Characterization of 70S Ribosome from *Rhodopseudomonas palustris* using an Integrated "Top-Down and "Bottom-Up" Mass Spectrometric Approach

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OVERVIEW

- **Integrating "Top-Down" and "Bottom-Up"**
  - We present a comprehensive mass spectrometric approach that integrates intact protein molecular mass measurement ("Top-Down") and proteolytic fragment characterization ("Bottom-Up") to characterize the 70S ribosome from *Rhodopseudomonas palustris*.
    - Identified 53 of the 54 orthologues to *Escherichia coli* ribosomal proteins by bottom up analysis.
    - Identified 42 intact masses from top down data.
    - Improved accuracy in distinguishing between isoforms.
    - Assigned the amino acid positions of several post-translational modifications.
    - Checked and validated the gene annotations for three ribosomal proteins predicted to possess extended C-termini.

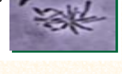
INTRODUCTION

Rhodopseudomonas palustris (**Figure 1**) is considered to be one of the most metabolically diverse organisms studied to date. This organism can grow in the presence or absence of oxygen and is capable of existing under different growth conditions in response to changes in environment. In order to better understand the network of complexes responsible for this metabolic diversity, a detailed understanding of each complex is needed. To this end, we present a proteomic study involving a comprehensive mass spectrometric approach that integrates intact protein molecular mass measurement (Top-Down) and proteolytic fragment identification (Bottom-Up) to characterize one of the most highly conserved and well studied complexes, the 70S ribosome.

The ribosome is the universal macromolecular machine involved in translating the genetic code into proteins. Although this complex has been studied extensively in both eukaryotic and prokaryotic organisms, a majority of this research has been dedicated to characterizing the *E. coli* (prokaryotic) ribosome. The *E.coli* (70S) ribosome consists of two noncovalently associated subunits; a small subunit (30S) consisting of 21 (S1-S21) proteins and a single rRNA (16S) and a large subunit consisting of 33 (L1-L36) proteins and 2 rRNAs (23S and 5S respectively). Interestingly, biological studies of prokaryotic ribosomes from different organisms indicate remarkable similarity in composition and structure to the *E.coli* ribosome. Furthermore, PTMs of homologous ribosomal proteins from various organisms are generally conserved with the only difference being the corresponding modification position. With this in mind, the 70S ribosome is an ideal model to develop our methodology of comprehensively analyzing complexes from this organism.

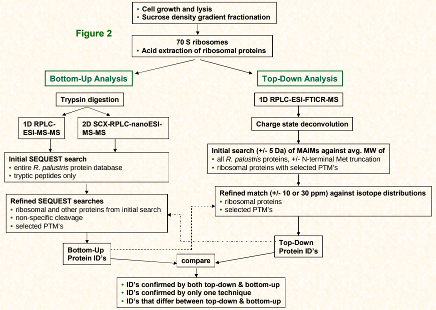
Figure 1 *Rhodopseudomonas palustris* CGA009

- **Purple nonsulfur phototrophic bacteria, member of the α-subgroup of the proteobacteria**
- **Genome sequenced by the DOE, fully assembled**
- **5.5 Mbp, high GC**
- **High metabolic versatility**



STRATEGY FOR TOP-DOWN BOTTOM-UP ANALYSIS

Figure 2



- **Bottom-up Analysis (Figure 2)**
  - 1D LC-MS/MS experiments were performed with an Ultimate HPLC (LC Packings, a division of Dionex, San Francisco, CA) coupled to an LCQ-DECA or LCQ-DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray source. Injections were made with a Famos (LC Packings) auto-sampler onto a 50ul loop. Flow rate was 4 ul/min with a 160 minute gradient for each injection.
    - A Vydac (Grace-Vydac, Hesperia, CA) C18 column (300μm id x 25 cm, 300Å with 5μm particles) was directly connected to the electrospray source.
    - For all 1D LC/MS/MS experiments, the LCQ was operated in the data dependent mode with dynamic exclusion enabled, where the top 4 peaks in every full MS scan were subjected to MS/MS analysis.
    - To increase dynamic range, separate injections were made with over 3 smaller m/z ranges.
  - 2D LC-MS/MS experiments were performed using a Famos/Swichos/Ultimate 2D HPLC system (LC Packings) coupled to an LCQ-DECA-XP ion trap equipped with a Finnigan nanospray source.
    - Peptides were injected into a dual column system containing a strong cation exchange (SCX) (LC Packings) column (500 μm id x 15mm), a LC Packings C18 precolumn (300μm id x 5mm, 300Å PepMap) and a C18 analytical column Vydac (75μm id x 25 cm, 300Å with 5μm particles).
    - 11 step gradient salt "bumps" ranging from 25mM-2M were used to elute peptides from the SCX column onto a precolumn. After desalting the precolumn a RP gradient was run for 160 minutes to elute peptides from the C18 column into the mass spectrometer.

