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Blackbody infrared radiative dissociation of larger (42 kDa) multiply charged proteins

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Abstract

Blackbody infrared radiative dissociation (BIRD), demonstrated originally with ions as large as 17 kDa, has been applied to larger proteins in a 6 T Fourier-transform mass spectrometer. For carbonic anhydrase (29 kDa), ThiF (27 kDa), and thiazole kinase (29 kDa), ion cell temperatures of 60–110 °C give mostly uninformative H_2O loss, but 145 °C gives extensive backbone dissociation. For carbonic anhydrase ions at 70 °C, H_2O loss continues for >240 s; thermalizing ions for ~30 s reduces H_2O loss eightfold. For thiaminase I (42 kDa), H_2O loss is not observed, with backbone dissociation occurring above 150 °C. For these proteins, BIRD has effected cleavages of 34, 41, 23, and 28, respectively, backbone bonds. Although most are the same as those cleaved by infrared multiphoton dissociation and collisionally activated dissociation, some BIRD cleavages do provide additional and complementary sequence information. Carbonic anhydrase also shows extensive H_2O loss from its fragment ions that compromises their validity for sequencing. (Int J Mass Spectrom 210/211 (2001) 203–214) © 2001 Elsevier Science B.V.

Keywords: Fourier-transform ion cyclotron resonance; Blackbody infrared radiative dissociation; Multiply charged proteins; Protein sequencing; Electrospray

1. Dedication

Where in our field of molecular mass spectrometry can we find great research achievements combined with a more wonderful person? Nico Nibbering knows personally almost everyone in the field, has gone out of his way to help a large fraction of these, and has done successful (and fun) collaborative research with a record number of us. One of the authors (F.W.M.) has been a primary beneficiary of Nico's great research ideas, enthusiastic research cooperation, and outstanding friendship for more than three decades. The application of ion cyclotron resonance (ICR), ion dissociation mechanisms, and biomolecule structural characterization of this article owe a great deal to the pioneering research of Professor Nibbering.

2. Introduction

With the development of electrospray ionization (ESI) [1] and matrix-assisted laser desorption/ionization (MALDI) [2], the application of mass spectrometry to the analysis of biomolecules has undergone

Dedicated to Professor Nico Nibbering on the occasion of his retirement.

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explosive growth in the last decade. ESI is particularly promising for larger proteins (>2 kDa) because it can produce multiply charged ions that are more easily dissociated. In addition, the unique analytical capabilities of Fourier transform mass spectrometry (FTMS) [3] make the combination of ESI with FTMS especially advantageous for direct protein characterization without prior proteolysis or other molecular degradation. In this "top down" approach [4], molecular and fragment ion dissociation in the mass spectrometer (MS/MS, MS^n) can provide information on sequence [5,6], location of post-translational modifications [7–9], derivatized active sites [10] and gas-phase conformation [11–13]. Energetic dissociation techniques that increase the ion's internal energy include nozzle-skimmer (NS) dissociation [14], collisionally activated dissociation (CAD) [15–17], surface-induced dissociation [18–20], and laser ultraviolet (193 nm) [21] and infrared multiphoton dissociation (IRMPD) [22]. A complementary new method, electron capture dissociation (ECD) [23], causes local fast ("nonergodic") cleavages of covalent bonds, with "activated ion" (AI) ECD required for larger proteins (>17 kDa) [24].

Blackbody infrared dissociation (BIRD) is another energetic dissociation technique for biomolecule ions demonstrated by the pioneering research of Williams and co-workers [25–29]. In BIRD, ions trapped in the ion cyclotron resonance (ICR) cell of FTMS can be dissociated by absorption of infrared blackbody photons emitted from the heated vacuum chamber walls. Of special advantage, the rate of photon absorption and emission for larger ions can greatly exceed the dissociation rate, so that a Boltzmann distribution of internal energies can be established in seconds [25,28,29]. Such BIRD of small and weakly bound cluster ions has provided unique dissociation kinetic measurements [30,31]. From the temperature dependence of the unimolecular dissociation rate constants, Arrhenius activation parameters in the zero-pressure limit could be obtained for both small and large molecules [25,26,28,31–33].

BIRD has so far been successfully applied to proteins as large as myoglobin (17 kDa) [26,27,34] and DNA molecules (≤3 kDa) [35]; these spectra show fragmentation similar to that obtained by IRMPD and CAD. Advantages found for BIRD versus other thermal excitation methods include controllable energy deposition and fragmentation, no blind spots in the product spectrum, fragmentation of off-axis ions, low expense, and easy implementation [26]. However, low energy dissociations are favored, including the rearrangement loss of small molecules that provide no sequence information [26,27]. Here, we report the extension of this promising technique to far larger proteins (42 kDa).

3. Experimental

Carbonic anhydrase was obtained from Sigma, and ThiF, thiazole kinase, and thiaminase I from Professor Tadhg Begley. The solutions were prepared as 20 μM in $CH_3OH/H_2O/CH_3COOH$ (49:49:2 for carbonic anhydrase, 80:18:2 for thiazole kinase, and 70:26:4 for ThiF and thiaminase) and electrosprayed at 1–50 nL/min with a nanospray emitter. The resulting ions were guided through a heated metal capillary, skimmer, and three radio frequency-only quadrupoles into a 6 T Finnigan FTMS with the Odyssey data system [36]. The outer trapping electrodes, conventionally used for electron containment [23], were used for extra ion trapping [24]. Typically, these trapping electrodes were held at 4.5 and 5.5 V and the source/analyzer traps plates at 1 V. Pulsed nitrogen ($\sim 10^{-6}$ Torr) was used to assist trapping. For BIRD experiments, the trapping electrodes were usually set at 2 V during the delay for IR photon exposure and at 1 V prior to broadband excitation and detection. The vacuum chamber surrounding the cell was resistively heated, waiting 1 h for the cell to reach temperature equilibrium (Omega temperature controller, Stanford, CT, Model 4001-JC) before ion introduction. Spectra collected are averages of 15–30 scans between *m/z* 500–1500. Fragment ion masses and compositions were assigned with the computer program THRASH [37].

