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Simultaneous Kinetic Characterization of Multiple Protein Forms by Top Down Mass Spectrometry

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Top down mass spectrometry, using a Fourier transform instrument, has unique capabilities for biomolecule kinetic studies, in that the concentration of large molecules in a reaction mixture can be monitored simultaneously from its mass spectrum produced by electrospray ionization. This is demonstrated with enzyme modifications occurring in the biosynthesis of the thiazole moiety of thiamin phosphate. The formation rate of ThiS-thiocarboxylate from ThiS was determined from the relative abundance of the corresponding *m/z* 10162 and 10146 isotopic peak clusters for all the observable charge states in the mass spectra measured at different reaction times. Even without measuring standard ionization efficiencies, the rate and precision of $0.018 \pm 0.004\ min^{-1}$ agree well with the $0.027 \pm 0.003\ min^{-1}$ obtained with a radiochemical assay, which requires a separate derivatization step. To illustrate the simultaneous characterization of the reaction kinetics of a native enzyme and its mutant, the imine formation rate of ThiG and its substrate DXP was compared between the native protein (M_r = 26803.9) and its E98A (M_r = 26745.9) or D182A (M_r = 26759.9) mutant in the same reaction mixture. The kinetic data show clearly that neither the E98 nor the D182 residues participate in the imine formation. The high resolution and MS/MS capabilities of FTMS should make possible the extension of this kinetics approach to far more complicated systems, such as simultaneous monitoring of 24 native, intermediate, and reduced forms in the reductive unfolding of a mixture of ribonuclease A and the five isoforms of ribonuclease B. Stable intermediates with different S—S bonding (same molecular weight) can be differentiated by MS/MS, while molecular ions differing by only 2 Da are distinguished clearly by synthesizing isotopically depleted proteins. (J Am Soc Mass Spectrom 2005, 16, 1052–1059) © 2005 American Society for Mass Spectrometry

Chemical transformations of proteins are basic to the function of all biological systems. A kinetic study of such chemical changes often can provide a deeper understanding of a biological mechanism. For example, characterization of the specific effect of a single enzyme mutation can require quantitative determination as a function of time of the mixed native and mutant enzymes and their modifications. The recent proteomics revolution has brought the development of powerful new techniques for characterizing the sequence and posttranslational modifications of individual proteins that are isolated from biological systems. The advantages of mass spectrometry (MS) for kinetic studies of small molecules are well known [1–4], but the application of MS to larger biomolecule kinetics has been limited to a single modification of a single protein without isotopic resolution [5, 6]. Here we describe how "top down" tandem MS using a Fourier transform (FT) [7, 8] instrument can be applied to important kinetics problems involving multiple protein measurements, including direct activity comparison of multiple enzymes in the same solution.

Classical methods for kinetic monitoring of enzymatic product formation, such as use of a characteristic fluorescence wavelength, must be specific for the monitored chemical modification; multiple modifications require multiple probes. Often this necessitates derivatization of the protein with an appropriate chromophore, which adds concern for its influence on the reaction studied as well as complexity to the analysis. Measuring the kinetic behavior of a wild-type protein and its mutant, or of a number of protein isoforms, together in the same reaction mixture provides the most accurate comparison, but is seldom attempted because this multiplies the number of probes required.

In the top down MS approach [9, 10] to protein characterization, electrospray ionization (ESI) of a protein mixture yields a mass spectrum of protein molecular ions.

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FTMS is the favored instrumentation, as its high resolving power and mass accuracy can derive accurate (±1 Da) molecular weight values (M_r) for more than 10 proteins from such a spectrum [11, 12]. Even a simple chemical modification such as methylation will make an easily distinguishable change (+14 Da) in the M_r value. Isomeric proteins (identical M_r values), such as those methylated at different sites, can often be distinguished by MS/MS dissociation of their molecular ions [12].

