reactivity differences to conformational changes, molecular dynamics calculations were used to provide information on the three-dimensional structures of the peptide ions.

2. Experimental

2.1. Mass spectrometry

A Bruker BioAPEX 47e FT-ICR (Billerica, MA) with an external ESI source (Analytica of Branford, Branford, CT) was employed for this work. The peptides were prepared in 50 μM solutions with a solvent system of 49.5/49.5/1 vol % of water/methanol/acetic acid. Reduced peptides, with S–S bonds replaced by two –SH groups, were produced by dissolving the intact peptides in 0.05 M dithiothreitol (Cleland's reagent [27]) and reacting at 100 °C for 40 min. Samples were introduced into the ESI source with a syringe pump at a flow rate of 45–60 μL/h and electrosprayed from a grounded needle to a capillary biased at -4 kV. The ESI drying gas was CO_2 at 225 °C. Ions generated in the ESI source were first trapped with a hexapole ion guide and then transferred to the ICR cell by electrostatic focusing. The pressure in the

cell was maintained to be less than 2×10^{-10} Torr prior to ion/molecule reactions.

The highest charge state generated by ESI was studied for each peptide: $[M + 3H]^{3+}$ of somatostatin-14 and TGF-α (34–43) (rat), and $[M + 2H]^{2+}$ of Arg^8-vasopressin (free acid). Deprotonation reactions involved reference bases at pressures of $(4–1000) \times 10^{-9}$ Torr. H/D exchange utilized d_4-methanol pressures of 1.7×10^{-7} and 3.3×10^{-7} Torr. To allow for exchange on instrument surfaces, d_4-methanol was added to the vacuum chamber more than 10 h prior to the start of reactions. The ion gauge was calibrated against an ion/molecule reaction with a known reaction rate coefficient ($CH_4^+ + CH_4$) and corrected for reactant gas ionization efficiency [28,29].

For deprotonation reactions, rate constants were obtained by fitting reactant ion intensity as a function of time to a pseudo-first-order equation. Reported reaction efficiencies are ratios of the experimental rate constants to the theoretical thermal capture rate constants [30,31]. For H/D exchange reactions, deconvoluted mass spectra were obtained using the maximum entropy method of Zhang et al. [32]. This method allowed separation of peaks due to deuterium replacement from ^{13}C isotopic peaks. H/D exchange was also found to follow pseudo-first-order kinetics. The H/D exchange rate was calculated by fitting the early portion of the formation curve, which is a plot of number of deuterium exchanged atoms versus reaction time. For both proton transfer and H/D exchange, three or more trials of each experiment were performed. The values obtained from various trials generally deviated by no more than 20% from the reported average.

All peptides were obtained from Bachem Bioscience (King of Prussia, PA) and were of high purity ($>$ 99%). Reference bases and d_4-methanol (D content 99.8%) were obtained from Aldrich (Milwaukee, MI). All chemicals were used without further purification.

2.2. Molecular dynamics calculations

Molecular dynamics calculations were performed with HyperChem 5.0 (HyperCube, Gainesville, FL) running on a personal computer under the WINDOWS NT operating system. Peptide structures were built using amino acid residues downloaded from the HyperChem database. All the basic residues (arginine, lysine, histidine, amino terminus) were protonated and the C-terminus was neutralized by adding a proton. An initial 200 step geometry optimization was performed to obtain a reasonable starting structure. The Bio+ force field, which is an implementation of the CHARMM force field [33], was utilized for molecular dynamics calculations. The ions were heated from 0 to 500 K in 1.0 ps, followed by 12 ps of dynamics simulations with a step size of 0.001 ps, and then cooled to 300 K in 1 ps. Geometry optimization was carried out on the final structures to assure that energy-minimized conformations were obtained. This process was conducted until the difference in energy with respect to displacement for two consecutive cycles was $<$ 0.05 kcal mol^{-1} $Å^{-1}$. Structures obtained from each molecular dynamics/geometry optimization procedure were used as starting structures for the next series of calculations. At least fifteen calculated structures were obtained for each ion.

3. Results and discussion

3.1. Deprotonation reactions

The sequences of the three peptides, with disulfide linkages intact, are given in Fig. 1. The ions under study were “fully” protonated, with the protons assumed to reside on the basic side chains of arginine, lysine, and histidine residues and on the terminal amino group.

