Kinetic Intermediates in the Folding of Gaseous Protein Ions Characterized by Electron Capture Dissociation Mass Spectrometry

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Received August 23, 2000

Abstract: Alternative mechanisms propose that protein folding in solution proceeds either through specific obligate intermediates or by a multiplicity of routes in a "folding funnel". These questions are examined in the gas phase by using a new method that provides details of the noncovalent binding of solvent-free protein ions. Capture of an electron by a multiply charged cation causes immediate dissociation (ECD) of a backbone bond, but with negligible excitation of noncovalent bonds; thus ECD of a linear protein ion produces two measurable fragment ions only if these are not held together by noncovalent bonds. Thermal unfolding of 9+ ions of cytochrome *c* proceeds through the separate unfolding of up to 13 backbone regions (represented by 44 bond cleavages) with melting temperatures of <26 to 140 °C. An 0.25 s laser IR pulse induces unfolding of 9+ ions in <4 s in six of these regions, followed by their refolding in 2 min. However, for the 15+ ions a laser IR pulse causes slower unfolding through poorly defined intermediates that leads to far more ECD products (63% increase in bond cleavages) after 1 min, even more than heating to 140 °C, with refolding to a more compact conformation in 10 min. Random isomerization appears to produce a dynamic mixture of conformers that folds through a variety of pathways to the most stable conformer(s), consistent with a "folding funnel"; this might also be considered as an extension of the classical view to a system with a far smaller free energy change yielding multiple conformers. As cautions to inferring solution conformational structure from gas-phase data, no structural relationship between these gaseous folding intermediates and those in solution is apparent, consistent with reduced hydrophobic bonding and increased electrostatic repulsion. Further, equilibrium folding of gaseous ions can require minutes, and even momentary unfolding of an intermolecular complex during this time can be irreversible.

Introduction

The understanding of protein folding has commanded extensive research studies over many years.[1−5] However, how this vital natural process efficiently selects the one native conformer from a vast number of possibilities is explained by two general mechanisms or combinations thereof. In the classical view,[1,4] folding proceeds through intermediates with specifically formed partial native structures, while the "new" view[2] proposes a folding funnel in which random noncovalent associations lead to ensembles of individual chain conformations, which in parallel events across a complex energy landscape may all lead to the most stable conformer. Characterizing the short-lived (e.g., milliseconds) intermediates expected in the classical mechanism and the even faster structural fluctuations expected for the folding funnel have presented formidable experimental challenges,[3] although hydrogen/deuterium (H/D) exchange experiments with cytochrome *c* (Cyt *c*) followed by NMR have recently been interpreted as providing strong support for the classical view.[4] Reported here are details for the far slower folding of gaseous (solvent free) Cyt *c* ions formed by electrospray ionization (ESI) and characterized by mass spectrometry (MS).[5] A new ion fragmentation method, electron capture dissociation (ECD),[6] is shown here to be uniquely capable of characterizing the thermal dissociation of noncovalent

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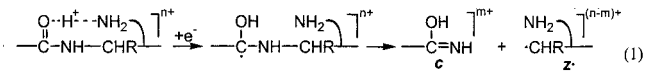
10.1021/ja003143u CCC: $20.00
Published on Web 09/14/2001

bonding in ionized proteins, including kinetic intermediates in laser pulse-induced unfolding and refolding.

ECD is also used here to address a related major question: To what extent are solution noncovalent bonding characteristics retained in the corresponding gaseous ions formed by ESI? H/D exchange[5] and ion cross section[7] studies with gaseous Cyt *c* ions earlier provided evidence for multiple nonnative conformers; solvent removal can greatly decrease hydrophobic and van der Waals bonding and enhance hydrogen bonding.[5,8] However, minimal aqueous/gaseous differences are reported in numerous research studies, especially those of intermolecular complexes.[8−10] A recent careful review reaches a qualified conclusion: "ESI/MS results may be classified as...suggestive or supportive" (of the solution noncovalent structure),[8] although another states "These observations strongly suggest that the process of ionization does not perturb protein conformation."[10]

ECD. The high amount (~6 eV) of energy released on capture of an electron by a multiply charged protein ion can effect immediate ($<10^{-12}$ s, nonergodic) dissociation of a backbone bond, producing mainly ***c*** and ***z*•** ions (eq 1).[6] Even



(1)

with randomization of the remainder of this 6 eV energy over the thousands of degrees of freedom of the ion, other bonds will not be appreciably energized;[11] thus any weak noncovalent bond between the newly formed complementary ***c*** and ***z*•** ions will keep them together, with this complex product exhibiting the same mass as the reduced molecular ion. (It is possible that local noncovalent bonding would interfere with the eq 1 mechanism, also reducing ECD.) In fact, separate ergodic precleavage of noncovalent bonds by collisional activation[6b] or IR photoexcitation is necessary to produce any ECD of larger (>17 kDa) proteins ("activated ion" ECD).[6d] In contrast, for peptide ions with minimal noncovalent bonding, temperature has little effect on their rate of ECD, as expected for a nonergodic dissociation.[6] Thus any new ECD cleavages produced by heating should be indicative of noncovalent bonds ruptured, as will be tested here.