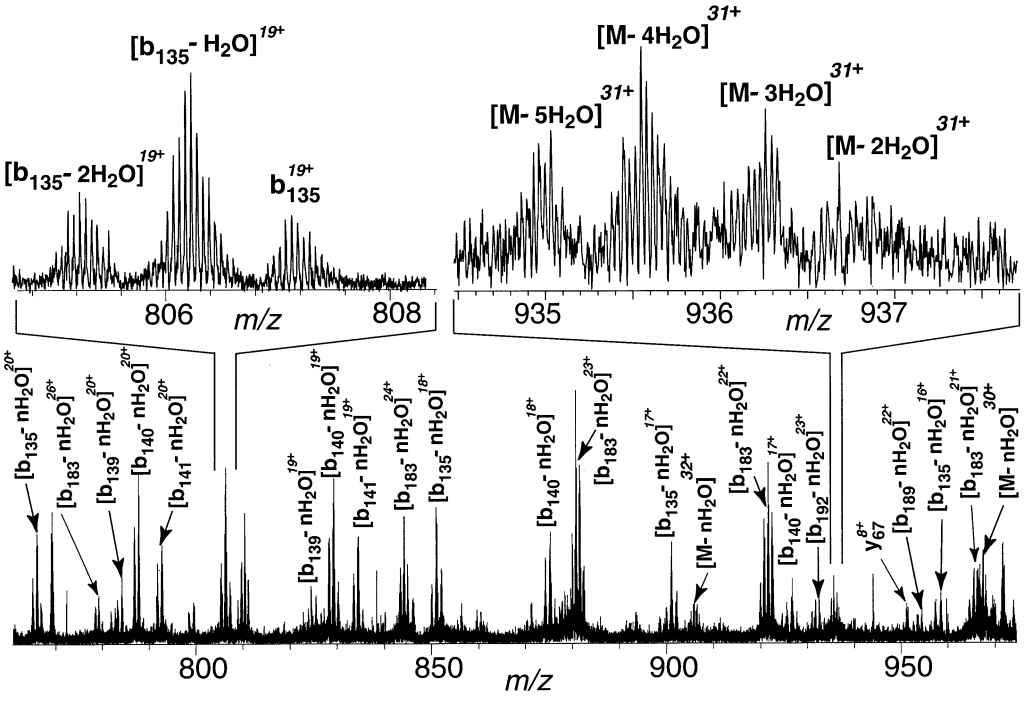

Fig. 1. BIRD spectrum of ions from ESI of carbonic anhydrase B, reaction delay 30 s, vacuum chamber 140 °C. Approximate intensities for losses of 2–5 H_2O molecules are 10%, 30%, 40%, and 20%.

4. Results and discussion

Of all the thermal excitation methods for ion dissociation, BIRD and IRMPD add energy in the smallest increments. Laser IRMPD adds energy much faster than BIRD, for which the excitation rate depends on the cell temperature. The resulting threshold energy reactions are exemplified by H_2O loss, examined in detail for the protein that showed the highest tendency for this undesirable effect.

4.1. Carbonic anhydrase, 29 kDa

The BIRD spectrum from a 30 s exposure at 60 °C cell wall temperature shows mainly undissociated molecular ions. At 90 °C, significant H_2O loss is observed, and at 140 °C, substantial backbone fragmentation ions accompany the H_2O loss (Fig. 1). The mass values for the multiple loss peaks are consistent with mass 18 losses, but minor losses of 17 (NH_3) are possible. BIRD spectra collected at even higher temperature for a 30 s delay time showed complete dissociation of the molecular ion and its H_2O loss products.

As reported by Price and Williams [29], for these ions the Boltzmann distribution of the internal energy should be established in far less than 30 s at room temperature. These times should be even shorter at higher temperatures due to the higher energy density of blackbody emission (which scales as T^4) and the improved overlap of higher frequency vibrational modes. Thus for 70 °C BIRD, delays >30 s reflect the kinetics of the H_2O loss process (Fig. 2). For all charge states, H_2O loss increases rapidly during the first 150 s before leveling off. The BIRD spectrum at 70 °C for 150 s delay resembles that at 90 °C for 30 s delay. Although of relatively low enthalphy requirements, H_2O loss is entropically unfavorable [29]. Thus misleading H_2O loss data can be minimized by limiting the delay before measuring BIRD spectra.

