To my family and wife for continued love and endless support
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# TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................................................. 4

LIST OF TABLES .......................................................................................................................... 8

LIST OF FIGURES .......................................................................................................................... 9

LIST OF ABBREVIATIONS ............................................................................................................ 11

ABSTRACT ..................................................................................................................................... 15

CHAPTER

1 LITERATURE REVIEW ................................................................................................................. 17

   Introduction ................................................................................................................................ 17
   26S Proteasomes .......................................................................................................................... 17
      20S Core Particle ...................................................................................................................... 17
      19S Regulatory Particle ............................................................................................................ 19
      Proteasome Activating Nucleotidase ..................................................................................... 21
   Ubiquitin ...................................................................................................................................... 22
      Ubiquitylation Reaction .......................................................................................................... 23
         Ubiquitin-activating enzyme (E1) ....................................................................................... 23
         Ubiquitin-conjugating enzymes (E2s) ................................................................................. 24
         Ubiquitin ligases (E3s) ......................................................................................................... 26
   Deubiquitylation ......................................................................................................................... 29
   Topology of Ubiquitin Conjugates .............................................................................................. 31
   Degradation Signals of Ubiquitin System .................................................................................. 32
      N-degrons and N-end rule ....................................................................................................... 32
      Phosphodegron ....................................................................................................................... 33
      Misfolded protein .................................................................................................................... 34
   Ubiquitin-like Proteins ............................................................................................................... 35
      Ubiquitin-related Modifier 1 (Urm1) ..................................................................................... 36
      Small Archaeal Modifier Protein (SAMP) ............................................................................. 37
      Prokaryotic Ubiquitin-like Protein (Pup) .............................................................................. 39
   Methionine Sulfoxide Reducing System .................................................................................... 41
      Methionine Sulfoxide Reductase A/B (MsrA/B) ................................................................. 42
      Free Methionine-(R)-sulfoxide Reductase (fMRsr) ............................................................ 44
      Biotin Sulfoxide Reductase (BisC) ....................................................................................... 45
      Methionine Sulfoxide Reductase PQ (MsrPQ) ..................................................................... 46
   Project Rational and Objectives ................................................................................................. 46

2 MATERIALS AND METHODS .................................................................................................... 51

   Chemicals, Strains, and Culture Conditions ............................................................................. 51
Chemicals and Reagents ................................................................. 51
Strains and Culture Conditions ........................................................ 51
DNA Techniques ........................................................................ 54
Cloning ......................................................................................... 54
Site-directed Mutagenesis ............................................................... 54
DNA Electrophoresis ..................................................................... 54
Sanger Sequencing ......................................................................... 55
Plasmid Extraction and Transformation ........................................... 55
Generation of Markerless Deletion Mutants ....................................... 55
RNA Techniques ........................................................................... 56
RNA Isolation ................................................................................ 56
qRT-PCR ......................................................................................... 56
Protein Techniques .......................................................................... 57
  Whole Cell Lysate Preparation ....................................................... 57
  Two-Dimensional Gel Electrophoresis ............................................. 58
  Immunoblotting ........................................................................... 58
  Chase Assay ................................................................................ 59
  Streptactin Affinity Chromatography ............................................. 59
  Nickel Affinity Chromatography .................................................... 60
  Gel Filtration Chromatography ...................................................... 61
  Pull-down Assay ........................................................................... 61
  In vitro Reconstitution of MsrA-dependent Ubl-bond Formation ..... 62
  MSO Reductase Assay .................................................................. 63
Mass Spectrometry ........................................................................... 64
  Determining Protein Mass by ESI-TOF .......................................... 64
  Mapping Phosphorylation Sites by MS/MS ..................................... 64

3 UBIQUITIN-LIKE PROTEASOME SYSTEM REPRESENTS A EUKARYOTIC-
LIKE PATHWAY FOR TARGETED PROTEOLYSIS IN ARCHAEA .................. 71

Introduction .................................................................................... 71
Results and Discussion ................................................................. 73
  TBP2 Abundance is Increased by Mutation of the SAMP-proteasome System ........................................... 73
  TBP2 Abundance is Increased by Chemical Inhibition of 20S Proteasomes and Cdc48/p97-type ATPases .................................................. 74
  TBP2 Abundance is Increased by Depletion of 20S proteasomes .............................................................. 74
  TBP2 is Stabilized by Mutation of the SAMP-proteasome System .............................................................. 75
  SAMP2 is not Targeted for Degradation by Archaeal Proteasomes ............................................................ 75
  Detection of TBP2 Conjugates Modified by SAMPs .................................................................................. 76
  Two Apparent Forms of “Unsampylated” TBP2 ...................................................................................... 78
  TBP2 is a Mixture of N-terminal Met1 Minus and Intact Forms ................................................................. 79
  Ser2 of TBP2 is Phosphorylated ........................................................................................................... 80
  TBP2 Migration and Abundance are Altered by Substitution of Ser2 ...................................................... 81
Conclusion ....................................................................................... 82
4 METHIONINE SULFOXIDE REDUCTASE ESSENTIAL FOR MILD OXIDANT INDUCED UBIQUITIN-LIKE MODIFICATIONS ................................................................. 96

Introduction ................................................................................................................................. 96

Results and Discussion .................................................................................................................. 97

- MsrA is Essential for SAMP-conjugates Induced by DMSO in vivo .................. 97
- MsrA is Essential for SAMP-conjugates Induced by MSO in vivo .................. 99
- Differential Effect of Chemicals on Stimulating SAMP-conjugates in vivo ........ 99
- Schema of MsrA-dependent Ubl-protein Modification .............................................. 100
- MsrA is Essential to Form SAMP-conjugates in vitro ........................................ 101
- DTT is not Needed to Form MsrA-dependent SAMP-conjugates in vitro ........ 102
- ATP and DMSO are Essential to Form MsrA-dependent SAMP-conjugates
  in vitro ................................................................................................................................. 102
- Robust MsrA-mediated SAMP-conjugates is Reconstituted in vitro ................. 103
- DMSO Stimulates MsrA-dependent SAMP-conjugates in a Dose Dependent
  Manner in vivo and in vitro ............................................................................................... 104
- MsrA/B are Active MSO Reductases ........................................................................... 104
- MsrA/B are not Essential for MSO Assimilation ....................................................... 106

Conclusion .................................................................................................................................. 106

5 SUMMARY AND FUTURE PERSPECTIVES ........................................................................... 125

- Summary of Findings ............................................................................................................ 125
- Future Directions ................................................................................................................. 126

LIST OF REFERENCES ............................................................................................................... 128

BIOGRAPHICAL SKETCH ........................................................................................................ 153
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>List of strains used in this study.</td>
<td>67</td>
</tr>
<tr>
<td>2-2</td>
<td>List of plasmids used in this study.</td>
<td>68</td>
</tr>
<tr>
<td>2-3</td>
<td>Primers used in the study.</td>
<td>69</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Side and top views of crystal structure of 20S proteasome core particle (CP) from <em>Homo sapiens</em>.</td>
<td>49</td>
</tr>
<tr>
<td>1-2</td>
<td>Protein ubiquitylation: its conjugation mechanism and function.</td>
<td>50</td>
</tr>
<tr>
<td>3-1</td>
<td>TBP2 abundance is increased by mutation of the SAMP-proteasome system.</td>
<td>83</td>
</tr>
<tr>
<td>3-2</td>
<td>Increased TBP2 abundance by mutation of the SAMP-proteasome system is not due to differences in transcript levels.</td>
<td>84</td>
</tr>
<tr>
<td>3-3</td>
<td>TBP2 abundance is increased by chemical inhibition of 20S proteasomes and Cdc48.</td>
<td>85</td>
</tr>
<tr>
<td>3-4</td>
<td>TBP2 abundance is increased by genetic depletion of 20S proteasomes.</td>
<td>86</td>
</tr>
<tr>
<td>3-5</td>
<td>TBP2 is stabilized by mutation of the SAMP-proteasome system.</td>
<td>87</td>
</tr>
<tr>
<td>3-6</td>
<td>Detection of TBP2 conjugates modified by SAMPs.</td>
<td>89</td>
</tr>
<tr>
<td>3-7</td>
<td>Mobility shift of TBP2 in SPS deletion strains compared to wt.</td>
<td>91</td>
</tr>
<tr>
<td>3-8</td>
<td>TBP2 is a mixture of N-terminal Met1 minus and intact forms based on ESI-MS analysis.</td>
<td>92</td>
</tr>
<tr>
<td>3-9</td>
<td>TBP2 is phosphorylated at its N-terminal Ser2.</td>
<td>93</td>
</tr>
<tr>
<td>3-10</td>
<td>TBP2 migration and abundance are altered by substitution of Ser2.</td>
<td>94</td>
</tr>
<tr>
<td>3-11</td>
<td>Model of regulated turnover of TBP2 by the archaeal SAMP-proteasome system.</td>
<td>95</td>
</tr>
<tr>
<td>4-1</td>
<td>MsrA is required for the Ubl-protein conjugates induced by DMSO.</td>
<td>109</td>
</tr>
<tr>
<td>4-2</td>
<td>MsrA active site residues are required for the Ubl-protein conjugates induced by DMSO.</td>
<td>111</td>
</tr>
<tr>
<td>4-3</td>
<td>MsrA is required for the Ubl-protein conjugates induced by MSO.</td>
<td>112</td>
</tr>
<tr>
<td>4-4</td>
<td>Differential effect of chemical agents on stimulating Ubl-conjugate levels in <em>Hfx. volcanii</em>.</td>
<td>113</td>
</tr>
<tr>
<td>4-5</td>
<td>Model of MsrA-dependent sampylation.</td>
<td>115</td>
</tr>
<tr>
<td>4-6</td>
<td><em>In vitro</em> reconstitution of Ubl-bond formation with MsrA.</td>
<td>116</td>
</tr>
</tbody>
</table>
In contrast to MSO reductase activity, DTT was not needed for MsrA-dependent \textit{in vitro} reconstitution of Ubl-bond formation. ........................................ 117

Hydrolyzable ATP and DMSO were required for MsrA-dependent Ubl-conjugates via \textit{in vitro} reconstitution. ........................................................................................................ 118

\textit{In vitro} reconstitution of robust SAMP-conjugates by MsrA. ........................................ 120

DMSO stimulated MsrA-dependent Ubl-conjugates in a dose dependent manner \textit{in vitro} and \textit{in vivo}. ........................................................................................................ 121

MsrA/B are catalytically active MSO reductases. ................................................................. 122

MsrA/B are not required for MSO assimilation................................................................. 124
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-gel</td>
<td>Two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>5-FOA</td>
<td>5-Fluoroorotic acid</td>
</tr>
<tr>
<td>AAA⁺</td>
<td>ATPase associated with various cellular activities</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Ampr</td>
<td>Ampicillin resistance</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenylyl-imidodiphosphate</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CA</td>
<td>Casamino acids</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CP</td>
<td>Core particle or 20S core particle</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (atomic mass unit)</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMS</td>
<td>Dimethyl sulfide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO₂</td>
<td>Dimethyl sulfone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitylating enzyme</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin-activating enzymes</td>
</tr>
</tbody>
</table>
E2 Ubiquitin-conjugating enzymes
E3 Ubiquitin ligase
EDTA Ethylenediaminetetraacetic acid
ESI Electrospray ionization
$g$ Gravitational force
$h$ Hour
HvJAMM JAB1/MPN/MOV34 metalloenzyme of *Haloferax volcanii*
HVO *Haloferax volcanii*
IB Immunoblot
IEF Isoelectric focusing
IP Immunoprecipitation
IPTG Isopropyl-β-D-thiogalactopyranoside
kDa Kilodalton
Km' Kanamycin resistance
LB Luria-Bertani broth
LC Liquid chromatography
M Molar (mol·liter$^{-1}$)
MAP Methionine aminopeptidase
MES 2-(N-morpholino)ethanesulfonic acid
MOAC Metal oxide affinity chromatography
μM Micromolar (μmol·liter$^{-1}$)
mA Milliampere
min Minutes
ml Milliliter
mM Millimolar (mmol·liter$^{-1}$)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>Nov&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Novobiocin resistance</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical Density at 600 nm</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAN</td>
<td>Proteasome activating nucleotidase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Pup</td>
<td>Prokaryotic ubiquitin-like protein</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RP</td>
<td>19S regulatory particle</td>
</tr>
<tr>
<td>Rpt</td>
<td>AAA ATPases subunits of RP</td>
</tr>
<tr>
<td>Rpn</td>
<td>non-ATPase subunits of RP</td>
</tr>
<tr>
<td>SAMP</td>
<td>Small archaeal modifier protein</td>
</tr>
<tr>
<td>Sampylation</td>
<td>Process of attaching SAMP to target proteins</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPS</td>
<td>Sampylation-proteasome system</td>
</tr>
<tr>
<td>TBP2</td>
<td>TATA binding protein 2</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>UbaA</td>
<td>Ubl-activating enzyme of archaea</td>
</tr>
<tr>
<td>Ubl</td>
<td>Ubiquitin-like</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>Vh</td>
<td>Volt hour</td>
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<td>v</td>
<td>Volume</td>
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<tr>
<td>w</td>
<td>Weight</td>
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</table>
Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

UBIQUITIN-LIKE PROTEINS OF ARCHAEA: ROLE IN TARGETED PROTEOLYSIS
AND PROTEIN MODIFICATION INDUCED BY MILD OXIDATION

By
Xian Fu

December 2016

Chair: Julie A. Maupin-Furlow
Major: Microbiology and Cell Science

Ubiquitin (Ub) and Ubiquitin-like (Ubl) proteins play a fundamental role in numerous cellular processes. Compared to Ubl proteins in eukaryotes, the role of archaeal Ubl proteins SAMPs (small archaeal modifier proteins) and the mechanism of Ubl bond formation in archaea (sampylation) are not well characterized. In this study, we advanced the fundamental knowledge of signal-guided proteolysis in archaea by the discovery of the sampylation-proteasome system (SPS) and uncovered a novel function of archaeal MsrA, a methionine sulfoxide (MSO) reductase, in guiding Ubl modification of proteins upon mild oxidative stress. We demonstrated that TBP2, a TATA-binding protein (TBP) modified by ubiquitin-like isopeptide bonds, was targeted for degradation by SPS in *Haloferax volcanii*. The key components of archaeal SPS that are responsible for rapid turnover of TBP2 included UbaA (the E1/MoeB/ThiF homolog of archaea), AAA ATPases (Cdc48/p97 and Rpt types), a type 2 JAB1/MPN/MOV34 metalloenzyme (JAMM/MPN+) homolog (JAMM2), as well as 20S proteasomes. In support of the model that attachment of ubiquitin-like tags targets proteins for degradation by proteasomes, we showed SAMP2 stimulated the degradation of TBP2 and SAMP2 modified TBP2
was accumulated in SPS mutants. Moreover, we provided evidence that phosphorylation of TBP2 Ser2 serves as the degradation signal to regulate SPS-mediated proteolysis. In addition, we showed that MsrA represents a moonlighting protein that may undergo a DMSO/MSO-induced switch from an MSO reductase to a protein factor required for sAMPylation during mild oxidative stress. MsrA required the conserved active site residues C13 and E56 for MSO reductase and Ubl-bond formation activities. MsrA was specific for the SAMP-conjugates formed only during mild oxidative stress (not severe oxidative stress or proteasome inhibition). Overall, our data provided new insights into the role of SAMPs in targeted proteolysis and oxidative stress response in archaea.
CHAPTER 1
LITERATURE REVIEW

Introduction

This literature review is designed to present the most up-to-date scientific literature concerning proteasomes, Ub/Ubl proteins, and MSO reductases. This review will highlight the Ub conjugation machinery and ubiquitin-proteasome system (UPS) mediated proteolysis pathway. Ub/Ubl proteins across domains of life and MSO reducing systems will also be discussed.

