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Analysis of intact proteins on a chromatographic time scale by electron transfer dissociation tandem mass spectrometry

An Chi¹, Dina L. Bai¹, Lewis Y. Geer², Jeffrey Shabanowitz¹, and Donald F. Hunt^{1,3}

¹Department of Chemistry, University of Virginia, Charlottesville, VA 22904 USA

²National Center for Biotechnology Information, National Library of Medicine, NIH, Bethesda, MD 20894 USA

³Department of Pathology, Health Sciences Center, University of Virginia, Charlottesville, VA 22908 USA

Abstract

Direct analysis of intact proteins on a chromatographic time scale is demonstrated on a modified linear ion trap mass spectrometer using sequential ion/ion reactions, electron transfer and proton transfer, to dissociate the sample and to convert the resulting peptide fragments to a mixture of singly and doubly charged species. Proteins are converted to gas-phase, multiply-charged, positive ions by electrospray ionization and then allowed to react with fluoranthene radical anions. Electron transfer to the multiply charged protein promotes random fragmentation of amide bonds along the protein backbone. Multiply charged fragment ions are then deprotonated in a second ion/ion reaction with even-electron benzoate anions. *M/z* values for the resulting singly and doubly charged ions are used to read a sequence of 15–40 amino acids at both the N-terminus and the C-terminus of the protein. This information, along with the measured mass of the intact protein, are employed to identify known proteins and to detect the presence of post-translational modifications. In this study, we analyze intact proteins from the *Escherichia coli* 70S ribosomal protein complex and identify 46 of the 55 known unique components in a single, 90 min, on-line, chromatography experiment. Truncated versions of the above proteins along with several post-translational modifications are also detected.

1. Introduction

In an earlier paper [1], we described modifications to a Finnigan LTQ mass spectrometer that make it possible to use ion/ion chemistry, electron transfer dissociation (ETD), to fragment and sequence peptides, particularly those with labile post-translational modifications (PTMs). Multiply protonated peptides are injected at one end of the instrument and allowed to react with fluoranthene radical anions generated at the opposite

end of the instrument. Transfer of one or more electrons from the fluoranthene radical anions to the multiply charged peptide promotes fragmentation along the peptide backbone and generates a series of ion of type c and z (Figure 1). Subtraction of the *m/z* values for the fragments within a given ion series that differ by a single amino acid affords the mass and thus the identity of the extra residue in the larger of the two fragments. The complete amino acid sequence of a peptide is deduced by extending this process to all homologous pairs of fragments within a particular ion series.

*Corresponding author: Jeffrey Shabanowitz University of Virginia Department of Chemistry McCormick Road Charlottesville VA 22904-4319 USA Tel. USA 434 924 7994; Fax USA 434 982 2781 e-mail js4c@virginia.edu

ETD also proceeds with high efficiency on intact proteins but generates highly charged fragments that are difficult or impossible to resolve on the benchtop LTQ mass spectrometer. Methodology involving sequential ion/ion reactions has been implemented to solve this problem [2]. Following the electron transfer reaction, fluoranthene radical anions are removed and replaced by even-electron, benzoate anions. These anions remove protons from the multiply charged fragment ions and, thus, reduce their charge. The result of this second ion/ion reaction, PTR, (proton transfer/charge reduction) is a simplified spectrum that is enriched in singly charged fragments.

Here we apply the above technology to the analysis of intact proteins that constitute the E. coli ribosome. Two subunits make up this 2.3 million Da particle [3,4]. The small 30S, or S subunit, contains 22 proteins involved in mRNA binding. The 50S, or L subunit, contains 35 proteins, binds to tRNA and mediates peptidyl transfer. In a single automated LCMS experiment, we identify 46 of the 55 known unique proteins and detect a number of post-translational modifications. Most importantly, we show that the time scale for recording ETD/PTR-MS/MS spectra on intact proteins is identical to that currently employed for acquiring collision activated dissociation spectra on mixtures of tryptic peptides.