Of course it is only when conventional techniques are inadequate that this expensive FTMS technique should be considered. Here we show that its M_r values can conveniently provide definitive and semi-quantitative data for kinetic studies in protein mixtures. Using more rigorous MS techniques, it is generally possible to obtain far more accurate quantitation and sensitivity [13], but these were not necessary for the studies described here. For example, M_r values provide highly specific probes for protein intermediates in the biosynthesis of the thiazole moiety of thiamin phosphate. Resolved M_r values (~14 kDa) and MS/MS also make possible the simultaneous monitoring of 24 native, intermediate, and reduced forms in the reductive unfolding of the six isoforms of ribonuclease A and B [14]. Possible top down MS kinetic studies in other important biological systems are discussed.

Experimental

Overexpression of Proteins

E. coli BL21(DE3) containing each plasmid was grown in LB media with ampicillin (50 μg/ml media) shaking at 37 °C until its OD_{600} reached 0.8, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 500 μM, and growth was continued for another 8 h at 37 °C. For the copurification of ThiSG and ThiF, cultures containing pCLK820 and pCLK431 were prepared in 1000 and 500 ml LB media, respectively, mixed and harvested together. Cultures for *B. subtilis* YrvO were prepared in 1L LB media.

Purification of Proteins

The cells prepared above were resuspended in 5 ml of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and lysed by sonication (Heat Systems Ultrasonics model W-385 sonicator, now Misonix, Farmingdale, NY, 2s cycle, 50% duty, 4 min). All proteins used in this study were purified using Ni-NTA resin following the manufacturer's instructions (Qiagen, Valencia, CA). After elution, protein solutions were buffer exchanged using a PD-10 column (Amersham, Piscataway, NJ, 50 mM Tris-HCl, pH 8.0).

Kinetics for the Formation of ThiS-Thiocarboxylate

2 mM cysteine, 4 mM ATP, 10 mM $MgCl_2$, and ThiSGF (3600 μg) were preincubated for 10 min before adding 150 μl YrvO (540 μg) in 200 mM Tris-HCl pH 7.8 (total volume 900 μl). Aliquots of the reaction mixture (90 μl) were quenched at different time points by desalting via reverse-phase protein traps (Michrom Bioresources, Auburn, CA), washed with 1:98:1 (MeOH: H_2O: AcOH), and eluted with 70:26:4 (MeOH: H_2O: AcOH).

Samples representing ~0.1 μg of ThiSGF were analyzed by ESI-FTMS. The resulting intensities of ThiS-thiocarboxylate were imported into sigmaplot and fitted to an exponential function.

Kinetics on the Effect of Mutation on Imine Formation with ThiG

A typical experiment was carried out by overexpressing ThiSG, ThiSG(E98A) and ThiSG(D182A) as separate cultures. The ThiSG culture (1L) was then harvested (by centrifugation) together with 1 L of ThiSG (E98A) or ThiSG (D182A) and stored at −20 °C until use. The proteins were then purified and incubated with 1-deoxy-D-xylulose-5-phosphate (DXP) (500 μM) to give a total volume of 512 μl of 25 mM Tris-HCl, pH 7.7 containing thiazole synthase (5 mg/ml, as ThiSG) and mutants. The reaction mixture was then reduced by treatment with $NaBH_4$ (200 mM) at various time points. The foam generated during the reduction was spun down in a clinical centrifuge for 2 min and 90 μl of this solution was gel filtered using biospin columns (BIO-RAD Tris, Hercules, CA pH 7.4, 0.02% NaN_3) to remove excess substrate and boron salts. The resulting protein solution was frozen and stored at −80 °C until it could be analyzed by ESI-FTMS. The resulting normalized intensities of molecular ions of the ThiG and ThiG +200 Da were plotted against time in sigmaplot and fitted with an exponential function. The molecular ions for ThiSG (E98A) and ThiG (E98A)+200 Da as well as ThiG (D182A) and ThiG (D182A)+200 Da were plotted in a similar fashion.

