Conformations of Gas-Phase Ions of Ubiquitin, Cytochrome *c*, Apomyoglobin, and *β*-Lactoglobulin Produced from Two Different Solution Conformations

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At low pH in solutions of 50% methanol, proteins form expanded denatured states (the "H" state). In 90% methanol, proteins form expanded helical denatured states with artificial *α*-helices (the "H_c" state). Gas-phase ions of ubiquitin, cytochrome *c*, apomyoglobin, and native and disulfide-reduced *β*-lactoglobulin were formed by electrospray ionization (ESI) of the proteins from the H and H_c states in solution. Both states in solution produce the same charge states in ESI. The conformations of the ions were studied with cross section measurements and gas-phase H/D exchange experiments. The cross sections show that the ions retain considerable folded structure. For a given protein and given charge state, ions produced from the H and H_c states showed the same cross sections (within ~1%). Ions of cytochrome *c*, apomyoglobin, and native and reduced *β*-lactoglobulin of a given charge state showed no differences in H/D exchange level when produced from the H or H_c state. However, ubiquitin ions produced from the H_c state consistently exchange fewer (~13%) hydrogens than ions produced from the H state, suggesting that in this case the gas-phase protein ions retain some memory of their solution conformations. (J Am Soc Mass Spectrom 2008, 19, 1906–1913) © 20082008 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

The conformations of gas-phase protein ions continue to be of interest [1, 2]. One question is whether solution conformations of proteins affect the properties of gas-phase ions formed by electrospray ionization (ESI). Comparisons of the conformations of gas-phase protein ions produced by ESI from different solution conformations can be difficult because different solution conformations often produce quite different charge state distributions [3, 4]. Because ions in different charge states have different degrees of Coulomb repulsion, their properties can differ. Proteins in solutions of high alcohol content provide the opportunity to study the properties of gas-phase protein ions formed from two different solution conformations that produce the same charge states. At low pH and in 50% alcohol, proteins can form denatured conformations (termed the "H" state) with the loss of tertiary structure. In 90% alcohol, proteins can form helical denatured states (termed "H_c" states). The helical denatured states have higher *α*-helix content (up to 90% to 100%), distinct CD spectra, and lower solution H/D exchange levels than those of the H state and acid or urea denatured conformations [5–10]. Because of their lower H/D exchange levels in solution, the H_c states are sometimes called the helical denatured "protected" states. The H and H_c states both produce high charge state distributions in ESI, similar to the high charge state distributions produced by acid denatured proteins [9, 10]. In this case, a direct comparison of the properties of the gas-phase protein ions produced from two different solution conformations is possible.

Mao et al. [4] studied the conformations of lysozyme ions formed from two different solution conformations at pH 2.0, the native conformation in water, and a helical denatured conformation in a 2/8 water/methanol solution. The two conformations produced the same charge states in ESI. No differences in cross sections of ions produced from the two conformations were seen. Ions produced from the helical denatured conformation exchanged nine fewer hydrogens than ions produced from the native conformation. This difference in exchange level was close to the combined uncertainties of the separate measurements. In this work, we extend the work of Mao et al. by examining conformations of the gas-phase protein ions produced from the H and H_c states of four proteins in solution: ubiquitin, cytochrome *c*, apomyoglobin, and *β*-lactoglobulin. Both the disulfide intact and disulfide reduced forms of *β*-lactoglobulin are studied. These proteins represent a range of *β*-sheet and *α*-helical tertiary structure, and protein sizes (Table 1). Their conformations in solutions of high

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Table 1. Proteins studied

				Native conformation	
Protein	m.w.	Amino acids	Disulfide bonds	α-helices	β-sheets
Ubiquitin	8,565	76	0	1	4
Cytochrome *c*	12,384	104	0	6	0
Apomyoglobin	16,951	153	0	8	0
β-Lactoglobulin	18,366	162	2	1	8
Reduced β-lactoglobulin	18,370	162	0	-	-

alcohol content have been studied previously by optical spectroscopy [5–8] and mass spectrometry [9, 10]. The H states were formed in 50% methanol and the H_c states in 90% methanol at the pH values shown in Table 2. Table 3 shows the measured values of $[\theta]_{222}$ and the percent α-helix for the solution conditions used here. Table 4 shows the radii of gyration, r_g, determined from small angle X-ray scattering for the native (N), alcohol denatured (H), and urea denatured (U) states of these proteins in solution. The solution "cross sections," A_g, shown in Table 4 are calculated from the radius of gyration from $A_g = 5\pi r_g^2/3$ [8, 15]. Also shown are collision cross sections, σ_N, for the native conformations of ubiquitin, cytochrome *c*, and apomyoglobin calculated from the crystal structures. The "solution cross sections" estimated from the radii of gyration of the proteins in their native conformations and the collision cross sections of the native conformations agree within about 15%. The radii of gyration show that the H and U states are unfolded compared with the native state and that the H and U states have different degrees of folding. Table 4 also shows the number of hydrogens exchanged for deuterium in solution after 45 min for the N, H, and H_c states. The H_c state shows significantly less H/D exchange than the H state because hydrogens are involved in hydrogen bonding in artificial α-helices. It has been shown that α-helices can be unusually stable in the gas phase [19] and so the artificial α-helices, induced by the change of solvent in solution, might conceivably survive in the gas-phase ions.