2. Experimental

2.1 Materials

All salts, buffers, iodoacetamide, glacial acetic acid, fluoranthene, and benzoic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dithiothreitol (DTT, electrophoresis grade) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). HPLC grade acetonitrile from Mallinckrodt, Inc. (Phillipsburg, NJ) and NANOPure water from Barnstead (Dubuque, IA) were used for all LC-MS analysis. Fused silica tubing (360 μm , od) was purchased from Polymicro Technologies (Phoenix, AZ). YMC C₈ ODS-AQ (5 μm , 300A $^\circ$) reverse phase packing material was obtained from Waters Corp. (Milford, MA).

2.2 Sample Preparation

Ribosomal proteins from E. coli were purified by sucrose, density-gradient fractionation as described previously [5]. The resulting sample (~100 pmol) was reduced with 1 mM DTT at 51°C for 1 h, carboxyamidomethylated with 2 mM iodoacetamide in the dark at room temperature for 45 min, and then brought to pH-3-4 with glacial acetic acid.

2.3. Chromatography

An aliquot of reduced, alkylated and acidified E. coli ribosomal proteins (~5 pmol) was pressure loaded onto a self-assembled, reverse-phase, pre-column (360 μm od $\times$ 75 μm id) containing 4 cm of C₈ beads. After washing the sample with 0.1% acetic acid to remove salts, the pre-column was connected via a Teflon sleeve to an analytical column (360 μm od $\times$ 50 μm id) packed with 10 cm of C₈ beads and equipped with an integrated electrospray ionization emitter [6]. Online protein separations were performed on an Agilent 1100 series binary HPLC system (Palo Alto, CA, USA) interfaced with a modified LTQ mass spectrometer (Thermo Electron Corp., San Jose, CA, USA). Proteins were eluted from the reverse-phase C₈ column to the mass spectrometer at a flow rate of 60 nl/min using a linear gradient of 0-60% B in 75 min, 60%-100% in 10 min, 100%B for 2 min, 100%-0%B in 3 min (A = 0.1M acetic acid, B = 70% acetonitrile in 0.1M acetic acid).

2.4 Instrument modification and operation

All experiments were performed on a modified LTQ mass spectrometer. Modifications to the LTQ that make it possible to use gas phase ion/ion chemistry for the identification of proteins have been described previously [1,2,7,8]. Positive ions from protein samples are generated in a micro-electrospray ionization source placed at the front of the instrument. Negative ion reagents for electron transfer dissociation (ETD) (fluoranthene radical anion) and proton transfer/charge reduction (PTR) (benzoate anion) are generated in a Finnigan 4500 chemical ionization source [9] placed at the rear of the instrument. For on-line chromatography experiments, the instrument was operated in the data-dependent mode and cycled through acquisition of a full MS spectrum followed by ETD/PTR-MS/MS spectra on the 6 most abundant ions recorded in the initial full MS spectra every <5 s (mass window = 3 Da; dynamic exclusion = 45 s; repeat count = 1). Full scan mass spectra were acquired over m/z range = 300–2000. Instrument control software was modified to accommodate the following sequence of events after precursor-ion selection and storage as described previously [2], but with the following modifications: (1) anion injection (~ 2 ms); (2) fluoranthene anion isolation (m/z 202, ~10 ms); (3) reaction of the fluoranthene radical anion with the precursor cation (10~45 ms); (4) removal of excess fluoranthene anions and storage of ETD products; (5) injection of benzoate anions (~2 ms); (6) application of selective waveform to remove m/z 202 and other background anion species (~5 ms); (7) reaction of purified benzoate anions (m/z 121) with ETD product ions (0~150 ms); (8) removal of excess benzoate anions and mass analysis of product ions.