Mass Spectrometric Analysis

Protein samples were desalted by reverse-phase protein traps (Michrom Bioresources, Auburn, CA), washed with 1:98:1 (MeOH:H_2O:AcOH), and eluted with 70:26:4 (MeOH:H_2O:AcOH). Solutions were electrosprayed at 1–50 nl/min with a nanospray emitter. The resulting ions were guided through a heated capillary, skimmer, and three radio frequency-only quadrupoles into a 6 T modified FTMS (ThermoFinnigan, San Jose, CA) with the Odyssey data system [15]. The normalized peak intensities for different ion signals were obtained with computer program THRASH [16]. The mass difference (in units of 1.00235 Da) between the most abundant isotopic peak and the monoisotopic peak is denoted in italics after each M_r value.

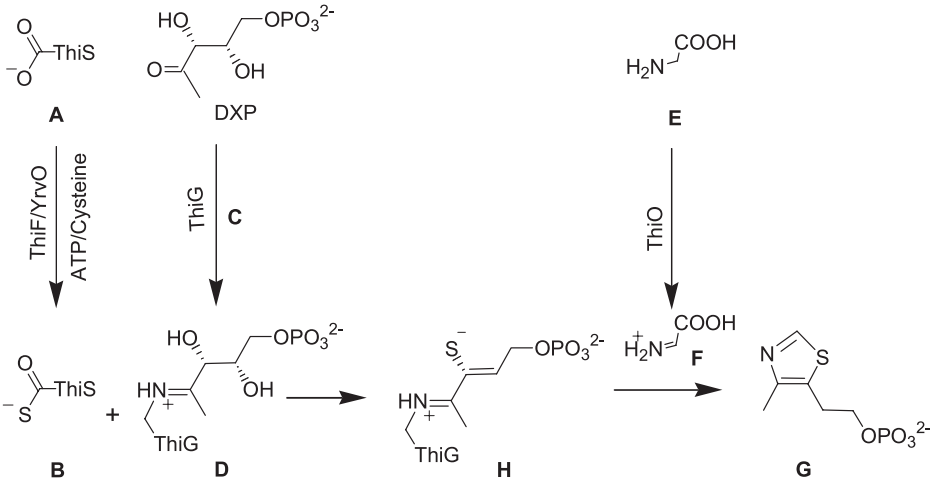

Figure 1. Biosynthesis of thiazole phosphate in *B. subtilis*.

Results and Discussion

Thiamin phosphate is an essential redox cofactor in all living systems where it functions to stabilize the acyl carbanion synthon [17]. It is composed of two subunits, the pyrimidine moiety and the thiazole moiety. Recently, the biosynthesis of its thiazole moiety was successfully reconstituted in *B. subtilis* [18] from glycine (**E**), cysteine, and deoxy-D-xylulose-5-phosphate (DXP) using enzymes ThiS (**A**), YrvO, ThiF, ThiG (**C**), and ThiO. As shown in Figure 1, thiazole phosphate (**G**) is formed by a reaction of the sulfur carrier protein ThiS-thiocarboxylate (**B**), ThiG-DXP imine (**D**) and dehydroglycine (**F**). The formation of ThiS-thiocarboxylate from ThiS was achieved by incubating ThiS carboxylate with YrvO, cysteine, ThiF, and ATP; while the oxidation of glycine to dehydroglycine was catalyzed by ThiO. In this paper, top down mass spectrometry is used to monitor the rate of the formation of ThiS thiocarboxylate and the effect of two ThiG mutations on the rate of reaction of ThiG and DXP.

Kinetics of ThiS-Thiocarboxylate Formation

ThiS-carboxylate (**A**) was incubated with ATP, cysteine, YrvO, ThiF, and ThiG (copurifies with **A**) at room temperature. Samples were taken at designated time intervals (Figure 2a), the reaction quenched in a reverse phase desalting column, and the ESI spectrum measured. The normalized abundance for all the observable charge states of ThiS thiocarboxylate (**B**, M_r = 10162.4) and (**A**, M_r = 10146.4) from the computer program TRASH were summed, plotted, and fit to a single exponential function, giving a rate of 0.018 ± 0.004 min^{-1} (Figure 2b). Radiochemical assay gave 0.027 ± 0.003 min^{-1}, so that the precision of the methods is comparable [18]. This small difference in rate constants could arise from differences in experimental parameters (e.g., "room temperature", with months between experiments), but probably not from the difference in ESI ionization efficiency caused by substituting −SH for −OH in the 10 kDa protein (although this was not measured).