ECD is applied here to the 9+, 15+, and 16+ gaseous ions of Cyt *c*,[5] one of the most thoroughly studied of all proteins.[4,5,7,12] The most abundant charge states from ESI of the native and denatured states of Cyt *c* are 9+ and 16+, respectively.[13] For solution unfolding of Cyt *c*, NMR studies[4] identified four separable cooperative units (bottom, Figure 1): the overlapped N- and C-terminal helices, the 60 s helix and an Ω loop on the other side of the protein, a central Ω loop, and an Ω loop adjacent to the C-terminal helix. In the two-state solution folding process, formation of the N/C helical complex is proposed as the rate-limiting (milliseconds) high-energy transition state, followed by the lower energy formation of the other cooperative units. However, initial unfolding at pH >5 can yield three-state kinetics and far slower (~0.1 s) folding when the Met-80 bound to the heme-Fe is replaced by a peripheral His to produce an off-pathway misligation.[4] In solution, pH 5.5 Cyt *c* molecules carry a net charge of ~9+ (pH <2 for 15+).[12]

Experimental Section

Cytochrome *c* was obtained from Sigma and methanol (HPLC grade) and acetic acid (99%) from Aldrich. Cyt *c* (20−50 μM in v/v 50:47:3 CH_3OH:H_2O:CH_3COOH) was introduced via a nanospray ESI emitter into a modified 6T Finnigan Fourier transform (FT) ion cyclotron resonance mass spectrometer,[6] with ion current ~200 pA and ion trapping aided by a 20 ms N_2 gas pulse (~10^{-6} Torr). After SWIFT isolation of the desired ion charge state, these ions were activated by either blackbody infrared (IR) irradiation from the ion chamber walls[14] (temperature equilibration for ~40 s) or a 0.25 s IR laser (10.6 μm) pulse.[15] For ECD spectra, electrons (<0.3 eV, ~0.2 μA) from a heated filament were introduced into the ion cell for 4 s and spectra from multiple (20−40) experiments were summed.[6] After the laser pulse, the quantity of Cyt *c* ions fell more rapidly than usual, so that ECD spectra taken after 1 min delay were of low signal/noise; the tight e^- collimation along the magnetic axis may make ECD spectra unusually sensitive to off-axis ion scattering. [6] Only ***c***, ***z*•** ions (eq 1) of m/z >500 are included; ***a*•**, ***y*** products[6] are ~10% of these. The computer program THRASH[16] was used to assign the fragment masses and compositions. The ECD spectra shown are averages of at least three separate series of measurements.

Results and Discussion

ECD Characterization of Noncovalent Bonding. Heating the FTMS ion cell and allowing the introduced protein ions to equilibrate thermally (absorption and re-emission of IR photons) has been shown by Williams and co-workers to produce a Boltzmann distribution of ion internal energies characteristic of the cell temperature.[14] Heating the 9+ Cyt *c* ions to 140 °C (Figure 1) increases the number of ECD cleavages yielding separated products from 23 to 44 (of the 103 possible) and the fragmentation yield from 14% to 31%.

The increased specific cleavages yielding ***c***, ***z*•** ions should not be due appreciably to the temperature effect on the bond dissociation energy (eq 1); not only is this dissociation energy decrease small compared to the large amount (up to 6 eV) of energy available from the e^- capture, but all cleavages would be affected similarly. ECD product yields from a specific bond cleavage increase with temperature because either the probability of H• attack (eq 1) at that site increases or the probability of the ***c***, ***z*•** products separating increases; both of these should be due to a loss of local conformational structure. No appreciable effect of temperature on ECD spectra was found for peptides,[6] consistent with minimal noncovalent bonding. Also, increasing the temperature from 25 to 125 °C greatly increases the ECD cleavages for 6+ and 7+ ions of ubiquitin (76 residues), but has no effect on the 13+ ions in which all basic residues are protonated.[17]

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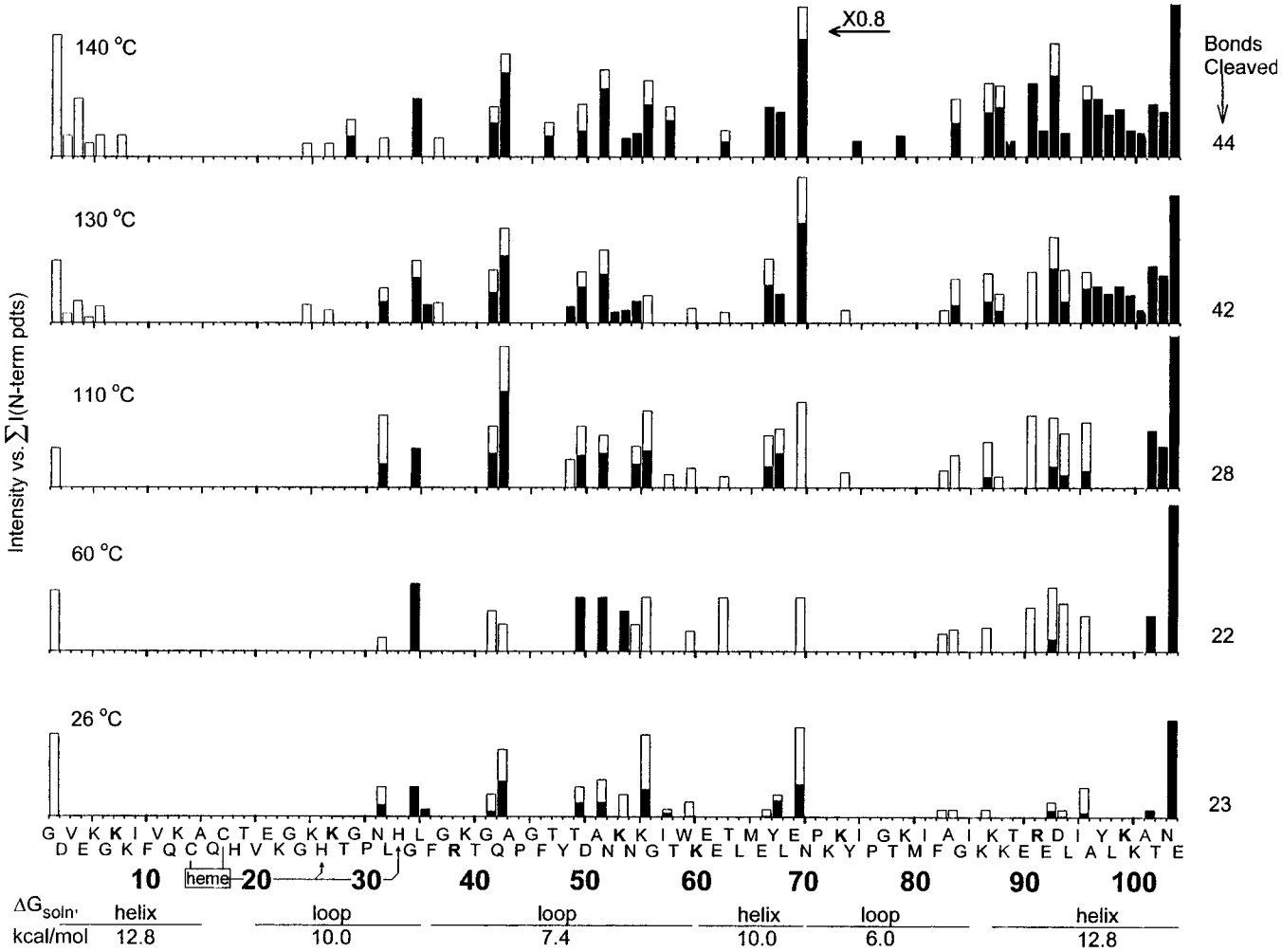