2.5 Data analysis

MS/MS spectra acquired on intact proteins were searched against the *E. coli* database (National Center for Biotechnology Information, <ftp://ftp.ncbi.nih.gov/genbank/>) using OMSSA software (1.0.5), <http://pubchem.ncbi.nlm.nih.gov/omssa> [10]. Search parameters were set to consider a static modification of + 57 Da on Cys and differential modifications of: +16 on Met for possible oxidation, +14 on Lys or the protein N-terminus for possible methylation, + 42 on the protein N-terminus or Lys for possible acetylation/trimethylation, +80 on Ser for possible phosphorylation, and –131 for the deletion of Met from the N-terminus of the gene sequence. Fragment ion mass tolerance was set as “exact mass” for considering monoisotopic masses of both C_{12} and C_{13} (mass window ± 0.5 Da). Protein sequence assignments in Table 1 were all validated by manual verification of OMSSA results or manual interpretation of the corresponding ETD/PTR mass spectra.

3. Results and Discussion

Shown in Figure 2A is the base peak chromatogram from a 90 min, automated, online LCMS experiment to identify 70S ribosomal proteins in a mixture of intact proteins obtained by sucrose gradient fractionation of *E. coli* bacteria. Analysis of the intact proteins was performed with a LTQ mass spectrometer modified for gas phase ion/ion chemistry. The LTQ was operated in the data dependent mode and cycled through acquisition of a full mass spectrum and ETD/PTR-MS/MS spectra on the six most abundant ions every 3 sec. Throughout the automated LCMS experiment, the ion-ion reaction times for ETD and PTR were set for 35 and 135 msec, respectively. Under these conditions, the resulting spectra are dominated by singly charged ions.

Displayed in Figure 2B, is the ESI spectrum recorded on Peak I in the total ion chromatogram. Signals in the observed charge envelope carry 8–13 positive charges and correspond to a protein having an average molecular weight of 5382. The ETD/PTRMS/MS spectrum recorded on the $(M+11H)^{11+}$ ion (m/z 490.3) in this cluster is shown in Figure 2C. Ions of type c and z in the spectra are labeled as such and define the first 15 and last 17 amino acids in the 50S ribosomal

protein L34. The observed sequence coverage for this protein is shown above and below the structure in Figure 2C. Because the experimental and calculated average molecular weights for this protein are in agreement (5382 and 5381, respectively), we conclude that protein L34 in Peak I is not post-translationally modified.

Three minutes later in the chromatogram (Peak II), the instrument records an ETD/PTR – MS/MS spectrum (Figure 2D) on another $(M+11H)^{11+}$ ion (m/z 492.9). Ions of type z in this spectrum occur at m/z values that are identical to those in Figure 2C. This result suggests that the protein in Peak II is a modified form of the 50S ribosomal protein, L34. All ions of type c ($c_2 - c_{15}$) in Figure 2D occur at m/z values that are 28 Da higher than those in Figure 2C. We conclude that this version of the L34 protein contains an N-terminus and Lys2 side chain that are both monomethylated or an N-terminus or Lys2 side chain that is either formylated or dimethylated. From the observed ion currents, we estimate that modified and unmodified versions of the L34 protein are present in a ratio of 1/20.

Also detected in the analysis of the *E. coli* sample were a number of protein truncations. One example is shown in Figure 3. Spectra in panels A and B were generated from two proteins that elute 6 min apart in the chromatogram. For the early eluting protein, an ETD/PTR-MS/MS spectrum was recorded on the $(M+7H)^{7+}$ ion (m/z 476.0). This corresponds to a protein MW_{Exp} of 3325. For the late eluting protein, an ETD/PTRMS/MS spectrum was recorded on the $(M+12H)^{12+}$ ion (m/z 527.3). This corresponds to a protein MW_{Exp} of 6316. Both spectra contain ions of type z ($z_3 - z_{17}$) that occur at identical m/z values and define an amino acid sequence that matches the last 17 amino acids in the 50S ribosomal subunit, L32. Ions of type c in Figure 3B define the first 17 amino acids of L32 and indicate that the N-terminal methionine residue in the gene sequence has been deleted. Ions of type c ($c_3 - c_{17}$) in Figure 3A define a N-terminal sequence that begins with Ser29 in the gene sequence for L32. We conclude that this truncated form of L32 is missing the N-terminal methionine plus another 27 amino acids. It is noteworthy that these two forms of this protein differ in abundance by a factor of 1000.