26S Proteasomes

The 26S proteasome, the most complex of proteases, is the primary nanomachine that functions in targeted and energy-dependent protein turnover in eukaryotes (1). Proteasomes are found in eukaryotes, archaea and actinobacteria (2). In eukaryotes, 26S proteasomes are composed of 33 distinct subunits associated in subcomplexes including the 19S regulatory particle (RP) and the 20S core particle (CP) that contains the proteolytic active sites (3). The RP plays an important role in recognition and translocation of substrates into the 20S CP for degradation (4). Owing to the essential role of 26S proteasomes, considerable efforts are focused on elucidating the molecular architecture of 26S proteasomes [reviewed in (5)]. Recently, the cryo-EM structure of the human 26S proteasome, a 2.5 MDa molecular machine, at atomic resolution has been determined (6, 7). Below, I describe the structure and function of proteasomes.

20S Core Particle

CPs from archaea and eukaryotes share high similarity in their overall structures (8-10). The 20S CP is composed of four stacked heptameric rings that form a barrel-like
structure (Figure 1-1) (10, 11). The outer rings contain seven different α-subunits (α1-α7) and the inner rings consist of seven distinct β-subunits (β1-β7) in the eukaryotic CP (Figure 1-1) (10). The N-terminal threonine residues of the β1, β2, and β5 subunits are sequestered within the inner space of the CP and are found to confer proteolytic activity to the proteasomes (12). The β1, β2, and β5 subunits are produced as proenzymes and undergo maturation to expose the catalytic threonine residue at the new N-terminus upon CP assembly to form the mature β subunits (13, 14). A number of specific CP inhibitors such as bortezomib, which reversibly binds to the catalytic site of CPs with high specificity and affinity (15), have been developed as chemotherapy agents based on the crystallographic study of CPs [reviewed in (16)]. The α-subunit rings form the gate of proteasomes to prevent nonspecific proteolysis (Figure 1-1) (17, 18). In addition, the pockets formed by interfaces of α-subunits serve as the docking sites for the RP (19).

Proteasomal CPs in archaea and eukaryotes are similar in overall structure but differ in subunit composition. Eukaryotic CPs are composed of seven different α-subunits (α1–α7) and seven distinct β-subunits (β1–β7). By contrast, archaeal CPs are simpler in composition. For example, only one α-subunit and one β-subunit form the four stacked homoheptameric rings of proteasomal CPs in Thermoplasma acidophilum (20, 21). In addition, two catalytic β-subunits and a single α-subunit are encoded from Sulfolobus acidocaldarius genome and Hfx. volcanii synthesizes single β-subunits and two α-subunits (22, 23). Although proteasomal CPs in archaea are simple in the subunit composition, different types of proteasomal CPs can be produced in archaeal cells. For instance, the presence of two α subunits enables Hfx. volcanii to synthesize more than
one type of 20S CPs (24). The physiological importance of archaeal CPs subpopulations remains largely unknown, however, the protein and transcript levels of CPs subunits are found to be dramatically altered during the growth phase of *Hfx. volcanii* (25).

Proteasomal CPs are essential for cell viability in archaea and eukaryotes. Genetic depletion of the *psmB* gene encoding the single β-subunit of *Hfx. volcanii* renders non-viable cells (26). Although the genes encoding the α1 or α2 subunit can be deleted individually in *Hfx. volcanii*, conditional depletion of the α1 subunit together with markerless deletion of the gene encoding α2 subunit prevent cells from growing (26). In yeast, proteasomal CPs are required for the proliferation of cells based on the gene disruption experiments (27, 28). Moreover, missense mutations in the *PRE2* gene encoding the β5 subunit of the yeast 20S CP lead to a temperature-sensitive growth phenotype (29).

**19S Regulatory Particle**

In eukaryotes, 19S regulatory particles (RPs) are often assembled with 20S CPs to form 26S proteasomes for Ub-mediated proteolysis in an ATP-dependent manner (3). The 19S RP can be separated into lid and base subcomplexes, and dissociation of the lid and base of the 19S RP is observed by deletion of the non-ATPase subunit Rpn10 of the RP or upon purification of wide-type proteasomes with high salt (30, 31). Regulatory particle AAA ATPases 1-6 (Rpt1-6) belonging to ATPases Associated with a variety of cellular Activities (AAA) family are located in the base of the RP to form the AAA-ATPase module of proteasomes which can unfold and translocate protein substrates into the CPs when coupled by a mechanism with ATP hydrolysis [reviewed in (32, 33)]. The C-terminal tail of Rpt3 and Rpt5 are found to be inserted into the pockets formed
between adjacent α-subunits of the CPs (7), thus, stabilizing the interactions between the 19S RP and the 20S CP. Three of the six Rpt subunits (Rpt2, Rpt3, and Rpt5) harbor the C-terminal HbYX motif (where Hb is a hydrophobic residue, Y is tyrosine, and X is any amino acid), which is important to trigger the opening of CPs (34). Besides Rpt1-6, the 13 additional RP non-ATPase subunits (Rpn) are required for assembling the RP and assisting in various steps of UPS-mediated proteolysis. For example, the large Rpn1 and Rpn2 subunits of the RP base serve as scaffolds to interact with different proteasomes associated factors (35). Rpn10 and Rpn13 bind to Ub with high affinity and serve as receptors of poly-Ub chains with different architectures (36-39).

Rpn11, a metalloprotease subunit in the lid subcomplex, plays an essential role in removing the poly-Ub chains on protein substrates, prior to or coincident with translocation of unfolded substrates (40, 41). Rpn11 is a promiscuous deubiquitinase with no Ub linkage specificity when assembled in an Rpn11-Rpn8 dimer, which explains the broad substrate specificity of Rpn11 in 26S proteasomes (42).

In addition to subunits that are constitutively present in the 19S RP, some other protein factors have been found to be associated with the 19S RP in a transient way. Shuttling receptors: Rad23, Dsk2, and Ddi1 interact with 19S RP though their N-terminal Ub-like (UBL) domain and recognize Ub modified substrates by the Ub associated (UBA) domain for targeting substrates to proteasomes (35, 43-46). Furthermore, the deubiquitylating enzyme (DUB) Ubp6 in yeast or its mammalian ortholog Usp14 is another proteasome-associated protein (35, 47) and plays an important role in controlling proteasome-mediated degradation via both enzymatic and non-enzymatic activities independent of ATP (48-50). The Ub ligase Hul5 has also been
shown to be associated with proteasomes and affect the half-life of various cellular proteins through its proteasome-dependent conjugation activity (51).

**Proteasome Activating Nucleotidase**

The Rpt1-6, AAA-ATPase module of 26S proteasomes, is thought to have evolved from archaeal proteasome-activating nucleotidase (PAN) through gene duplication (52). Distantly related AAA-ATPase modules in actinobacteria and *Mycobacterium* are referred to AAA+ ATPase forming a ring-shaped complex (ARC) and mycobacterial proteasome ATPase (Mpa), respectively. These AAA-ATPases from archaea and bacteria are proposed to interact with CPs, stimulate the opening of CPs, and unfold and translocate substrates into CPs for efficient protein turnover by proteasomes (34, 53, 54).

Great advances have been made in understanding the molecular details of AAA-ATPase module assisted proteolysis by proteasomes via biochemical and structural studies using archaeal PAN as a relatively simple model [reviewed in (8)]. Archaeal PAN forms a hexameric complex with an N-terminal coiled-coil domain, a C-terminal ATPase domain, and a linker segment that connects the N-terminal and C-terminal domains (55). N-terminal tentacle-like coiled-coil pairs (three per hexameric ring) protrude from the central pore formed by an oligonucleotide-binding fold. The PAN N-terminal domain is thought to recognize protein substrates and are found to have the chaperone activity (56). Interestingly, the coiled-coil domains of *Mycobacterial Mpa* are needed to interact with prokaryotic ubiquitin-like protein (Pup) to induce the formation of an α-helix in Pup (57). An aromatic–hydrophobic (Ar–φ) loop within the narrowest region of the ATPase is apparent in archaeal PAN (55), which stimulates protein unfolding through repetitive pulling forces driven by ATP hydrolysis (58). The conserved C-
terminal HbYX motif in PAN is found to trigger the conformational changes of the CP that are needed to open the CP gate for efficient protein turnover by proteasomes (54, 59).

The physiological importance of PAN has been studied in archaea by genetic studies. *Hfx. volcanii* genome encodes two PAN proteins, and the level of PAN1 protein is relatively constant while PAN2 is increased in its abundance from log-phase to stationary phase (25). Although the *Hfx. volcanii* CP is essential, both PAN1 and PAN2 can be simultaneously deleted (26), suggesting additional AAA-ATPase modules exist in archaea for functional redundancy. Consistent with this hypothesis, AAA+ ATPase homologs of the Cdc48-type with HbYX motifs are widespread among all archaeal genomes (60), and can interact with proteasomal CPs *in vitro* and stimulate substrate degradation by CPs in the presence of ATP (60-62). Similarly, five of seven AAA-ATPase modules with HbYX motifs in *Methanosarcina mazei* can also enhance the activity of CPs *in vitro* (63), which suggests that a complicated network of AAA ATPase modules (Cdc48 and Rpt-like PANs) exist in archaea to enhance the capacity and functional flexibility of proteasome-mediated proteolysis.

**Ubiquitin**

Ubiquitin (Ub), a small protein with only 76 amino acids, is a post-translational modifier found in all eukaryotes. The process by which Ub is covalently attached to target proteins by a cascade of enzymes is termed ubiquitylation (Figure 1-2). Ub has a C-terminal diglycine motif that is needed to form the isopetide linkage between its C-terminal carboxyl group and a lysine side chain(s) of the protein substrate (64). Ub adopts a distinct β-grasp fold revealed by its crystal structure (65, 66). In addition to lysine residues, Ub also can be covalently linked to the N-terminal amino group in
higher eukaryotes (67, 68) and to threonine, serine, or cysteine residues (69-71). In eukaryotic cells, Ub plays a fundamental role in almost all cellular processes including intracellular protein homeostasis, protein trafficking, cell division, DNA repair, transcriptional regulation, endocytosis, signaling transduction, and protein quality control [reviewed in (72-75)]. The Ub system has become one of the most extensively studied systems in the biological and biomedical fields since its discovery in 1975 (76) and some breakthrough findings around 1980s [reviewed in (77)]. Considering the essential role of Ub, it is not surprising to see several threatening human diseases are related to malfunctions of the Ub system (e.g., Parkinson disease, Alzheimer's disease, Angelman syndrome, the Johanson-Blizzard syndrome, and several cancers) (74, 77). Below, I describe ubiquitylation and deubiquitylation reactions, topology of Ub chains, and UPS mediated proteolysis.

**Ubiquitylation Reaction**

Ubiquitylation is mediated by a cascade of enzymes consist of an Ub-activating enzyme E1, Ub-conjugating enzymes E2s, and Ub ligases E3s (Figure 1-2). Generally, the E1 readies the Ub C-terminus for protein modification by activating Ub at the expense of ATP, while E2s and E3s guide Ub modification on protein substrates (72). Below, the Ub conjugation machinery (E1, E2s, and E3s) will be the focus of the discussion.

**Ubiquitin-activating enzyme (E1)**

The E1 enzyme is required for the first step of ubiquitylation and cell viability ranging from yeast to mammals (78-80). The E1 enzyme activates Ub by adenylylating its C-terminus with release of PPI from ATP, followed by transfer of Ub to the catalytic Cys residue of the E1 enzyme to form an E1~Ub thioester intermediate (81, 82).
Subsequently, a second Ub molecule non-covalently binds the E1 prior to adenylation. Thus, the current model is that two Ub molecules are asymmetrically loaded at distinct sites of the E1 in the first step of ubiquitylation, through which E1 is thought to adopt a favourable conformation for adenylation (83). Structural studies of E1 provide valuable insights into the mechanism of E1-mediated Ub activation. The E1 enzyme consists of three domains: the canonical E1 adenylation domain, a catalytic Cys domain and a C-terminal E2-binding Ub-fold domain (UFD). The adenylation domain of E1 enzyme is needed to bind Ub and initiate the adenylation reaction by using ATP (83) through a mechanism similar to that of the MoeB-MoaD complex from bacteria (84). The catalytic Cys attacks the Ub-AMP via conformational changes to form an E1~Ub thioester intermediate (85). The UFD of E1~Ub directly binds to E2 and undergoes dramatic rotation to allow the E1 catalytic Cys to be in close proximity to the acceptor Cys of E2 (86). Currently, only a single E1 is found present in lower eukaryotes, while two E1 enzymes are found to exist in vertebrates and sea urchins (87).