- **Top-Down Analysis (Figure 2)**
  - All FT-ICR experiments were performed using an Ultimate HPLC (LC Packings) coupled to an IonSpec (Irvine, CA) 9.4-T HiRes electrospray Fourier transform ion cyclotron mass spectrometer (ES-FTICR-MS).
    - A Vydac (Grace-Vydac, Hesperia, CA) C4 column (300μm id x 15 cm, 300Å with 5μm particles) was directly connected to the electrospray source.
    - Ions were generated with an Analytica source.
    - Mass calibration was accomplished with standard proteins (ubiquitin or myoglobin).
    - The high resolution mass measurement enables isotopic resolution of multiply charged ions. Thus the charged state of an ion can be determined solely by its isotopic spacing.
    - Molecular masses were generated from deconvoluted spectra using the IonSpec software.
  - **Subset of PTMs included in Search**
  - For PTM identification we included PTMs previously reported to occur in ribosomal proteins of either *E. coli* or eukaryotic organelles thought to have evolved from bacteria by endosymbiosis.
    - By Bottom-Up analysis:
      - Methionine truncations
      - +14 on KR (mono-methylation)
      - +28 on KR (di-methylation)
      - +42 on K (acetylation or tri-methylation), static +42 modifications on N-terminal residues (acetylation or tri-methylation).
      - +46 on D (β-methylthiolation)
    - By Top-Down analysis any combination of methionine truncation, acetylation and methylation.

RESULTS AND DISCUSSION

Table 3 PTMs of *R. palustris* ribosomal proteins

Protein	Modification	Residue(s)
RRP-L3[a]	methylation	K155 or K158
RRP-L7/L12[b]	A: 2 methylations and 1 methylation	K69, K86
	B: 3 methylations or acetylation	K86, K89
RRP-L11	Acetylation or 3 methylation	K40
RRP-L30	methylation	N-terminus or K3[c]
RRP-L33	methylation	N-terminus or K3[c]
RRP-S12[d]	β-methylthiolation	D88

[A] The spectrum localizes the modification to either K155 or K158
[B] Present as two isoforms A and B.
[C] Insufficient data to distinguish between methylation at the N-terminus vs. K3.
[D] Present in modified and unmodified forms.

- 53 out of 54 *E. coli* orthologues to ribosomal proteins were identified by Bottom-Up analysis using either 1D or 2D LC MS/MS **(Table 1)**. 40 of these were identified with greater than 60% sequence coverage.
- 42 intact masses, representing modified and unmodified ribosomal proteins, were identified by Top-Down analysis **(Table 2)**.
- Combining both approaches allowed improved accuracy in distinguishing between isoforms and assigning the amino acid position of several PTMS **(Table 3)**.
- **Figure 3** illustrates the advantage of integrating both techniques. Panel A and B represent fragmentation spectra of modified peptides acquired by Bottom-Up data and Panel C and D represent the measured and calculated isotopic distributions respectively. The intact mass for RRP- L7/L12A matched this ribosomal protein with two modifications identified by fragmentation spectra (corresponding to di-methylation at K69 and mono-methylation at K86).
- Without these combined approaches we would not have been able to definitively assign these two PTMs to a single isoform of RRP-L7/L12.
- The combined mass spectrometry data also allowed us to check and validate the gene annotations for three ribosomal proteins predicted to possess extended C-termini.
- In particular, we identified a highly repetitive C-terminal "alanine tail" on RRP-L25 **(Figure 4)**. This type of low complexity sequence, common to eukaryotic proteins, has previously not been identified at the protein level in prokaryotic proteins. The other two include:
  - RRP-L9
  - RRP-S2
- To our knowledge, this is the most comprehensive analysis to date that integrates two MS techniques.