The three pairs of disulfide-intact and -reduced peptide ions were deprotonated by a series of reference compounds with gas-phase basicities (GBs) ranging from 198.2 to 219.7 kcal/mol^{-1} [34]. Deprotonation reaction efficiencies are summarized in Table 1. Reactions with stronger bases were highly exoergic and quite efficient. Information about steric effects of peptide ions on deprotonation reactions is more ap-

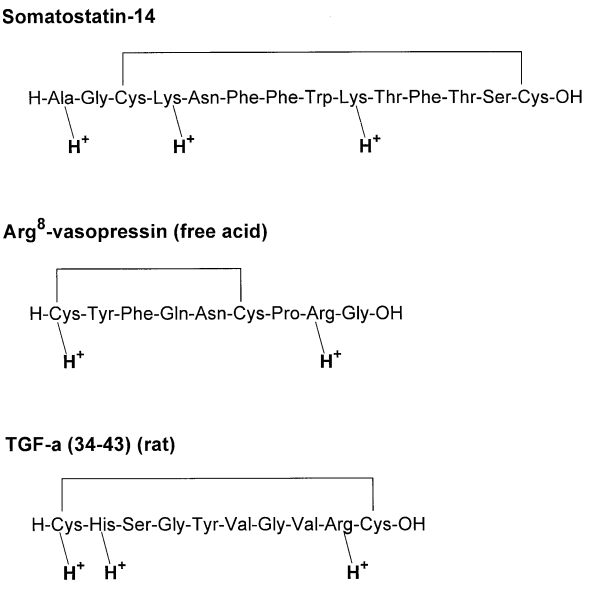

Fig. 1. Sequences of small peptides involved in this study.

parent for slower reactions, which are on the border between exoergic and endoergic processes.

Disulfide-intact and -reduced $[M + 3H]^{3+}$ of somatostatin-14 underwent deprotonation at near equal rates. Thermoneutral (or even slightly endoergic) reactions with 2-fluoropyridine occur more readily for the intact form; however, with the other reference bases, these ions were virtually indistinguishable by proton transfer reactions. For Arg^8-vasopressin $[M + 2H]^{2+}$, the intact ion was slightly faster to deprotonate than the reduced ion. In contrast, for reactions with lower basicity compounds (i.e. more thermoneutral reactions) the reduced form of $[M + 3H]^{3+}$ from TGF-α (34–43) reacted more rapidly than the intact form. However, for all three peptides, the cleavage of the disulfide bond has a relatively small effect on the peptide ion's ability to undergo proton transfer, as measured by its apparent gas-phase acidity (GA_{app}). Using a reaction efficiency of 0.10 to assign GA_{app}'s [35], for somatostatin-14 $[M + 3H]^{3+}$ and Arg^8-vasopressin $[M + 2H]^{2+}$ both the intact and reduced forms have GA_{app}'s of 205.9 ± 4.1 kcal mol^{-1}. $[M + 3H]^{3+}$ from TGF-α (34–43) deprotonate more readily, with both forms having GA_{app}'s in the range of 201.0 ± 4.8 kcal mol^{-1}.

Relative to the other peptide ions in this study, the Arg^8-vasopressin ions have unique reactivities. First, during deprotonation with 3-fluoropyridine and *n*-propylamine, the kinetics do not follow pseudo-first-order behavior. This suggests the presence of at least two ion structures and occurs for both the disulfide-intact and -reduced forms, with the faster-reacting population accounting for approximately 80% of the ions. Second, attachment of the reference base (B) to produce an adduct, $[M + 2H + B]^{2+}$, is a major pathway for reactions with *n*-propylamine and dieth-

Table 1
Reaction efficiencies[a] for the deprotonation of small peptide ions, $[M + nH]^{n+}$

Peptide: GB,[b] kcal mol^{-1}:	*n*	S–S[c]	Acetophenone 198.2	2-Fluoropyridine 203.8	3-Fluoropyridine 208.0	*n*-Propylamine 211.3	Diethylamine 219.7
Somatostatin-14	3	I		0.016 ± 0.001	0.14 ± 0.02	0.24 ± 0.05	0.44 ± 0.04
		R		0.0026 ± 0.0002	0.21 ± 0.02	0.27 ± 0.05	0.31 ± 0.03
Arg^8-vasopressin	2	I		0.000 12 ± 0.000 04	0.19 ± 0.02 (83%)[d] 0.032 ± 0.003 (17%)	0.046 ± 0.010 (78%)[e] 0.033 ± 0.010 (22%)	0.64 ± 0.06[e]
		R		0.000 08 ± 0.000 05	0.12 ± 0.03 (78%) 0.017 ± 0.004 (22%)	0.010 ± 0.003[e]	0.18 ± 0.08[e]
TGF-α (34–43)	3	I	0.038 ± 0.006	0.27 ± 0.03	0.48 ± 0.04	0.66 ± 0.07	0.60 ± 0.06
		R	0.068 ± 0.009	0.40 ± 0.03	0.32 ± 0.04	0.42 ± 0.12	0.70 ± 0.07