Here, the gas-phase conformations of the ions formed from the H and H_c states are studied with measurements of collision cross sections and gas-phase H/D exchange. For a given protein and given charge, no significant differences (within ~1%) between collision cross sections of ions formed from the H and H_c solution states are seen. Ions of cytochrome *c*, apomyoglobin, and native and reduced β-lactoglobulin produced from the H and H_c states in a given charge state show the same H/D exchange levels. In contrast, ions of ubiquitin produced from the H_c state appear to show slightly less (~13%) gas-phase H/D exchange than ions formed from the H state. In this case the gas-phase ions appear to retain some memory of their solution-phase conformations.

Table 2. Solutions producing the H and H_c states

		% Methanol	
Protein	pH	H	H_c
Ubiquitin	2.0	50	90
Cytochrome *c*	2.8	50	90
Apomyoglobin	4.0	50	90
β-Lactoglobulin	2.0	50	90
Reduced β-lactoglobulin	2.0	50	90

Experimental

Solutions, Reagents

Horse heart cytochrome *c* (C-7752), horse heart myoglobin (M-1882), bovine milk β-lactoglobulin A (L-7880), bovine red blood cell ubiquitin (U-6253), and glacial acetic acid (99.9+%) were from Sigma Chemical Co. (St. Louis, MO); methanol (HPLC grade) and hydrochloric acid were from Fisher Scientific (Fair Lawn, NJ); D_2O (99.9% D) was from Cambridge Isotope Laboratories (Andover, MA).

Table 3. Summary of literature CD spectra of the four proteins studied here

Protein	pH	% MeOH	$-[\theta]_{222}$[a]	% α-helix[f]
Ubiquitin	2.0	50	6.3 [11]	29
		100[b]	12.7 [11]	49
Cytochrome *c*	3.0[c]	50	11.5 [7]	51
		80[b]	16 [7]	61
Apomyoglobin	4.0	50	22.4 [10]	82
		90	31 [10]	~100
β-Lactoglobulin	2.0	50	23 [12]	84
		80[b]	28 [12]	~100
β-Lactoglobulin	2.0	20[e]	20 [6]	74
Disulfide reduced[d]		40[e]	25 [6]	90

[a] deg·cm^2·dm^{-1} x 10^{-3}
[b] Slightly different H_c methanol conditions from our conditions (90 % MeOH).
[c] Slightly different H and H_c pH conditions from our conditions (pH 2.8).
[d] Trifluoroethanol (TFE) was used for both disulfide intact β-lactoglobulin and disulfide reduced β-lactoglobulin.
[e] 20% and 40% TFE are shown here as an example of the change in α-helical content when higher concentrations of alcohol are used. Very little change in $[\theta]_{222}$ was observed at >40% TFE.
[f] Estimated % α-helix (f_H) is calculated from $[\theta]_{222} = ([\theta]_H^n - [\theta]_R)f_H + [\theta]_R$ [13], where $[\theta]_H^n$ is the α-helical elipticity, $[\theta]_R$ is the unordered elipticity and the estimated coefficients used are $([\theta]_H^n - [\theta]_R) = -30{,}300$ and $[\theta]_R = -2340$ [14].

Table 4. Protein properties in solution

Protein	Radius of gyration r_g (Å)[a,b]			Solution cross sections (Å^2)[a]			Cross sections	Solution H/D exchange[a,c]		
	r_{gN}	r_{gH}	r_{gU}	A_{gN}	A_{gH}	A_{gU}	σ_N	N	H	H_c
Ubiquitin	13.2	28.4	26.3	912	4223	3622	912[g]	72	112	75
Cytochrome c	14.6[d]	31.7[d]	32.1[d]	1116	5262	5395	1340[h]	126	165[e]	130[e]
Apomyoglobin	19.7	30.6	34.2	2032	4903	6124	1773[i]	135[f]	220[f]	165[f]
β-Lactoglobulin	18	39.5	44.4	1696	8169	10322	-	116	183	124

[a]N = native state; H = denatured state; H_c = denatured protected state; U = urea denatured state.
[b]Radii of gyration from [8] except cytochrome *c*.
[c]The number of H/D exchanges from [9] except apomyoglobin.
[d]From [7].
[e]The H and H_c states of cytochrome *c* formed from 2% acetic acid solution (pH 2.8) showed 166 and 130 exchanges, respectively.
[f]From [10].
[g]From [16].
[h]From [17].
[i]From [18].