Following the formation of ThiS-thiocarboxylate, the addition of DXP causes conversion of ThiS-thiocarboxylate back to ThiS-carboxylate (Figure 3) at a rate (0.20 ± 0.01 min^{-1}) ten times faster than that of the ThiS-thiocarboxylate **B** formation. This suggests that the role of **B** is the facile donation of sulfur to the ThiG-DXP imine **D** (Figure 1) to yield the key intermediate **H**.

Kinetics of Imine Formation by ThiG and its Mutants

ThiG (**C**), the thiazole-phosphate synthase, forms an imine (**D**) with its substrate DXP (Figure 1, M_r = 26803.9). This imine has been localized to the $-NH_2$ group of K96 of the ThiG by top down mass spectrometry analysis [19]; K96 is found to be absolutely conserved by sequence alignment with other thiazole biosynthetic genes from phylogenetically diverse microorganisms. Mutation of K96 to an alanine resulted in a thiazole synthase (M_r = 26746.9) that cannot form either the imine **D** or thiazole phosphate **G** [20]. Two additional residues E98A and D182 are also absolutely conserved; E98 mutant of **C** showed a 38-fold decrease in the rate of formation of thiazole phosphate **G** and the D182A mutant was unable to form thiazole phosphate in vitro [20]. To find out if the activity loss results from the direct involvement of these residues in imine **D** formation, or in the conversion of the imine **D** to the thiazole **G**, the corresponding kinetic data were measured for both these mutants.

When DXP was added to a 1:1 mixture of ThiG and the D182A ThiG mutant at room temperature, aliquots of sample were immediately reduced to irreversibly trap the DXP-protein-imine **D** at different time points. The relative amounts of these products from wild ThiG and its E98A mutant plotted against time (Figure 4),

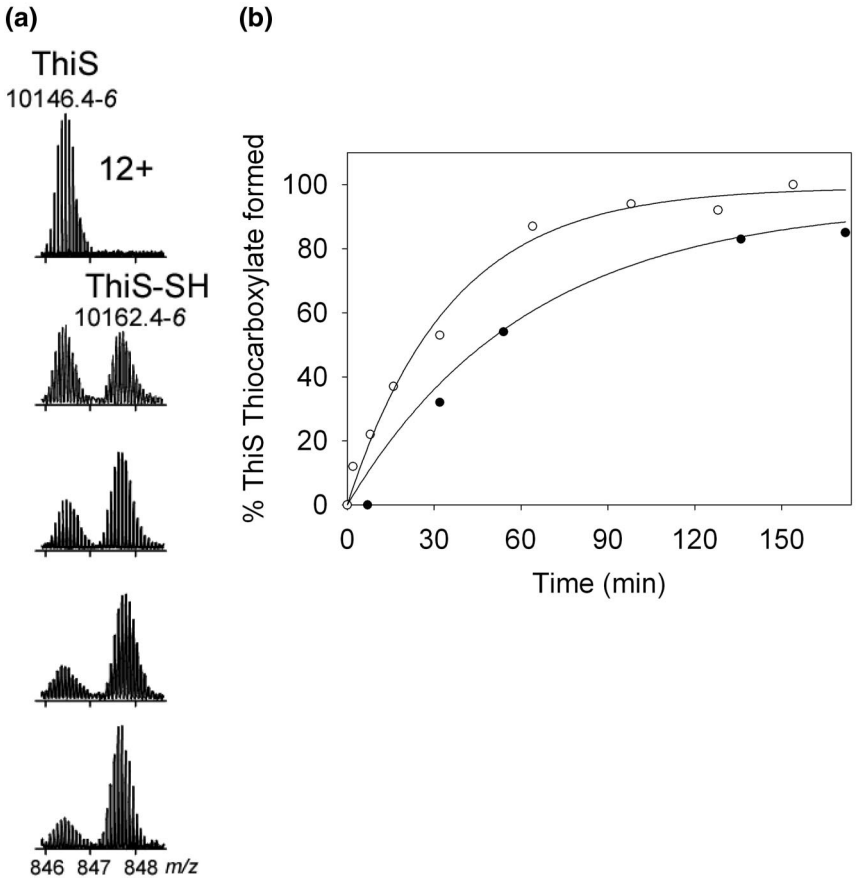

Figure 2. (**a**) ESI-FTMS spectra of the time dependent formation of ThiS thiocarboxylate. (**b**) Data from mass spectrometry (filled circles) and autoradiography (open circles).