**Ubiquitin-conjugating enzymes (E2s)**

Although E2 enzymes are small proteins (usually twice the size of Ub), they have multiple roles and are essential in ubiquitylation (88). A total of 11 and 37 E2 enzymes have been found in *Saccharomyces cerevisiae* and *Homo sapiens*, respectively (89, 90). Generally, the catalytic Cys of an E2 enzyme accepts the Ub from the Ub~E1 thioester intermediate to form an Ub~E2s thioester via a transthiolation reaction (88, 91). Based on more than 32 E2s with determined structure, E2s all contain an Ub-conjugating (UBC) domain that adopts an α/β-fold with variable N- and/or C-terminal extensions [reviewed in (88)]. This E2 UBC core has the active site Cys and binds to E1 and E3 enzymes with an overlapping region, while the extension regions of different
types of E2s confer additional specificity to function (88). Consistent with the
directionality of the ubiquitylation reaction, the binding affinity of E1 with uncharged E2
is higher than the Ub~E2 thioester intermediate, thus the loaded Ub~E2 is released
after catalysis by uncharged E2s (91, 92). Similarly, E3s bind to E2~Ub with higher
affinity than the uncharged E2s, which facilitates the successive attachment of Ub to the
substrates from ubiquitylation enzymes (93). For RING E3 and E3-independent
ubiquitylation, E2s play the determining role in product formation, regulation of
processivity of chain formation, and control of Ub chain topology (88). For example, the
E2 variant Mms2 of the Mms2-Ubc13 heterodimer plays the key role in keeping the
Lys63 of the acceptor Ub in the appropriate position for catalysis by the active site of the
E2 Ubc13 (94). In the E3-independent ubiquitylation, the transfer of Ub from the E2~Ub
intermediate to the protein substrates can occur via the intrinsic reactivity towards
nucleophiles containing molecules like free Lys and Cys residues (92). Interestingly,
both mono- and poly-Ub can be mediated by the same E2s. For instance, the E2
heterodimer Rad6/Ubc2 in yeast can catalyze monoubiquitylation of histone H2B as well
as poly-Ub chain formation in certain situations (95, 96).

The physiological importance of different types of E2s has been studied.
Cdc34/Ubc3 has been found to be the only one out of eleven yeast E2s required for cell
viability (97). Later study disclosed that the inability to destroy the cyclin-dependent
kinase inhibitor Sic1, whose degradation is needed for cells transition from G1 to S
phase, accounts for the essential role of the E2 Cdc34 (98). In addition, E2-dependent
proteolysis has been shown to control several protein quality control pathways. For
example, Ubc6, a E2 anchored into the ER membrane, is required to synthesize K11-
linked poly-Ub chains and regulate the stability of numerous cellular proteins through the endoplasmic reticulum-associated degradation (ERAD) pathway (99). The E2s Ubc4 and Ubc5 function similarly to ensure timely conjugation of Ub onto abnormal proteins for proteasome-mediated protein turnover (100), and deletion of both Ubc4 and Ubc5 genes render yeast cells inviable (101).

**Ubiquitin ligases (E3s)**

Ubiquitin ligases (E3s) are a large family of enzymes involved in the last step of ubiquitylation to confer substrate specificity. A total of 60-100 and over 600 E3s are predicted from yeast and human genomes, respectively (89, 102). Although different types of E3s differ in their ways of Ub transfer, they all harbor an E2~Ub-binding domain. E3s are divided into three major groups: RING (really interesting new gene), HECT (homologous to E6AP C-terminus) and RBR (RING-between-RING) based on their structural features and Ub transfer mechanisms (Figure 1-2). RING E3s are the most abundant E3 and facilitate the transfer of Ub from E2~Ub thioesters to the substrate by bringing the charged E2~Ub in close proximity to the substrate (Figure 1-2). RING E3s are featured by their zinc-containing RING domain, which serves as the minimal region for interacting with E2~Ub and stimulating Ub transfer (103). RING E3s are revealed to be very diverse in their architectures. RING E3s can form monomer, homodimer, heterodimer, and multi-subunit complexes such as E3 cullin-RING ligases (CRLs) and anaphase promoting complex/cyclosome (APC/C) [reviewed in (104)]. CRLs harbor the cullin protein which functions as a scaffold to connect the substrate receptor domain with RING domain (105). CRLs can recognize a huge number of different substrates via utilization of diverse substrate receptors (104). Recent structural studies provide insights into the mechanism of priming Ub for transfer mediated by
RING E3s. In the current model, the binding of the RING E3 stabilizes the closed conformations of the E2~Ub thioester intermediate which otherwise is very flexible (106). In the RING E3 bound closed conformation of E2~Ub, the RING domain interacts with both the Ile36 surface of Ub and the E2 UBC domain, through which the E2~Ub is presented in a favourable configuration for nucleophilic attack by the lysine residue of the protein substrate (107). The cellular function of some RING E3s has also been determined. For instance, Ubr1, the first identified E3, recognizes the N-terminal amino acids of protein substrates with differential affinities for controlling the half-life of cellular proteins by the N-terminal rule (108). The E3 San1 regulates nuclear protein quality control by ubiquitylating aberrant nuclear proteins and targeting them for proteasome-mediated proteolysis (109).

By contrast to the RING E3s, the HECT and RBR E3s have a catalytic cysteine that attacks the E2~Ub to form an E3~Ub thioester intermediate prior to the transfer of Ub to the protein substrate (Figure 1-2). HECT E3s consist of an N-lobe for E2 binding, a C-lobe with the catalytic Cys, and a substrate binding domain (110). In the current model of HECT E3 mediated Ub transfer, the N-lobe first recruits the E2~Ub, which is then in close proximity to the catalytic Cys of the C-lobe upon rotation of the hinge to form HECT E3~Ub thioester (111, 112). Following transthiolation, the E3~Ub is positioned to contact the substrate Lys residue through the rotation of the C-lobe with this conformation further stabilized by the interaction between the N-lobe and the C-lobe (113). Compared with RING and HECT E3s, the mechanism of the RBP E3 catalyzed Ub transfer is less understood. RBR E3s, which also referred to 'RING/HECT hybrid' enzymes, such as PARKIN are shown to share structure features with both RING and
HECT E3s and adopt autoinhibited conformations in their free forms (114, 115). In the current model, the typical RING domain RING1 of RBR functions in recognizing E2~Ub and the RING2 domain harboring the catalytic Cys plays a role in forming the RBP E3~Ub intermediate (104). Recent study demonstrates the HOIP/E2~Ub complex adopts a conformation that is notably distinct from auto-inhibited RBRs, thus, providing a new insight into the general mechanism of RBR E3 via a dramatic conformational change (116). Although HECT and RBR E3s are less abundant than RING E3s, they play an essential role in physiology. For example, Hul5, a HECT E3, plays an essential role in degradation of short-lived misfolded proteins under heat-shock stress response (117). In addition, numerous studies demonstrate that RBR E3 PARKIN is the key enzyme linked with Parkinson’s disease (118).

Interestingly, evidence shows that bacteria and viruses can synthesize different effectors proteins with E3-like activities that modulate the host Ub pathways (119). For example, the effector protein termed AvrPtoB from the pant pathogen Pseudomonas syringae adopts a remarkable structural homology to the RING domain in its C-terminal domain and shows Ub ligase activity during bacterial virulence (120). In the human pathogen Salmonella, the C-terminal domain of SopA has been shown to be a structural homolog of the N- and C-lobes of the HECT domain (121) and can interact with human E2 UbcH7 to catalyze Ub transfer (122). Surprisingly, a family of bacterial effectors in Legionella pneumophila have been recently found to ubiquitylate several human Rab small GTPases independent of host E1 an E2 enzymes (123). In contrast to the canonical ubiquitylation via transthioilation mechanism that requires use of ATP as the energy source, this novel Ub modification depends on nicotinamide adenine
dinucleotide as the cofactor and likely activates Ub by formation of an ADP-ribosylated Ub (123).

**Deubiquitylation**

The hydrolysis of the isopeptide bond linkage between Ub and protein targets catalyzed by deubiquitylating enzymes (DUBs) is termed deubiquitylation (124). DUBs are responsible for: (i) processing the C-terminal tail of pro-Ub to synthesize mature Ub by exposing its diglycine motif; (ii) editing and trimming Ub chains to program the topology of Ub conjugates; (iii) regulating protein activity or stability by removing Ub chains from the modified targets (89, 124). Yeast cells encode twenty DUBs belonging to four families including Ub C-terminal hydrolases (UCH), Ub-specific protease (USP), ovarian tumor (OTU), and JAB1/MPN/MOV34 (JAMM) domain family (89). Besides the four DUB families present in yeast, the human genome encodes an additional DUB family termed the Machado-Josephin domain proteases (MJDs) (124). MINDY (motif interacting with Ub-containing novel DUB family), a new family of DUBs found in all eukaryotes, was discovered very recently and shown to prefer to cleave the K48-linked poly-Ub chain (125). Although DUBs are very diverse in terms of their functions, cellular locations, Ub linkage specificity, and enzyme structures, they can be classified into two main classes based on the deubiquitylation enzymology: the cysteine proteases and the metalloproteases containing only the JAMM domain proteases (124). For the cysteine DUBs, diads (His-Cys) or triads (His-Cys-Asn/Asp) of active site residues catalyze the cleavage of the isopeptide bond. The polarized His is needed to lower the pKa of the catalytic Cys to enable its nucleophilic attack of isopeptide bonds (126). In contrast, the JAMM metalloprotease contains a metal ion such as zinc in the active site. The
conserved Glu in the active site serves as an acid-base catalyst to polarize the nucleophilic water for bond hydrolysis (127).

DUB mediated cleavage of the Ub bond is responsible for variable cellular functions. For instance, Ubp8, a DUB subunit of the SAGA acetylation complex, regulates gene transcription via deubiquitylation of histone H2B (128). AMSH-like protein (associated molecule with the SH3 domain of STAM), a JAMM domain DUB, specifically hydrolyzes the K63-linked poly-Ub chain and functions in regulation of receptor trafficking (129, 130). The complex structure of AMSH-LP bound to Lys 63-linked di-Ub is determined and reveals the mechanism of AMSH-mediated Ub linkage selectivity (131). Rpn11 and Ubp6 are the best-studied DUBs and play an essential role in recycling Ub by removing it from protein targets prior to proteasome-mediated degradation (89). Rpn11 must be incorporated into the lid subcomplex of 26S proteasomes for efficient DUB activity and functions in cleaving the poly-Ub chain from Ub modified protein targets independent of linkage specificity (40, 42, 132). The requirement of Rpn11 for UPS mediated proteolysis is due to its role in removing Ub chains which otherwise sterically block translocation of protein substrates into the proteasome chamber for degradation, and mutation of Rpn11 causes loss of cell viability (40). Although Ubp6 is not essential for viability, its deletion leads to accelerated rates of Ub degradation, suggesting its role in preventing Ub from turnover by an Ub recycling mechanism (133). Ubp6 can also rescue proteins engaged by proteasomes by shortening poly-Ub chains on protein substrates prior to entering into the proteasomal CPs (49).
Topology of Ubiquitin Conjugates

The fate of cellular proteins modified by Ub largely relies on the topology of Ub conjugates (Figure 1-2). Ub itself has seven lysines (K6, K11, K27, K29, K33, K48, and K63) and all of these lysine residues can be modified by a second Ub molecule (134). The attachment of a single Ub onto protein substrate is referred to monoubiquitylation, while the attachment of more than one Ub onto a substrate protein is termed multiubiquitylation or polyubiquitylation (Figure 1-2). Multiubiquitylation refers to the attachment of multiple Ubs onto only one protein as a single moiety of Ub at different lysine residues, while polyubiquitylation means the formation of Ub chains on a single lysine of a substrate protein. Generally, mono- or multiubiquitylation plays a non-proteolytic role such as intracellular trafficking, protein-protein interaction and modulation of enzyme activity (135, 136); however, exceptions do exist (137).

Polyubiquitylation is well characterized compared with mono- or multiubiquitylation and mediates various cellular processes, often for targeted protein turnover by proteasomes (99, 134, 138). The K48 poly-Ub chains haven been long considered as the canonical chain topology serving as a common signal for proteasome-mediated protein degradation. In contrast, K63-linked poly-Ub chains usually play a non-proteolytic role (e.g., DNA repair, immune response, and apoptosis) (139, 140) and formation K63 polyubiquitylation was recently shown to be linked to oxidative stress responses (141). Quantitative proteomics reveals that poly-Ub through K11, K48, and K63 chains are the most abundant in unperturbed yeast cells (99). Genetic engineering of yeast cells expressing Ub variants (K to R) has been shown to be a valuable strategy to determine the physiological importance of the different types of poly-Ub chains, through which the K48 poly-Ub chain was found to be essential for cell
viability and to mediate proteolysis by proteasomes (142), the K63 poly-Ub chain was shown to be needed for DNA damage and oxidative stress responses (140, 141), and the K11 poly-Ub chain was suggested to be linked with the ERAD pathway (99). Furthermore, heterogeneous chains consisting of both Ub and the Ubl protein SUMO (small Ubl modifier) were discovered by quantitative proteomics study (143). Interestingly, in addition to poly-Ub chains linked by isopeptide bonds, N-terminally-linked head-to-tail poly-Ub chains could be synthesized by an Ub ligase complex in vitro (144).

**Degradation Signals of Ubiquitin System**

Degradation signals (degrons) specifically guide the ubiquitylation on protein targets for their turnover by 26S proteasomes. Below, three common degrons of the Ub system will be highlighted for discussion.

**N-degrons and N-end rule**

The N-end rule is a selective proteolysis pathway by which the half-life of cellular proteins is determined by the identity of the N-terminal-residue (145). The degradation signals following an N-end rule are referred to N-degrons. The N-degrons are one of the first discovered and most systematically studied degradation signals and are found to regulate the stability of numerous proteins (145, 146). The Arg/N-end rule and the Ac/N-end rule pathways are two branches of N-end rule in eukaryotes (145). In 1986, Varshavsky and colleagues comprehensively tested the half-life of β-galactosidase with twenty different N-terminal residues after removal of an N-terminal Ub domain and found a remarkable range of turnover rates among these proteins (from a few minutes to more than twenty hours) (146). The E3 ligase Ubr1 is responsible for recognition of N-terminal primary destabilizing residues (Phe, Tyr, Trp, Leu, Arg, Lys, His, and Ile) on
protein substrates in the Arg/N-end rule branch (145, 147). Gln, Glu, Asp, and Asn can be converted to Arg through deamidation and/or arginylation for recognition by Ubr1 to enhance the functional capacity of the Arg/N-end rule (145). Ubr1 also recognizes N-terminal Met when it is followed by a hydrophobic residue and targets multiple normal and aberrant proteins with N-terminal Met for UPS-mediated proteolysis (148). On the other hand, the E3 ligase Doa10 in the Ac/N-end rule pathway selects proteins with N-terminal acetylation for degradation (149). Eukaryotic proteins with an uncharged and nonbulky amino acid at the second position (e.g., Ala, Ser, Cys, Thr, and Val) are often co-translationally modified by N-terminal acetylation after N-terminal Met1 is removed by a Met-aminopeptidase (150, 151). Based on the complementarity of the Arg/N-end rule and Ac/N-end rule pathways, it seems that most proteins harbor N-degrons from the moment of their birth. Considering the fact that many proteins are metabolically stable, evidence suggests that the N-degrons can be shielded by rapid and appropriate folding during translation or masked by protein partners (152).