Sample Preparation and pH Measurements

Protein solutions (20–50 μM) were prepared in either 50% or 90% methanol with 0.1% acetic acid. The pH was measured with an Accumet pH meter (model 15; Fisher Scientific) and adjusted using HCl. The exceptions were the H and H_c solution conditions of cytochrome *c* for gas-phase H/D exchange experiments, which contained 2% acetic acid (no HCl) for a final pH of 2.8, while for the cross section experiments the H and H_c solutions were prepared using 0.1% acetic acid and the pH was adjusted to 3.0 using HCl. Both these solutions form the H and H_c states depending on the concentration of methanol (Table 4) [7, 9]. Solutions used and the number of trials performed for each experiment are shown in Table 5.

Reduction of Disulfide Bonds

To reduce the disulfide bonds of β-lactoglobulin, 1 mM protein in 10 mM Tris-HCl (pH 8.4) was mixed with 20 mM dithiothreitol (DTT) and incubated at 60 °C for 12 h. The solution was then dialyzed extensively against 20 mM HCl using 3500 or 8000 MWCO Spectra/Por membranes (Spectrum, Gardena, CA) before MS analysis.

Collision Cross Sections

Collision cross sections of the protein ions were measured with kinetic energy loss experiments with a triple quadrupole mass spectrometer [20–22]. Protonated protein ions, generated by ESI, pass through an orifice in a curtain plate (880 V) and through a dry nitrogen "curtain gas" (~1 L/min), an orifice (0.25 mm diameter, 200 V), a skimmer (0.75 mm orifice diameter, 120 V), and enter a radio frequency (RF) only quadrupole ion guide (Q_0) with a DC offset of 117.5 V (frequency 768 kHz). The pressure in the ion guide is ~4 mTorr, measured using a precision capacitance manometer (model 120AA; MKS Instruments, Andover, MA). In Q_0, ions cool to ion translational energies and energy spreads of about 1 to 2 eV per charge. Ions then pass through a quadrupole (Q_1) operated in RF-only mode and are injected into a quadrupole collision cell (Q_2) with an initial kinetic energy, E_0, of 12.5 eV per charge, determined by the potential difference between the rod offsets of the first RF quadrupole (Q_0) and the collision cell (Q_2). The collision cell is filled with argon at pressures between 0 and 1 mTorr. The kinetic energies, E, of ions leaving Q_2 are determined from stopping curves generated with the rod-offset potential of Q_3,

Table 5. Solutions producing the H and H_c states and number of trials

Protein	Concentration	Number of trials			
		H (50% methanol)		H_c (90% methanol)	
	(μM)	σ[a]	H/D[b]	σ[a]	H/D[b]
Ubiquitin	20	3	4	3	4
Cytochrome *c*	20	4	4	4	3
Apomyoglobin	50	3	3	3	4
β-Lactoglobulin	50	3	4	3	4
disulfide reduced β-Lactoglobulin	50	3	3	3	3

[a]Collision cross sections.
[b]H/D Exchange. Ions produced from the H and H_c states were run on the same days.

operated as a mass filter. Cross sections (σ) are calculated by fitting E to

$$\frac{E}{E_0} = \exp\left(\frac{-C_d n m_2 l \sigma}{m_1}\right), \quad (1)$$

where C_d is a drag coefficient for diffuse scattering [21, 22], n is the gas number density, m_1 is the mass of the protein ion, m_2 is the mass of the collision gas, and l is the length of the collision cell (20.6 cm). For the conditions of these experiments, values of C_d varied from 2.3 to 2.5, depending on the ion mass and charge state. These values of C_d give cross sections that are 13% to 20% smaller than cross sections calculated from hard sphere scattering (C_d = 2.0) [22].