[a] Mean ± standard deviation.
[b] Gas-phase basicities are from [34].
[c] Disulfide linkage: I for intact and R for reduced (cleaved).
[d] In cases where an ion reacts at multiple rates, the percentage of the ion population with each reaction efficiency is shown in parentheses.
[e] Adduct formation is also observed.

ylamine. In particular, the dominance of adduct formation with *n*-propylamine results in anomalously low rates of proton transfer. Adduct formation has previously been reported in reactions of other peptide ions [36–39] with the rate and number of attachments being dependent upon the sequence. The exact structural features that influence this process are unknown, but it is often most prevalent at near thermoneutral deprotonation conditions where the adduct may be considered as the reaction intermediate for proton transfer. The Arg^8-vasopressin $[M + 2H]^{2+}$ reactions with diethylamine are unusual in that adduct formation occurs even when proton transfer is rapid and presumably exoergic.

Because the amino acid sequences and intrinsic acidities/basicities of the sites being deprotonated are the same for a pair of disulfide-intact and -reduced peptide ions, the difference in deprotonation efficiency depends on the Coulomb repulsion among the charge-site protons, as well as their accessibility and hydrogen bonding. In a compact conformation, protonation sites might be less accessible for attack by a neutral molecule and may be more highly involved in intramolecular hydrogen bonding. Both of these factors would limit exchange. However, a more compact conformation yields charge sites that are closer together, leading to a higher Coulomb repulsion that promotes faster proton transfer [35]. These effects are offsetting, which is consistent with our experimental observation that cleavage of the disulfide bond does not dramatically impact the gas-phase deprotonation reactions of these peptide ions.

3.2. Hydrogen/deuterium exchange reactions

Table 2 summarizes the measured H/D exchange rate constants and the maximum number of exchanges observed for each of the peptide ions under study. For somatostatin-14 $[M + 3H]^{3+}$, the disulfide-reduced form exchanges slightly faster than the intact ion. Although, the measured exchange rate constants are virtually the same, reactions performed on the same day under the same experimental conditions clearly show that the reduced form reacts slightly faster. To illustrate the difference in reactivity between the two

Table 2
Hydrogen/deuterium exchange kinetics for small peptide ions

	Disulfide linkage[a]	k_{HD}[b] (10^{-13} cm^3 atom^{-1} s^{-1})	Maximum number of exchanges observed	Number of labile hydrogens
Somatostatin-14	I	2.5 ± 1.1	15	27
$[M+ 3H]^{3+}$	R	3.1 ± 0.8	18	29
Arg^8-vasopressin	I	2.7 ± 0.8	14	21
$[M+ 2H]^{2+}$	R	1.3 ± 0.9	16	23
TGF-α (34–43)	I	2.9 ± 1.8	6	22
$[M+ 3H]^{3+}$	R	47 ± 11	16	24

[a] Disulfide linkage: I for intact and R for reduced (cleaved).
[b] Mean ± standard deviation.

forms of somatostatin-14, the number of H/D exchanges as a function of reaction time is displayed in Fig. 2. There are 27 labile hydrogen atoms (hydrogens bonded to N, O, and S atoms) in the disulfide-intact ion and 29 in the disulfide-reduced form. The maximum number of exchanges observed (15 for the intact form and 18 for the reduced form) is smaller than the total labile hydrogen atoms, suggesting that the structure is not fully open and some hydrogens may be engaged in intramolecular interactions such as hydrogen bonding.