Table 1 Bottom-Up Analysis

Name	Sequence Coverage 1D	Sequence Coverage 2D	# Peptide Identifications* 1D	# Peptide Identifications* 2D
RRP-L1	66.0%	80.0%	25	43
RRP-L2	46.0%	58.0%	12	19
RRP-L3	79.0%	92.0%	23	45
RRP-L4	68.0%	78.0%	22	19
RRP-L5	54.0%	45.0%	10	14
RRP-L6	36.0%	50.0%	9	19
RRP-L7/L12	51.0%	60.0%	16	18
RRP-L9	90.0%	56.0%	13	17
RRP-L10	91.0%	91.0%	27	45
RRP-L11	61.0%	69.0%	10	20
RRP-L13	81.0%	87.0%	17	24
RRP-L14	80.0%	84.0%	11	16
RRP-L15	78.0%	85.0%	20	24
RRP-L16	65.0%	77.0%	14	26
RRP-L17	66.0%	76.0%	13	22
RRP-L18	73.0%	73.0%	12	27
RRP-L19	78.0%	72.0%	18	24
RRP-L20	51.0%	57.0%	10	11
RRP-L21	58.0%	22.0%	5	4
RRP-L22	63.0%	57.0%	12	18
RRP-L23	44.0%	86.0%	5	13
RRP-L24	89.0%	89.0%	11	14
RRP-L25	74.0%	62.0%	20	30
RRP-L27	63.0%	87.0%	8	15
RRP-L28	77.0%	55.0%	13	13
RRP-L29	45.0%	32.0%	5	5
RRP-L30	91.0%	91.0%	6	6
RRP-L31	80.0%	100.0%	6	9
RRP-L32	58.0%	58%	2	2
RRP-L33	56.0%	66.0%	4	9
RRP-L34	0	0	0	0
RRP-L35	27.0%	49.0%	3	5
RRP-L36	22.0%	0	1	0
RRP-S1	37.0%	39.0%	12	16
RRP-S2	72.0%	87.0%	29	41
RRP-S3	57.0%	68.0%	16	26
RRP-S4	78.0%	85.0%	20	29
RRP-S5	72.0%	70.0%	22	25
RRP-S6	63.0%	69.0%	19	21
RRP-S7	72.0%	82.0%	22	28
RRP-S8	87.0%	71.0%	17	21
RRP-S9	84.0%	74.0%	18	27
RRP-S10	39.0%	66.0%	4	5
RRP-S11	86.0%	33.0%	10	13
RRP-S12	67.0%	63.0%	11	11
RRP-S13	33.0%	72.0%	6	21
RRP-S14	36.0%	50.0%	6	7
RRP-S15	87.0%	99.0%	14	17
RRP-S16	44.0%	56.0%	8	16
RRP-S17	83.0%	79.0%	12	10
RRP-S18	58.0%	61.0%	7	11
RRP-S19	98.0%	84.0%	12	19
RRP-S20	36.0%	23.0%	5	3
RRP-S21	23.0%	51.0%	3	9

* number of different peptide identifications including +1, +2 and +3 charges states for identical peptides