H/D Exchange of Gas-Phase Ions

Experiments were performed with a linear quadrupole ion trap reflectron time-of-flight mass spectrometer system (LIT-TOF) described previously [23, 24]. Protonated protein ions pass through a 5 mm diameter aperture in a curtain plate (1000 V), pass through a dry nitrogen "curtain" gas (~1 L/min), an orifice (0.25 mm diameter, 170 V), a skimmer (0.75 mm diameter aperture, 20 V) (typical orifice skimmer voltage difference ΔV_{OS} = 150 V), and enter a chamber containing two RF-only quadrupoles, Q_0 (length 5 cm, field radius r_0 = 4.17 mm) and Q_1 (length 20 cm, field radius r_0 = 4.17 mm). Quadrupole Q_1 is operated as a linear ion trap (LIT). Ions are confined radially by the RF potentials (frequency 768 kHz) on the quadrupole, and axially by timed DC stopping potentials applied to the entrance lens (Q_0/Q_1, 4.0 mm aperture covered with a 90% transmitting 50 mesh grid) and exit lens (L_1, 0.7 mm aperture, which also acts as the differential pumping orifice). Ions were trapped with an RF voltage of 250 $V_{0\text{-}p}$ (pole-to-ground) giving q-values between a maximum of 0.386 for cytochrome c +20 and a minimum of 0.138 for ubiquitin +5. These low q values were chosen because trapping ions near the q = 0.908 stability boundary can cause heating of the ions with changes of the H/D exchange levels [25]. Ions then leave the LIT through a stack of focusing lenses with 1.3 mm separation (2 mm diameter orifices) with potentials that can be independently controlled, and enter the source region of a TOF (SCIEX, Concord, ON, Canada) used for mass analysis (mass resolution ca. 2000 at m/z = 609). Ions are detected by a dual microchannel plate (MCP) detector (40 mm diameter; BURLE Electro-Optics, Sturbridge, MA). The timing sequence for the ion storage and TOF acquisition is controlled by an arbitrary waveform generator (AWG-344; PC Instruments, Akron, OH), which triggers home-made power supplies for the LIT entrance and exit plates, as well as a pulse generator (model 500; Berkeley Nucleonics, San Rafael, CA) to control the TOF-MS detection. Typical trapping conditions: 50 ms drain, 50 ms injection, 1–5000 ms trap, 50 ms detection. A 5-point adjacent average smoothing function was applied to each spectrum before converting time of flight to m/z, by assigning the square root of the known m/z ratios of the electrosprayed ions to the appropriate time of flight in the control experiment and creating a calibration curve. The slope of this curve was then used to calibrate the m/z of the accompanying D_2O experiments. The base pressure in the trap chamber was 2.0 mTorr of N_2. For H/D exchange experiments, D_2O at a pressure of 5.0 mTorr was flowed through the chamber (total pressure 7.0 mTorr). In control experiments, nitrogen was added to the chamber to give a total pressure of 7.0 mTorr or a gate valve between the chamber and turbopump was partially closed to give a final trap chamber pressure of 7.0 mTorr.

Results and Discussion

Charge State Distributions

Figure 1 shows the mass spectra of ions produced from the solution H and H_c states of ubiquitin, cytochrome c,

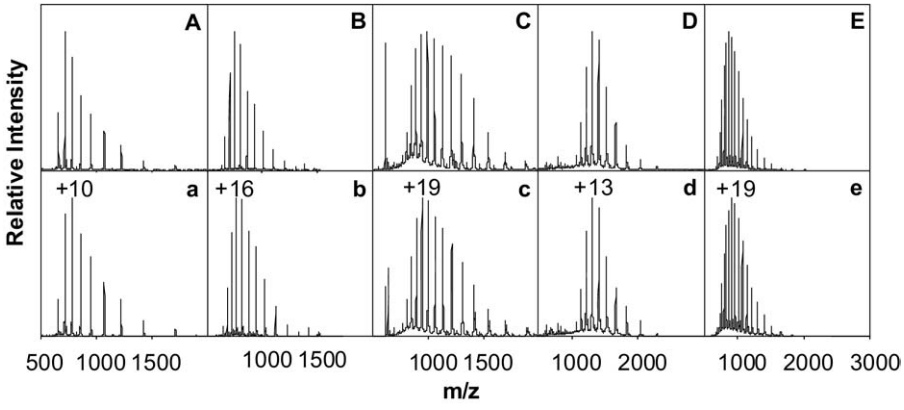

Figure 1. ESI mass spectra of (**a**) ubiquitin, (**b**) cytochrome c, (**c**) apomyoglobin, (**d**) disulfide intact β-lactoglobulin, and (**e**) disulfide reduced β-lactoglobulin in 50% methanol (H conformation) (upper case) and 90% methanol (H_c conformation) (lower case). Notation: +10 is $+10H^+$.

apomyoglobin, β-lactoglobulin, and disulfide reduced β-lactoglobulin. In all cases, no significant differences in charge state distributions are observed between ions formed from the H and H_c solution states. The reduction of the disulfide bonds in β-lactoglobulin causes the ESI charge state distribution to shift to higher charges as expected [26, 27], from a distribution with a maximum at +13, to a distribution with a maximum at +19. The spectra of ubiquitin, cytochrome *c*, apomyoglobin, and both disulfide intact and reduced β -lactoglobulin are similar to those found by Babu et al. [9, 10]. Charge state distributions for all the proteins observed with the LIT-TOF and the triple quadrupole mass spectrometer were similar.