Additional data from the present study on *E. coli* 70S ribosomal proteins is presented in Table 1. Detected proteins are listed in columns 1 and 2 by accession numbers and common names, respectively. Calculated average molecular weights for these entries, with and without the N-terminal methionine residue from the gene sequence, but including 57 Da per Cys residue (carboxyamidomethylation), are shown in columns 3 and 4. The experimental MW determined from the full scan ESI mass spectrum is reported in column 5. Detected post-translational modifications and/or truncations are reported in column 6. Columns 7 and 8 show the number of amino acid residues at the N- and C-termini of the protein that are defined by observed ions of type c and z , respectively.

ETD/PTR-MS/MS spectra recorded on ions from the forty-six proteins in Table 1, all contain a series of ions of type c or z that define at least the first or last 12-20 amino acids in the precursor molecule. This alone makes it possible to identify the protein from the *E. coli* database. For 39 of the 46 proteins, the experimental average molecular weights determined from the full scan ESI spectra either match those calculated for the database sequence, with or without the N-terminal methionine residue, or differ by amounts that can easily be explained by the presence of simple post-translational modifications. These, in turn, are all localized to specific residues at the N- or C-terminus by noting the observed mass shifts in the corresponding c or z ions. Four of the 46 proteins (S12, L1, L13, L16) appear to contain post-translational modifications, as the measured and calculated average molecule masses for these proteins do not match. Unfortunately none of the fragment ions of type c and z below m/z 2000 undergo a mass shift that would specify the type and location of the modification. Since the upper mass limit of the LTQ is presently 2000, we are unable to fully characterize these proteins. Finally, three of the 46 proteins (S3, S10, L3), despite being identified by a series of

c or z ions, were of too low abundance to allow for molecular weight determination. As a result, we cannot comment on the possibility that these proteins might be post-translationally modified.

Of all the different forms found for the 46 proteins identified Table 1, fourteen retain their N-terminal methionine residue, thirty are missing their N-terminal methionine, ten are detected in truncated forms, three (S11, L16 and L33) are monomethylated on either the N-terminus or Lys2, two (S5, S18) are acetylated/trimethylated on the N-terminus, one (L34) is either monomethylated on both the N-terminus and side chain of Lys2, or dimethylated/formylated on either the N-terminus or side chain of Lys2, and one (S6) is mono, di, and tri-glutamylated. Some modifications that have been previously characterized or detected include: (a) glutamylation of S6 at or near the C-terminus [11], monomethylation on the N-terminus of L16 [12,13] and L33 [13,14], acetylation on the N-terminus of S5 and S18 [15], thiomethylation of Asp88 in S12 [16], and methylation of Lisoaspartate in S11 [17].

The E. coli 70 ribosomal protein complex contains 55 unique proteins. We identify the 46 shown in Table 1 in a single 90 min LCMS experiment using a C8 column and a total of 5 pmols sample (~100 fmol for each protein). Three of the remaining 9 proteins, S2, S8, and L14, can be found by targeted analysis and appear as broad peaks at the very end of the gradient. We have no evidence for the presence of the remaining 6 proteins, S1, L4, L9, L11, L15, and L21. Possible explanations could be chromatographic or modifications to both the N- and C-termini obfuscating identification.