Figure 1. Thermal unfolding of 9+ ions of Cyt *c* as shown by their ECD spectra at different ion cell temperatures. Changes in the intensities of ***c*** (filled bars) and ***z***• (open bars) (eq 1) indicate the extent of denaturing of the noncovalent bonding at the site. Bottom: ΔG values for solution denaturing of specific unfolding intermediates.[4]

The "energetic" MS/MS techniques such as CAD, blackbody infrared dissociation (BIRD),[14] and IR multiphoton dissociation (IRMPD)[15] of course show increased protein cleavages with increasing temperature. ECD is quite the opposite, with separated products expected from cleavages between almost all unbound residue pairs (except the N-terminal side of Pro) or near sites of higher H• affinity, vide infra),[6] even making possible de novo sequencing of ubiquitin.[18] For 8+ to 18+ Cyt *c* ions excited by collisional activation, ECD yields ***c*** and ***z***• ions from cleavage of all bonds in the central region, residues 24 to 90, except for four X-Pro pairs.[6d] For 15+ Cyt *c* ions excited by an IR laser pulse, ECD yields separated products from all but five of these cleavages (vide infra). Thus thermal denaturation appears to be the only logical basis for the ECD spectral changes resulting from ion cell or laser heating, as discussed further below.

For a broad region near the Fe(III) heme (bonds 8 through 23), no ECD is observed at any temperature for the 9+, 15+, and 16+ ions (vide infra). This is consistent with the far higher (+1 eV) H• affinity of the Fe(III) heme versus the backbone amide carbonyls;[6b] thus ECD provides no useful information on the noncovalent bonding in this region that represents 15% of the protein.

ECD of Gaseous 9+ Cyt *c* Ions. The ECD spectrum of the 9+ Cyt *c* ions at 26 °C (Figure 1) shows ***c***, ***z***• ions resulting from cleavage between 23 pairs of the 104 residues. For all these cleavages except those of bonds 1 and 103, the transition-state intermediate of solution Cyt *c* folding cannot be present. In this intermediate the N- and C-terminal helices (bottom, Figure 1) are formed and complexed to each other by hydrophobic bonding; this head-to-tail connection would obviously prevent separation of any ***c*** and ***z***• ions formed by interior backbone cleavage. This nonnative structure for 9+ Cyt *c* ions was assumed previously from high values for both H/D exchange[5] and cross section[7] of these ions.

Several ECD products are also inconsistent with heme-Fe ligation to Met-80-S or His-33; the latter is a cause of the slow 3-state folding in solution.[4] Two of the largest peaks of the ECD spectrum, those representing cleavages at bonds 1 and 103, could result from repulsion by the central charges of the terminal proton sites (e.g., Lys-5 or -7, Lys-99 or -100) toward the corresponding chain ends[19] so that these protons would be near the appropriate (eq 1) carbonyl groups when e^- capture occurred (or for intersystem crossing from the initial Rydberg state).[6c]

Thermal Denaturation of 9+ Cyt *c* Ions. At 26 °C the most intense ECD products result from cleavages at bonds 1, 55, 69, and 103; these remain intense at higher temperatures (Table

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Table 1. Melting Temperatures (°C) at Bonds of 9+, 15+, and 16+ Cyt *c* Ions[a]

9+ ions	15+ ions	16+ ions
1: <26	1[b]: <26	*1*[b]: <26
2−7: 140		
24−28: 140	24−26: <26	24−26: <26
	27[c]: 130	
31-***42***: 90	34−38: <26	34−38: <26
	39[c]: 120	39[c]: 120
46 (**51**)−54: 40	46[c]: 100	4*c*[c]: 110
55: <26	***49***[b]: <26	***49***: <26
66,67:	80	
69: <26	***69***[b]: <26	***69***: <26
73, 74: 100		
82−87: 80	*87*[b]: <26	*87*[b]: <26
90−95: 50	*90*[b]: <26	**92**: 60
96−102: 120	101[c]: 120	101[c]: 130
103: <26	***103***[c]: <26	**103**[c]: 120

[a] Bonds in italics and bold: the five dominant cleavages at 26 and 140 °C, respectively. [b] Bond in the salt bridge region whose relative cleavage is reduced with increasing temperature. [c] Bond on the N-terminal side of Thr whose relative cleavage is increased with temperature; this effect is evident, but smaller, at bonds 57, 62, and 77 for 15+ ions and 57 and 62 for 16+ ions.

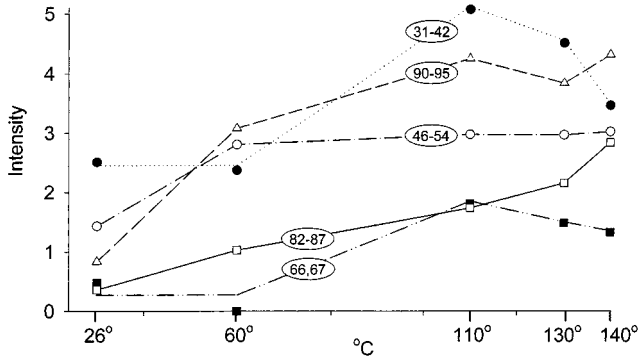

Figure 2. Unfolding in bond regions (Table 1) of 9+ Cyt *c* ions versus temperature from Figure 1 data.

