pH Dependence of the Chemical Labeling of *Thermus thermophilus* HB8 Ribosomes

William E. Running and James P. Reilly
Indiana University, Department of Chemistry, Bloomington, IN 47405

Introduction

S-methylthioacetimidate (SMTA) is a reagent designed to selectively modify lysine residues under physiologically relevant solution conditions, and to be less disruptive of protein structure than acetyl groups, as it preserves the positive charge on a lysine residue. SMTA produces a characteristic 41 Da mass shift for every amino group (lysine or protein N- terminus) derivatized according to the scheme below[1-2].

Past results using SMTA to modify surface accessible lysine residues in the ribosomes of several bacterial species showed excellent agreement between predicted extents of reactivity based on inspection of ribosome crystal structures and the observed extent of reaction[3-6]. We have extended these results by investigating the effect of changing pH on the reactivity of *Thermus thermophilus* HB8 ribosomal proteins.

Methods

- Sample Preparation
  - Cells grown as shown in Figure 1
  - Cell lysis with a French press
  - Ribosomes isolated by ultracentrifugation
- Chemical modification
  - Ribosomes reacted with SMTA in 1 M buffer solution
  - 3 pH values: 6.3 (MES), 7.5 (HEPES), 8.3 (tris)
  - Reactions quenched with acid
- Whole Protein Analysis
  - 2DLC fractionation (SCX, C4 RPLC), LC-MS with a QToF
  - 1DLC nano-ESI (C4 RPLC), mass analysis with an LTQ-FT
- Peptide Analysis
  - Proteins from C4 RPLC fractions digested with trypsin
  - Nano-ESI of peptides, mass analysis with an LTQ-FT
- Inspection of published crystal structures
  - 2J00, 2J02 (30S), 2J01, 2J03, 3HUX (50S)[7-8]
  - Predict maximum extent of modification of native proteins
  - Rationalize observed variations in reactivity with pH

Figure 1: A culture of *T. thermophilus* HB8. Cells were grown in a 70 °C incubator cabinet in ATCC #697 medium, supplemented with inorganic salts.

Table 1: A summary of the observed large and small subunit proteins from *T. thermophilus* HB8 ribosomes. The mass and modifications columns are the result of measurements with a Micromass QToF Micro from 2DLC experiments. Columns under the FT-ICR masses heading are measurements made with a Thermo LTQ-FT; the hyphenated number appended to the observed mass indicates the most intense isotopomer in the spectrum. Sequence coverage is the maximum coverage observed in LC-MSMS experiments.

	QToF Data		FT-ICR Data			% Sequence
Protein	obs. mass	modifications	calc. mass	obs. mass	Δ ppm	Coverage
L1	24702.2	-Met	24699.34	24699.31 - *15*	1	51%
L2	30340.4	-Met	30338.67	30338.71 - *20*	2	62%
L3	22438.0	+2(CH_2)	22434.19	22434.21 – *12*	1	50%
L4 (L1e)	23237.9		23230.56	23230.60 - *10*	2	70%
L5	20899.8	-Met	20891.38	20891.45 – *6*	4	69%
L6	19402.6	-Met	19399.88	19399.81 – *11*	3	78%
L7/L12	12937.1	-Met	12935.23	12935.24 – *7*	1	40%
L9	16398.7		16396.19	16396.19 – *9*	<1	63%
L10	18435.2	-Met	18431.16	18431.09 - *8*	3	20%
L11	15674.4	+12(CH_2)	15672.48	15672.48 – *9*	<1	39%
L13	15896.4		15894.85	15894.75 – *10*	5	68%
L14	13303.7		13302.31	13302.31 – *8*	<1	82%
L15	16282.1		16281.08	16280.86 – *10*	13	55%
L16	15964.1		15961.63	15961.64 – *9*	1	55%
L17	13716.0		13714.76	13714.75 – *8*	1	61%
L18	12481.1	-Met	12480.07	12480.07 – *7*	<1	57%
L19	17153.0		17149.43	17149.43 – *8*	<1	65%
L20	13611.7	-Met	13610.67	13610.57 - *7*	7	54%
L21	11048.1		11046.32	11046.33 – *6*	<1	77%
L22	12781.3		12779.16	12779.12 – *7*	3	62%
L23	10737.4		10736.11	10736.13 – *6*	2	64%
L24	12056.0		12051.74	12051.74 - *7*	<1	70%
L25	23207.0		23195.27	23195.36 - *5*	4	54%
L27	9377.6	-Met	9376.16	9376.16 - *5*	<1	9%
L28	10847.7	-Met	10846.31	10846.30 - *6*	<1	62%
L29	8650.8		8648.92	8648.92 - *4*	1	68%
L30	6654.2	-Met	6652.99	6652.89 - *3*	<1	83%
L31	8285.6		8281.12	8281.13 - *5*	1	0%
L32	6570.7	-Met	6569.44	6569.44 - *4*	<1	0%
L33	6482.6	-Met	6480.44	6480.44 - *4*	<1	0%
L34	6107.2		6108.58	6108.57 – *3*	1	0%
L35	7353.1	-Met	7352.29	7352.30 - *4*	<1	67%
L36	4421.3		4418.41	4418.41 - *2*	<1	0%