both fit a first order function well, giving rates of imine formation of 2.6 ± 0.1 min^{-1} and 3.1 ± 0.1 min^{-1} respectively. When the same methodology was applied to a 1:1 mixture of ThiG and its E98A mutant to form the respective imine **D**, the rates of 2.4 ± 0.1 min^{-1} and 1.9 ± 0.1 min^{-1}, respectively (Figure 5), implied that mutation lowered the efficiency slightly. However, these differences can hardly account for the great reduction observed in the rate for thiazole-phosphate **G** formation. Based on these kinetic data obtained under identical experimental conditions, the mutations at residues E98 and D182 have a far larger effect on **D** + **F** → **G** than on DXP + ThiG → **D**, in contrast to the K96 mutation that stopped **D** formation.

Possible Extensions of Top Down MS Kinetic Studies

From the ESI mass spectrum of a poly(ethene/propene) glycol copolymer exhibiting 130 M_r values, the sequences of five oligomer molecular ions of ~1% relative abundance were successfully characterized by MS/MS [21]. Thus, top down MS and MS/MS kinetic studies of far more complicated protein systems should be feasible. As a possible system, a recent top down MS study characterized the intermediates in the reductive unfolding of bovine pancreatic ribonuclease A (RNase A) and five isoforms of RNase B, each with a different, naturally occurring carbohydrate moiety [14]. The kinetic parameters of the reductive unfolding measured by HPLC showed little difference between the A and B. However, HPLC could not separate the five isoforms of RNase B to determine the kinetics of each individual isoform. A kinetic study of the simultaneous unfolding of RNase A and B would require simultaneous detection of the six folded and six completely unfolded forms; five M_r values of the latter are easily characterized in the ESI/FTMS spectrum of Figure 6. Stable intermediates with one S—S bond cleaved were identified by derivatization of the new −SH groups that modified the M_r values, while MS/MS differentiated the two stable isomers with a different S—S bond reduced. Thus, such a simultaneous kinetic study of six protein isoforms, measuring for each its native, two intermediate, and reduced forms, a total of 24 compounds should be possible by top down ESI/FTMS.

Without −SH derivatization, disulfide bond formation only involves a 2 Da mass decrease, so that the resulting isotope distribution pattern will overlap that of the native molecular ion, reducing the abundance accuracy for the determination of these compounds. Stable intermediates from further disulfide bond cleav-