**Phosphodegron**

Post-translational modification such as phosphorylation can serve as a reversible degron. Compared with the non-phosphorylated form, phosphorylated protein substrates can be recognized with higher affinity by specific E3 ligases, usually of the SKP1-CUL1-F-box (SCF) family (153). The F-box subunit of SCF E3 ligase is the substrate acceptor domain and responsible for recognizing the target phosphodegron. The best example is the selective degradation of cell cycle inhibitor Sic1 by SCF\textsuperscript{Cdc4}, a SCF ligase with Cdc4 functioning as the F-box protein (154). In detail, cell cycle inhibitor Sic1 is phosphorylated by the G1 and S-phase kinases Cln2/Cdc28 and Clb5/Cdc28 to develop the phosphodegron, which is then identified by SCF\textsuperscript{Cdc4} to initiate UPS-
mediated protein turnover for appropriate cell-cycle transition (98, 155-157). In addition, the phosphodegron can be removed by phosphatase to regulate protein stability. For instance, phosphatase PP1 prevents SRC coactivator from turnover by dephosphorylation of two Ser residues to regulate oncogenic cell proliferation (158).

**Misfolded protein**

Misfolded proteins are thought to be recognized by the Ub system, leading to the formation of poly-Ub chains for targeted proteolysis by proteasomes. Ub-dependent protein quality control (PQC) is essential for selective degradation of polypeptides harboring biosynthetic errors (159). Endoplasmic reticulum (ER) constitutes one of the primary sites for Ub mediated PQC, and the endoplasmic reticulum associated degradation (ERAD) system can recognize misfolded proteins in the ER and ubiquitylate them for destruction by proteasomes in the cytosol (160). Misfolded proteins can be repaired by chaperone assisted refolding, and chaperone mediated PQC serves as an alternative strategy to get rid of aberrant proteins if they are beyond repair (154). In the best-studied case, the chaperone Hsp70 and Hsp90 can recognize misfolded proteins and recruit the E3 ligase CHIP (carboxyl terminus of Hsc70-interacting protein) to promote the formation of poly-Ub chains on chaperone client proteins for proteasome-dependent degradation (161, 162). Oxidative stress and heat-shock can also cause protein misfolding with exposure of the hydrophobic amino acid residues (163, 164). Generally, surface-exposed hydrophobic regions are proposed to be common degrons with the mechanisms poorly defined. Insights into the hydrophobic stretches serving as a degron to regulate protein stability have been gained by the study of the yeast MATα2, a key transcription factor that controls yeast cell types (165-167). The N-terminal hydrophobic region of MATα2 is recognized by an E3 ligase Doa10.
which functions with two types of E2s (Ubc6 and Ubc7) for selective degradation by UPS (166, 168). In addition, the hydrophobic degron of MATα2 can be shielded by formation of a protein dimer with MATα1 (165).

**Ubiquitin-like Proteins**

Since the discovery of Ub, different types of Ubl proteins have been found in all three domains of life. In eukaryotes, although Ubl proteins differ in their amino acid sequence, they have common Ub features such as forming protein conjugates, the overall structure, the type of bond linkage, and the enzymology of the Ubl bond formation (169-172). Eukaryotic Ubl proteins that function as posttranslational protein modifiers include FAT10, Nedd8, ISG15, Ufm1, SUMO, Atg12 and others (171). The Ub-related modifier 1 (Urm1) serves as the first example of an Ubl protein that can act as both protein modifier and sulfur carrier for tRNA thiolation in eukaryotic cells (173-175). Similar to Urm1, Ubl proteins with dual functions like SAMP and TtuB (tRNA-two-thiouridine B) have been recently found in the archaeon *Halofax volcanii* and thermophilic bacterium *Thermus thermophiles*, respectively (176-178). In archaea, the Ubl protein SAMP adopts a Ub-fold and is isopeptide-linked to protein targets catalyzed by an E1-like enzyme [reviewed in (170)]. In bacteria, Ubl proteins including MoaD, ThiS, and CysO resemble Ub in their overall structure but only serve as sulfur carriers in various biosynthetic pathways (179-181). Prokaryotic ubiquitin-like protein (Pup) is the first bacterial protein modifier identified. In *Mycobacterium tuberculosis*, Pup functions in targeting proteins to proteasomes for degradation [reviwed in (182)]. Below, Urm1 in eukaryotes, SAMP from archaea, and Pup of actinobacteria are highlighted for discussion.
Ubiquitin-related Modifier 1 (Urm1)

Urm1 was discovered in *Saccharomyces cerevisiae* due to its sequence similarity with MoaD and ThiS, the known bacterial Ubl proteins that adopt a β-grasp fold and have a C-terminal diglycine motif (174). Later study revealed that the solution structure of Urm1 shares common features with proteins of the Ub/Ubl superfamily, especially for MoaD (183). The E1-like enzyme Uba4 was found to interact with Urm1 via a yeast two-hybrid screen using Urm1 as bait and was shown to be essential to form protein conjugation by Urm1 (174). In the ubiquitylation pathway, Ub is first adenylated by E1 and then forms an E1~Ub thioester intermediate in the activation step (72). However, a Urm1~Uba4 thioester has yet to be identified; instead, evidence is provided that the rhodanese domain of Uba4 can mediate the delivery of sulfur to the C terminus of Urm1 in vitro, leading to the formation of a thiocarboxylated Urm1 (184). Later work confirmed the presence of a Urm1 thiocarboxylate in vivo (185), and several independent studies demonstrated that Urm1 is activated by Uba4 to act as the sulfur carrier for the formation of 2-thiouridine in eukaryotic cells (175, 185, 186). Thus, the Urm1-mediated sulfur relay mechanism is reminiscent of that adopted by prokaryotic sulfur carriers such as MoaD. This evidence suggests Urm1 is a molecular fossil in the evolutionary link between eukaryotic Ubl proteins and prokaryotic sulfur carriers.

Compared with the clear role of Urm1 as a sulfur carrier for tRNA thiolation, its function as a protein modifier in the Urm1 conjugation pathway is less understood. Urm1 protein conjugation was first observed in 2000 (174). The C-terminal diglycine motif of Urm1 is required for protein conjugation (173, 174). Van der Veen et al. provided evidence that a thioester intermediate is formed in the Urm conjugation pathway, and demonstrated that a Urm1 thiocarboxylate (COSH) but not a Urm1...
carboxylate (COOH) is required for conjugate formation (173). The level of Urm1 conjugates can be dramatically increased by oxidative stresses (H_{2}O_{2} and diamide), and dozens of proteins are likely modified by Urm1 in mammalian cells based on MS/MS results (173). Interestingly, some proteins involved in the Urm1-dependent tRNA thialation pathway are potential urmylation substrates including MOCS3 (Uba4 and UbaA homologs in Homo sapiens and Hfx. volcanii, respectively) and ATPBD3 (Ncs6p and NcsA homologs in Homo sapiens and Hfx. volcanii, respectively) (173). Uba4, the E1 homolog, is the only enzyme that has been identified to be required in Urm1 conjugation pathway (173, 174, 187), and no Urm1-specific conjugating and ligating enzymes (E2, E3) have been identified. In addition, the enzyme that is responsible for cleavage of the Urm1 conjugates is not known. Future work is needed to determine the mechanism used to differentiate pathways between sulfur mobilization and Ubl protein modification and confer the substrate specificity.

**Small Archaeal Modifier Protein (SAMP)**

The Ubl protein SAMP was first discovered in archaea by study of Hfx. volcanii (176), and total three SAMPs (SAMP1/2/3) have been disclosed in this organism (188). Expression of SAMPs fused with an N-terminal Flag-tag in Hfx. volcanii has been found to form Ubl protein conjugates, which are resistant to boiling in the presence of reducing reagents and SDS, revealing the covalent bond formation (176). Similar to ubiquitylation, MS/MS analysis has demonstrated the formation of isopeptide linkages between the C-terminal glycine of SAMP and the side chain of lysine residues on various cellular proteins (176, 188, 189). Interestingly, evidence suggests both homogenous and heterogeneous poly-SAMP chains are generated in the cell (176, 188, 189). In addition, archaeal SAMPs resemble Ub in terms of the overall 3D structure
referred as a β-grasp fold (190, 191) and the C-terminal digycine motif required for Ubl protein conjugation (170). The process by which SAMPs are attached to protein substrates is termed as sampylation which is catalyzed by an E1-like enzyme in an ATP-dependent manner (192, 193). While E1 homologs are widespread in archaeal genomes, canonical E2 or E3 enzymes are not predicted based on primary sequence comparisons. Desampylation, a process in which SAMPs are released from Ubl protein conjugates through cleavage of the isopeptide bond, is catalyzed by HvJAMM1 in Hfx. volcanii, a homolog of the JAMM/MPN+ domain type DUBs (194, 195).

Our knowledge regarding the cellular function and physiological importance of the SAMP system in archaea are expanding. The level of sampylation has been found to be regulated by different environmental factors including nitrogen-limitation and mild oxidative stress conditions (176, 188, 189). In addition, sampylation is suggested to be associated with proteasomes-mediated proteolysis based on finding: (i) SAMP conjugates are increased in their abundance by genetic depletion or chemical inhibition of proteasome CPs (176, 188), (ii) free SAMP homologs can be degraded by CPs in vitro (22), and (iii) SAMP1 can weakly associat with an N-terminal peptide derived from PAN2 in vitro (196). Evidence also suggests SAMP and HvJAMM1 work together to control the availability of an enzyme active site (195). Furthermore, SAMP1/2 in Hfx. volcanii are proposed to serve as sulfur carriers to mediate sulfur relay in MoCo biosynthesis and tRNA thiolation, respectively (192). The detailed biochemical study of the E1-like enzyme UbaA in Hfx. volcanii provides new insights into key residues used for differentiating sulfur mobilization and/or Ubl protein modification in archaea (197). Unlike the essential role of Ub in cell viability, all three SAMPs can be deleted together
with the E1-like enzyme UbaA in *Hfx. volcanii* (192). Consistent with the proposed role of SAMP1 in MoCo biosynthesis, UbaA and SAMP1 are essential for anaerobic growth with DMSO as the terminal electron acceptor (192). Moreover, reduced cell growth is observed at high temperatures for SAMP2 and UbaA deletion strains (192), probably because of the inability to form the thermal stable tRNA by tRNA thiolation (198), a post-transcriptional modification required for cell growth at high temperatures (199). Overall, the SAMP system in archaea is thought to function in multiple cellular pathways.

**Prokaryotic Ubiquitin-like Protein (Pup)**

Pup is a small protein modifier identified in *M. tuberculosis* (200). Pupylation, a posttranslational modification by which the C-terminal glutamate of Pup is covalently attached to the lysine residue of substrate proteins, is present in *Actinobacteria* and *Nitrospirae* based on a comparative genomics study (201). In analogy to UPS mediated protein degradation, Pup serves as a signal to bring proteins to the proteasome for turnover in mycobacteria (200, 202). Two distinct features of Pup have been disclosed to explain the Pup-dependent protein destruction by proteasomes. First, the N-terminal region of Pup is essential for its recruitment by coiled-coil domains of *Mycobacteria* Mpa, the AAA-ATPase module, which promotes the unfolding and translocation of substrates into proteasome chamber for degradation (203, 204). In this regard, Pup is proposed to initiate the substrate degradation by engaging itself into the Mpa pore (203). Secondly, unstructured Pup undergoes a conformational change to form the α-helix structure upon binding with Mpa, probably for stabilizing the interaction between pupylated substrates and Mpa (57). To date, numerous proteins have been found to be modified by Pup based on proteomics studies (205, 206), and the Pup-proteasome system is linked to pathogenesis of *M. tuberculosis* (207). A recent study by Darwin and
coworkers discovered that Rv1205, an enzyme that catalyzes the production of cytokinins, is a pupylated proteasome substrate in M. tuberculosis (208). Accumulation of cytokinin breakdown products is proposed to cause Pup-proteasome mutant strains of M. tuberculosis to be sensitive to nitric oxide, a chemical produced by the host to inhibit growth of M. tuberculosis (208). Although pupylation is responsible for proteasome-mediated proteolysis, not all the pupylated substrates are targeted for destruction by proteasomes (205). For example, Mpa complexes can be disassembled into monomers upon Pup modification (209).

Pup is functionally analogous to Ub; however, pupylation is distinct from ubiquitylation in many aspects. Firstly, Pup forms an intrinsically disordered structure in its unbound state (210, 211), while Ub and other Ubl proteins adopt a β-grasp fold (172). Furthermore, Pup has an additional glutamine or glutamate residue after its C-terminal diglycine residues which is a common feature among Ub/Ubl proteins. The C-terminal glutamate residue is required for pupylation (200, 212). The deamidation of the C-terminal glutamine to glutamate residue is mediated by Dop (deamidase of Pup) with ATP as a cofactor (213). Besides the deamidase activity, Dop can function as a depupylase that cleaves the isopeptide bond between Pup and protein substrates, thus making pupylation a reversible modification (214, 215). Moreover, unlike ubiquitylation, which is mediated by cascade of enzymes (E1-E2-E3), pupylation is catalyzed by PafA (proteasome accessory factor A) after exposure of the Pup C-terminal glutamate (213). PafA had been known as a protein cofactor for proteasome-mediated proteolysis before the discovery of Pup (216), and a later study revealed PafA catalyzes pupylation by formation of a phosphorylated Pup intermediate with release of ADP (217). Interestingly,
Dop and PafA are close structural homologs with the active site located on the concave surface of a β-sheet (218). The crystal structure of Pup in complex with its ligase PafA discloses the binding-induced mechanism for pupylation activation by PafA (219).

