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

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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.
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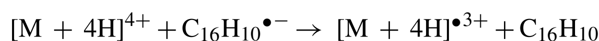
Keywords: ETD; PTR

1. Introduction

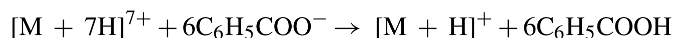
In an earlier paper [1], we described modifications to a Finnigan LTQ mass spectrometer that make it possible to use ion/ion chemistry, electron transfer dissociation (ETD), to fragment and sequence peptides, particularly those with labile post-translational modifications (PTMs). Multiply protonated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



$$[M + 4H]^{4+} + C_{16}H_{10}^{\bullet-} \rightarrow [M + 4H]^{\bullet 3+} + C_{16}H_{10}$$

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 types c and z (Fig. 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.

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, proton transfer/charge reduction (PTR) is a simplified spectrum that is enriched in singly charged fragments.



$$[M + 7H]^{7+} + 6C_6H_5COO^- \rightarrow [M + H]^+ + 6C_6H_5COOH$$

Abbreviations: ETD, electron transfer dissociation; PTR, proton transfer charge reduction

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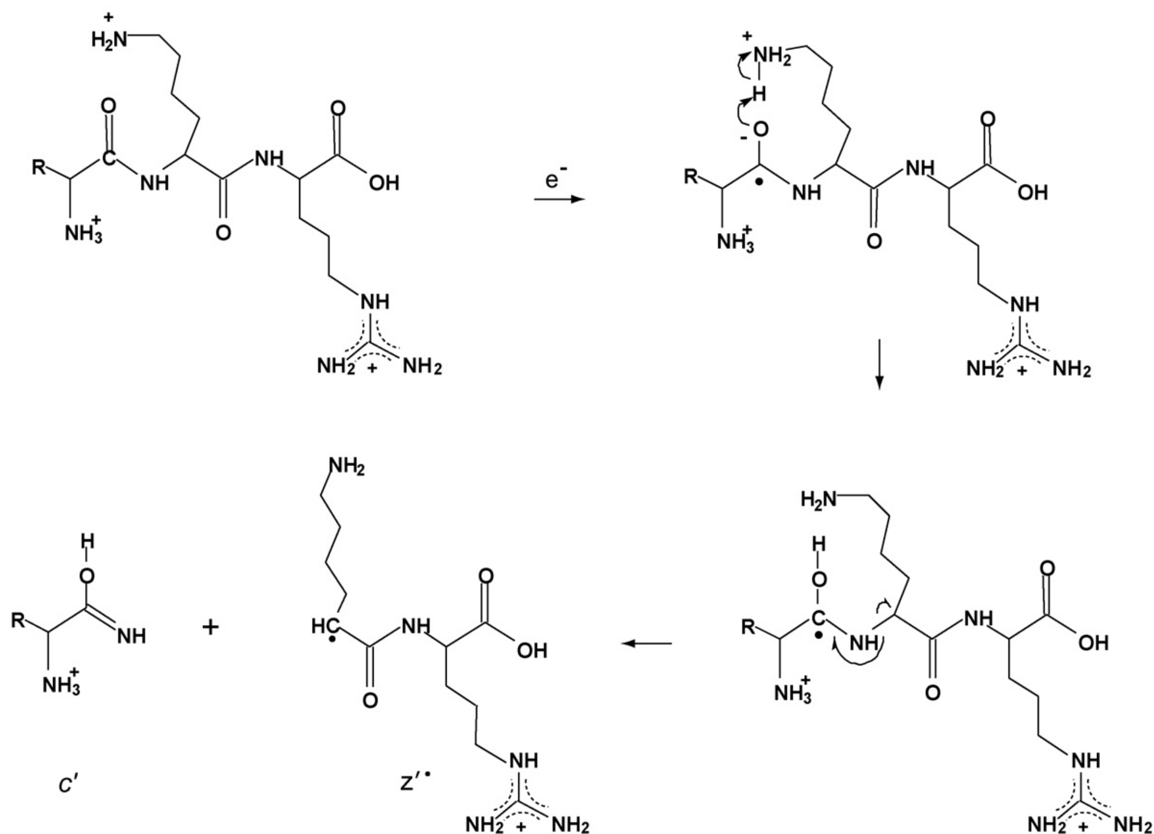

Fig. 1. Fragmentation scheme for production of ions of types c and z after a multiply charged peptide receives an electron from the radical anion of fluoranthene.