Choosing a 30 s delay time, the kinetics of BIRD H_2O loss were studied further, measuring total H_2O

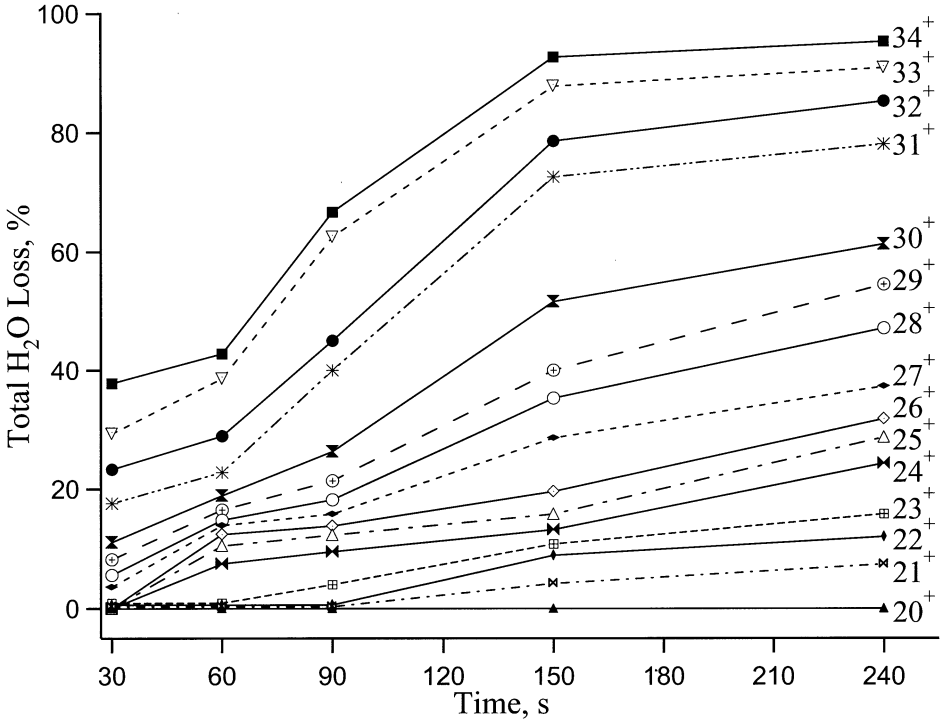

Fig. 2. Effect of delay time on the total H_2O loss from carbonic anhydrase ions by BIRD at 70 °C; *y* axis, $[(M - nH_2O)^{z+}]/\{[M^{z+}] + [(M-nH_2O)^{z+}]\}\times 100\%$; $n = 1$–5, $z = 20$–34.

loss as a function of temperature and charge state (Fig. 3). This loss can involve as many as five H_2O molecules (Fig. 1); details on $[M^{z+}]$, $[(M-H_2O)^{z+}]$, and $[(M-3H_2O]^{z+}]$ are given in Fig. 4. The rate of H_2O loss increases from 60 to 130 °C, as expected, but falls off above this temperature from competition by the backbone dissociation process. The data for the loss of one and three H_2O molecules (Fig. 4), and that for two, four, and five (not shown) are consistent with similar rate constants for each subsequent loss. Thus, the single H_2O loss increases first with temperature but then decreases as the double H_2O loss increases, and so forth. For the 31+ (Fig. 1) and 34+ ions, this can be observed through five H_2O losses from 60 to 140 °C. Thus H_2O loss is the only low energy dissociation process that occurs before sufficient energy can be added to the ions to cause competitive backbone fragmentation.

Loss of H_2O also increases substantially with increasing charge (Figs. 2–4). As reported previously [26], H_2O loss presumably involves proton transfer from basic residues such as Arg, His, and Lys to the Ser and Thr side chain hydroxyl groups and/or Asp and Glu carboxyl groups; note that H/D exchange of such protein ions adds D_2O to a protonated site that rearranges and then loses HDO [11]. Not only should the rate of this rearrangement increase with an increasing number of protons, but the probability that the hydroxyl and proton are within bonding distance should increase with disruption of their secondary and tertiary noncovalent bonding resulting from the increase in coulombic repulsion between the protonated sites [38–40]. Higher charge state ions have been shown to have more open conformations [10–13,41] in which the protons and hydroxyl groups are less involved in the noncovalent bonding of the secondary and tertiary structure. Heating has a similar effect on this noncovalent structure [13,24,41].

The behavior of the 4+ to 9+ ions of ubiquitin [26] is in apparent disagreement. Williams and co-