1). However, heating the 9+ Cyt *c* ions to 140 °C greatly increased the products resulting from most of the other ECD cleavages found at 26 °C (Table 1), presumably by more extensive dissociation of the noncovalent bonding that prevents separation of the ***c*** and ***z***• fragment ions. Consistent with more open ion conformations, heating of ions from 25 to 110 °C increased H/D exchange by 30%, although inexplicably this value dropped to 20% at 130 °C; only a single conformer was observed over this temperature range.[5d] Products from the cleavages of neighboring bonds that appear to undergo similar intensity changes are assumed to represent coordinated unfolding intermediates (Table 1). Plotting the summed product ion intensities versus ion temperature provides approximate melting temperature values, with plots for five bond groups of values 40 to 80 °C shown in Figure 2. Three more groups for cleavages at bonds 2−7, 24−28, and 96−102 (Table 1) show melting temperatures ≥120 °C.

The ECD data show that the terminal regions 2−7 and 96−102 of thermally denatured gaseous 9+ Cyt *c* ions would have the highest tendency to fold, in parallel to the initial folding of the termini of Cyt *c* in solution. Again, here the resemblance ends, with all other ECD cleavages inconsistent with the head-to-tail complexation of these N- and C-terminal helices in solution that yields the cyclic template for further folding to the native structure.[4] With D_2O, trapped 9+ Cyt *c* ions exchange 128 ± 2 of their 198 exchangeable hydrogens;[5] because an α-helical structure presumably would show H/D exchange mainly for its 98 side-chain H atoms, a structure combining α-helical[20] and *β*-sheet sections was suggested.[5d] Thus such sections could correspond to the regions of Table 1. Even without specific structural data, the gaseous folding of the 9+ ions does appear to proceed through as many as 13 specific intermediates of increasing free energy requirements, far more than those identified in solution studies.[1−5] Within the rather large experimental error, it is possible that these represent only three ensembles of parallel events occurring at <26, 40 to 100, and >120 °C (Table 1).[2] However, the large intensity variation between the ***c***, ***z***• products in each ECD spectrum could arise from averaging the ECD spectra of many conformers. To study the interdependency of these intermediates, kinetic data on unfolding and refolding were sought.

Laser Pulse Denaturation of 9+ Cyt c Ions. Photoexcitation by a 0.25 s IR laser pulse causes unfolding (Figure 3) that is sufficiently fast (<4 s) to be evident in the "0 s delay" ECD spectrum; its 4 s period of electron capture was initiated with the laser event. This increases ECD ***c***, ***z***• product intensities (Figure 4) in the protein regions affected by heating the ion cell from 40 to 100 °C (Table 1), and so represents only partial unfolding. Most of these six regions have unfolded in a few seconds and refolded to near their original intensity values in ∼120 s. Denaturing of the noncovalent bonding in the 90−95 region appears slower than that of the other regions of Figure 4, despite its relatively favorable melting temperature (Table 1); possibly prior unfolding of the neighboring 82−87 region is necessary to destabilize the 90−95 region. The effect may also work in reverse, with the 90−95 region appearing to refold faster than the others of Figure 4. These six regions (each possibly similar to an α-helix or *β*-sheet, vide supra)[5d,20] represent the majority of bonds in the center of the protein chain (cleavage at 37 bonds between bonds 31 and 95), separated by bonds 55 and 69 of dominant 26 °C cleavage.

In solution, the reversibility of Cyt *c* folding in six distinct subsequences (bottom, Figure 1) is cited [4] as strong support for the classical folding theory. Figure 4 shows similar behavior for six gas-phase regions of partially unfolded Cyt *c* ions, even though these regions show no clear structural relationship to those in solution. This gaseous folding is far slower than the milliseconds Cyt *c* two-state folding in solution, and the rates for the six gaseous regions are similar; thus these regions could also be viewed as folding in an ensemble of parallel events.[2] Any excited vibrational states of the Cyt *c* ions populated by the laser IR pulse should re-radiate IR and re-establish temperature equilibrium with the cell walls within a few seconds.[14] The reactants have far longer lifetimes; the initial laser pulse appears to cause random cleavage of noncovalent bonds to produce a complex mixture of additional conformers. Recalling the search of configurational space in the "new" view,[2] dynamic flexing of the protein chain should result in continuing isomerizations producing new conformers that finally result in the energetically favorable folded regions (vide infra).

ECD of 15+ and 16+ Cyt *c* Ions. The 26 °C ECD spectrum of the 15+ (Figure 5) is quite similar to that of the 16+ ions (not shown); these contain some of the prominent peaks of the 9+ spectrum (Table 1). However, no differences correlate clearly with the gas-phase conformer states assigned by H/D exchange.[21] The 15+ and 16+ spectra show ∼70% more cleavages than that of the 9+ ions; the increased electrostatic repulsion[19] apparently has weakened the noncovalent (as well

(20) Theoretical calculations indicate that $\alpha(4_{13})$ oligoAla helices adapt the 3_{10} conformation in the gas phase: Topol, I. A.; Burt, S. K.; Deretey, E.; Tang, T.-H.; Perczell, A.; Rashin, A.; Csizmadia, I. G. *J. Am. Chem. Soc.* **2001**, *123*, 6054−6060.