**Methionine Sulfoxide Reducing System**

Cells are constantly threatened by (ROS) reactive oxygen species, which can be synthesized during cellular metabolism or can form upon exposure to oxidative agents like hydrogen peroxide and sodium hypochlorite, and extreme environmental conditions such as desiccation and ionizing radiation (220). ROS can cause damage to proteins, DNA, lipids as well as other biomolecules in both reversible and irreversible ways (221). Mildly oxidized proteins can be repaired by chaperone-assisted refolding and the enzyme-mediated repairing including methionine sulfoxide (MSO) reductases, protein L-isoaspartyl methyltransferases, and proline isomerases (222). The sulfur-containing amino acid residues (cysteine and methionine) are the primary targets for protein oxidation (223). Oxidation of cysteine residues usually leads to the formation of disulfide bonds which can be reduced by glutaredoxin or thioredoxin systems (224). Both free and protein-bound methionines can be oxidized to form a diastereomeric mixture of S- and R-forms of methionine sulfoxide (MSO). In order to deal with the methionine oxidation, cells have evolved different types of MSO reductase enzymes to reduce MSO back to methionine as residues of proteins and in free amino acid forms (225). Besides repair of oxidized proteins by specialized enzymes, redox controlled proteolysis serves as a mechanism of last resort to ensure the timely degradation of oxidized proteins that are beyond repair (222, 226). Below, I discuss all four methionine sulfoxide reductases (Msrs) including MsrA/B, fRMsr, BisC, and MsrPQ, their enzymatic mechanisms, as well as their functions and physiological importances.
Methionine Sulfoxide Reductase A/B (MsrA/B)

MsrA and MsrB, the most widespread types of Msrs, are present in most organisms across all domains of life, but are absent in many hyperthermophiles and some intracellular parasites (227, 228). MsrA is specific for reduction of free and protein-based S-form of MSO, whereas MsrB can only reduce the protein-based R-form of MSO (225). An MsrA enzyme from partially purified cell extracts was first discovered in 1981 with reduction activity for MSO residues in ribosomal protein L12 (229). The gene encoding MsrA was cloned form E. coli and mammalian cells in 1990s (230, 231), and purified MsrA was shown to have stereospecific activity for reduction of protein-bound methionine-S-sulfoxide and organic compounds containing the sulfoxide group such as DMSO (231, 232). Compared with MsrA, MsrB was identified much later. Two independent studies disclosed a new type of enzyme that differed from MsrA and was required for efficient reduction of for a mixture of the S- and R-forms of free MSO or MSO residues of calmodulin (233, 234). Later biochemical studies demonstrated MsrB is specific for reducing protein-based methionine-R-sulfoxide from different organisms (235, 236). Methionine oxidation results in the formation of a mixture of S- and R-forms of MSO; thus, MsrA and MsrB with different stereospecific enzyme activities need to work together to fully repair methionine oxidation. Consistent with this idea, msrA and msrB genes are often found within genomic neighborhoods (237), and an MsrA-MsrB fusion protein (MsrAB) is found in archaeon Thermococcus kodakaraensis (238). In addition, the number of msrA and msrB genes is variable among different organisms (225, 228). For example, mammalian cells encode one msrA and three msrB genes (225), while Staphylococcus aureus have three msrA genes and a single msrB gene (239).
Crystal structure of MsrA/B types of enzymes have been determined from different organisms across domains of life (240-242). Although the overall structures of MsrA and MsrB differ, they have a mirror-image active site (242), suggesting convergent evolution of these two enzymes. In addition, MsrB has two CxxC (x indicates any amino acid) motifs that are involved in zinc binding probably for a structural function (236). Consistent with the mirror-like relationship of MsrA/B at their active sites, MsrA and MsrB use a similar mechanism for reduction of MSO diastereomers. The general catalytic mechanism of MsrA/B includes three steps: (i) the catalytic Cys attacks the sulfoxide moiety of the substrate to generate Cys sulfenic acid with the concomitant release of methionine; (ii) the sulfenic acid intermediate is attacked by a recycling Cys in the active site to generate a disulfide bond between the catalytic and recycling Cys residues; (iii) reduction of the disulfide bond to free the catalytic Cys by the reducing power provided at the expense of NADPH through thioredoxin or glutaredoxin system in vivo, while high levels of dithiothreitol (DTT) can also be effective in vitro (243-245). MsrA can be further classified into different groups depending on how many recycling Cys residues are involved in the second step. For example, MsrA from E. coli has two recycling Cys residues in addition to the catalytic Cys (240), while M. tuberculosis MsrA only has a single resolving Cys to recycle the sulfenic acid intermediate (246). Interestingly, selenoprotein MsrA usually lacks the recycling Cys and shows very robust MSO reductase activity with its enzymatic mechanism still unclear (247).

MsrA/B enzymes play an important role in oxidative stress resistance and reversible regulation of protein activity [reviewed in (225)]. Numerous studies of different
organisms have shown that overexpression of MsrA/B type enzymes confer cells with high resistance to different types of oxidative agents and deletion of genes encoding these enzymes causes reduced cell viability under oxidative stress conditions (248-250). Furthermore, MsrA/B enzymes have been found to regulate the activities of various cellular proteins by reversible modification of methionine oxidation. For instance, HypT (hypochlorite-responsive transcription factor), a transcription factor require for protecting E. coli cells from hypochlorite, was found to be activated by methionine oxidation and inactivated by MsrA and MsrB (251). MsrA/B enzymes have also been implicated in the process of aging as well as some neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases (225). Consistent with the idea that Msrs function in delaying aging by scavenging ROS, MsrA/B type enzymes clearly regulate lifespan of different organisms by several independent studies (252-254). The oxidation-induced aggregation of protein is proposed to be one of the main reasons leading to neurodegenerative disorders, which is under the control by MsrA/B enzymes (255).

**Free Methionine-(R)-sulfoxide Reductase (fRMsr)**

Msrs that can specifically reduce the free form of methionine-\(R\)-sulfoxide were hypothesized to exist before the discovery of fRMsr based on the observations that MsrA/B double mutant E. coli strains still show apparent MSO reductase activity for free MSO (256). The fRMsr from E. coli was first discovered by Lowther and colleagues through the fractionation method followed by proteomic analysis, which represents the first protein belonging to GAF domain family to show enzymatic activity (257). Later comparative genomics suggested that fRMsr was present in many prokaryotes and unicellular eukaryotes (258). As implied by the nomenclature, fRMsr has specific activity for the free \(R\)-form of MSO and serves as the major enzyme responsible for reducing
free R-form of MSO in *S. cerevisiae* (257, 258). In *S. cerevisiae*, deletion of the gene encoding fRMsr results in enhanced sensitivity to oxidative stress, and overexpression of this enzyme leads to increased resistance to oxidative agents (258). All the Cys residues of fRMsr from *S. cerevisiae* have been comprehensively studied by in vivo complementation growth assay, through which Cys125 has been shown to be the catalytic residue (259). The general catalytic mechanism of fRMsr is proposed to be very similar to that of MsrA/B type enzymes with conformational changes at the active site based on biochemical studies and crystal structures of fRMsr in free and substrate-bound forms (260, 261).

**Biotin Sulfoxide Reductase (BisC)**

As implied in the name, BisC was first identified as a molybdenum cofactor-dependent biotin sulfoxide reductase that catalyzes the conversion of biotin sulfoxide to biotin (262). The *bisC* gene was cloned and sequenced from *E. coli* KS302 in 1990 (263). The *bisZ* gene encoding a BisC homolog has been found in *E. coli* K-12, and its deletion can fully abolish the background activity for reduction of biotin sulfoxide in *bisC* mutant strains (264). The BisC homolog from *Rhodobacter sphaeroides* has been found to reduce a variety of substrates such as free MSO and DMSO with higher activity than its reduction of biotin sulfoxide in vitro (265). BisC activity for reduction of MSO is supported by later genetic study, by which BisC was found to be required for methionine auxotroph cells lacking both MsrA and MsrB to grow when the S-form of MSO was provided as the sole Met source (266). In addition, BisC can only reduce the free form of the methionine-S-sulfoxide stereoisomer by biochemical study using purified enzyme (266).
Methionine Sulfoxide Reductase PQ (MsrPQ)

MsrPQ has been discovered very recently and represents a novel MSO reductase consisting of MsrP, a periplasmic molybdopterin-containing oxidoreductase, and MsrQ, a haem-binding membrane protein (267). The discovery of MsrPQ was initiated by the finding that a suppressor mutation of a gene encoding the kinase of the YedV/YedW two-component system can enable an E. coli Met auxotroph lacking all of the cytoplasmic Msrs to grow when MSO serves as the sole source of Met (267). Interestingly, YedY, an oxidoreductase reduces a variety of substrates such as DMSO and MSO in vitro (268), and YedZ, the putative membrane redox partner of YedY, are encoded in close vicinity to the suppressor mutation site (267), suggesting YedY and YedZ (now referred as MsrP and MsrQ respectively) are responsible for the suppressor phenotype. MsrPQ synthesis is induced by hypochlorous acid in E. coli (267), which is consistent with the previous finding that the MsrPQ homologs of Azospira suillum confer resistance to hypochlorous acid (269). Unique features of the MsrPQ enzymes are that MsrP can non-stereospecifically reduce MSO in both free and protein-bound forms, and MsrPQ seems to use electrons from the respiratory chain, not the typical thioredoxin system (267). Semi-quantitative proteomic analysis reveals dozens of potential MsrPQ substrates with confirmation of SurA, the major periplasmic chaperone, which is essential for cell resistance to hypochlorous acid (267). Furthermore, MsrPQ is suggested to play an important role in maintaining the envelope integrity under severe oxidative stress condition (267).

Project Rationales and Objectives

In this study, we aim to advance fundamental knowledge of targeted proteolysis in archaeal cell biology by examining the sanylation-proteasome system (SPS) (an
analog of the ubiquitin-proteasome system or UPS) and determining its associated degradation signals (degrons). All archaea encode functional proteasomes and the structure of archaeal proteasomes are highly related to those of eukaryotes (2, 8). In eukaryotes, the Ub serves as the tag to select protein substrates for targeted proteolysis by 26S proteasomes (1). Thus, one of the key open questions in archaeal cell biology is how are cellular proteins targeted to proteasomes for turnover. Interestingly, UPS gene homologs are widespread in archaeal genomes (270, 271), suggesting the existence of an ancient pathway for targeted proteolysis pathway in archaea. In particular, the discovery of the Ubl protein SAMP in Hfx. volcanii (176) and the finding that the level of sampylation is increased by genetic depletion and chemical inhibition of proteasomes (176, 188), suggests SAMP tags serve as signals for proteasome-mediated degradation in archaea. TATA binding protein 2 (TBP2), which was found to be modified by Ubl isopeptide bonds (176), served as the model substrate to determine the molecular mechanism of SPS-mediated proteolysis. TBP2 was examined for its steady-state protein and transcript levels and Ubl modification by use of biochemical, molecular biology, and proteomic techniques.

The second major objective of this study is to identify and characterize protein factors required for sampylation. Ubiquitylation is mediated by E1-E2-E3-type enzymes (272). In archaea, E1-type enzymes (e.g. UbaA) are demonstrated (188); however no E2 or E3 homologs are predicted in most archaea. In Hfx. volcanii, the E1-like enzyme UbaA has dual functions in protein conjugation and sulfur mobilization (192). Over 50 protein targets with diverse functions and structures are modified by the SAMPs in an UbaA-dependent manner under different environmental conditions (176, 188, 189). At
At this stage, the downstream mechanisms to differentiate pathways between sulfur mobilization and Ubl bond formation in archaea are not clear, and protein factors required for sAMPylation in response to diverse stresses are also not widespread in archaea. Thus, we aim to determine additional enzymes beyond the E1-like UbaA which may be involved in Ubl protein modifications induced by environmental stresses. In particular, our focus was on understanding the downstream components required for sAMPylation under mild oxidative stress. Previous study discovered that SAMP conjugates are stimulated by mild oxidants (188). The role of MsrA/B-type MSO reductases in Ubl modification of proteins was studied because both MsrA and MsrB are modified by SAMPs under mild oxidative stress (189) and the thalidomide binding domain of cereblon is a structural homolog of MsrB (273, 274). Cereblon functions in ubiquitylation as a flexible substrate presenting subunit of the many diverse DCAFs (DDB1 and CUL4-associated factors) that function with E3 cullin 4-RING ligase CRL4 complexes (273, 274).
Figure 1-1. Side and top views of crystal structure of 20S proteasome core particle (CP) from *Homo sapiens* (PDB: 5LE5) (275). The 20S CP is composed of four stacked heterohexameric rings to form a barrel-like structure. The two central rings formed by seven distinct β-subunits (β1-β7) contain the catalytic sites of 20S proteasome. The central gate of CP is created by the outer rings containing seven different α-subunits (α1-α7).
Figure 1-2. Protein ubiquitylation: its conjugation mechanism and function. Ubiquitin (Ub) is activated by an Ub-activating enzyme (E1) using ATP to form adenylated Ub, which is then linked to the active cysteine of E1 to form a thioester intermediate. Ub is next transferred to an Ub-conjugating enzyme (E2) by formation of a thioester bond with a cysteine of E2, and finally is covalently attached to protein substrates usually on the lysine residue under the help of Ub ligase (E3). Substrate selectivity depends on E3. Right panel describes the E3 catalytic mechanisms based on RING, HECT, and RBR domain E3. Generally, RING domain E3 assists the transfer of Ub from E2 to protein substrate, while HECT and RBR domain E3s mediate Ub transfer by forming a thioester intermediate. Ub conjugates can be cleaved by deubiquitinating enzymes (DUBs) to make ubiquitylation a reversible process. Different topologies of Ub conjugates are existed. Mono- and multi-ubiquitylation usually involves in non-proteolytic functions. In most cases, poly-ubiquitylation is a common signal for proteasome-mediated degradation, while K63-linked poly-Ub chain plays a non-proteolytic role. Right panel is based on (104).
CHAPTER 2
MATERIALS AND METHODS

Chemicals, Strains, and Culture Conditions

Chemicals and Reagents

Organic and inorganic chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Marietta, GA) and Bio-Rad Laboratories (Hercules, CA) unless otherwise indicated. Desalted oligonucleotides were bought from Integrated DNA Technologies (Coralville, IN). Phusion High Fidelity DNA Polymerase, restriction endonucleases and DNA-modifying enzymes were from New England BioLabs (Beverly, MA). QuikChange II XL Site-Directed Mutagenesis Kit was from Agilent Technologies (Santa Clara, CA). RNeasy Mini Kit, QuantiTect SYBR Green RT-PCR Kit, QIAprep Spin Miniprep Kit, MinElute PCR Purification Kit, the mouse anti-StrepII polyclonal antibody, and Strep-Tactin Superflow resin were from Qiagen (Hilden, Germany). TURBO DNA-free Kit and Zeba Spin Desalting Columns were from Thermo Fisher Scientific (Waltham, MA). The goat anti-mouse IgG (whole molecule)-alkaline phosphatase-linked antibody, the α-Flag M2 monoclonal antibody, α-Flag resin and Flag peptide were from Sigma-Aldrich. Strep-Tactin column and HisTrap HP column were from GE Healthcare (Chicago, IL).