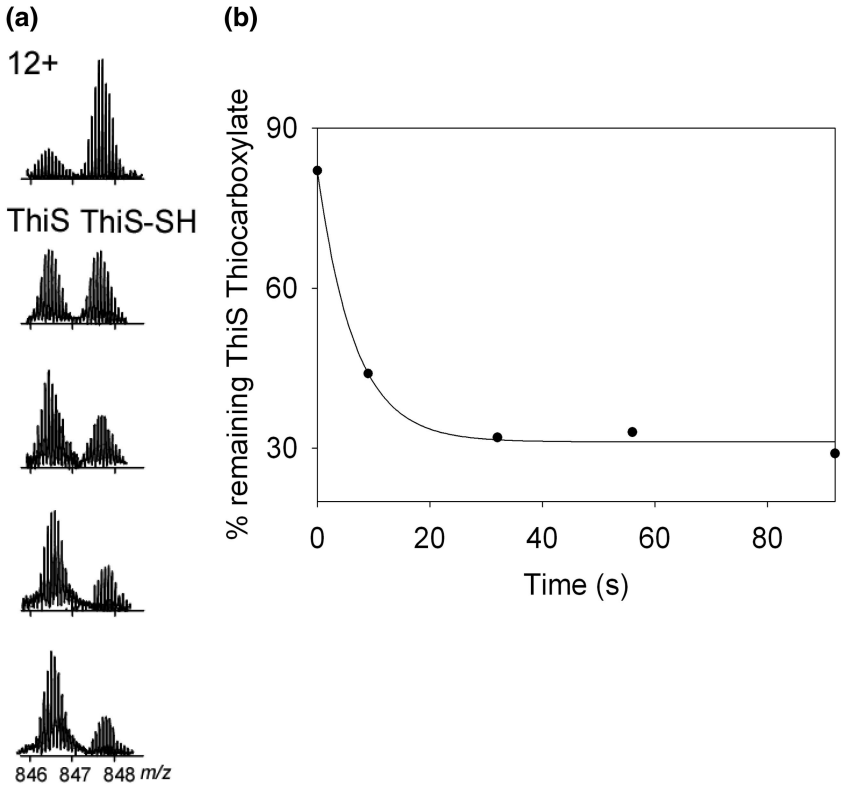

Figure 3. (**a**) ESI-FTMS spectra and (**b**) plot of the time dependent conversion from ThiS thiocarboxylate back to ThiS-carboxylate.

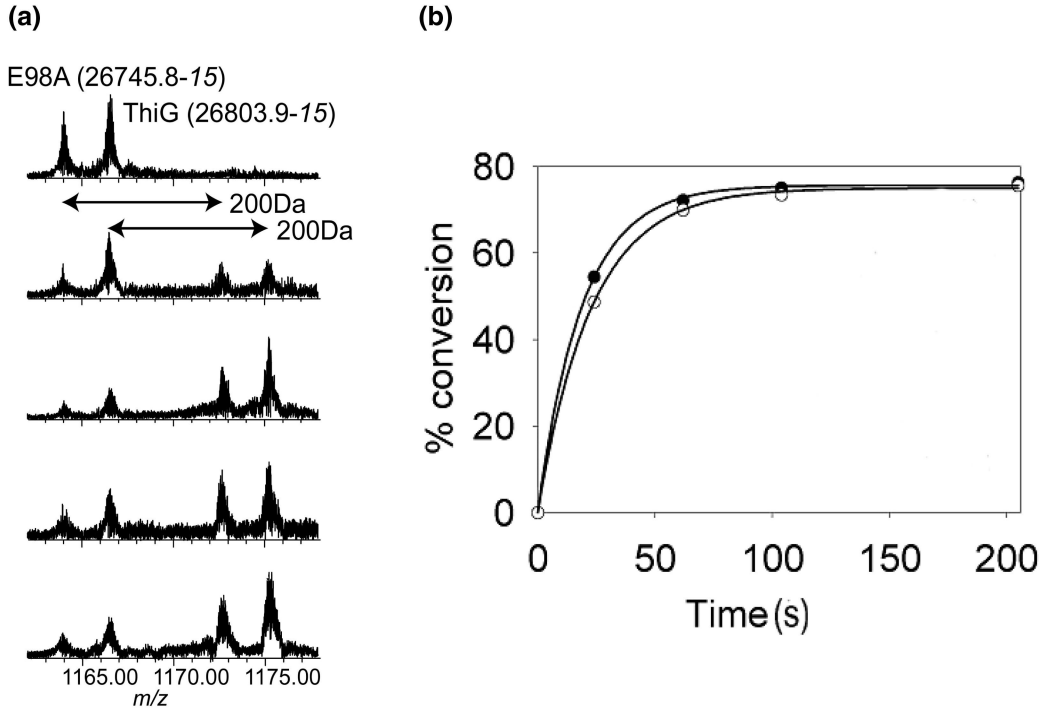

Figure 4. (**a**) ESI-FTMS spectra and (**b**) plots of the simultaneous time dependent formation of imine adduct **D** from wild type ThiG (open circles) and its E98A mutant (filled circles).