4. Conclusions

Sequential ion/ion reactions, ETD and PTR, in combination with a data dependent LCMS experiment, have been employed to record MS/MS spectra on intact proteins from the E. coli 70S ribosomal particle. A full ESI spectrum is employed to define the average molecular weight of each eluting protein. Then, ETD/PTR-MS/MS spectra are recorded in the data dependent mode to provide sequence information at the N- and/or C-termini of the protein. This allows the protein to be identified in the E. coli database. We find 42 examples where the calculated and experimental average molecular weights of the proteins disagree and assign the differences to: deletion of the N-terminal methionine in the gene sequence, truncations at either the N- or C-terminus of the protein, and post-translational modifications such as methylation, dimethylation/formylation, trimethylation/acetylation, and glutamylation. Use of sequential ion/ion chemistry on the benchtop LTQ mass spectrometer makes it possible to analyze mixtures of intact proteins on a chromatographic time scale that is identical to that used for acquiring collision-activated-dissociation, MS/MS spectra on a mixture of tryptic peptides.

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Abbreviations

ETD, electron transfer dissociation; PTR, proton transfer charge reduction.

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Figure 1.

Fragmentation scheme for production of ions of type c and z after a multiply charged peptide receives an electron from the radical anion of fluoranthene.

Figure 2.

Analysis of *E. coli*, 70S ribosomal proteins by LC-ESI-ETD/PTR tandem mass spectrometry. (A) Base peak ion chromatograph observed for the fractionation of *E. coli* ribosomal proteins by C8 HPLC. (B) Single scan ESI mass spectrum recorded on the protein eluting under peak I in Panel A. (C) Single scan ETD/PTR-MS/MS spectrum of the protein eluting under peak I in Panel A. Observed ions of type c and z define sequences at the N- and C-termini, respectively, that matches that of 50S ribosomal protein L34. Lines above and below the protein sequence indicate the amino acid sequence coverage defined by ions in the spectrum. (D) ETD/PTR-MS/MS spectrum of a protein (peak II) eluting ~3 min after peak I, in Panel A. Spectra in Panel C and D contain an identical set of ions of type z. Ions of type c in the two spectra differ in mass by 28 Da. This suggests that the L34 isoform in panel D is either monomethylated on both the N-terminus and side chain of Lys2, or dimethylated/formylated on either the N-terminus or side chain of Lys2

Figure 3. ETD/PTR-MS/MS spectra recorded on the *E. coli* 70S ribosomal protein, L32. Spectra in Panels (A) and (B) correspond to truncated and intact forms, respectively, that elute ~ 6 min apart in the chromatogram. Lines above and below the sequences represent the coverage defined by ions of type c and z. Note that the z- ions in the two spectra occur at identical m/z values. Ions of type c in the two spectra are completely different and indicate that the truncated fragment is missing the first 29 residues of the intact protein.