	QToF Data		FT-ICR Data			% Sequence
Protein	obs. mass	modifications	calc. mass	obs. mass	Δ ppm	Coverage
S2	29148.6	-Met	29143.46	29143.35 - *16*	4	46%
S3	26572.3	-Met	26563.74	26563.61 - *10*	5	55%
S4	24193.3	-Met	24195.06	24195.18 - *21*	5	55%
S5	17427.8	-Met	17425.44	17425.41 - *10*	2	48%
S6	11973.6		11971.33	11971.33 - *6*	<1	89%
S7	17886.6	-Met	17885.57	17885.55 - *12*	1	50%
S8	15838.8		15836.73	15836.72 - *9*	1	77%
S9	14383.7		14382.80	14382.81 - *9*	<1	68%
S10	11799.6		11925.61	11925.45 - *3*	13	74%
S11	13584.4	-Met	13582.29	13582.26 - *9*	2	51%
S12	14514.9	-Met, +SCH_3[h]	14513.29	14513.23 - *8*	4	37%
S13	14174.8	-Met	14172.99	14172.95 - *8*	2	62%
S14	7008.5	-Met.	7003.89	7003.90 - *4*	2	0%
S15	10423.9	-Met	10422.76	10422.76 - *6*	<1	62%
S16	10387.4	Also modified[i]	10386.60	10386.50 - *6*	10	46%
S17	12167.2	-Met	12169.88	12169.84 – *11*	3	62%
S18	10101.3	-Met	10101.96	10101.83 – *8*	13	45%
S19	10450.9	-Met	10448.72	10448.71 - *5*	1	68%
S20	11572.5	-Met	11573.83	11573.80 - *9*	2	53%
THX	3206.5	-Met		No appearance		0%

Results

- Extent of modification of proteins changes as a function of pH
  - Figure 2 shows representative data
  - Less modification is seen at pH 6.6, more at pH 7.5 and 8.3
  - Maximum modification is as predicted from structures
  - Data for all proteins is summarized in Figure 7
- Peptide LC-MSMS localizes amidination sites
  - Figures 3 and 4 present examples
  - Extent of lysine amidination increases with pH
  - Location of amidinations correlates with 3° or 4° structure
- Differentially modified lysines share common features
  - Figures 5 and 6 are structural rationalizations of labeling
  - Proximity to carboxylate residues (D or E)
  - Proximity to rRNA backbone phosphates

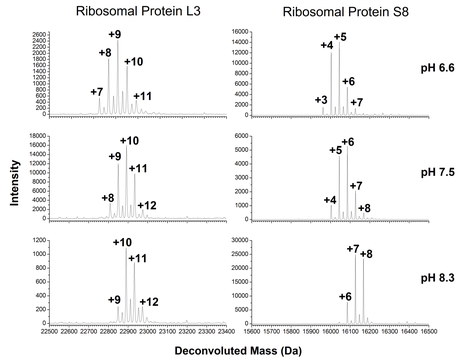


Figure 2: Deconvoluted MS spectra of large subunit protein L3 (left column) and small subunit protein S8 (right column). As the reaction pH increases, the extent of modification increases

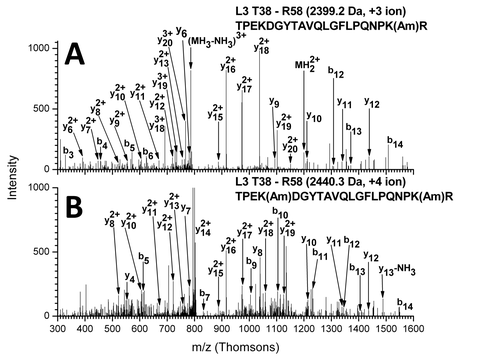


Figure 3: MSMS spectra of a tryptic peptide from large subunit protein L3. Panel A shows a spectrum of a peptide from a reaction at pH 6.3. Panel B shows a spectrum from a reaction at pH 7.6. The masses of the singly charged, high m/z b- and y-ions show the 41 Th mass shift from SMTA modification, indicating that K41's reactivity increases as pH is raised.