Collision Cross Sections

The collision cross sections of ubiquitin, cytochrome *c*, apomyoglobin, and disulfide intact β-lactoglobulin ions in different charge states produced from the H and H_c states, are shown in Figure 2. The cross sections vary from 816 $Å^2$ for the ubiquitin +5 ions to 2776 $Å^2$ for the apomyoglobin +20 ions. For all of the proteins, the higher charge states show higher cross sections as expected [1, 2, 4, 22, 28]. There are no significant or systematic differences in the cross sections between ions formed from the solution phase H and H_c conformers within ~1%. The cross sections of apomyoglobin and cytochrome *c* are the same, within a few percent as the cross sections for ions of the same proteins formed from a denaturing solution of 50% water/50% acetonitrile and 0.1% acetic acid [22]. Table 6 lists the collision cross sections of all the protein ions. For a given charge state, ions of disulfide reduced β-lactoglobulin have cross sections similar to ions of the disulfide intact protein. The cross sections in Table 6 are the average of at least three separate measurements (Table 5). Uncertainties in Table 6 are the standard deviations of the means $((sd)/\sqrt{N})$ where *sd* is the standard deviation of the individual measurements and *N* is the number of measurements.

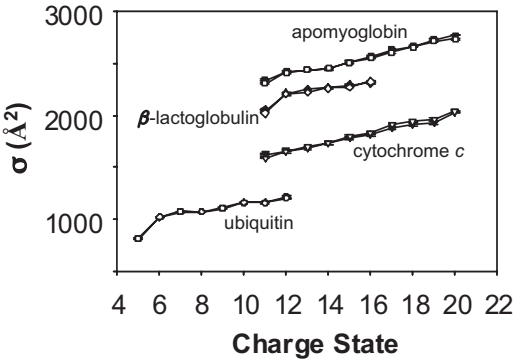

Figure 2. Cross sections of ions of ubiquitin, cytochrome *c*, apomyoglobin, and disulfide intact β-lactoglobulin produced from solutions with 50% methanol (H conformation) (open symbols) and 90% methanol (H_c conformation) (closed symbols).

The cross sections of the ubiquitin ions vary from 816 $Å^2$ for the +5 ions to 1223 $Å^2$ for the +12 ions (Figure 2). The cross section calculated from the radius of gyration of the native conformation in solution (N state) is 912 $Å^2$ ($A_g = 5\pi r_g^2/3$) [8, 15]. The same cross section has been estimated from the crystal structure [16] (Table 4). The cross section of the H state in solution, calculated from the radius of gyration, is 4223 $Å^2$, and that of the U state is 3622 $Å^2$. This calculation assumes a spherical structure, which probably does not apply to the H and H_c states. Nevertheless, the increased radii of gyration for the H and H_c states show the protein has unfolded considerably in solution. The cross section of ubiquitin ions unfolded into a "string" has been calculated to be 2180 $Å^2$ [16]. The ubiquitin ions formed from the H and H_c solution conformations have gas-phase conformations roughly similar in "size" to the N state in solution and substantially smaller than the H or U states in solution, or the string conformation in the gas phase. Cross sections for cytochrome *c* and apomyoglobin ions unfolded into strings have been calculated to be 3453 $Å^2$ [17] and 4944 $Å^2$ [18], respectively. Cytochrome *c* and apomyoglobin ions have gas-phase cross sections that are only ~20% greater than those calculated from the solution phase N state radius of gyration or the calculated cross section from the crystal structure (Table 4). Similarly, β-lactoglobulin ions are only about 20% greater than the "solution cross section" calculated from the radius of gyration of the native conformation. The gas-phase protein ions retain considerable folding. Protein ions formed from the H and H_c states in solution appear to refold from the expanded solution structures, to structures with smaller cross sections similar to the native compact structure, on the time scale required for the protein to travel through the triple quadrupole mass spectrometer for the cross section measurements (ca. 1–2 ms). The net effect is that the gas-phase protein ions produced from different solution conformations mostly lose memory of the solution properties. None of the proteins show systematic differences in the cross sections of the ions produced from the H and H_c states. For each of the charge states of the proteins, the ratio of cross section of the ions produced from the H state to the cross section produced from the H_c state was calculated. These ratios are one within the uncertainties of the data, and Figure 2 shows there is no systematic trend with charge state.