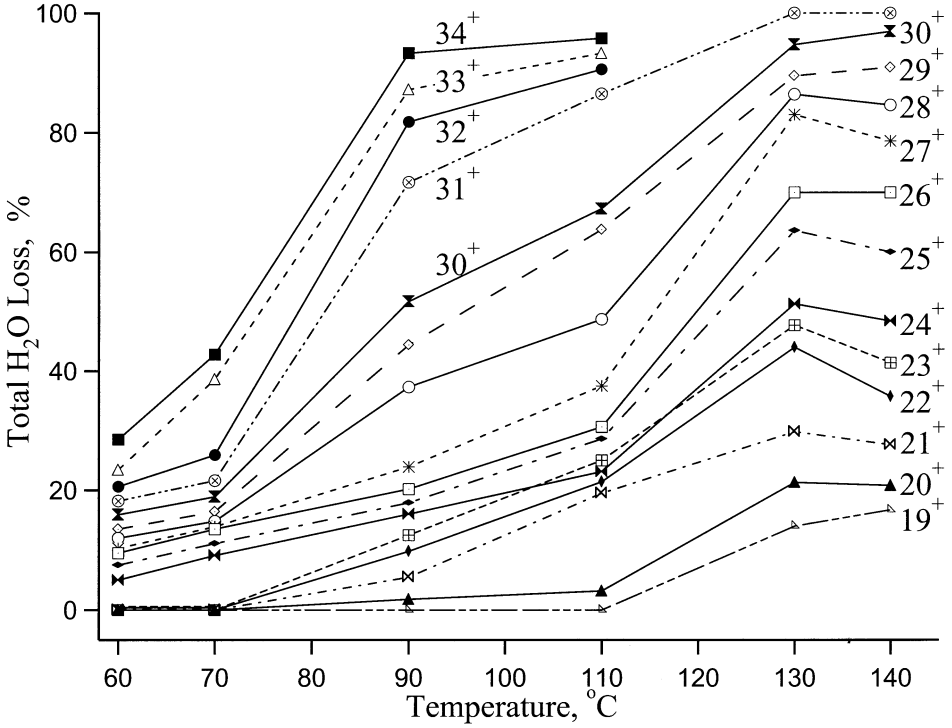

Fig. 3. For a 30 s delay time before BIRD, the effects of charge state and temperature on total water loss from carbonic anhydrase molecular ions; y axis, $[(M - nH_2O)^{z+}]/\{[M^{z+}] + [(M - nH_2O)^{z+}]\} \times 100\%$, $n = 1$–5, $z = 20$–34.

workers reported that the extent of water loss decreases with increasing charge state of the ion [25–29]. Their explanation of "increasing the number of charges decreases the dissociation threshold for backbone cleavages, making these dissociation processes more competitive" is certainly logical. This discrepancy is clarified in Fig. 5 showing molecular ion water loss for the 5+ to 13+ charge states of ubiquitin; although our 5+ to 9+ ions were kept at 165 °C for 90 s versus 200 °C for 20 s [26], the data for these charge states are in agreement. The tightly folded charge state of 5+ is very resistant to backbone cleavage. On the other hand, the increase in H_2O loss for the 11+ to 13+ ions is dramatic, a far greater effect than just the proportional increase in the number of protons, and one that also reflects the effect in Fig. 3. This increase in electrostatic repulsion that disrupts the noncovalent structure [13] increases the probability that a proton will be adjacent to a hydroxyl group, and thus could result in the greatly increased H_2O loss. Unusual noncovalent bonding involving threonine in the 15+ and 16+, but not the 9+, ions of cytochrome *c* [12] could be related to the H_2O loss tendencies noted here.

Extensive backbone fragmentation of carbonic anhydrase is evident in the BIRD spectrum at 140 °C for 30 s delay time (Fig. 1), and more extensive at 150 and 160 °C (Fig. 6). However, these BIRD spectra at different temperatures do display 10%–30% unique backbone cleavages (Table 1). There are 22/258 bond cleavages (14 ***b*** and 12 ***y*** ions) in the BIRD spectrum at 140 °C, with 9/22 unique compared to those at 150 and 160 °C. Of the 22/258 bond cleavages at 150 °C (8 ***b*** and 14 ***y*** ions), 5 are unique; and of 16/258 bond cleavages at 160 °C (6 ***b*** and 8 ***y*** ions), 3 are unique. Combined, the three BIRD spectra give 34/258 bond cleavages (Fig. 5), a >50% increase over any single spectrum. In contrast, the CAD spectra of carbonic anhydrase gave 45 ***b*** and 30 ***y*** fragment ions [4,42,43] and IRMPD gave 23 ***b***, ***y*** ions [44]. The spectrum

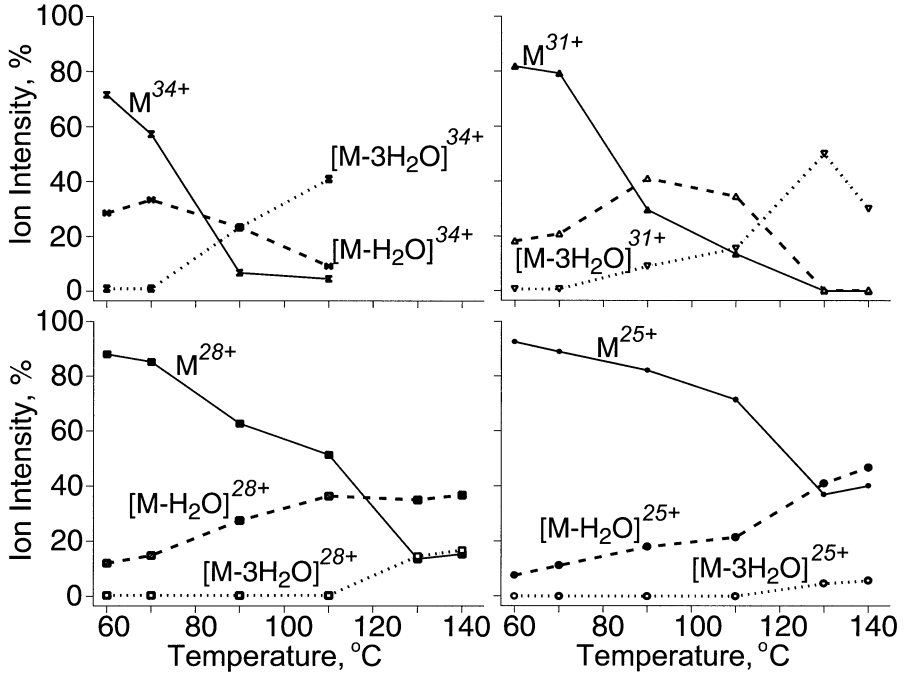

Fig. 4. For the Fig. 3 BIRD spectra (30 s delay) of carbonic anhydrase molecular ions ($z = 34+, 31+, 28+, 25+$), intensity data on these and their single and triple H_2O loss ions normalized vs. $\{[M^{z+}] + [(M-nH_2O)^{z+}]\}$.