Here we apply the above technology to the analysis of intact proteins that constitute the *Escherchia 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 LC–MS 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, o.d.) was purchased from Polymicro Technologies (Phoenix, AZ). YMC C_8 ODS-AQ (5 μm, 300 Å) 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 o.d. × 75 μm i.d.) containing 4 cm of C_8 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 o.d. × 50 μm i.d.) packed with 10 cm of C_8 beads and equipped with an integrated electrospray ionization emitter [6]. On-line 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 C_8 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 and 100–0% B in 3 min (A: 0.1 M acetic acid, and B: 70% acetonitrile in 0.1 M 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 (fluoranthene radical anion) and proton transfer/charge reduction (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-depended mode and cycled through acquisition of a full MS spectrum followed by ETD/PTR-MS/MS spectra on the six 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 Fig. 2A is the base peak chromatogram from a 90 min, automated, on-line LC–MS 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 s. Throughout the automated LC–MS experiment, the ion–ion reaction times for ETD and PTR were set for 35 and 135 ms, respectively. Under these conditions, the resulting spectra are dominated by singly charged ions.

Displayed in Fig. 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/PTR-MS/MS spectrum recorded on the $(M+11H)^{11+}$ ion (*m/z* 490.3) in this cluster is shown in Fig. 2C. Ions of types 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 Fig. 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 (Fig. 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 Fig. 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 Fig. 2D occur at *m/z* values that are 28 Da higher then those in Fig. 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 Fig. 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/PTR-MS/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 Fig. 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 Fig. 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 miss-

Table 1
E. coli, 70S ribosomal proteins detected by LC-ESI-ETD/PTR tandem mass spectrometry

Accession numbers[a]	Protein subunits[b]	MW_{Calc}[c]	$MW - Met_{Calc}$[d]	MW_{Exp}[e]	Post-translational modifications[f]	Number of amino acid residues identified[g]	
						c ions	z ions
P02352	S3	25,983	25,852	nd	na	nd	16
P02354	S4	23,526	23,395	3392	Truncated N-terminus, -Met	17	18
P02356	S5	17,604	17,472	17,514	+42 Da on N-terminus, -Met	17	nd
P02358	S6	15,187	15,056	15,184	None	15	nd
				15,316	+ (n)129 Da[h]	15	nd
P02359	S7	20,019	19,888	17,475	Truncated N-terminus, -Met	16	15
P02363	S9	14,856	14,725	14,727	-Met	15	15
P02364	S10	11,736	11,604	nd	na	14	nd
P02366	S11	13,959	13,828	13,840	+14 Da on the N-terminus/Lys2, -Met	17	nd
P02367	S12	13,965	13,834	13,939	-Met, ns	17	16
P02369	S13	13,157	13,025	13,026	-Met	18	17
P02370	S14	11,638	11,506	11,507	-Met	17	18
				4357	Truncated N-terminus, -Met	12	12
				1532	Truncated C-terminus	5	18
P02371	S15	10,269	10,138	10,138	-Met	nd	16
P02372	S16	9,191	9,059	9193	None	16	19
P02373	S17	9,819	9,687	9688	-Met	17	17
				9822	None	17	17
P02374	S18	9,044	8,912	8957	+42 Da at the N-terminus, -Met	14	15
P02375	S19	10,430	10,299	10,301	-Met	17	16
P02378	S20/L26	9,684	9,553	9555	-Met	17	18
P02379	S21	8,557	8,426	8428	-Met	16	16
P28690	S22	5,096	4,965	5097	None	16	18
P02384	L1	24,730	24,599	15,144	Truncated N-terminus, -Met, ns	16	nd
P60422	L2	29,918	29,786	2975	Truncated C-terminus	15	15
P60438	L3	22,244	22,112	nd	na	17	nd
P02389	L5	20,359	20,228	20,230	-Met	13	nd
P02390	L6	18,961	18,830	18,828	-Met	20	17
P02392	L7/L12	12,295	12,164	12,165	-Met	16	nd
				12,206	ns	nd	nd
				12,296	None	nd	nd
P02408	L10	17,769	17,638	17,636	-Met	18	20
				1372	Truncated C-terminus	12	13
P02410	L13	16,019	15,887	16,025	ns	14	15
P02414	L16	15,281	15,150	15,310	+14 Da on N-terminus, ns	14	nd
				3107	Truncated C-terminus	17	18
P02416	L17	14,422	14,291	14,422	None	15	nd
P02419	L18	12,770	12,639	12,769	None	16	18
				1816	Truncated N-terminus	15	14
P02420	L19	13,133	13,002	13,004	-Met	15	16
P02421	L20	13,497	13,366	13,367	-Met	15	nd
P61175	L22	12,226	12,095	12,228	None	17	17
P02424	L23	11,199	11,068	11,203	None	16	18
P60624	L24	11,316	11,185	11,185	-Met	17	17
P02426	L25	10,693	10,562	10,694	None	17	17
P02427	L27	9,182	9,050	9053	-Met	18	17
P02428	L28	9,064	8,932	8936	-Met	17	17
P02429	L29	7,273	7,142	7274	None	16	18
P02430	L30	6,542	6,411	6412	-Met	18	17
P02432	L31	8,099	7,968	8100	None	16	17
				7986	ns	16	17
				8075	ns	16	17
P02435	L32	6,446	6,315	6316	-Met	17	17
				3325	Truncated C-terminus	17	17
P02436	L33	6,372	6,240	6254	+14 Da on N-terminus/Lys2, -Met	18	16
P02437	L34	5,380	5,249	5382	None	16	17
				5410	+28 Da on N-terminus/Lys2	16	17
P07085	L35	7,346	7,215	7214	-Met	16	18
P21194	L36	4,536	4,404	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 45 ms/PTR 135 ms reaction time: nd, not detected.
[h] Addition of (n)129 Da observed where $n = 1, 2, 3$.