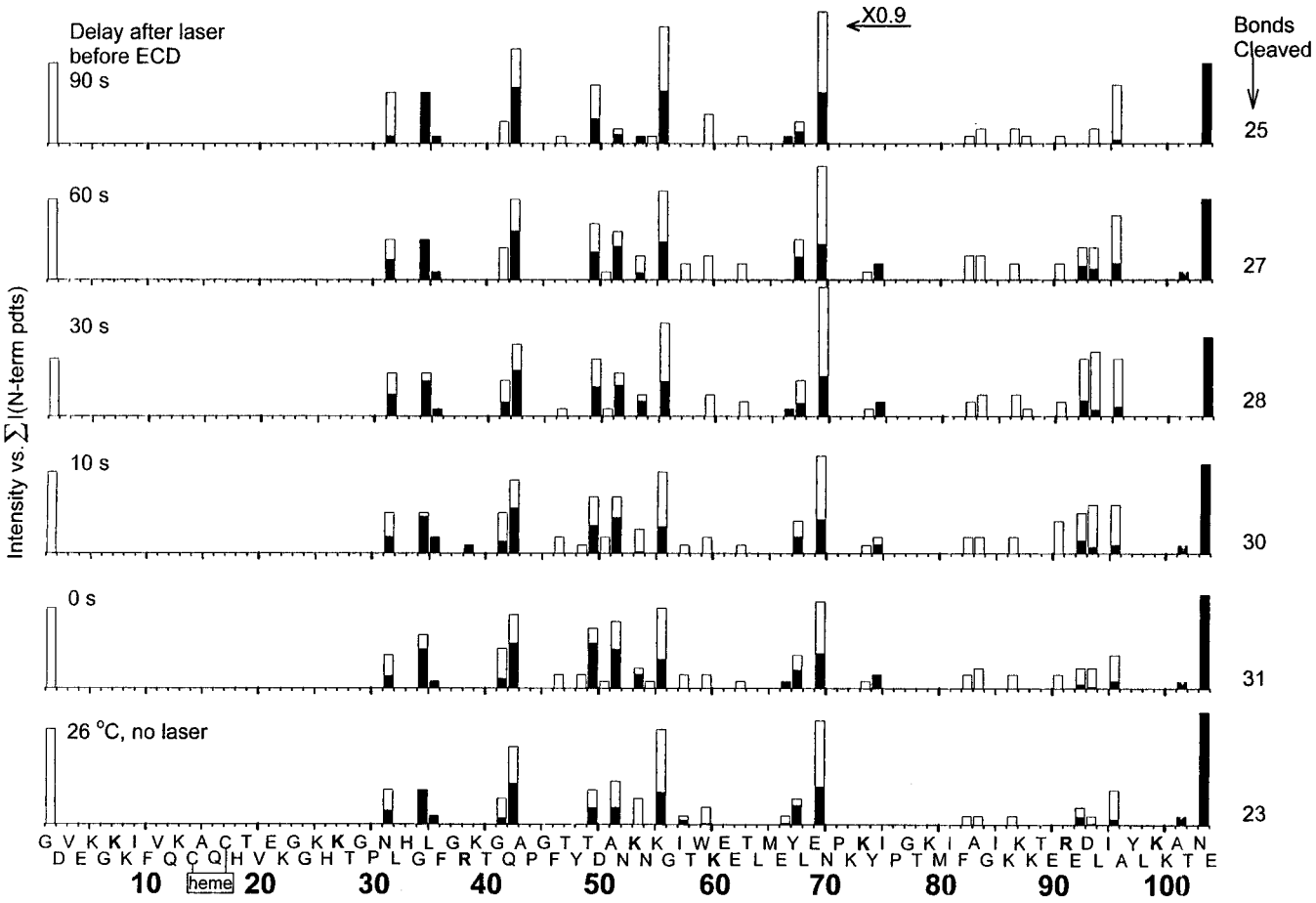

Figure 3. IR laser pulse excitation of 26 °C 9+ Cyt*c* ions, with ECD spectra (4 s e^- exposure) started after the indicated delay.

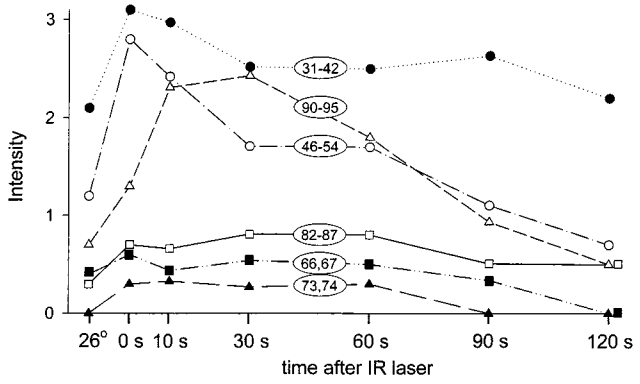

Figure 4. Unfolding and folding by laser excitation of bond regions of 9+ Cyt *c* ions from Figure 3 data.

as the covalent) bonding, as observed also for ubiquitin ions.[17] The prominent cleavages of the 9+ ion ECD spectrum at bonds 1, 69, and 103 still produce significant products in the 15+ and 16+ ion spectra, although the 55 bond cleavage has inexplicably disappeared. The cleavage at bond 69 and those at bonds 49, 87, and 90 are all on the edge of salt bridge regions. Such regions of Cyt *c* ions were also found to be uniquely resistant to H/D exchange of their amide hydrogen atoms.[5d]

Thermal Denaturation of 15+ and 16+ Cyt *c* Ions. Ion cell heating[14] of these more highly charged ions to 60 °C and to 110 °C produces relatively few new ***c***, ***z*•** products from ECD cleavages (Figure 5). In contrast to the 9+ ion behavior, the intensity increases are mainly characteristic of individual sites and correlate poorly with regional cooperative denaturing of intermediates. However, higher temperatures cause a ~50% increase in the number of bonds cleaved; the conformer structure has been extensively denatured. The most dramatic type of product increase (Table 1) arises from a specific cleavage on the N-terminal side of many Thr residues (bonds 27, 39, 46, 57, 62, 77, and 101); this is accompanied by a product reduction from most cleavages at the edges of salt bridge regions (bonds 1, 49, 69, 87, and 90). The local noncovalent bonding (possibly to the Thr hydroxyl group) that is weakened thermally to allow the Thr cleavage could somehow then stabilize the salt bridge region; however, the latter cleavage reduction could also just result from thermal destabilization of all noncovalent binding that makes most probabilities of ***c***, ***z*•** product separation after ECD cleavage more similar. The 15+ and 16+ ions should have a relatively more open tertiary structure than the 9+ ions,[7a,19,22] but also they have a substantial increase in secondary noncovalent bonding from solvation of their additional side-chain protons to the electronegative carbonyl groups of the backbone.[5d,19] Thus thermal rupture of this additional secondary structure is imposed on any cooperative unfolding of α-helix/β-sheet regions. Note that H/D exchange indicated that laser IR heating of the 15+ State V ions converted them to State II,