Table 2 Top-Down Analysis

Protein	Modification	Calc. Mass[a]	Meas. Mass[a]	Mass error (ppm)[b]
L1	loss of Met	23877.832	23877.449	*16.0*
L3	plus Methyl	25622.463	25622.159	*11.9*
L5	plus 2 Methyl	21064.992	21064.576	*19.7*
L6	loss of Met	19272.408	19272.674	*-13.8*
L7/L12	loss of Met + 3 Methyl	12754.07	12754.089	*-1.5*
L9	none	21178.022	21178.268	*-11.6*
L10	loss of Met	19067.739	19067.617	*6.4*
L11	loss of Met+Acet+ 9 Methyl	15507.107	15507.246	*-9.0*
L14	none	13488.498	13488.645	*-10.9*
L15	none	16836.243	16836.259	*-1.0*
L17	plus 3 Methyl	15716.353	15716.056	*18.9*
L18	loss of Met	12904.93	12905.157	*-17.6*
L19	none	14296.764	14296.899	*-9.4*
L21	loss of Met	13358.081	13358.533	*-33.8*
L22	loss of Met	13826.007	13825.6447	*26.2*
L23	none	10907.949	10908.021	*-6.6*
L24	loss of Met	10998.226	10998.231	*-0.5*
L24	loss of Met + Methyl	11012.241	11012.146	*8.6*
L29	loss of Met	7849.213	7849.239	*-3.3*
L30	loss of Met	7092.967	7092.988	*-3.0*
L31	none	8566.315	8566.334	*-2.2*
L32	loss of Met	6860.73	6860.636	*13.7*
L33	loss of Met + Methyl	6248.504	6248.45	*8.6*
L35	loss of Met	7415.278	7415.278	*0.0*
L36	none	5063.971	5063.952	*3.8*
S4	loss of Met + Methyl	23441.536	23441.69	*-6.6*
S5	loss of Met	20522.086	20522.411	*-15.8*
S7	loss of Met	17556.27	17556.629	*-20.4*
S8	loss of Met	14477.6316	14477.683	*-3.6*
S8	loss of Met+Acet+4Methyl	14575.704	14575.619	*5.8*
S10	none	11667.363	11667.404	*-3.5*
S11	loss of Met + Methyl	13760.215	13760.314	*-7.2*
S12	none	13874.799	13875.167	*-26.5*
S13	loss of Met	14313.985	14313.596	*27.2*
S14	loss of Met	11331.399	11331.9	*-44.2*
S15	loss of Met	10010.563	10010.562	*0.1*
S16	loss of Met	12017.595	12017.575	*1.7*
S17	loss of Met	9553.253	9553.316	*-6.6*
S18	plus 6 Methyl	9178.219	9177.834	*41.9*
S19	loss of Met	10087.371	10087.379	*-0.8*
S20	loss of Met	9577.324	9577.387	*-6.6*
S21	none	10062.669	10062.722	*-5.3*

[a] MAIM (most abundant isotopic mass)
[b] Larger mass errors are associated with species measured at low-signal: noise

Figure 3 RRP-L7/L12A

ADLQKIVDDLSSLTVLEAAELAKLLEEKWGVSAAAAVAVAAAPGAGGAAAPAEEK
TEFTVVLASAGDK*KIEVIKEVRAITGLGLK*EAKDLVEGAPKPLKEGVNKEEAEKV
KAQLEKAGAKVELK

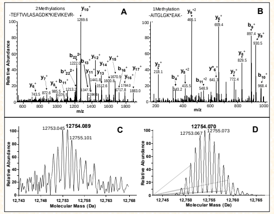


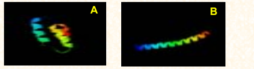
Figure 4 RRP-L25

MTSVLELATARPKSGKGAARAERRAGRVPGVIYGDNQSPLPISVEEKELRLRLILAGRFLTTV
FDVVLDGKKHRVIPRDYHLDPVRDFPIHVDFLRLGAGATIRVSVVPLHLKGLEVAPGVKRGGT
FNIVTHTVELEAPAENIPQFIEADVSTLDIGVSLHLSDIALPTGVKSVSRDDVTLVTIVPPSGYNE
DKAAAGAAPAAAAAPAAAAKAPAAAAKAPAAAAPAAKKK

Below represents the two most likely structures of the highlighted sequence based on optimal threading alignment between the target sequence and a template (templates were obtained by searching NCBI database). The threading algorithm used was PROSPECT (structures done by Dr. Bo Yan at ORNL).

A. Up and down four helix bundle secondary motif.

B. An extend alpha helix.



Conclusion

- As illustrated in Figure 3, integrating both MS approaches provided the advantage of using FT-ICR to identify the aggregate intact mass for a protein with PTMs and the use of fragmentation spectra to identify the exact modification positions.
- This method improved the accuracy in assigning isoforms. It is difficult to identify isoforms using only Bottom-Up analysis because modified peptides that map to different regions of a protein may be from the same protein or isoforms of that protein.
- This method also allowed the use of both types of MS data for checking and validating gene annotations with unusual DNA predicted sequences. The identification of peptides corresponding to the entire sequence outlined in Figure 4 indicates that this C-terminal extension is a part of the RRP-L25 structure. This predicted C-terminal sequence could have been an error that resulted because the stop codon was not correctly identified during the gene annotation process.
- Although either MS method could be individually used to identify PTMs, the data presented here clearly illustrate the advantage of using both MS approaches for comprehensively characterizing a biological complex.

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