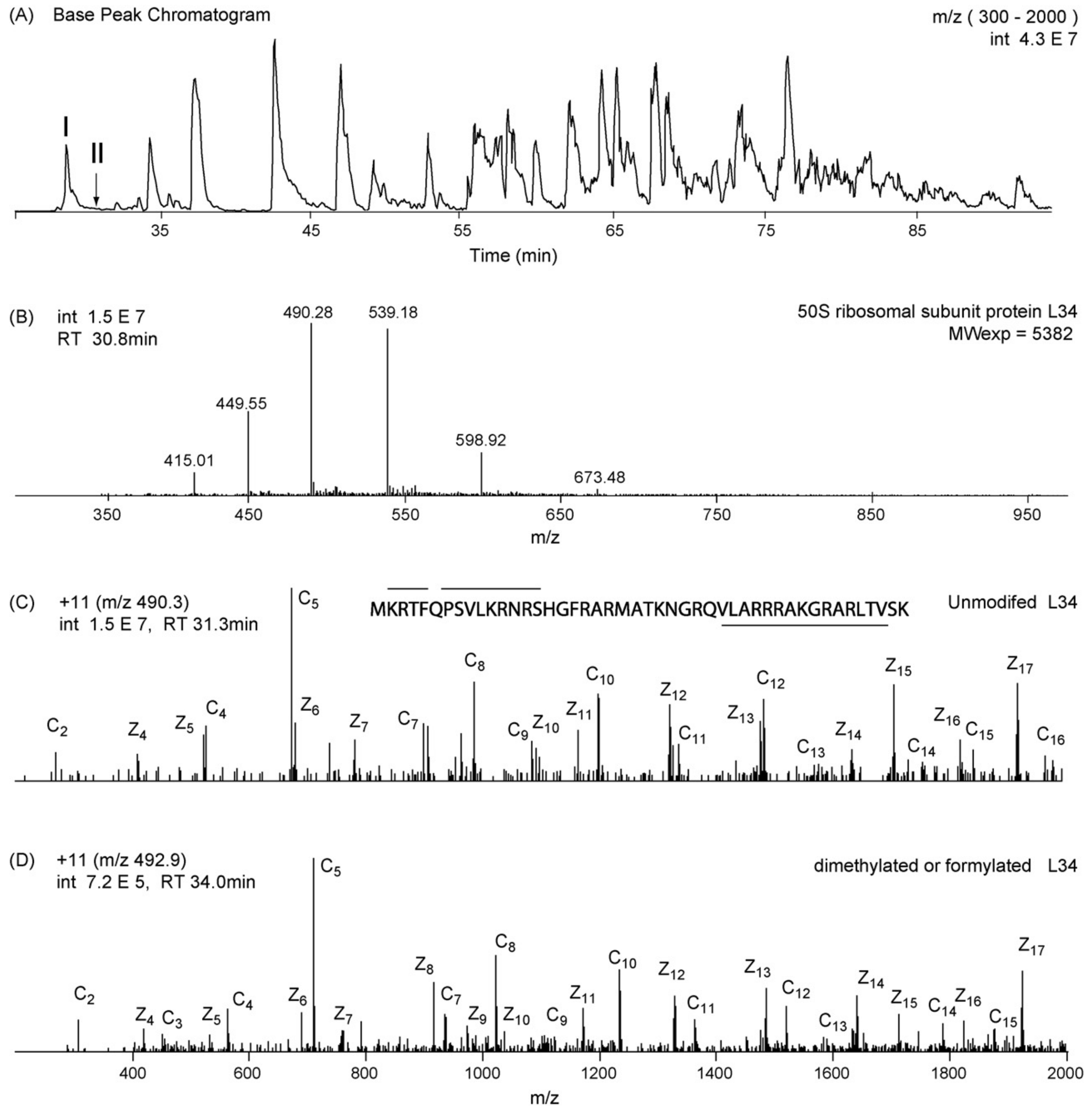

Fig. 2. Analysis of *E. coli*, 70S ribosomal proteins by LC-ESI-ETD/PTR tandem mass spectrometry. (A) Base peak ion chromatograph observed for the fractionation of *E. coli* ribosomal proteins by C_8 HPLC. (B) Single scan ESI mass spectrum recorded on the protein eluting under peak I in panel (A). (C) Single scan ETD/PTR-MS/MS spectrum of the protein eluting under peak I in panel (A). Observed ions of types 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 panels (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.

ing 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 types c and z, respectively.