(21) The 15+ Cyt *c* ions produced by ESI consist of the single State V conformer, the 16+ ions of a 70:30 mixture of States I and II, and 9+ ions of State II only; heating does not change the number of conformers. For a given charge state, State I is the most compact of these, with D_2O exchanging ~10 H atoms less than State V, which exchanges ~10 H atoms less than State II.[5d] The 16+ Cyt *c* ions of conformer state II have 14 less exchangeable H atoms than do the 9+ ions.[5d]

(22) Hudgins, R. R.; Woenckhaus, J.; Jarrold, M. F. *Int. J. Mass Spectrom. Ion Processes* **1997**, *165/166*, 497−507.

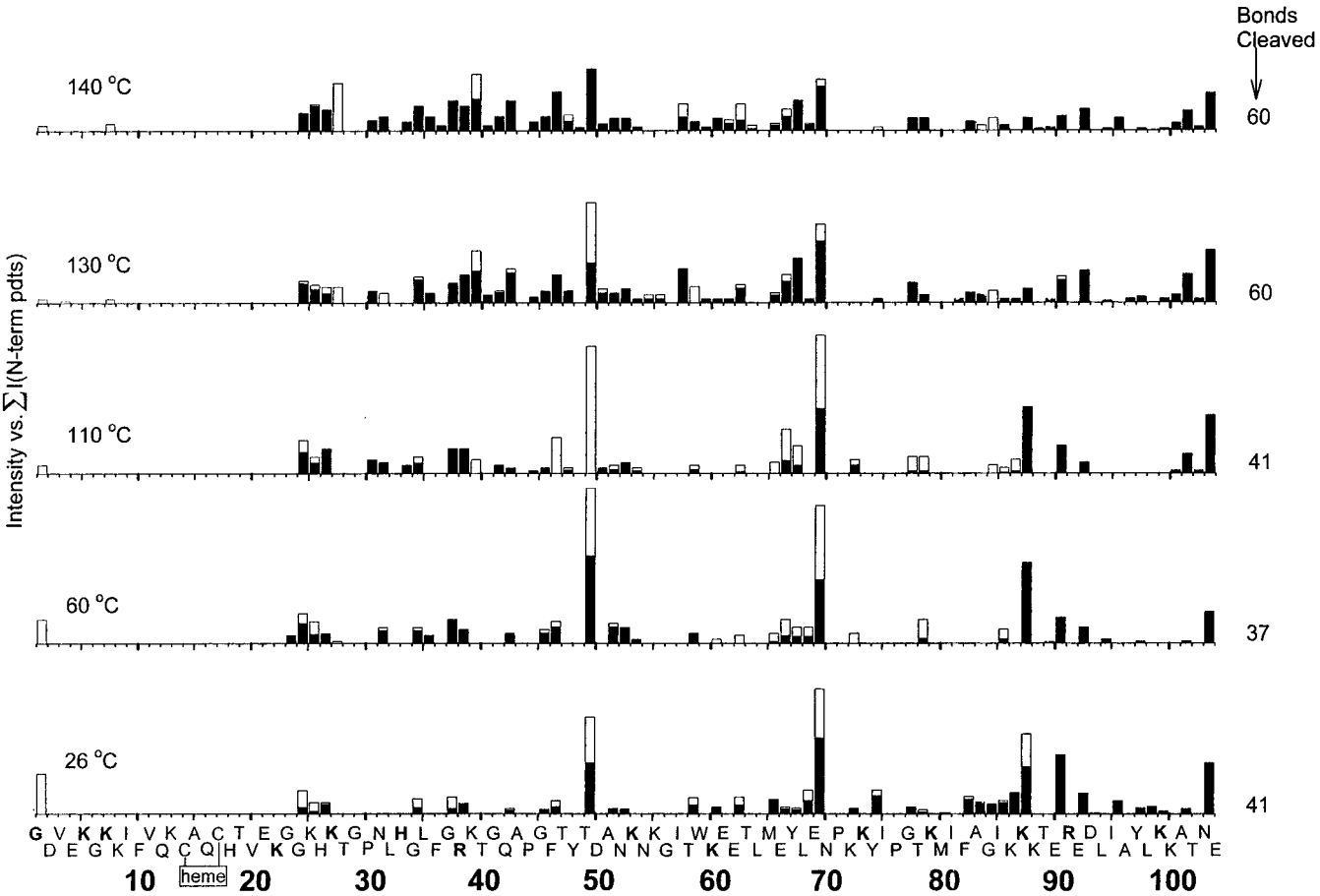

Figure 5. Thermal denaturing of 15+ Cyt *c* ions; see Figure 1 legend. The maximum intensity (I_{max}) is 5.6 units (Figure 2).

and that of 16+ States I and II ions to States V and II.[5b,d,21] Details of these mechanisms are under further investigation.

Laser Pulse Denaturation of 15+ Cyt *c* Ions. As found for the 9+ ions, the laser IR excitation (Figure 6) of 15+ ions is not sufficient to denature regions of melting temperatures above ~100 °C (Table 1); this excitation of the 15+ ions has little effect on the bonds on the N-terminal side of Thr or on the salt bridge bonds. Despite the higher electrostatic repulsion, their ECD spectra respond much more slowly to the laser pulse than do those of the 9+ ions. Only after 5 s is there a general increase in intensity for the minor products in the central regions (bonds 24−86), but as yet with a negligible increase in the number of bonds cleaved. However, in time the latter increases dramatically, with 58% more cleavages in the 60 s spectrum than that before the laser excitation. Some peak intensities have decreased in the 60 s spectrum, suggesting that some refolding has begun, but signal/noise levels at longer delay times were too low for definitive characterization of regional refolding. This is a far greater change in the ECD spectrum than that between the 26 °C spectra of the 15+ and 16+ ions, and thus is not due to laser isomerization of the 15+ conformer V.[21] Excluding the bonds on the N-terminal side of Pro, for which ECD is ineffective,[6] 58 bonds of the possible 63 in the central regions (bonds 24 to 90, 4 Pro residues) are cleaved; only 47 of these are cleaved at 140 °C. All 63 of these bonds were cleaved in the ECD spectrum of 8+ to 18+ Cyt *c* ions excited by collisional activation just prior to electron capture,[6d] suggesting a similar denaturing mechanism for "in beam" ECD.