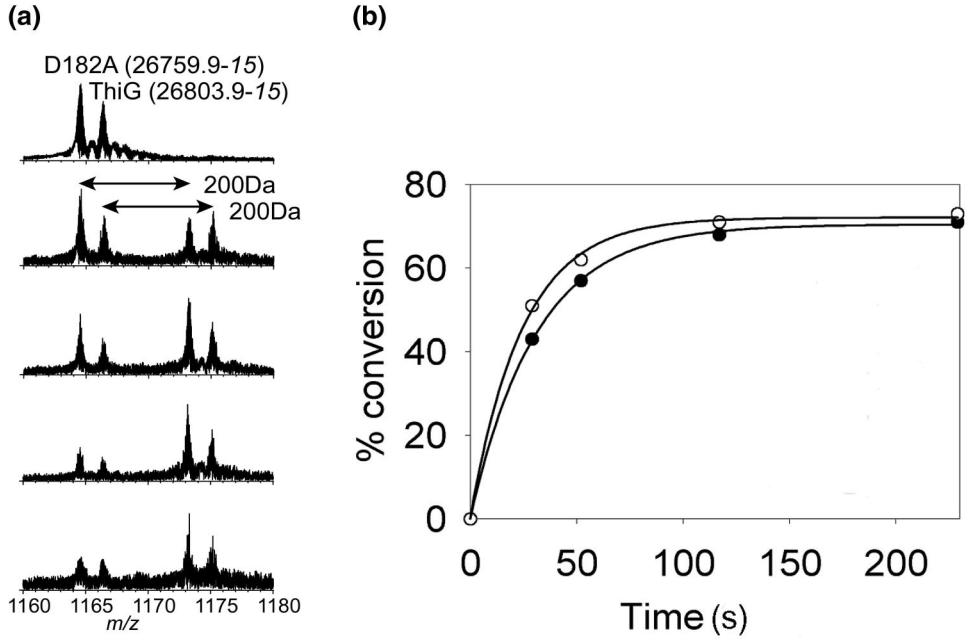

Figure 5. (**a**) ESI-FTMS spectra and (**b**) plots of the simultaneous time dependent formation of imine adduct **D** from wild type ThiG (open circles) and its D182A mutant (filled circles).

age would, in the same way, give molecular ions with isotopic peak distributions at lower mass. The complexity of the resulting isotopic peak distribution can be greatly reduced, as well as signal intensity improved, by preparing the sample with its heavy isotopes depleted [22]. Figure 7 illustrates the change in isotopic distribution for ThiS grown from ^{13}C and ^{15}N depleted media, in which the monoisotopic peak has become the most abundant of the molecular ions. This could provide relatively accurate determination of intermediates whose M_r values differ by only 2 Da [23].

Other possible systems amenable to this top down ESI-FTMS kinetic approach include phosphorylation and acylations of histones [24], thioester formation of polyketide and non-ribosomal peptide biosynthetic systems [25, 26], disulfide bond formation during protein folding [27], and adenylation and sulfurylation of the sulfur carrier proteins such as the ones found in thiazole-phosphate biosynthesis [28]. McLoughlin and Kelleher have just recently done a similar FTMS kinetic study that simultaneously follows seven covalent modifications to the PCP1 active site peptide Val^{1396}-Met^{1580} (M_r = 20705) from apo-HMWP2 of Yersiniabactin biosynthesis [29].

Conclusions

Top down ESI/FTMS has been able to compare directly, under identical experimental conditions, the reaction kinetics of a native enzyme and its mutants. Further, the