Strains and Culture Conditions

Strains used in this study are summarized in Table 2-1. Escherichia coli TOP10 was used for cloning and E. coli GM2163 was used to produce plasmid DNA without methylation. E. coli strains were grown aerobically in Luria-Bertani medium at 37 °C or on plates [solid medium with 1.5% (w/v) agar]. Ampicillin (0.1 mg·ml⁻¹) and kanamycin (50 μg·ml⁻¹) were added as needed for culturing E. coli.
**Hfx. volcanii** strains were grown at 42 °C in ATCC974, glycerol minimal (GM), and casamino acids (CA) media as previously described (276). Cells were grown aerobically in liquid medium with rotary shaking at 200 rpm or on plates (solid medium with 2% (w/v) agar). Novobiocin (0.2 μg·ml⁻¹), uracil (50 μg·ml⁻¹), methionine (Met, 5 mM) or methionine sulfoxide (MSO, 5 mM) were added as needed for culturing *Hfx. volcanii* strains. Uracil was dissolved in 0.5 M sodium hydroxide (NaOH) at a final concentration of 50 μg·ml⁻¹. *Hfx. volcanii* cells were routinely streaked onto ATCC974 agar plates from -80 °C in 20% (v/v) glycerol stocks, and freshly isolated colonies were inoculated and grown to log phase in 3-4 ml ATCC974 medium for future analysis.

To monitor the effect of bortezomib (Fisher) on TBP2 abundance, log-phase cells were subcultured into 4.4 ml fresh ATCC974 medium and grown to log phase (OD₆₀₀ 0.5 to 0.7). Bortezomib [2.2 μl of 200 mM bortezomib stock dissolved in DMF at 99.8% (w/v)] and DMF solvent alone [2.2 μl DMF at 99.8% (w/v)] were added to log-phase cells (final concentrations at 100 μM bortezomib and 25 mM DMF). For regulation of synthesis of proteasomal CPs, log-phase cells were subcultured into 4.4 ml ATCC medium with 2.5 mM tryptophan and grown to log phase (OD₆₀₀ 0.5 to 0.7). Cells were harvested by centrifugation (10,000 × g, 6 min, 25 °C), washed twice with ATCC974 medium, and resuspended in 4.4 ml fresh ATCC974 medium with or without 2.5 mM tryptophan.

To monitor DMSO-stimulated Ubl protein modifications, log-phase cells were subcultured into fresh ATCC974 medium (4 ml) supplemented with 0.18-0.7% (v/v) or 25-100 mM DMSO and grown to stationary phase (OD₆₀₀ 2.0-3.0). Similar methods were used for treatment of cells with DMS (25 mM) and DMSO₂ (25 mM). To monitor
MSO-stimulated sampylation, log-phase cells were washed with GM and inoculated (0.1 ml) into fresh GM medium (4 ml) for growth to log-phase (OD$_{600}$ 0.4-0.6). The log-phase cells were subcultured into 4 ml of fresh GM medium with or without 25 mM MSO and grown to stationary phase (OD$_{600}$ 1.2-1.5). To monitor NaOCl-stimulated sampylation, log-phase cells were subcultured into 4 ml fresh ATCC974 medium and grown to log phase (OD$_{600}$ 0.4 to 0.6). NaOCl (Sigma) was then added to the log-phase cells (final concentrations at 8 mM) for 1 hour treatment with shanking (200 rpm) at 42 °C.

For growth assay for methionine auxotroph strains, log-phase cells were washed with GM and inoculated (0.1 ml) into fresh GM medium (4 ml) for growth to log-phase (OD$_{600}$ 0.4-0.6). The log-phase cells were subcultured into 4 ml of fresh GM medium supplemented with 5 mM Met or MSO. Cell density was monitored after 120 hours at OD$_{600}$.

For survival assays under oxidative stress conditions, log-phase cells were subcultured into 4 ml of fresh ATCC974 medium and grown to early log-phase (OD$_{600}$ 0.4-0.6). Cultures were supplemented with or without potent oxidizing agents (5-13 mM H$_2$O$_2$ and 16-20 mM NaOCl) (Sigma) for 30 min. Cells were diluted to 0.04 OD$_{600}$ and spot plated in 10-fold serial dilution onto ATCC974 agar medium for 5 days.

The culture conditions of _Hfx. volcanii_ cells to generate markerless gene deletion mutants were similar as previously described (277). CA medium supplemented with 2% (w/v) agar was used to select _Hfx. volcanii_ uracil prototrophs with the non-replicating deletion plasmids integrated onto the chromosome. Cells for counter-selection were grown in ATCC974 liquid medium before plated on ATCC974 plates supplemented with 100 μg·ml$^{-1}$ 5-fluoroorotic acid (5-FOA). 5-FOA was dissolved in 100% (v/v) DMSO.
DNA Techniques

Cloning

The plasmids constructed by cloning in this study are listed in Table 2-2. The genes were amplified by PCR by using Phusion® High Fidelity DNA Polymerase (New England Biolabs). The primers used in this study to amplify target genes by PCR are listed in Table 2-3. PCR-amplified products were digested by appropriate restriction endonucleases (New England Biolabs), purified by MinElute PCR Purification Kit (Qiagen), and ligated by T4 DNA ligase (New England Biolabs) according to manufacturer’s protocols. Alternatively, sequence- and ligation-independent cloning (SLIC) was utilized for cloning as previously described (278). The SLIC and ligation products were routinely transformed into *E. coli* TOP10 chemically competent cells.

Site-directed Mutagenesis

Site-directed mutagenesis (SDM) was performed using a QuikChange II XL Site-Directed Mutagenesis Kit according to the supplier (Agilent Technologies). Primers used in SDM are listed in Table 2-3.

DNA Electrophoresis

PCR products, RNA, and plasmids were analyzed by electrophoresis (100 V, 30-35 min) using 0.8-2% (w/v) agarose gels with ethidium bromide (0.25 μg·ml⁻¹) in TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5). Hi-Lo DNA molecular weight markers served as the standards (Minnesota Molecular, Minneapolis, MN). DNA signals within gels were visualized by a Mini visionary imaging system (FOTODYNE, Hartland, WI).
Sanger Sequencing

The fidelity of PCR-generated products was confirmed by Sanger automated DNA sequencing using an Applied Biosystems model 3130 genetic analyzer (ICBR Genomics Division, University of Florida).

Plasmid Extraction and Transformation

Plasmids were isolated using QIAprep Spin Miniprep kit according to the supplier’s protocols (Qiagen). As for *Hfx. volcanii* transformations, plasmid DNA was purified from *E. coli* GM2163 and transformed into *Hfx. volcanii* competent cells as previously described (279).

Generation of Markerless Deletion Mutants

Markerless deletion mutants were generated by using established *pyrE2*-based pop-in/pop-out method developed for *Hfx. volcanii* (277, 280). The PCR products of the target gene with its ~500 bp downstream and upstream region were ligated with pTA131 to generate the pre-deletion plasmids. Inverse PCR was used to remove the coding region of the genes (start to stop codons) by using pre-deletion plasmids as the templates. The inverse PCR products were self-ligated by using T4 DNA ligase (New England Biolabs) to generate the deletion plasmids. *Hfx. volcanii* cells were transformed with the deletion plasmid and plated on CA medium agar plates without uracil. Colonies growing on CA medium were patched onto CA agar medium for PCR screening to select cells that successfully incorporated the plasmid onto the chromosome. The confirmed pop-in cells were inoculated into ATCC974 medium, grown to log-phase (OD$_{600}$ 0.4-0.6) and plated using a serial dilution on ATCC974 agar plates supplemented with 5-FOA (100 μg·ml$^{-1}$). Markerless deletion mutants were identified by
PCR screening using primers specific for the target gene and ~700 bp upstream and downstream region of the gene.

**RNA Techniques**

**RNA Isolation**

Total RNA was isolated from *Hfx. volcanii* cells by using an RNeasy Mini Kit according to the supplier (Qiagen). DNA was removed by using a TURBO DNA-free Kit according to the recommendations of the supplier (Thermo Fisher Scientific). The level of contaminating DNA after TURBO DNase digestion was below the detection limit of electrophoresis after PCR amplification. RNA concentration was determined by using a Nanovue Plus Spectrometer instrument (GE Healthcare Life Sciences, Uppsala, Sweden). The integrity of RNA was determined by 2% (w/v) agarose gel electrophoresis as mentioned above.

**qRT-PCR**

Primers used for qRT-PCR with PCR efficiency between 95% and 105% are listed in Table 2-3. 100 ng RNA served as the template per reaction (25 μl). One-step quantitative reverse transcriptase PCR (qRT-PCR) was performed using QuantiTect SYBR Green RT-PCR Kit following the protocol described in the handbook of the supplier (Qiagen). One-step qRT-PCR was performed under conditions of 50°C for 30 min; 95°C for 15 min; and 40 cycles at 94°C for 15 s, 51°C for 30 s, and 72°C for 30 s, followed by determination of the melting curve by using a CFX96 real-time C1000 thermal cycler (Bio-Rad). A single peak revealed by the melting curve indicated a single product. The TBP2 mRNA levels were normalized to the internal standard *ribL* based on a previous study (281). A standard curve was generated by using a QuantiTect SYBR green PCR kit (Qiagen) following the manufacturer’s protocol. Genomic DNA and
pJAM2201 served as the template to test ribL and tbp2 primers for PCR efficiency respectively.

**Protein Techniques**

**Whole Cell Lysate Preparation**

Cells were harvested by centrifugation (10,000 × g, 6 min, 25 °C). Cell pellets were suspended in lysis buffer [50 mM Tris-HCl, pH 6.8, 2 M urea, 2% (w/v) SDS, and 0.1 mg·ml⁻¹ EDTA-free protease inhibitor cocktail (Roche)] with glass beads (Chemglass Life Sciences). Cells were lysed with glass disruptor beads (0.1 mm dia.) by vigorous vortexing for 5-7 times (1 min each time with 1 min incubation on ice between vortexings). Cell debris and glass beads were removed by centrifugation (14,000 × g, 20 min, 4 °C), and supernatants were transferred into 1.5 ml Eppendorf tubes on ice. Protein concentration of cell lysate was measured by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockville, IL) according to the supplier using bovine serum albumin (BSA, Thermo Scientific) as the protein standard. Cell lysates were mixed with equal volume of SDS reducing buffer [100 mM Tris-Cl buffer at pH 6.8 with 4% (w/v) SDS, 20% (v/v) glycerol, 0.6 mg·ml⁻¹ bromophenol blue, and 5% (v/v) β-mercaptoethanol] and boiled for 10 min. Equivalent protein amount determined by BCA assay was separated by SDS-PAGE (12%) for each lane. For rapid lysis purpose, the harvested cells were resuspended in SDS reducing buffer and boiled 3 times (5 min each time with 30 sec vortex between each time). Equivalent protein loading was determined by OD₆₀₀ of cell culture (0.08 units per lane) and confirmed by staining parallel gels with Coomassie blue. Samples were immediately separated by SDS-PAGE or stored at 4 °C until needed.
Two-Dimensional Gel Electrophoresis

Proteins were extracted using a Trizol-based method according to a protocol described previously (282) followed by acetone precipitation. Protein pellets were solubilized in 2D-sample buffer [8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.2% (w/v) SDS, and 10 mM Tris-Cl, pH 8.5]. Protein concentration was measured using EZQ Protein Quantitation kit according to manufacturer’s instructions (ThermoFisher Scientific). Isoelectrofocusing (IEF) was carried out in an IPGphor3 unit (GE Healthcare) on 18 cm immobilized pH gradient (IPG) strips, pH range 3.5-4.5, using a cup-loading method to load the sample (GE Healthcare). The following conditions were used for IEF: initial voltage was set at 300 V for 300 Vh, ramped up to 1000 V in 1000 Vh, ramped up to 10000 V in 20,000 Vh, and finally held and focused at 10000 V for 60,000 Vh until the current reached steady state at around 20 mA. The strip was mounted onto a 20 x 20 cm 8-16% polyacrylamide Tris-glycine gel (Jule Biotechnologies INC) after IEF. Electrophoresis was carried out at 12°C as follows: 10 mA/gel for 1 h and then overnight at a constant current of 12 mA/gel with a limit of 150 V until the dye reached to the bottom of the plate. Immediately after gel electrophoresis, the gel was transferred onto a PDVF membrane for IB as described below.

Immunoblotting

Proteins were separated by 12% SDS–PAGE and electroblotted onto PVDF membranes (Amersham) using transfer buffer (25 mM 2-[N-morpholino]ethanesulfonic acid [MES] buffer, pH 6.0 with 10% (v/v) methanol) at either 30 V for 800 min or 90 V for 180 min at 4 °C. Membranes after transfer were washed 3 times (5 min each time) by Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) supplemented with 0.05% (v/v) Tween-20 and then incubated with TBS with 0.05% (v/v) Tween-20 and 5%
(w/v) dry non-fat milk for 2h at room temperature or 12-16 h at 4 °C with rocking. StrepII affinity tagged proteins were detected by mouse anti-StrepII polyclonal antibody (Qiagen) followed by goat anti-mouse IgG (whole molecule)-alkaline phosphatase-linked antibody (Sigma). N-terminal His tagged proteins were detected by mouse anti-His polyclonal antibody (GE Healthcare) followed by goat anti-mouse IgG (whole molecule)-alkaline phosphatase-linked antibody (Sigma). Flag affinity tagged proteins were detected by alkaline phosphatase-linked anti-Flag M2 monoclonal antibody (Sigma). Targeted proteins on PVDF were visualized by chemiluminescence using CDP-Star (Applied Biosystems) with X-ray film (Hyperfilm; Amersham Biosciences). The intensity of protein bands was quantified by Image as previously described (283).