Conformational Cooling. In attempts to check these 15+ data nearly one year later, the 26 °C ECD spectrum (no laser) showed products from 55 cleavages. However, good spectra at much longer delays after IR laser pulse were found possible,[23] and a 10 min delay reduced the cleavages that yielded $\boldsymbol{c}$, $\boldsymbol{z}^{\bullet}$ products to 24. In a single similar experiment with 16+ ions, the 10 min delay reduced cleavages from 43 to 11; even after correcting for ion losses in 10 min, more than half of the ECD cleavages are no longer measurable. The pulsed gas collisions used for translational cooling for initial trapping of the ions in the cell appear to have broken noncovalent bonds, so that ECD spectra taken soon after the ion collection are subject to variations in ion introduction and trapping conditions. Vibrationally excited ions produced by collisional or photoactivation should cool by IR emission in a few seconds.[14] Thus for the dissipation of internal energy added to these larger ions, the dominant process appears to "conformational cooling", in which the more open conformers produced must fold to intermediate lower energy conformers that are thus vibrationally excited and capable of emitting IR photons. The extent of gaseous D_2O H/D exchange[5d] of laser IR excited ions after 10 min of cooling will be determined for further evidence of such conformational cooling.

Solution versus Gas-Phase Unfolding. Two-state solution folding of Cyt *c* proceeds through an initial rate-limiting barrier formed in "a lengthy energetically uphill conformational search for a set of interactions that can pin the chain into some native-like transition state topology".[4] For the gaseous 9+ and 15+ Cyt *c* ions studied here, it now appears clear that the electrostatic repulsion of the protonated sites[19] does not allow the protein chain to cyclize to form this nativelike transition state. However,

(23) The new electron gun could be producing a higher diameter electron beam, offsetting any *x*,*y* expansion of the trapped ions caused by the laser pulse.

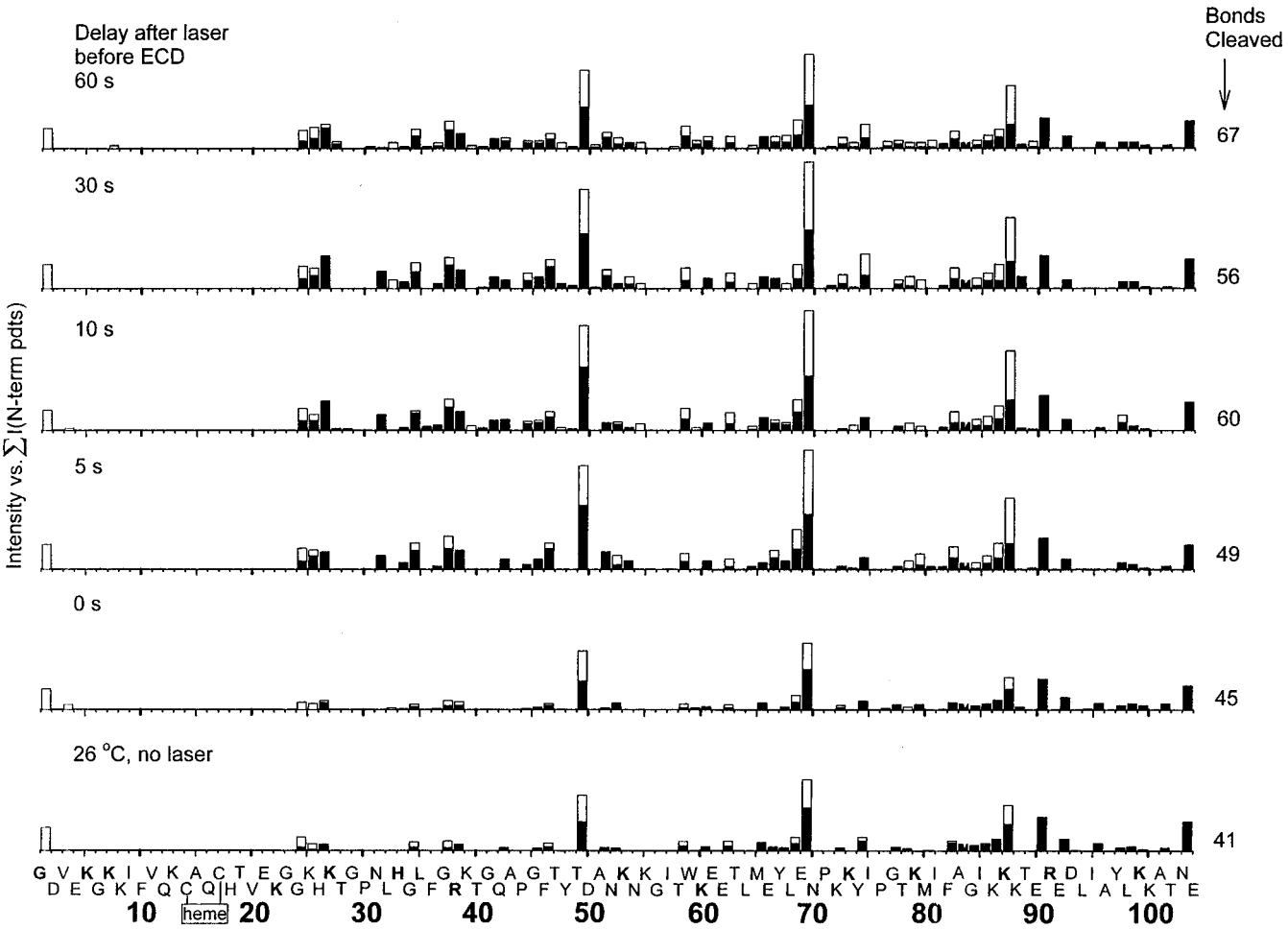

Figure 6. IR laser pulse excitation of 26 °C 15+ Cyt *c* ions with ECD spectra. I_{max} = 8.0 units.