Gas-Phase H/D Exchange

Mass spectra of the +7 ions of ubiquitin, produced from 50% methanol at pH 2.0 trapped for various times in the presence of D_2O vapor, are shown in Figure 3. The mass spectrum of ubiquitin with no trapping in the presence of 7 mTorr N_2 is also shown (Figure 3a). As the ubiquitin ions exchange hydrogen for deuterium, the width of the isotopic distribution increases, and the peak shifts to higher *m/z*. As the exchange nears completion, the isotopic distribution narrows. The levels of

Table 6. Gas-phase collision cross sections and number of hydrogens exchanged in the gas-phase

Protein	Charge	Cross sections (Å^2)		Labile hydrogens	Number of H/D exchanges	
		H	H_c		H	H_c
Ubiquitin	5	816 (5)	819 (8)	149	66.1 (2.4)	62.0 (3.0)
	6	1020 (4)	1020 (9)	150	84.7 (2.9)	76.3 (2.8)
	7	1078 (2)	1076 (5)	151	73.8 (2.8)	67.9 (2.6)
	8	1072 (2)	1074 (4)	152	64.5 (2.1)	60.5 (2.5)
	9	1113 (12)	1110 (6)	153	64.3 (2.3)	58.0 (2.5)
	10	1161 (6)	1166 (3)	154	58.7 (3.0)	54.3 (4.0)
	11	1164 (1)	1160 (1)	155	54.3 (1.6)	48.0 (1.8)
	12	1223 (3)	1205 (5)	156	41.7 (0.5)	30.5 (1.1)
Cytochrome *c*	11	1629 (20)	1594 (4)	209	80.2 (4.3)	83.9 (0.4)
	12	1662 (24)	1660 (31)	210	87.0 (4.7)	86.9 (2.6)
	13	1685 (24)	1694 (28)	211	89.5 (2.2)	88.7 (4.5)
	14	1734 (37)	1734 (36)	212	87.8 (1.4)	89.2 (3.4)
	15	1787 (21)	1798 (30)	213	85.0 (2.9)	88.1 (1.1)
	16	1816 (28)	1835 (34)	214	81.5 (3.0)	85.2 (3.6)
	17	1890 (45)	1909 (38)	215	78.5 (4.5)	79.4 (0.6)
	18	1919 (36)	1940 (27)	216	66.0 (1.9)	62.0 (1.5)
	19	1929 (39)	1962 (35)	217	59.8 (2.0)	63.0 (1.5)
	20	2037 (7)	2039 (18)	218	57.8 (3.1)	61.3 (2.0)
Apomyoglobin	11	2338 (15)	2308 (17)	273	102.2 (3.6)	101.1 (4.0)
	12	2425 (9)	2416 (18)	274	96.6 (6.4)	99.7 (1.9)
	13	2439 (13)	2438 (4)	275	98.3 (4.5)	96.1 (2.4)
	14	2452 (15)	2455 (9)	276	92.7 (2.3)	96.4 (4.1)
	15	2515 (17)	2508 (5)	277	103.2 (2.4)	102.3 (3.7)
	16	2573 (14)	2554 (11)	278	96.7 (4.3)	97.8 (0.7)
	17	2630 (15)	2611 (20)	279	99.7 (0.1)	105.0 (3.1)
	18	2670 (14)	2659 (7)	280	94.7 (0.1)	99.0 (1.9)
	19	2732 (11)	2719 (3)	281	97.3 (8.1)	100.1 (3.8)
	20	2776 (9)	2743 (14)	282	97.0 (2.8)	97.7 (5.4)
β-Lactoglobulin	11	2053 (1)	2023 (14)	293	105.7 (7.7)	101.8 (3.0)
	12	2215 (6)	2206 (20)	294	101.9 (1.8)	100.2 (3.3)
	13	2248 (10)	2223 (7)	295	107.9 (1.9)	102.3 (3.8)
	14	2267 (6)	2264 (14)	296	98.2 (5.7)	100.7 (3.7)
	15	2294 (7)	2274 (10)	297	104.8 (3.3)	101.5 (4.2)
	16	2313 (2)	2328 (7)	298	108.9 (0.7)	104.5 (2.6)
Disulfide reduced beta-lactoglobulin	12	2124 (19)	2184 (13)	294	101.5 (1.9)	98.6 (3.8)
	13	2267 (56)	2290 (50)	295	92.6 (2.0)	93.5 (2.3)
	14	2290 (54)	2333 (44)	296	92.9 (4.6)	92.4 (4.1)
	15	2333 (44)	2348 (45)	297	90.6 (2.5)	90.7 (2.5)
	16	2369 (35)	2372 (44)	298	79.7 (4.8)	81.1 (2.5)
	17	2412 (28)	2406 (42)	299	80.2 (3.1)	83.6 (2.6)
	18	2468 (15)	2454 (72)	300	74.0 (1.7)	79.7 (0.7)
	19	2541 (23)	2484 (70)	301	77.5 (2.7)	80.1 (2.8)
	20	2644 (65)	2604 (131)	302	86.8 (1.7)	85.0 (0.1)
	21	2729 (20)	2686 (16)	303	89.9 (2.9)	87.2 (5.0)