**Chase Assay**

*Hfx. volcanii* cells were aerobically grown in 4 ml ATCC974 medium to early log phase, subcultured into 5.1 ml of fresh ATCC974 medium, and grown to log phase (OD<sub>600</sub> 0.4-0.7). Cells were harvested by centrifugation (10,000 × g, 6 min, 25 °C), suspended in 4.2 ml concentrated salt water stock solution (4.1 M NaCl, 150 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 140 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 100 mM KCl, and 20 mM Tris-Cl, pH7.5) and treated with 20 μg·ml<sup>-1</sup> actinomycin D (Sigma) and 50 μg·ml<sup>-1</sup> anisomycin (Sigma). Cells were harvested by centrifugation (10,000 × g, 6 min, 4 °C) at various intervals (0 to 2 h). Cell pellets were frozen at -80 °C before analyzed by immunoblotting (IB).

**Streptactin Affinity Chromatography**

For purification of TBP2-StrepII, *Hfx. volcanii* cells (wt or SPS mutants) expressing pJAM2201 were grown to stationary phase in 4 x 1-liter ATCC974 medium at 42 °C. For purification of MsrA-StrepII from *Hfx. volcanii*, LR03 (Δsamp1 Δsamp2 Δsamp3 ΔmsrA ΔubaA) expressing pJAM3010 was grown to stationary phase in 4 x 1-
liter ATCC974 medium at 42 °C. For purification of MsrA-StrepII from E. coli, Rosetta (DE3) freshly transformed with expressing plasmid [pJAM3200 (MsrA wt) or pJAM2273 (MsrA C13S) or pJAM2284 (MsrA E56A)] was grown in 500 mL LB medium with Km at 37°C. MsrA expression was induced in the log phase (OD$_{600}$ of 0.6–0.8) by addition of 0.4 mM IPTG, and cultures were shifted to 25°C for 12 h prior to harvest. All cell types were harvested by centrifugation and lysed by French Press 4-6 times (24,000 psi) in lysis buffer [50 mM Tris-$\text{Cl}$, pH 7.4, 2 M NaCl, 1 mg·ml$^{-1}$ EDTA-free protease inhibitor cocktail (Roche)]. The resulting cell lysate was clarified by centrifugation and filtration (0.45 μm) prior to application to a Strep-Tactin column (1 ml bed volume, GE Healthcare) at a flow rate of 0.5 ml/min. Unbound proteins were removed by washing the column with 140 ml lysis buffer at a flow rate of 1.2 ml·min$^{-1}$, and MsrA proteins were eluted in Tris-$\text{Cl}$ buffer (2 M NaCl, 50 mM Tris-$\text{Cl}$, pH 7.4) supplemented with 5 mM d-desthiobiotin.

**Nickel Affinity Chromatography**

For purification of His$_6$-UbaA, Hfx. volcanii cells expressing pJAM1209 were grown to stationary phase in 4 x 1-liter ATCC974 medium. For purification of Flag-His$_6$-SAMP2 and His$_6$-HvJAMM1, E. coli Rosetta (DE3) carrying plasmid pJAM1132 or pJAM991 was grown to log phase (OD$_{600}$ of 0.6–0.8) in 500 mL LB medium with Km at 37°C. Protein expression was induced by addition of 0.4 mM IPTG, and cultures were shifted to 25°C for 12 h prior to harvest. All cell types were harvested by centrifugation and lysed by French Press 4-6 times (24,000 psi) in lysis buffer (50 mM Tris-$\text{Cl}$, pH 7.4, 2 M NaCl, 40 mM imidazole). The resulting cell lysate was clarified by centrifugation and filtration (0.45 μm) prior to application to a HisTrap HP column (5 ml bed volume, GE Healthcare) at a flow rate of 1 ml·min$^{-1}$. Unbound proteins were removed by washing the
column with 140 ml lysis buffer at a flow rate of 1.2 ml⋅min$^{-1}$, and His$_6$ tagged proteins were eluted in lysis bufer with 500 mM imidazole.

**Gel Filtration Chromatography**

Proteins purified by affinity chromatography (0.5 ml per run) were concentrated by Ultracel-0.5 centrifugal filtration (Millipore) and further applied to a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in 50 mM Tris-Cl, pH 7.5 buffer supplemented with 2 M NaCl at a flow rate of 0.3 ml⋅min$^{-1}$. Elution of protein was monitored by UV absorbance at 280 nm.

**Pull-down Assay**

For the StreplI pull-down assay, wt and SPS deletion strains carrying plasmid pJAM2201 were aerobically grown in 4 ml ATCC974 medium to early log phase. Cells were subcultured into 50 ml ATCC974 medium (in 250 ml baffled flasks at 42 °C, 200 rpm) to stationary phase. Cell pellets were harvested, resuspended in lysis buffer [2 M NaCl, 50 mM Tris-Cl, pH 7.4, 1 mg⋅ml$^{-1}$ EDTA-free protease inhibitor cocktail (Roche)], and lysed by French Press 4-6 times (24000 psi). Cell debris was removed by centrifugation and equivalent amount of cell lysate as determined by BCA protein assay was applied to 100 μl Strep-Tactin Superflow resin (QIagen) equilibrated in Tris-Cl buffer (2 M NaCl, 50 mM Tris-Cl, pH 7.4). Nonspecific proteins were removed by washing Strep-Tactin resin with 40 column volumes of lysis buffer. Bound proteins were eluted from the resin by addition of 30 μl of 5 mM $d$-desthiobiotin dissolved in Tris-Cl buffer. Elution fractions were mixed with equal volume of SDS reducing buffer and analyzed by IB.

SAMP2 modified TBP2 was enriched by Streptactin affinity purification followed by α-Flag immunoprecipitation (IP), wt and SPS deletion strains (ΔubaA and
Δpan1Δpan2) expressing plasmid pJAM2201 and wt cells expressing pJAM202c were grown to stationary phase in 2 × 1-liter ATCC974 medium at 42 °C. After cell lysis, an equivalent amount of total cellular proteins as determined by BCA protein was applied to a Strep-Tactin column as described above. A total of 3-ml pooled fractions of eluted proteins were subsequently used for IP by anti-Flag M2 affinity gel according to the supplier (Sigma). SAMP2-TBP2 conjugates were eluted in Tris-Cl buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4) supplemented with 150 ng·µl⁻¹ Flag peptide (Sigma) and analyzed by IB.

In vitro Reconstitution of MsrA-dependent Ubl-bond Formation

Cell lysate for assay was from Hfx. volcanii cells that expressed N-terminal Flag tagged (Flag-) Ubl SAMPs. Flag-SAMP1 was ectopically expressed from plasmid pJAM947 in Hfx. volcanii LR02 (Δsamp1 Δsamp2 Δsamp3 ΔmsrA) or XF124 (Δsamp1 Δsamp2 Δsamp3 ΔmsrA ΔmoaE). Flag-SAMP2 was ectopically expressed from plasmid pJAM949 in Hfx. volcanii LR02 or LR03 (Δsamp1 Δsamp2 Δsamp3 ΔmsrA ΔubaA). Cells (0.5-l) were grown to stationary phase in ATCC974 medium supplemented with Nv and 25 mM DMSO as needed. Cells were harvested by centrifugation for 10 min (3500 × g, 15 °C), suspended in 8 ml lysis buffer [2 M NaCl, 50 mM Tris-Cl, pH 7.5, 30 µg·ml⁻¹ DNase I from bovine pancreas (Sigma), 10 mM Mg²⁺, 100 µM bortezomib (LC Laboratories)] and lysed by French press (24000 psi). Cell debris was removed by centrifugation and filtration (0.2 µm). The supernatant composed of cell lysate was dialyzed against Tris-salt buffer (2 M NaCl, 50 mM Tris-Cl, pH 7.5) 3 times at 4 °C and used as source of the substrate (Flag-SAMP1/2, protein targets, and other enzyme components) for in vitro reconstitution of Ubl protein modifications. Reaction mixtures (100 µl total) consisted of 80 µl cell lysate and 5 µM purified MsrA-StrepII, 10 µM Flag-
His-SAMP2, 5 µM His-UbaA in assay buffer (4 mM ATP, 25 mM DMSO, 0.5 mM DTT, 2 M NaCl, 50 mM Tris-Cl, pH 7.5). To study the effect of different chemical agents on stimulation of Ubl-conjugation, buffer was supplemented with 4 mM ATP and 25 mM DMSO or 25 mM DMSO-related compounds including DMS, DMSO$_2$, and MSO as indicated. To study the effect of different nucleotide ligand(s) on \textit{in vitro} reconstitution for Ubl protein modifications, the buffer was supplemented with 25 mM DMSO and 4 mM nucleotide ligand(s) including ATP, ADP, AMP, and AMP-PNP. Reactions were incubated at 45 °C for 0 to 18 h. Cell lysate was incubated with protease inhibitors including 2 mM bortezomib, 2 mM PMSF, 10 mM ETDA, or 0.2 mg·ml$^{-1}$ EDTA-free protease inhibitor cocktail (Roche) to assess unbound SAMP hydrolysis. After assay, salts were removed from the reaction mixtures using Zeba Spin Desalting Columns (7K MWCO) according to the supplier (Thermo Scientific). Desalted samples (40 µL) were mixed with equal volume of SDS-PAGE loading buffer and boiled for 10 min prior to separation by 12% SDS-PAGE and analysis by anti-Flag, anti-StrepII, and anti-N-terminal-His IB.

\textbf{MSO Reductase Assay}

Methionine sulfoxide reductase activity of MsrA and cell extract was assayed by Jackob Moskovitz as previously described (284). In brief, reactions (100 µl) containing 1 µg pure enzyme or 400 µg cell extract, 200 µM dabsyl-MSO, 20 mM DTT, and 25 mM Tris-Cl, pH 7.5 were incubated for 30 min at 37°C. Protein concentration was measured by Bradford reagent (Bio-Rad), using BSA as the standard. Following the incubation period, the samples were treated with equal volume of acetonitrile and subjected to C-18 reverse phase chromatography using HPLC. The peak of dabsyl-Met was quantified and the specific activity was calculated accordingly. Cell extract for assay was prepared
(described as above) from *Hfx. volcanii* strains grown to stationary phase in ATCC medium with and without 100 mM DMSO and stored at -80 °C before enzyme activity measurement. One unit of activity is defined as 1 nmole dabsyl-Met generated per min at 37°C.

**Mass Spectrometry**

**Determining Protein Mass by ESI-TOF**

Purified TBP2-StrepII was dialyzed against deionized water 6 times for 2-4 h at 4 °C by use of mini dialysis tubing (1 kDa MW cut-off, GE Healthcare). Protein samples were analyzed for accurate mass by ESI mass spectrometry. Samples were treated with 50% (v/v) acetonitrile and 1% (v/v) formic acid and immediately loaded into an Agilent 6210 time-of-flight mass spectrometer (Agilent Technologies, Inc., Santa Clara, California) with electrospray ionization source in the positive mode. Signals were analyzed using Analyst QS software (AB Sciex Inc., CA).

**Mapping Phosphorylation Sites by MS/MS**

TBP2 purified by Strep-Tactin chromatography was separated by 12% SDS-PAGE and stained with Bio-Safe Coomassie (Bio-Rad). After destaining in double deionized water by 5 times, protein bands were excised from the gel, washed with water, and destained with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate buffer. Proteins in gel were reduced with DTT and alkylated with iodoacetamide similarly to what previously described (285). Samples were digested with chymotrypsin overnight or double digested with endoproteinase GluC (*Staphylococcus aureus* protease V8) overnight followed by 4 h incubation with trypsin at 37°C. Peptides were extracted with a mixture of 70% (v/v) acetonitrile and 0.1% (w/v) trifluoroacetic acid and lyophilized.
Phosphopeptides were enriched by TiO$_2$ NuTip micro columns (GlygenSci, Columbia MD) according to the method described previously (286). The lyophilized peptides (enriched and flow through) were solubilized in 10 μl of loading buffer [3% (v/v) acetonitrile, 0.1% (v/v) acetic acid and 0.01% (v/v) trifluoroacetic acid] and loaded onto a C18 capillary trap cartridge (LC Packings, USA). Peptides were separated on a 15 cm nanoflow analytical C18 column (PepMap 75 m id, 3 m, 100 Å) at a flow rate of 300 nl·min$^{-1}$ using a nanoLC ultra 1D plus system (ABsciex, USA). Solvent A composition was 3% (v/v) acetonitrile and 0.1% (v/v) acetic acid; whereas solvent B was 97% (v/v) acetonitrile and 0.1% (v/v) acetic acid. Peptide separation was performed using a linear gradient from 3-40% of solvent B for 20 min, followed by an increase to 90% of solvent B in 5 min and hold for 5 min. The flow was directly sprayed into an LTQ Orbitrap-XL mass spectrometer (ThermoFisher, Bremen, Germany). MS2 spectra were acquired in a data-dependent mode. An Orbitrap full MS scan (resolution: 3 X 10$^4$, mass range 400 to 1,800 Da) was followed by 10 MS$^2$ scans in the ion trap, which was performed by collision induced dissociation on the top 10 most abundant ions. The isolation window for ion selection was 3 Da. Normalized collision energy was set at 28%. Dynamic exclusion time was 20 s based on a previous study (287). Additionally, if a phosphate neutral loss of 98, 49, 32.66 and 24.5 m/z below the precursor ion mass was detected, an additional activation was included in the analysis. This Multi Stage Activation (MSA) event was repeated for the top five ions in a data-dependent manner provided the precursor exceeded a threshold of 500 ion counts (288). An inclusion list of the peptides for the TBP2 N-terminus was used in the method.
All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the database containing total proteome of *Hfx. volcanii* DS2 and TBP2-StrepII (8087 entries) assuming the digestion enzyme: 1) chymotrypsin or 2) Glu-C and trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Gln to pyro-Glu of the N-terminus, deamidation of Asn and Gln, oxidation of Met and phosphorylation of Ser, Thr and Tyr were specified in Mascot as variable modifications. Scaffold (v. 4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (289).
Table 2-1. List of strains used in this study.

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<th>Strains</th>
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^a JAMM1, HVO_2505; JAMM2, HVO_1016; PAN1, HVO_0850; PAN2, HVO_1957; PsmB, HVO_1562; Cdc48b, HVO_2700; Cdc48c, HVO_1327; SAMP1, HVO_2619; SAMP2, HVO_0202; SAMP3, HVO_2177; UbaA, HVO_0558; MoaE, HVO_1864; MsrA, HVO_A0230; MsrB, HVO_2234; MetE1, HVO_2742; MetE2, HVO_2743.
Table 2-2. List of plasmids used in this study.

