Introduction

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

Past results using SMIT 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 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 SMIT 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 RPC), LC-MS with a QToF
  - 1DLC nano-ESI (C4 RPC), mass analysis with an LTQ-FT

- Peptide Analysis
  - Proteins from C4 RPC fractions digested with trypsin
  - Nano-ESI of peptides, mass analysis with an LTQ-FT

- Inspection of published crystal structures
  - 2J00, 2J02 (30S), 2J01, 3HUX (50S)
  - Native structure is preserved across the pH range

Table 1: A summary of the observed large and small subunit proteins from Thermus thermophilus HB8 ribosomes. The mass and modifications columns are the result of measurements with a QToF Mass MicrOTOF 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 isotope component in the spectrum. Coverage is the maximum coverage observed in LC-MS/MS experiments.

<table>
<thead>
<tr>
<th>Protein</th>
<th>obs. mass</th>
<th>modifications</th>
<th>calc. mass</th>
<th>obs. mass</th>
<th>Δ ppm</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>24702.2</td>
<td>-Met</td>
<td>24702.2</td>
<td>6107.2</td>
<td>6108.58</td>
<td>3 1 0%</td>
</tr>
<tr>
<td>L35</td>
<td>7353.1</td>
<td>-Met</td>
<td>7352.30</td>
<td>4 &lt;1</td>
<td>67%</td>
<td></td>
</tr>
<tr>
<td>L36</td>
<td>4421.3</td>
<td></td>
<td>4418.41</td>
<td>2 &lt;1</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>29148.6</td>
<td></td>
<td>10448.72</td>
<td>5 &lt;1</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>S20</td>
<td>11572.5</td>
<td>-Met</td>
<td>11573.80</td>
<td>9 2</td>
<td>53%</td>
<td></td>
</tr>
<tr>
<td>THX</td>
<td>3206.5</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: A culture of Thermus thermophilus. Cells were grown in a 70 °C incubation cabinet in ATCC #697 medium, supplemented with inorganic salts.

Figure 3: MS/MS spectra of a tryptic peptide from large subunit protein L3. Panel A shows a spectrum from a reaction at pH 6.3. Panel B shows a spectrum from a reaction at pH 7.5. The masses of the singly charged, high m/z b- and y-ions show the 41 mass shift from SMIT modification, indicating that K41's reactivity increases as pH is raised.

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-MS/MS locizes amidination sites
  - Figures 3 and 4 present examples
  - Extent of lysine amidination increases with pH
  - Location of amidinations correlates with 3D or 4D 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 4: MS/MS 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 20.7% increase in the m/z of the doubly-charged ions is the result of residual K56's increased reactivity at higher pH.

Figure 5: Ribosomal L3 (in blue) bound to the large subunit of the ribosome (dark blue). Lysine 41 is shown in red with other lysine residues in yellow. Residues K41 and D42 are shown in orange. The K41's lower reactivity 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 rRNA backbone phosphates of adenosine 653 and guanosine 634. Disruption of these interactions leads to enhanced reactivity of K56 at elevated pH.

Conclusions

- SMIT 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)
- 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

B. B, D., Reilly, J., P. , F., J. Proteome Res. 2006, 5, 1374-1384
C. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
D. Li, F., F., J. Proteome Res. 2006, 5, 6880-6887
E. Lederer, M., Reilly, J., P., J. Proteome Res. 2008, 7, 8221-8228
G. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
H. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
I. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
J. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
K. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
L. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
M. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
N. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
O. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
P. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
Q. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
R. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
S. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
T. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
U. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
V. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
W. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
X. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
Y. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456
Z. El-K, M., L., J. Proteome Res. 2006, 5, 3451-3456