The rate constant for H/D exchange of the disulfide-intact Arg^8-vasopressin ions is a factor of 2 larger than the rate constant for the reduced ions. Again, this was confirmed by reactions performed on the same

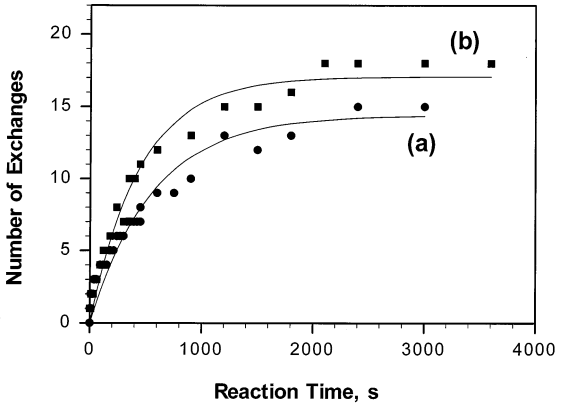

Fig. 2. Number of H/D exchanges for (a) disulfide-intact and (b) -reduced somatostatin-14 $[M + 3H]^{3+}$ plotted versus reaction time with CD_3OD at 3.3×10^{-7} Torr.

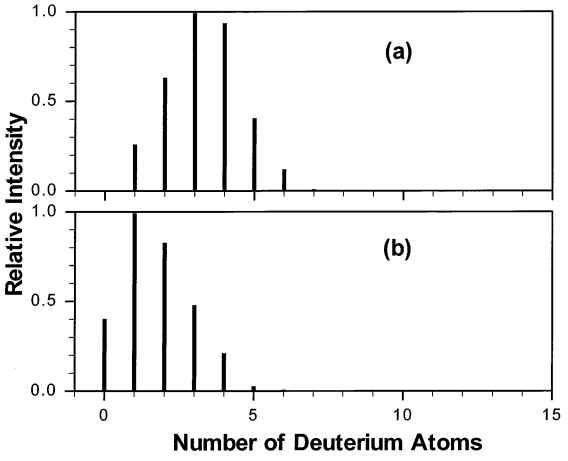

Fig. 3. Deconvoluted H/D spectra for (a) disulfide-intact and (b) -reduced $[M + 2H]^{2+}$ of vasopressin reacting with CD_3OD at 3.3×10^{-7} Torr for 600 s.

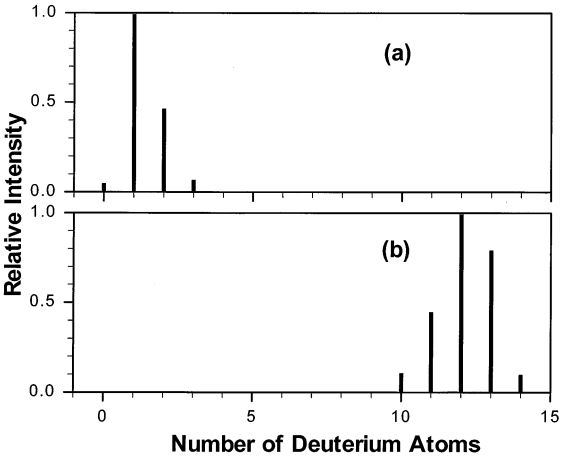

Fig. 4. Deconvoluted H/D spectra for (a) disulfide-intact and (b) -reduced $[M + 3H]^{3+}$ of TGF-α (34–43) reacting with CD_3OD at 3.3×10^{-7} Torr for 300 s.

day under the same conditions. The more rapid exchange for the reduced form is illustrated in Fig. 3, which shows the deconvoluted mass spectra for both ions after a 600 s reaction period. However, the maximum number of exchanges observed (14 for intact and 16 for reduced) is equivalent, when considering that the –SH groups of the reduced form yield 2 additional hydrogens.

The difference in H/D exchange between disulfide-intact and -reduced $[M + 3H]^{3+}$ of TGF-α (34–43) is striking: the reduced ion exchanges 16 times faster than the intact ion. This is illustrated in Fig. 4 for a reaction time of 300 s. In addition, the slow-reacting intact form exchanged only 6 of its 22 labile hydrogen atoms, while the fast-reacting reduced form exchanged 16 hydrogens. This suggests that a major difference in conformation occurs for TGF-α (34–43) following cleavage of the disulfide bond. In contrast, the much less pronounced differences in exchange behavior for ions of somatostatin-14 and Arg^8-vasopressin suggest that little conformational change has occurred.

The H/D exchange and deprotonation reaction results correlate well. For somatostatin-14 ions, the disulfide-reduced and -intact forms react in nearly identical manners. For Arg^8-vasopressin ions, the intact form reacts somewhat more rapidly in both experiments. In contrast, for TGF-α (34–43), both experiments reveal that the reduced form reacts more rapidly, although this is more dramatic for the H/D exchange results. This is consistent with H/D exchange being more sensitive to peptide conformation because it probes accessibility of labile hydrogens along the entire peptide chain. In contrast, the reactive sites for deprotonation are localized basic sites that have been protonated in the ESI process.