Uncertainties are in parentheses.

hydrogen exchange with D_2O of ubiquitin +7 ions produced from the H and H_c states at various trapping times are shown in Figure 4. Even without trapping, the ions exchange up to 15 hydrogens, and the peak broadens somewhat because ions accumulate in the quadrupole for 50 ms before the trapping begins. During this period, ions can undergo H/D exchange, as discussed previously [4, 29, 30]. Figure 4 shows that after 5 s of trapping with D_2O, the +7 ions produced from the H state exchange 73.8 ± 2.8 hydrogens, while the ions produced from the H_c state exchange 67.9 ± 2.6. Ions of cytochrome *c*, apomyoglobin, and both disulfide-intact and reduced β-lactoglobulin also showed similar trap time-dependent peak broadening and increases in *m/z*. Bimodal H/D exchange distributions were not observed for any of the protein ions. The maximum number of gas-phase H/D exchanges (after 5 s exchange) for all the protein ions are shown in Table 6. Table 6 also shows the numbers of labile hydrogens, which are determined by adding the total for the neutral protein (from the amino acid sequence) and the number of charges on the protein ion. The number of exchangeable hydrogens for cytochrome *c* also includes the two propionic acid residues in the heme.

Table 6 shows that for most of the proteins, there are no systematic differences in the H/D exchange levels of

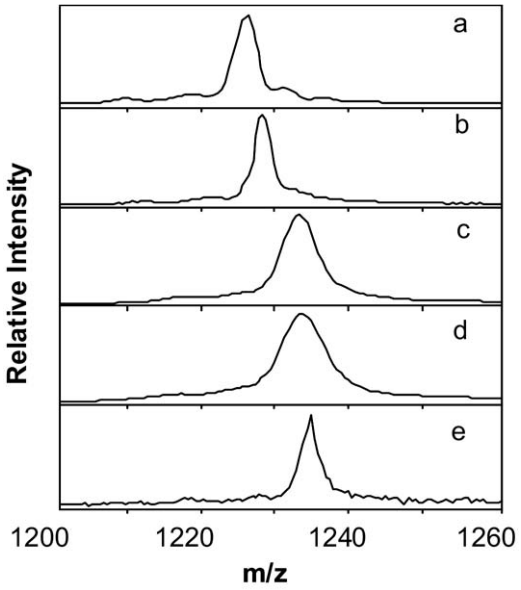

Figure 3. Mass spectra of ubiquitin +7 ions produced from 50% methanol at pH 2.0 at (**a**) and (**b**) 0, (**c**) 0.5, (**d**) 1, and (**e**) 5 s of trapping. The pressures in the trap chamber were (**a**) 7 mTorr N_2 and (**b**)–(**e**) 2 mTorr N_2 and 5 mTorr D_2O.

ions produced from the H and H_c states. Ubiquitin is an exception. The H/D exchange levels produced from the H and H_c states are shown in Figure 5. Ions +5 to +12 produced from the H_c states consistently show slightly lower exchange levels than ions produced from the H state. Apparently the H_c state of ubiquitin forms gas-phase ions that exchange about 13% fewer hydrogens on average than ions formed from the H state. Ubiquitin +12 ions show the greatest difference; ions produced from the H_c and H states exchange 30.5 and 41.7 hydrogens, respectively, a ratio of 1.37. Although the mechanism of H/D exchange in the gas phase for large ions is not well established and differs from that in

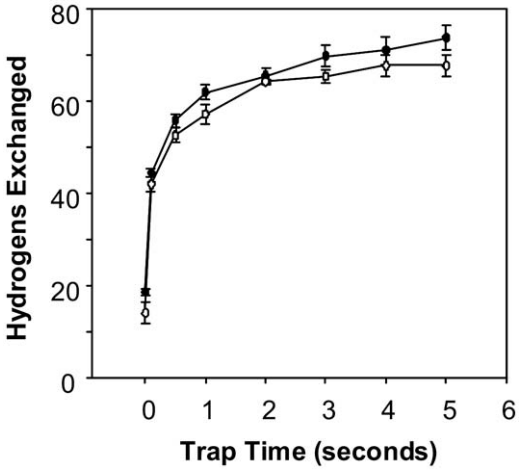

Figure 4. The number of hydrogens exchanged by ubiquitin +7 ions generated from solutions with 50% methanol (closed symbols) and 90% methanol (open symbols) at pH 2.0 versus trapping time.