E. coli, 70S ribosomal proteins detected by LC-ESI-ETD/PTR tandem mass spectrometry

Table 1

Accession numbers ^a	Protein subunits ^b	MW ^c Calc.	MW - Met ^d Calc.	MW ^e Exp.	Post-translational modifications ^f	Number of amino acid residues identified ^g	
						e- ions	z- ions
P02352	S3	25983	25852	nd	na	nd	16
P02354	S4	23526	23395	3392	Truncated N-terminus, -Met	17	18
P02356	S5	17604	17472	17514	+42 Da on N-terminus, -Met	17	nd
P02358	S6	15187	15056	15184	None	15	nd
				15316	+ (n)29 Da ^h	15	nd
P02359	S7	20019	19888	17475	Truncated N-terminus, -Met	16	15
P02363	S9	14856	14725	14727	-Met	15	15
P02364	S10	11736	11604	nd	na	14	nd
P02366	S11	13959	13828	13840	+14 Da on the N-terminus/Lys2, -Met	17	nd
P02367	S12	13965	13834	13939	-Met, ns	17	16
P02369	S13	13157	13025	13026	-Met	18	17
P02370	S14	11638	11506	11507	-Met	17	18
				4357	Truncated N-terminus, -Met	12	12
				1532	Truncated C-terminus	5	18
P02371	S15	10269	10138	10138	-Met	nd	16
P02372	S16	9191	9059	9193	None	16	19
P02373	S17	9819	9687	9688	-Met	17	17
				9822	None	17	17
P02374	S18	9044	8912	8957	+42 Da at the N-terminus, -Met	14	15
P02375	S19	10430	10299	10301	-Met	17	16
P02378	S20/L26	9684	9553	9555	-Met	17	18
P02379	S21	8557	8426	8428	-Met	16	16
P28690	S22	5096	4965	5097	None	16	18
P02384	L1	24730	24599	15144	Truncated N-terminus, -Met, ns	16	nd
P60422	L2	29918	29786	2975	Truncated C-terminus	15	15
P60438	L3	22244	22112	nd	na	17	nd
P02389	L5	20359	20228	20230	-Met	13	nd
P02390	L6	18961	18830	18828	-Met	20	17
P02392	L7/L12	12295	12164	12165	-Met	16	nd
				12206	ns	nd	nd
				12296	None	nd	nd
P02408	L10	17769	17638	17636	-Met	18	20
				1372	Truncated C-terminus	12	13
P02410	L13	16019	15887	16025	ns	14	15
P02414	L16	15281	15150	15310	+14 Da on N-terminus, ns	14	nd
				3107	Truncated C-terminus	17	18
P02416	L17	14422	14291	14422	None	15	nd
P02419	L18	12770	12639	12769	None	16	18
				1816	Truncated N-terminus	15	14
P02420	L19	13133	13002	13004	-Met	15	16
P02421	L20	13497	13366	13367	-Met	15	nd
P61175	L22	12226	12095	12228	None	17	17
P02424	L23	11199	11068	11203	None	16	18
P60624	L24	11316	11185	11185	-Met	17	17
P02426	L25	10693	10562	10694	None	17	17
P02427	L27	9182	9050	9053	-Met	18	17
P02428	L28	9064	8932	8936	-Met	17	17
P02429	L29	7273	7142	7274	None	16	18
P02430	L30	6542	6411	6412	-Met	18	17

Accession numbers ^a	Protein subunits ^b	MW ^c Calc.	MW - Met ^d Calc.	MW ^e Exp.	Post-translational modifications ^f	Number of amino acid residues identified ^g	
						c- ions	z- ions
P02432	L31	8099	7968	8100	None	16	17
				7986	ns	16	17
P02435	L32	6446	6315	8075	ns	16	17
				6316	-Met	17	17
P02436	L33	6372	6240	3325	Truncated C-terminus	17	17
P02437	L34	5380	5249	6254	+ 14 Da on N-terminus/Lys2, -Met	18	16
				5382	None	16	17
P07085	L35	7346	7215	5410	+28 Da on N-terminus/Lys2	16	17
P21194	L36	4536	4404	7214	-Met	16	18
				4537	None	16	17

^a Accession numbers of E.coli proteins from the non-redundant database (nr) (NCBI, ftp://ftp.ncbi.nih.gov/genbank/).

^b Common names of 70S E. coli ribosomal protein subunits.

^c Calculated MW (average mass) based on protein sequences obtained from the nr database.

^d Calculated MW (average mass) based on protein sequences obtained from the nr database after deletion of N-terminal Met.

^e Experimental MW (average mass) calculated from the ESI spectrum of the intact protein: nd, required ions not detected.

^f Post-translational modifications suggested from the mass differences observed between the calculated and experimentally determined MW values: na, not applicable; - Met, deletion of N-terminal Met; None, calculated and experimental MW's agree; ns, modification detected but not specified.

^g Number of amino acid residues identified from N- and C-terminus of the protein using ETD 45ms/PTR 135ms reaction time: nd, not detected.

^h Addition of (n)129Da observed where n= 1, 2, 3.