3.3. Molecular dynamics calculations

To aid in relating differences in gas-phase reactivity to conformations, molecular modeling was used to obtain low energy structures for these peptide ions. For somatostatin-14 $[M + 3H]^{3+}$, typical low energy structures are shown in Fig. 5. Both disulfide-intact and -reduced ions possess compact conformations. This is consistent with their near identical proton transfer and H/D exchange properties. Nevertheless, the intact form is slightly more compact than the reduced ion, especially at the peptide backbone. The average calculated Coulomb energy between the charge sites in the intact ion (102 kcal mol^{-1}) is higher than that of the reduced ion (83 kcal mol^{-1}). This higher Coulomb energy may be responsible for the tendency of the disulfide-intact ions to react more

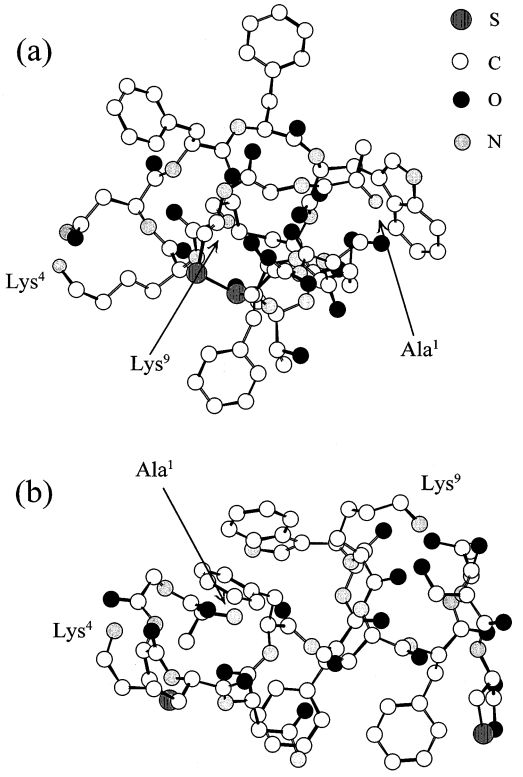

Fig. 5. Typical low energy conformations of (a) disulfide-intact and (b) -reduced somatostatin-14 $[M + 3H]^{3+}$.

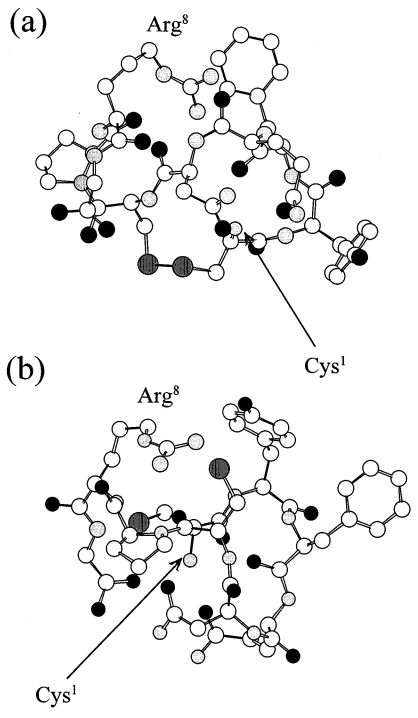

Fig. 6. Typical low energy conformations of (a) disulfide-intact and (b) -reduced vasopressin $[M + 2H]^{2+}$. Atom legends are the same as in Fig. 5.

readily in the slow (near thermoneutral) deprotonation reactions with 2-fluoropyridine.

As shown in Fig. 6, both forms of $[M + 2H]^{2+}$ for Arg^8-vasopressin have generally compact conformations. However, for this peptide, cleavage of the disulfide linkage results in a more flexible peptide backbone, which compacts slightly to enhance intramolecular hydrogen bonding. For example, in the structures of Fig. 6, the greatest distance between backbone heteroatoms in the disulfide-intact ion is 12.7 Å, which is slightly longer than the 11.5 Å distance of the reduced ion. The calculated conformations of Arg^8-vasopressin are consistent with the H/D exchange results, which show the more compact reduced form to exchange less rapidly. The average calculated Coulomb energy of the intact ions (45 kcal mol^{-1}) is nearly identical to that of the reduced ions (46 kcal mol^{-1}). However, for the intact ions, the protonation site on Cys^1 is more accessible because the geometry induced by the disulfide bond forces the N-terminal amino group away from the peptide backbone. Of the two protonation sites on this peptide (Cys^1 and Arg^8), Cys^1 is expected to be deprotonated first because of the gas-phase basicity of the amino acid cysteine is 33 kcal mol^{-1} less than that of arginine [34]. The increased accessibility of Cys^1 in the disulfide-intact ions may contribute to their slightly faster rate of deprotonation.