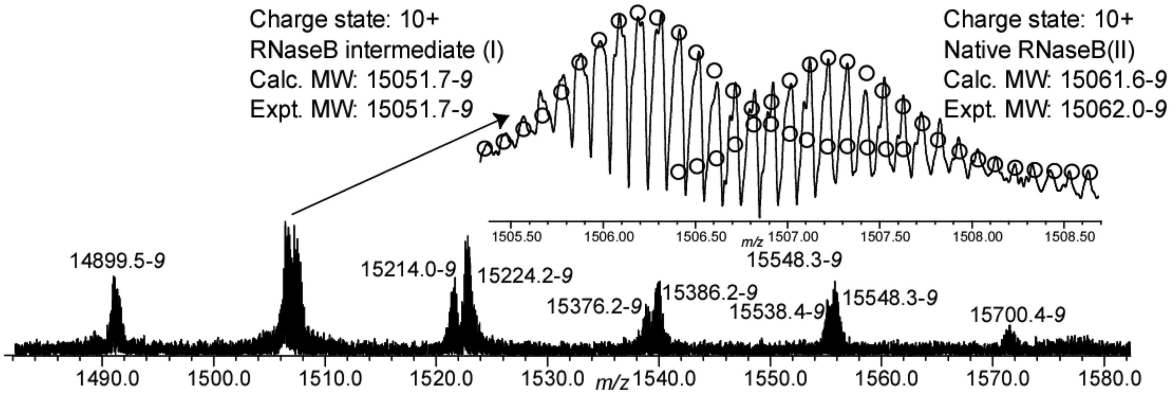

Figure 6. Partial ESI/FTMS spectra of the reductive unfolding mixture of RNase B, showing the overlapping peaks of the five isoforms (differing in the number of mannose molecule, ΔM_r = 162.2) of totally reduced RNase B and their three-disulfide-containing reductive intermediates. Dots on the expanded portions represent the theoretical abundance distribution of the isotopic peaks.

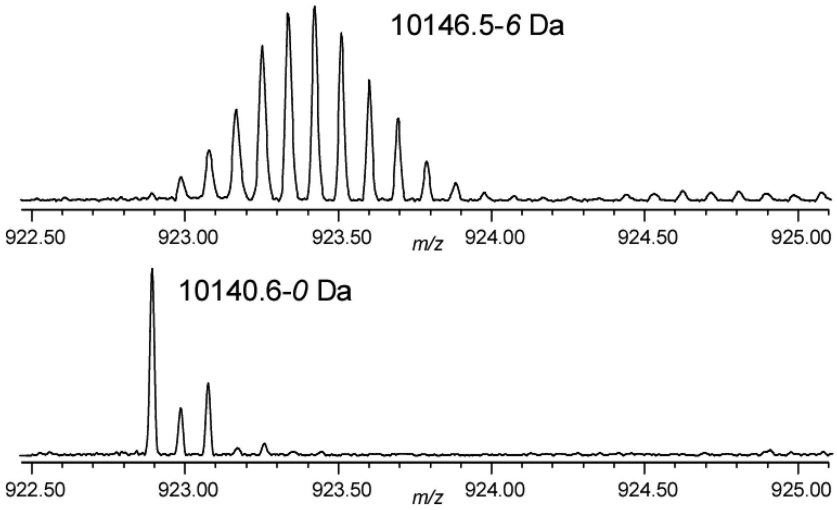

Figure 7. ESI/FTMS spectra of (**a**) normal ThiS protein and (**b**) ThiS grown in ^{13}C and ^{15}N depleted media.

isotopic resolution and MS/MS capabilities demonstrate a potential for ready extension to other systems of far greater complexity. The chief limitation of conventional methods for kinetic studies of proteins and other large biological molecules is the lack of highly specific probes that do not affect reactivity. With MS, the thousands of possible M_r values can serve as just such probes. Mutants or isoforms of an enzyme, and their reaction products, will give different M_r values. Small differences (e.g., 2 Da) in M_r values give lower accuracy because of overlapping isotopic distributions, but biosynthesis of the protein with depleted heavy isotopes alleviates this problem. Even constituents with the same M_r value, such as the derivatized RNase with two different S—S bonds reduced, can be monitored separately by MS/MS. Mutants and reaction products often have similar ionization efficiencies, making standardization unnecessary for relative comparisons. This capability to measure directly the kinetics of individual protein reactions in complex mixtures should find many valuable applications in biomedical research and drug development.

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The authors dedicate this study of protein kinetics in honor of Michael Bowers. His highly deserved ASMS Distinguished Contribution Award is for "his fundamental contribution to ion-neutral collision theory." Although the high level of physical chemistry and theory that made this landmark achievement possible can only be fully appreciated by true experts in that specialty, it has benefited all of us in mass spectrometry by providing a far more solid mechanistic foundation for our field.

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