ETD/PTR-MS/MS spectra recorded on ions from the 46 proteins in Table 1, all contain a series of ions of type c or z that

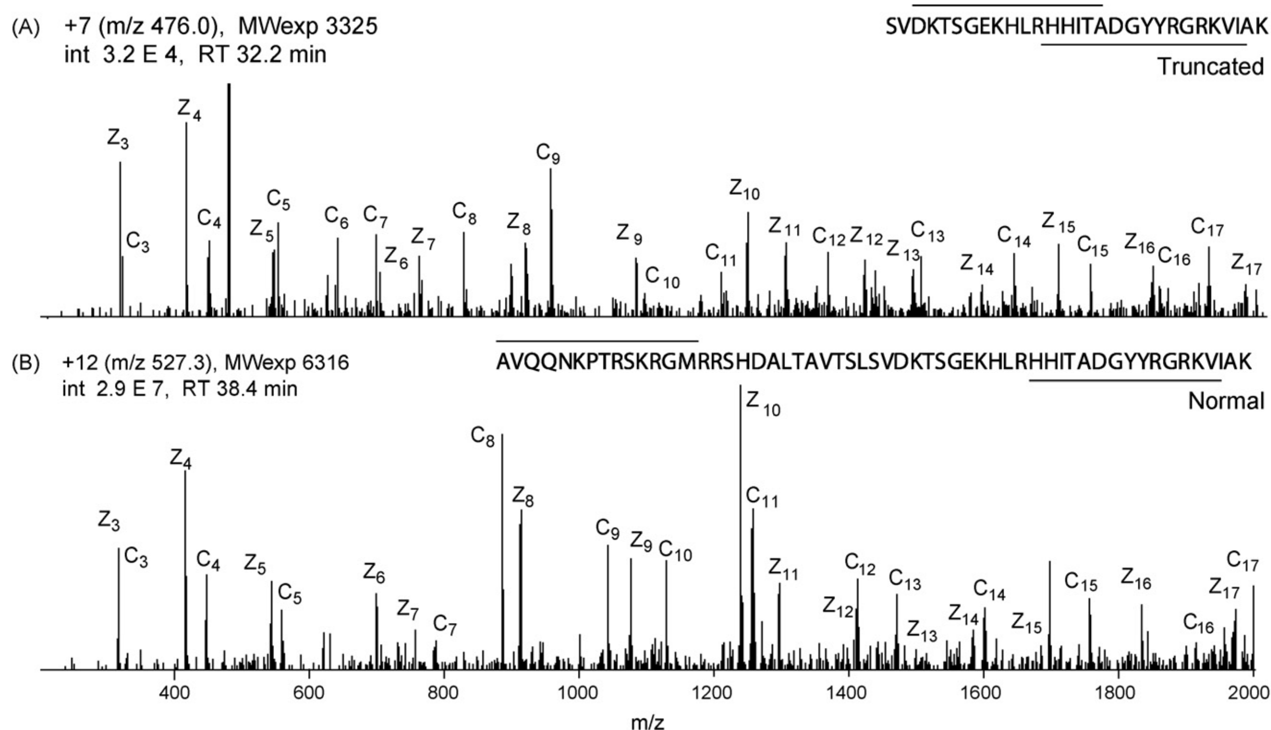

Fig. 3. ETD/PTR-MS/MS spectra recorded on the *E. coli* 70S ribosomal protein, L32. Spectra in panels (A and B) correspond to truncated and intact forms, respectively, that elute ∼6 min apart in the chromatogram. Lines above and below the sequences represent the coverage defined by ions of types c and 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.

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 types 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, 3 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, 14 retain their N-terminal methionine residue, 30 are missing their N-terminal methionine, 10 are detected in truncated forms, 3 (S11, L16 and L33) are monomethylated on either the N-terminus or Lys2, 2 (S5, S18) are acetylated/trimethylated on the N-terminus, 1 (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 1 (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 L-isoaspartate 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 LC–MS experiment using a C_8 column and a total of 5 pmols sample (∼100 fmol for each protein). Three of the remaining nine 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 six proteins, S1, L4, L9, L11, L15 and L21. It is possible these proteins were either not eluted off the column or both the N- and C-termini were modified and thus not identified by the search.

4. Conclusions

Sequential ion/ion reactions, ETD and PTR, in combination with a data dependent LC–MS 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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