TGF-α (34–43) is unique in that its disulfide linkage incorporates the entire peptide chain. As is shown in Fig. 7(a), when this linkage is intact, the structure is highly compact; the longest distance between backbone heteroatoms is 16.5 Å. Cleavage of this constraining bond has a dramatic effect on the conformation. As is obvious from Fig. 7(b), the reduced form of TGF-α (34–43) $[M + 3H]^{3+}$ has a highly extended conformation; the longest distance between heteroatoms is now 23.5 Å. The much more open structure is consistent with the reduced ions'

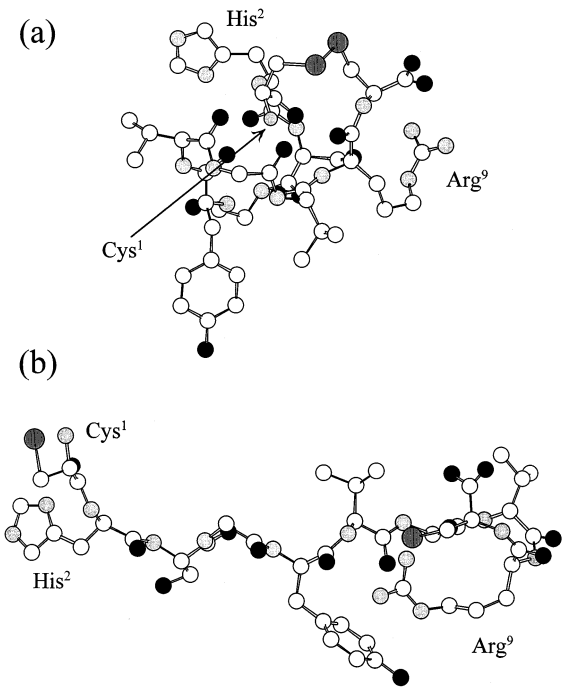

Fig. 7. Typical low energy conformations of (a) disulfide-intact and (b) -reduced TGF-α $[M + 3H]^{3+}$. Atom legends are the same as in Fig. 5.

considerably higher rate of H/D exchange. In addition, the almost linear structure for the 5 residues nearer the N-terminal in the reduced ion make 10 labile hydrogens highly accessible (the phenolic hydrogen on Tyr^5 is accessible in both structures). This is in excellent agreement with our H/D exchange experiments, where reduced TGF-α (34–43) ions exchanged 10 more hydrogens than their disulfide-intact counterparts.

The elongated structure of reduced TGF-α (34–43) ions also increases the accessibility of its least basic protonation site, Cys^1. With less steric hindrance, incoming molecules of reference bases should more readily abstract a proton. This may account for the greater rate of proton transfer by the reduced form of TGF-α (34–43). However, a reduction in Coulomb energy will somewhat offset this effect. Because elongation increases the distances between charge sites, the average calculated Coulomb energy of the reduced ions (94 kcal mol^{-1}) is lower than that of the disulfide-intact ions (115 kcal mol^{-1}).

4. Conclusions

The effects of disulfide cleavage on deprotonation reactions and H/D exchange were found to depend upon the sequence of the peptide. When a disulfide bond is broken, the peptide chain becomes flexible and may extend (to reduce Coulomb repulsion) or fold (to increase intramolecular interactions). Thus, reduction of a disulfide bond can result in a more extended structure [e.g. TGF-α (34–43)], a more compact structure (e.g. Arg^8-vasopressin), or have little effect on conformation (e.g. somatostatin-14). For the three peptides studied, the gas-phase reactivity and molecular modeling results correlate well. In all cases, the more diffuse form (as indicated by molecular modeling) reacts more readily by H/D exchange and deprotonation. However, H/D exchange was observed to be a more sensitive indicator of conformation than deprotonation. This is consistent with the fact that H/D exchange samples labile hydrogens along the entire peptide backbone while deprotonation focuses on removal of protons from a few selected basic sites.

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