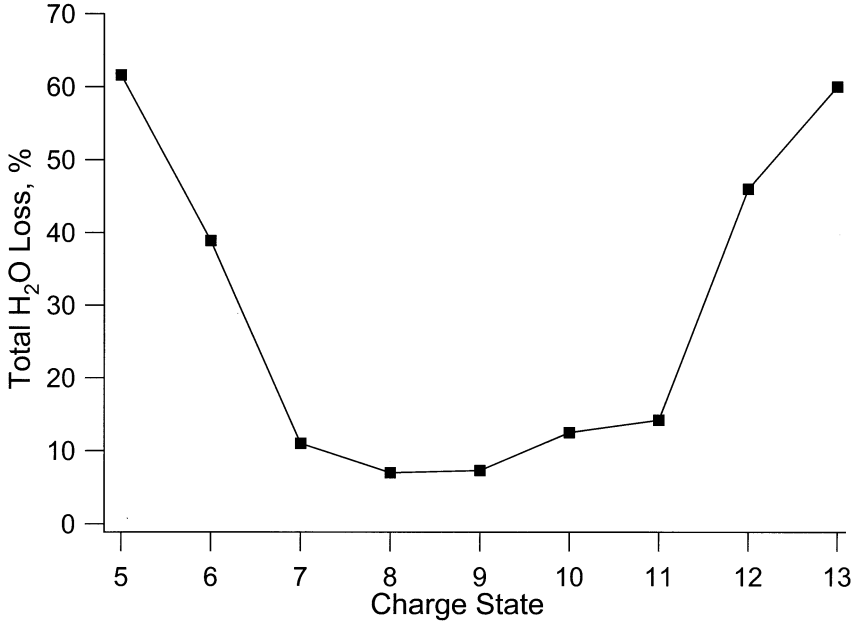

Fig. 5. H_2O loss from 5+ to 13+ ubiquitin molecular ions at 165 °C for 90 s.

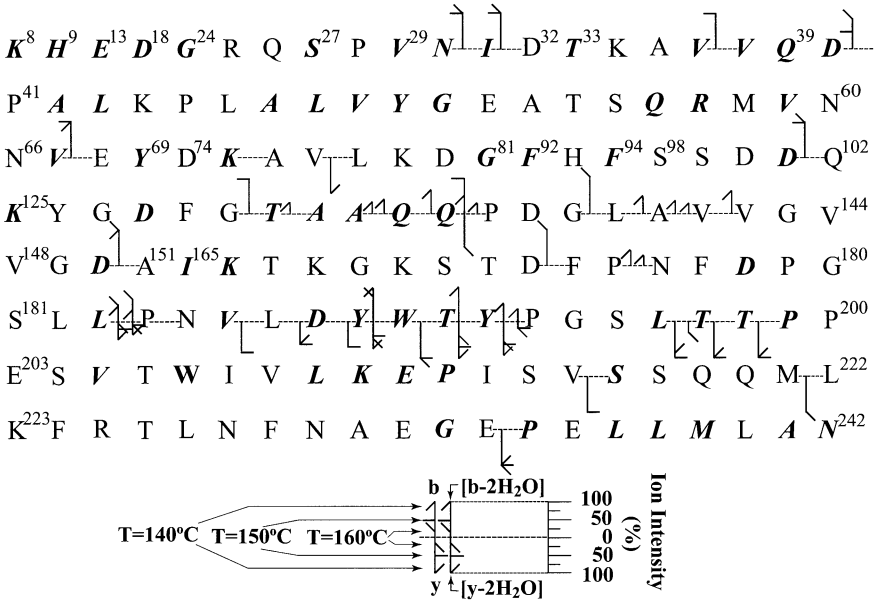

Fig. 6. Cleavages in BIRD spectra of carbonic anhydrase at 140, 150, and 160 °C. Only selected amino acids of the 259 total are shown. Letters in bold and italics stand for the CAD cleavages. Superscript numbers correspond to the amino acid order.

from swept frequency multiple excitation collisional activation (MECA) [15] is remarkably similar to the IRMPD spectrum. The recently developed technique, AI ECD, identified 116 bond cleavages [24]. Despite the fact that BIRD has affected mostly the same backbone cleavages as that of CAD or IRMPD, BIRD did produce seven additional informative cleavages. For both the molecular and fragment ions from BIRD of carbonic anhydrase, however, H_2O loss is more prevalent than that from CAD/IRMPD (Fig. 1), with the H_2O loss peak even more intense than that of its parent ion in some cases. As shown before [26], loss of H_2O could occur after as well as before fragmentation.