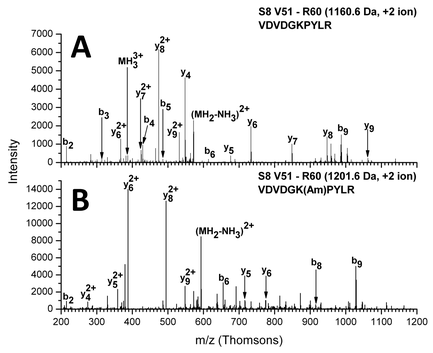


Figure 4: MSMS spectra of peptides from small subunit protein S8. Panel A shows the spectrum of a tryptic peptide from a sample modified at pH 6.3. Panel B shows a spectrum from a reaction at pH 8.3. The 20.5 Th increase in the m/z of the doubly-charged y-ions is the result of residue K56's increased reactivity at higher pH.

Figure 5: Ribosomal protein L3 (white) bound to the large subunit of the ribosome (dark blue). Lysine 41 is shown in red with other lysine residues in yellow. Residues E40 and D42 are shown in orange. The K41's lower reactivity with SMTA at pH 6.3 is consistent with a hydrogen bonding interaction between these residues, or a salt bridge with either carboxylate. Increasing pH disrupts these interactions with the lysine enhancing its reactivity.

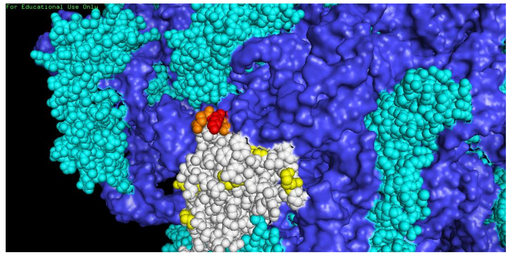
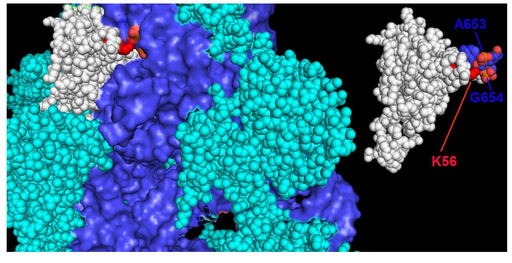
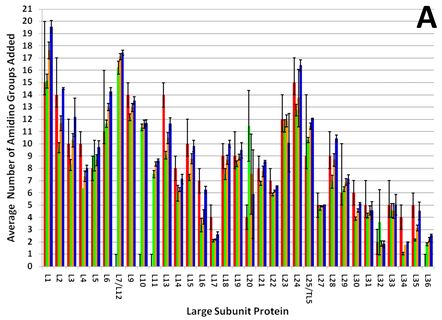


Figure 6: Ribosomal protein S8 bound to the small subunit. The isolated structure to the right shows details of lysine 56's interactions with the phosphates of adenosine 653 and guanosine 654. Disruption of these interactions leads to enhanced reactivity of K56 at elevated pH.





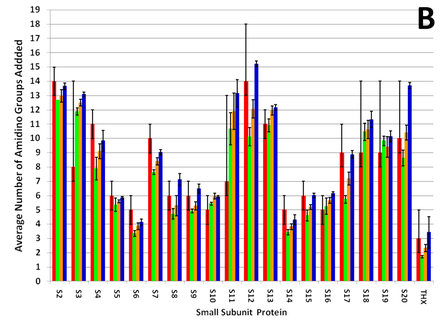


Figure 7: A summary of the pH dependence of ribosomal protein SMTA reactivity. Panel A shows the reactivity of large subunit proteins; panel B shows small subunit proteins. In each panel, the red bar is a prediction of maximum reactivity derived by inspection of crystal structures of each subunit. Experimental data are from reactions at pH 6.6 (green bars), 7.5 (orange bars), and 8.3 (blue bars).

Conclusions

- SMTA labeling can be extended to pH between 6.0 and 9.0
- Ribosomal proteins show distinct labeling patterns
  - Extent reactivity is predictable by inspection of crystal structures
  - Native structure is preserved across the pH range
  - Changing buffers or ionic strength does not denature ribosomes
- Proteins showing little change in reactivity from pH 6.6 to 8.3 are:
  - Exposed to solution and flexible (e. g. L7/L12)
  - Buried in rRNA (e. g. L27)
- Proteins showing large changes in reactivity have exposed lysines that:
  - Interact with adjacent carboxylates
  - Interact with the rRNA backbone
- Tertiary and quaternary interactions can be disrupted by pH changes
  - Structure alterations are non-denaturing
  - Structure alterations localize to flexible regions in a complex

References

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