the failure of this key step allows gas-phase observation of the kinetic conformational search process that instead leads to the multiplicity of nearly isoenergetic conformers indicated by H/D exchange.[5d]

As found for the 9+ ions, regions of both the 15+ and 16+ ions do unfold sequentially, at least for temperatures <26, 26−100, and >100 °C (Table 1). These regions could be viewed as folding intermediates, per the classical view,[1,4] but also as ensembles of parallel events, per the "new" view.[2] However, the laser IR excitation has caused an unfolding of the 15+ Cyt *c* ions that is even slower and more complex than that of the 9+ ions. The initial absorption of IR photons could have produced short-lived (<3 s)[14] excited vibrational states. Consistent with the minimal change in the "0 s delay" spectrum, redistribution of this energy to cleave noncovalent bonds is necessary to yield the increase of minor ECD product intensities in the "5 s delay" spectrum. However, for delay times of 10 s up to 60 s, these intensities stay relatively constant, but the number of cleavages continues to increase to a value even greater than that for 15+ ions whose conformers had equilibrated at 140 °C. Although the ions at 140 °C have surely attained a far higher average excitation energy, possibly their "conformational cooling" for 40 s at this higher temperature was sufficient to reach a near equilibrium of stable conformers (times longer than 40 s had little effect on the ECD spectrum). This suggests that the laser excitation has produced a long-lived dynamic interchange of opening and closing secondary noncovalent bonds in structures of nearly equal energies that are undergoing complex positional fluctuations. The configuration space explored must be unusually large (5 stable conformers have been demonstrated)[5d] and energetically flat (the laser pulse produced no conformational melting that requires >100 °C), so that an amazing several minutes appears to be necessary to explore this configuration space effectively. Thus at one moment in time ECD cleavage would give separable products for those backbone sites not then held together by noncovalent bonds, with the 4 s electron exposure allowing conformer isomerization for ECD to cleave other bonds. Conformer equilibration is slowed by many orders of magnitude not only by removal of the solvent, but also apparently by the far smaller free energy benefit of folding to conformer(s) not designed by natural selection. A "folding funnel" of randomly equilibrating conformers, sampled also at random by ECD, would appear to account for the Figure 6 data, but this could also be interpreted with the classical view for multiple reaction pathways of much smaller free energy change leading to multiple isoenergetic products.

Retention of Solution Conformational Structure in the Gas Phase. As postulated previously, removal of the aqueous solvent should increase hydrogen (and van der Waals) bonding and greatly decrease hydrophobic bonding.[5,8] Thus a noncovalent complex held together by hydrogen bonding in solution could retain its conformational structure in the electrospray process; these and other experimental factors have been covered extensively in the excellent recent review of Loo.[8]

None of the discrete unfolding intermediates of Cyt *c* ions found here appear to be similar to those found in solution.[4] As a critical factor in this, the isolated charges on a gaseous multiply

protonated protein repel each other far more than in aqueous solution with its far higher dielectric constant;[19] this makes cyclization to join the terminal regions of Cyt *c* ions much more difficult in the gas phase. These ECD kinetic studies also indicate a previously unrecognized factor: conformational isomerizations in the gas phase can occur nearly randomly over even minutes of time, so that the structures of the resulting gaseous conformer ions could depend critically on electrospray experimental conditions such as temperature, pressure, nozzle-skimmer voltage, ion trapping time, and delay before spectral measurement. Recent ion mobility data on model systems have lead to theoretically proposed conformer structures quite different than those found in solution.[7,24]

This study was concerned only with intramolecular noncovalent bonding, while the preponderance of reports proposing similar solution and gaseous structures are for intermolecular complexes.[8−10] For most of these the bonding will be in a much smaller region of the interior of the larger molecule (e.g., protein), so that the electrostatic repulsion of the relevant protonated sites will be increased less by transfer to the gas phase. A new concern, however, is the possible lengthy minutes of exploration of conformational space before the gaseous intermolecular complex is measured; even a very low probability combination of noncovalent bonds that releases the bound molecule will produce a misleading binding constant, as this loss is irreversible in the gas phase.

(24) Kohtani, M.; Kinnear, B. S.; Jarrold, M. F. *J. Am. Chem. Soc.* **2000**, *122*, 12377−12378. Counterman, A. E.; Clemmer, D. E. *J. Am. Chem. Soc.* **2001**, *123*, 1490−1498.

Conclusions

Electron capture dissociation is unique in providing information on the noncovalent bonding in gaseous protein ions, such as at 72 of the 103 interresidue sites of 15+ Cyt *c* ions (Figures 5 and 6). Consistent with the classical view, unfolding of these ions in the gas phase appears to proceed through a series of discrete intermediates,[1,4,20] although their unusually large number could indicate grouping in ensembles of parallel events. After partial unfolding from a laser excitation pulse, the 9+ ions show refolding of six intermediates in a minute, regenerating the same ECD spectrum. However, this pulsed excitation of the 15+ ions appears to produce noncovalent bond rearrangements that are far less discrete, representing a dynamic equilibrium of conformational states requiring minutes to refold to multiple stable conformers. This provides yet another caution against assuming that the electrospray process does not alter noncovalent bonding.

Acknowledgment. We thank Barry Carpenter, Blas Cerda, Ken Dill, Walter Englander, Ying Ge, Martin Karplus, and Harold Scheraga for helpful discussions, Melissa Allard for data analysis, and the National Institutes of Health (GM16609) for generous financial support.

JA003143U