4.2. ThiF, 27 kDa

The effects of temperature, delay time, and charge state observed in the BIRD spectra of ThiF resemble those of carbonic anhydrase. H_2O loss starts at 60 °C, and extensive backbone dissociation is observed at 135 °C. Here a 60 s IR exposure was used; 135 °C gives 20/250 bond cleavages (14 ***b*** and 6 ***y*** ions), with

Table 1
Techniques for dissociation of carbonic anhydrase ions

Techniques	Products	Backbone cleavages of 258 possible	Unique cleavages
BIRD			
140 °C	14 ***b***, 12 ***y***	22	9/34
150 °C	8 ***b***, 14 ***y***	22	5/34
160 °C	9 ***b***, 9 ***y***	16	3/34
Combined	22 ***b***, 17 ***y***	34	7/258
CAD	45 ***b***, 30 ***y***	66	13/258
AI ECD	59 ***c***, 45 ***z***, 15 ***a***, 23 ***b***, 18 ***y***	116	71/258
Combined techniques	59 ***c***, 45 ***z***, 15 ***a***, 60 ***b***, 43 ***y***	148	

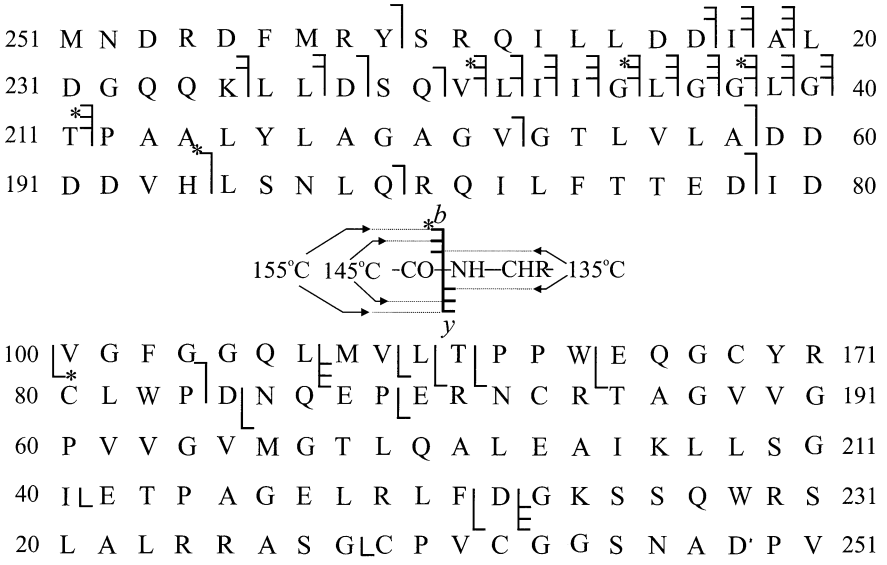

Fig. 7. Cleavages in BIRD spectra of ThiF at 135, 145, and 155 °C. An asterisk indicates the corresponding single H_2O loss.

5 unique compared to those at 145 and 155 °C (Fig. 7). The 145 °C spectrum showed 24/250 backbone cleavages (19 ***b*** and 5 ***y*** ions), with 6 unique cleavages; 155 °C generates 22 ***b*** and 7 ***y*** ions, with 10 unique. Combined, BIRD cleaved 41 different bonds out of the 250 bonds, with 19 cleavages unique versus other spectra. Of the 30 bond cleavages in CAD/IRMPD spectra, 6 are unique [45], and of the 65 in AI ECD spectra, 40 are unique. Unlike carbonic anhydrase, little (<10%) H_2O loss was observed for ThiF fragment ions (data not shown), comparable to that by CAD and IRMPD.

4.3. Thiazole kinase, 29 kDa

Similar to ThiF, the BIRD spectra of thiazole kinase resemble those of carbonic anhydrase in terms of the effect of temperature, delay time and charge state. Loss of H_2O starts at 80 °C, and extensive backbone dissociation is observed at 145 °C. A 70 s exposure was used here; at 145 °C 18/283 bonds (16 ***b*** and 3 ***y*** ions) are cleaved, with 6 unique compared to that at 155 °C (Fig. 8). The spectrum at 155 °C showed 17/283 backbone cleavages (12 ***b*** and 5 ***y*** ions), with 5 unique cleavages. Combined, BIRD produces 23 distinctive bond cleavages, with 6 unique versus NS CAD and AI ECD that cleave 22/283 and 60/283 bonds, respectively [24]. Comparable to that by CAD and IRMPD, about 20%–30% H_2O loss from the fragment ions is observed in BIRD.

4.4. Thiaminase I (42 kDa)

As a mixture of three isozymes, thiaminase I shows heterogeneity on the N-terminus consisting of an extra N-terminal Ala and Ala-Gly, respectively [46]. In great contrast to the other three proteins described above, no H_2O loss is observed in the BIRD spectra of thiaminase; possibly the rupture of the noncovalently bonded tertiary structure is now the dominant low energy process [24]. Backbone fragmentation is evident at 150 °C for 90 s delay time (Fig. 9), and is much more extensive at 175 and 182 °C (Fig. 10). The 150 °C spectrum shows 11/378 bond cleavages (4 **b** and 8 **y** ions), with 8 unique to those at 175 and 182 °C (Fig. 10). Complete dissociation of the molecular ions takes place at 175 °C; of the 14/378 bond cleavages (6 **b** and 8 **y** ions), 4 are unique, and of 14/378 bond cleavages at 182 °C (4 **b** and 10 **y** ions), 6 are unique. Combined, the three BIRD spectra give 28/378 bond cleavages, of which 15 are unique versus