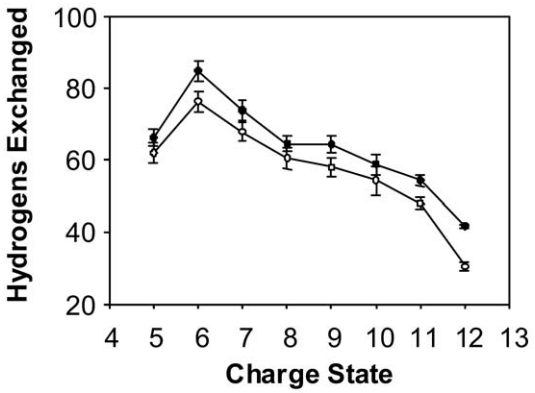

Figure 5. Number of hydrogens exchanged in 5 s by gas-phase ions of ubiquitin formed from the H state (closed symbols) and the H_c state (open symbols) in solutions at pH 2.0.

solution, it is plausible to assume that different conformations exchange different numbers of hydrogens. Ions formed from the helical denatured state of ubiquitin are apparently slightly more protected in the gas phase and have some conformation differences compared with ions formed from the H state in solution. A similar effect was seen with ions of lysozyme [4]. The cross sections show that the ions have refolded considerably from their solution conformations. Nevertheless the ions seem to retain some memory of their solution conformations. For all the proteins, no systematic trends in gas-phase H/D exchange with amino acid composition or native tertiary structure were observed.

The product of D_2O pressure (p) and trapping time (t) in these H/D exchange experiments ($pt = 2.5 \times 10^{-2}$ Torr s) is more than two orders of magnitude greater than those used in ion cyclotron resonance (ICR) studies of H/D exchange of ions of ubiquitin [31–33] and cytochrome *c* [34]. Despite this, for ubiquitin, and for charge states where comparisons are possible, our H/D exchange levels are *less* than those seen in the ICR experiments of Freitas et al. ($pt = 7 \times 10^{-4}$ Torr s) [32] although greater than those seen by Robinson and Williams ($pt = 3 \times 10^{-5}$ Torr s) [33] and Cassady and Carr ($pt = 4 \times 10^{-6}$ Torr s) [31]. Similarly, for cytochrome *c* our H/D exchange levels are *less* than those seen in the ICR experiments of Wood et al. ($pt = 1.8 \times 10^{-4}$ Torr s) [34]. Our cytochrome *c* exchange levels are about 30% greater than those seen in mobility experiments of [35], which had a product of pressure and time of $pt = 4 \times 10^{-4}$ Torr s, about 50 times lower than our experiments. Later mobility experiments by Valentine and Clemmer [36] used a higher product of pressure and time, up to $pt = 2 \times 10^{-2}$ Torr s, but found the same exchange levels (63) for the +5 and +9 ions as at the lower pressure–time products. As noted by others [35], H/D exchange levels appear to depend strongly on the instrumentation and operating conditions used.

Our cross section measurements have comparatively low resolution in comparison to high-pressure ion mobility experiments [1, 2]. Mobility experiments might

conceivably show some differences in cross sections of ubiquitin ions produced from the H and H_c solution states. The cross section measurements occur within about 1 to 2 ms of ion formation, whereas the H/D exchange experiments occur over 5 s. Possibly, ions unfold or refold on the longer time scale of the exchange experiments. Because the H_c state is more protected against H/D exchange in solution, it may seem that some of the protection in the gas phase derives from the survival of solution artificial helices in the gas-phase ions. However, the cross sections show the ions formed from the unfolded H and H_c states have considerable folded structure, and so it is not clear if any of the solution structure survives in the gas phase. Nevertheless, H/D exchange, at least in these experiments, reveals small differences in conformation not seen in cross section measurements.

Summary

Both the gas-phase collision cross sections and H/D exchange levels of ions of cytochrome *c*, apomyoglobin, and native and reduced β-lactoglobulin show no difference in the conformation of the ions formed from the H and H_c states in solution. The cross sections show that the gas-phase ions have structures roughly comparable in size to native conformations in solution. Ubiquitin, however, appears to show slightly more protection to gas-phase H/D exchange for ions produced from the H_c state compared with those produced from the H state.

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