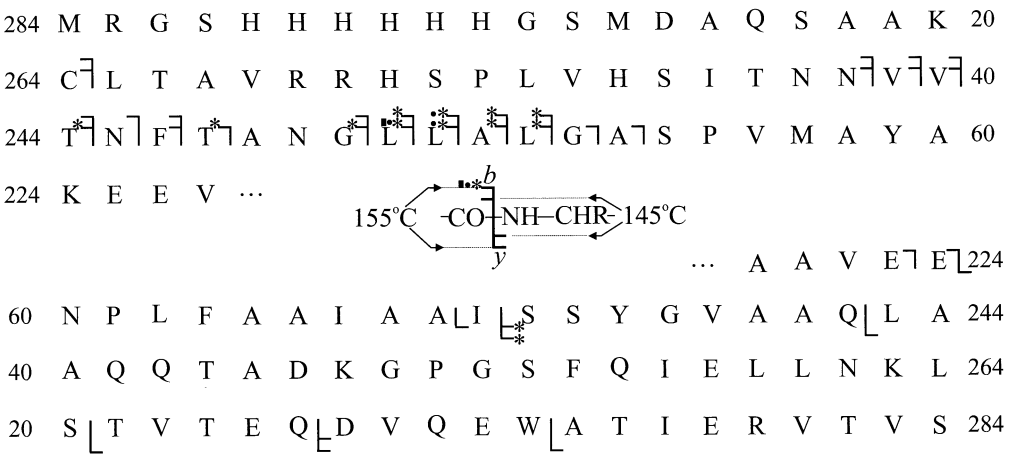

Fig. 8. Cleavages in BIRD spectra of thiazole kinase at 145 and 155 °C. An asterisk indicates the corresponding single H_2O loss, a circle for double H_2O loss, and a bar for triple H_2O loss.

the other spectra, whereas CAD/IRMPD generates 28 [46] and AI ECD 54 bond cleavages [24]. The absence of H_2O loss from the BIRD fragment ions and the relatively higher temperature and longer induction period necessary for backbone dissociation is consistent with the strong tertiary structure indicated by AI ECD [24].

4.5. BIRD loss of water

In contrast to CAD/IRMPD, rearrangement loss of H_2O is a major dissociation pathway in the lower temperature BIRD spectra for carbonic anhydrase, ThiF, and thiazole kinase. Evidently CAD/IRMPD deposits energy in the protein ions sufficiently fast to

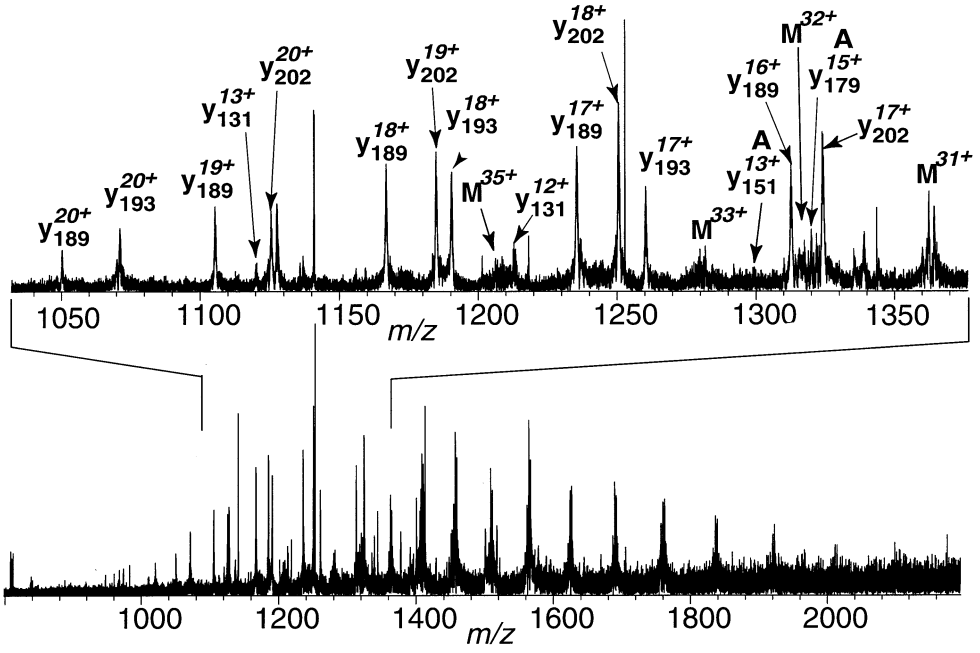

Fig. 9. BIRD spectrum of thiaminase I at 150 °C cell temperature for 90 s delay.

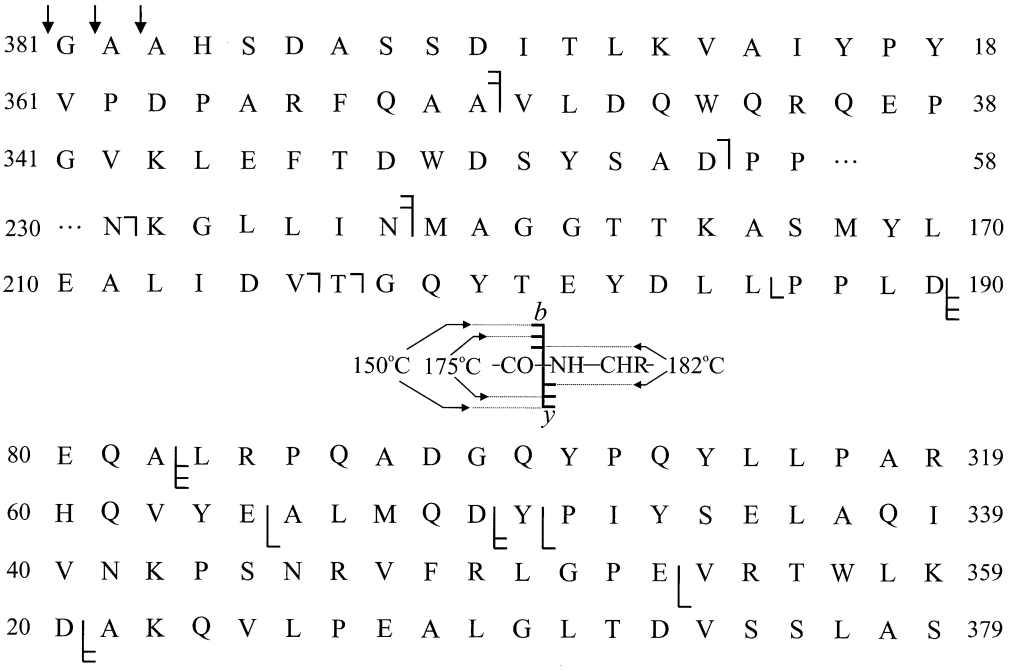

Fig. 10. Cleavages observed in BIRD spectra of thiaminase I at 150, 175, and 182 °C.

minimize the enthalpically favored rearrangement in favor of the entropically favored backbone fragmentation [47].

It is not clear why carbonic anhydrase is so much more vulnerable to H_2O loss than ThiF, thiazole kinase, and the 45% larger thiaminase. The number of hydroxyl containing amino acids (Ser:Thr:Asp:Glu) in these proteins is 16:14:21:11, 17:21:11:16, 12:15:20:9, and 28:12:31:18, respectively. Involvement of tyrosine in H_2O loss has been suggested [26]; here the numbers are 8, 3, 3, and 21. Recent ECD studies have provided detailed conformational information on the gaseous ions of cytochrome *c* [12] and ubiquitin [13]; these should be extended to the proteins examined here.

4.6. Amino acid influence on the fragmentation

The sequence information for these four proteins provided by BIRD (Figs. 4–7) is summarized in terms of the frequency of cleavage (%) on the C- and N-terminal sides of the different amino acids (Fig. 11). Here, useful temperatures for this varied from 135 to 182 °C, with greater sequence information resulting from spectra measured at several temperatures. In general, this bears a strong resemblance to earlier reports on BIRD [27,48] and CAD/IRMPD [6,11,49] spectra, as summarized recently in similar bar graph form [50]. Thus an extremely high tendency is observed for cleavage on the C-terminal side of Asp (more than three times the average), and also facile cleavage on the N-terminal side of Pro (Fig. 10). Low cleavage frequency is observed for the basic amino acids His, Lys, and Arg, presumably through stabilization by solvation of their protonated side chains to neighboring backbone carbonyl groups [6,11,48]. However, BIRD did produce unique cleavages compared to CAD/IRMPD; possibly BIRD dissociates the noncovalent tertiary structure of the much larger proteins more extensively due to its long reaction time frame. More convenient and rapid variation in cell temperature could provide longer reaction time for the entropically controlled activation and dissociation of the noncovalent bonds, followed rapidly by the higher temperatures necessary for backbone dissociation. As

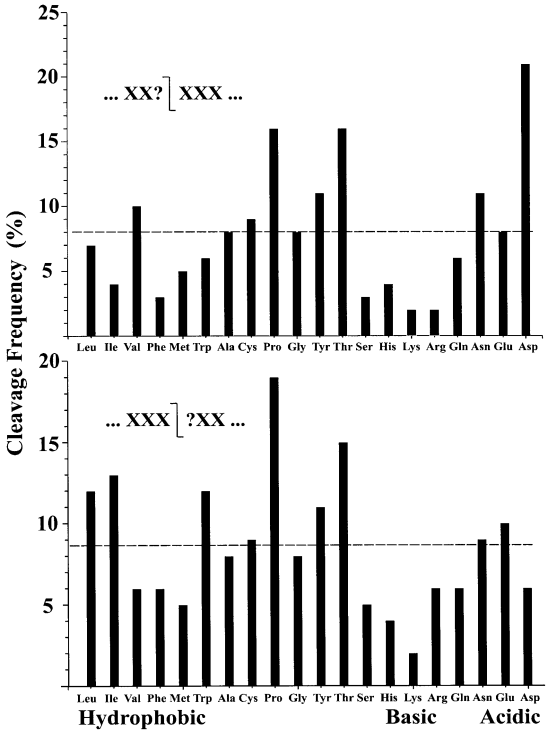

Fig. 11. Frequency of cleavage (%) on the C-terminal (above) and N-terminal (below) sides of the designated amino acids for the BIRD spectra of the four proteins studied here. The relative intensity of the product ion (***b***/***y***) resulting from cleavage of a specific backbone bond is credited to the amino acids on both the C-terminal side and N-terminal side of the cleavage, irrespective of the fragment ion abundance, and normalized to the number of occurrences of the amino acid.

described previously, BIRD is valuable for successive "boiling off" of the noncovalent adducts [35, 51].

5. Conclusions

BIRD is an inexpensive and easy to implement dissociation technique that is shown to be valuable for far larger proteins (up to 42 kDa). In comparison to other techniques, it suffers from more facile H_2O loss and more time-consuming temperature adjustment of the ion cell. However, if CAD/IRMPD and AI ECD do not yield sufficient structural characterization, BIRD can provide additional and complementary sequence information that is of increasing value for increasing protein size.

Acknowledgements

The authors thank Professor Tadhg Begley for the ThiF, thiazole kinase, and thiaminase samples and for his participation in their structural characterization; Kathrin Breuker, Aaron Frank, Blas Cerda, and Evan Williams for helpful discussions; and the National Institutes of Health (grant no. GM16609) for generous financial support.

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