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<td>(197)</td>
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<td>pET24b</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;, IPTG induced expression plasmid</td>
<td>Novagen</td>
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<td>pJAM2273</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;, pET24b carries msrA&lt;sub&gt;C13S&lt;/sub&gt;-streplII</td>
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<td>pJAM2284</td>
<td>Km&lt;sup&gt;a&lt;/sup&gt;, pET24b carries msrA&lt;sub&gt;E54A&lt;/sub&gt;-streplII</td>
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<td>pJAM1132</td>
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<td>(197)</td>
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<td>(194)</td>
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<sup>a</sup>Ap, ampicillin resistance; Nv, novobiocin resistance; Tet, tetracycline resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance. TBP2, HVO_1727; SAMP1, HVO_2619; SAMP2, HVO_0202; SAMP3, HVO_2177; UbaA, HVO_0558; JAMM1, HVO_2505; MoaE, HVO_1864; MsrA, HVO_A0230; MsrB, HVO_2234; MetE1, HVO_2742; MetE2, HVO_2743.
Table 2-3. Primers used in the study.

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<th>Primer Pair&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>TBP2 KpnI RV</td>
<td>5'-gggtaccGCGAGCAATGCGCAGTCCA AG -3'</td>
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<td>TBP2 S2A inv FW</td>
<td>5'-GGAGATATACATATGCGCAGACAGTCC-3'</td>
<td>TBP2 S2A SDM</td>
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<td>TBP2 S2A inv RV</td>
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<td>TBP2 S2E inv FW</td>
<td>5'-GGAGATATACATATGCGCAGACAGTCC-3'</td>
<td>TBP2 S2E SDM</td>
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<td>TBP2 S2E inv RV</td>
<td>5'-GGAGTCTCGCAGCCGATATGTATATCTCC-3'</td>
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<tr>
<td>MsrA NdeI FW</td>
<td>5'-aacatagtGAAGCGCAGACAGCTCGGACGTT-3'</td>
<td>amplify msa coding region</td>
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<td>MsrA inverse FW</td>
<td>5'-CCCTGCTGAGGGCGGCGTCG-3'</td>
<td>inverse PCR to generate msa pre-knockout plasmid</td>
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<td>MsrA inverse RV</td>
<td>5'-AGCGCTCTTCCGACGCTCC-3'</td>
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<tr>
<td>MsrA-700 FW</td>
<td>5'-GGCTCAAGAGGGGCGGCGGGA-3'</td>
<td>confirm ΔmsrA mutants by annealing 700 bp 5' and 3' of msa</td>
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<tr>
<td>MsrA-700 RV</td>
<td>5'-CGGTGCTCGCGAGACGCTCCGACGCGC-3'</td>
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<tr>
<td>MsrB NdeI FW</td>
<td>5'-aacatagtAGGAGCGCAGACGACTCCGACGTT-3'</td>
<td>amplify msa coding region</td>
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<td>MsrB KpnI RV</td>
<td>5'-ttagtaccGGGTCGCGACTCCGACGCTCC-3'</td>
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<td>MsrB-500 up FW</td>
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Table 2-3. Continued.

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<td>MsrA C48S SDM</td>
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<td>MsrA C48S RV</td>
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<tr>
<td></td>
<td>G-3’</td>
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</tr>
<tr>
<td>MsrA C162S FW</td>
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<td>MsrA E56A RV</td>
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<tr>
<td>MoaE-700 FW</td>
<td>5’-CAGCCCGCTATAGACGAGCAGCGG-3’</td>
<td>confirm ΔmoaE mutants by annealing 700 bp 5’ and 3’ of moaE</td>
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<td>MoaE-700 RV</td>
<td>5’-AGTCGCTGCTTCGGTTCTCCGCG-3’</td>
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<td>MetE inverse FW</td>
<td>5’-GGACGACGCCGCGCCGCGTTTC-3’</td>
<td>inverse PCR to generate 500 bp flanking 5’ and 3’ of metE&lt;sub&gt;1/2&lt;/sub&gt;</td>
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<td>MetE inverse RV</td>
<td>5’-CACCTCTCAGATAAGAAATGACGGGTATGATAATATTCTCGT-3’</td>
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<td>MetE-573 FW</td>
<td>5’-AGGAATTCGATATCCAGTGCAGCCGTCGCTCGG-3’</td>
<td>Confirm ΔmetE&lt;sub&gt;1/2&lt;/sub&gt; mutant and generate the pre-knockout plasmid.</td>
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<td>MetE-537 RV</td>
<td>5’-CGGTATACGATAAGCTGCCCCGCCGCCCACCACCCGCG-3’</td>
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<sup>a</sup>TBP2, HVO_1727; RibL, HVO_0484; MsrA, HVO_A2030; MsrB, HVO_2234; MoaE, HVO_1864; MetE1, HVO_2742; MetE2_2743.

<sup>b</sup>Oligonucleotide sequences introduced in primers for cloning are in lowercase with sites for restriction enzyme cleavage underlined and sites for site-directed mutagenesis italicized.
CHAPTER 3
UBIQUITIN-LIKE PROTEASOME SYSTEM REPRESENTS A EUKARYOTIC-LIKE PATHWAY FOR TARGETED PROTEOLYSIS IN ARCHAEA

Introduction

Highly selective and targeted protein turnover plays a pivotal role in intracellular homeostasis, signaling, transcription regulation, and protein quality control (72, 73, 293). In eukaryotes, the specificity and selectivity of proteolysis are mainly conferred by ubiquitylation, a post-translational modification by which Ub is covalently attached to target proteins by E1-E2-E3-type enzymes (272). Selective protein turnover often requires degradation signals or “degrons” in protein substrates. Degrons of protein substrates include: biosynthetic errors or misfolding (294), specific amino acid regions (154), reversible post-translational modifications like phosphorylation (158), co-translational modifications like Nα-acetylation (149), and N-terminal residue identity (146, 148). In eukaryotes, degrons are frequently recognized by the ubiquitylation system resulting in the formation of poly-Ub chains which serve as the signal for proteolysis by 26S proteasomes.

The 26S proteasomes are multicatalytic, energy-dependent proteases that process substrates of the UPS for cellular proteostasis in eukaryotes. These enzymes are nanocompartmentalized and consist of 19S RPs and 20S CPs harboring the proteolytic active sites (295). CPs are formed from four stacked heptameric rings of α- and β-subunits and are conserved in all three domains of life (8). Protein degradation by proteasomes is proposed to be initiated by unstructured regions on substrates (296).

Efficient protein degradation by CPs is assisted by AAA+ ATPases including the Rpt1–6 subunits of RPs in eukaryotes and the related archaeal PANs, which unfold protein substrates, stimulate the opening of CPs, and translocate unfolded substrates into the CP channel for proteolysis (34, 58, 297). One key feature of UPS mediated degradation is removal of the poly-Ub on protein substrates by a DUB (the Rpn11 subunit of 26S proteasomes), prior or coincident with translocation of unfolded substrates (40, 41, 132).

Homologs related to UPS are widely distributed across all the archaeal genomes, suggesting the existence of an ancient pathway for targeted proteolysis in archaea. Ubl SAMPs were discovered in archaea by study of *Hfx. volcanii* (176, 188). SAMPs are isopeptide linked to lysine residues of protein substrates by a mechanism termed sampylation which requires the noncanonical E1 enzyme, UbaA (170, 192). Desampylation, the reversed process of sampylation, is achieved by DUB homologs of the JAB1/MPN/MOV34 metalloenzyme (JAMM/MPN+) subfamily, such as HvJAMM1 (194, 195). Although evidence reveals sampylation is linked to archaeal proteasome function (22, 176, 188), little is known about the mechanism by which substrates are targeted for turnover by the SAMP-proteasome system (SPS, an analog of UPS), the components of SPS, and the degrons associated with the targeted proteolysis.

In this study, SPS mutant strains were generated for studying their roles in targeted proteolysis pathway in *Hfx. volcanii*. TATA-binding protein, TBP2 served as the model substrate and its steady-state protein and transcript levels, protein stability, protein modification, and formation of SAMP-conjugates were tested in SPS mutant and wt strains.


Results and Discussion

TBP2 Abundance is Increased by Mutation of the SAMP-proteasome System

TBP2 is one of four TATA-binding protein paralogs in *Hfx. volcanii* and is found isopeptide linked at lysine residues to the C-terminus of the Ubl protein SAMP2 (176). In analogy to Ub-tagging in eukaryotes, we hypothesized that sampylation in archaea serves as a signal for targeting proteins such as TBP2 for destruction by proteasomes or alters its association with protein partners through non-proteolytic mechanisms.

To initiate this study, the steady state levels of TBP2 were compared in wild type (wt) and SPS mutant strains. The abundance of TBP2 protein was found increased ~7-fold upon deletion of SPS genes including *ubaA*, *jamm2*, *pan2* and *cdc48c* (where UbaA is the E1-like SAMP activating enzyme, JAMM2 is a JAMM/MPN+ homolog, PAN2 is related to Rpt1-6 AAA ATPases and Cdc48c is a member of the Cdc48/p97 AAA ATPase family) (Figure 3-1). By contrast, deletion of the genes encoding the desampylase HvJAMM1 (*Δjamm1*) and other related AAA ATPases (*Δpan1* and *Δcdc48b*) had little if any influence on TBP2 abundance (Figure 3-1). qRT-PCR analysis revealed the increased level of TBP2 protein in the SPS mutant strains was not due to differences in transcript levels, as the transcripts specific for TBP2 were less abundant in SPS mutants than wt (Figure 3-2). Thus, the observed increase in TBP2 protein levels caused by SPS mutation was post-transcriptional and may have triggered an autoregulatory response that reduced levels of TBP2 transcript. Autoregulation by transcriptional repression of transcription factors is common (296) and is possible for TBP2 regulation of *tbp2-strepII*, as this gene was expressed from a TATA box-based promoter known to bind TBPs.
TBP2 Abundance is Increased by Chemical Inhibition of 20S Proteasomes and Cdc48/p97-type ATPases

I next examined whether the levels of TBP2 protein could be stabilized in archaea by addition of UPS inhibitors. TBP2 levels were analyzed in Hfx. volcanii cells treated with bortezomib (PS-341, velcade), which reversibly binds and inhibits the catalytic site of proteasomal CPs with high affinity and specificity (15) (IC50 of < 40 nM for 20S core particles of Hfx. volcanii, McMillian and Maupin-Furlow, unpublished results). The level of TBP2 protein was found to be increased by ~3.5 fold after 45-min treatment with bortezomib compared to the mock control (Figure 3-3A). Likewise, treatment of Hfx. volcanii cells with N\textsuperscript{2},N\textsuperscript{4}-dibenzylquinazoline-2,4-diamine (DBeQ), a selective and potent inhibitor the highly conserved p97/Cdc48 ATPases (299), increased the abundance of TBP2 by ~2-fold when compared with the mock control (Figure 3-3B). These results reveal that treatment of archaeal cells with UPS inhibitors can increase the abundance of target proteins, such as TBP2 which is a substrate of smpylation.

TBP2 Abundance is Increased by Depletion of 20S proteasomes

I next studied whether TBP2 abundance could be increased by genetic depletion of proteasomal CPs. To test this idea, TBP2 levels were analyzed in GZ138, a strain which harbors an insertion of the tryptophan-dependent tryptophanase (P\textsubscript{tnaA}) promoter upstream of psmB encoding the sole β subunit of proteasomal CPs, which are essential in Hfx. volcanii (26). Cells were cultured on medium with and without tryptophan, the inducer of psmB gene expression. TBP2 levels were found notably increased within 45 min after psmB expression was repressed by removal of tryptophan from the culture medium (Figure 3-4). By contrast, the steady state levels of TBP2 remained constant.
when the medium was supplemented with tryptophan (Figure 3-4). As an added control, the levels of TBP2 were found unaltered when tryptophan was removed from wt cells, in which psmB was regulated by its native promoter (Figure 3-4). Thus, TBP2 abundance was increased by genetic depletion of proteasomal CPs. Taken together, these data suggested TBP2 was targeted for degradation by proteasomes.

**TBP2 is Stabilized by Mutation of the SAMP-proteasome System**

In order to determine whether the accumulation of TBP2 protein observed in the SPS mutants was due to an increase in the rate of translation or a decrease in the rate of protein turnover, a chase assay was performed. Cells were treated with inhibitors of translation (anisomycin) and transcription (actinomycin D) to minimize synthesis of new protein, and TBP2 protein levels were monitored by immunoblotting and densitometric analysis. During the 1 h chase period, TBP2 was found to be degraded in wt cells; whereas, little if any TBP2 turnover was observed in the ΔubaA and Δjamm2 strains, and the half-life of TBP2 increased 7-16 fold in the Δpan2 and Δcdc48c strains (Figure 3-5A and 3-5B). SAMP2 was apparently required to target TBP2 for protein turnover based on finding that the protein levels of the trans-expressed TBP2 were not altered by deletion of ubaA when expressed alone but did accumulate when co-expressed with ‘extra’ SAMP2 (Figure 3-5C). Together, these results demonstrate that a major transcription factor of archaeal cells, TBP2, is degraded in an SPS dependent manner.

**SAMP2 is not Targeted for Degradation by Archaeal Proteasomes**

The Ub-like SAMP2 is correlated with TBP2 degradation but is itself not degraded. Further analysis of the SAMP2 degradation rate in the chase assay revealed SAMP2 was highly stable. Although TBP2 was clearly degraded in wt, SAMP2 turnover was not detected in either wt or SPS mutant strains (Figure 3-5A and 3-5B). Anjum et
al. (2015) proposed a model that SAMPs are degraded together with substrates by 20S CP proteasomes in archaea (22). This model was based on in vitro degradation of a linear fusion of a SAMP homolog to green fluorescent protein (GFP) (22) and by analogy to the disordered Pup tag of mycobacteria that is covalently bound to substrate proteins and co-degraded by ATPase-proteasomes (203). Here we provide evidence that the Ub-like SAMP2 is not targeted for degradation by the archaeal SPS and is instead recycled by the DUB-like protease, JAMM2. The Rpn11 JAMM/MPN+ metalloprotease homolog, JAMM2, is our proposed candidate for recycling SAMP2, as deletion of jamm2 stabilizes and increases the abundance of TBP2 in the cell.

**Detection of TBP2 Conjugates Modified by SAMPs**

If sampylation preceded proteasome-mediated turnover of TBP2, we hypothesized that this transcription factor would accumulate in Ubl-modified forms in SPS mutants that were dysfunctional in proteasome function but not sampylation. To test this hypothesis, TBP2 was enriched by anti-StrepII affinity chromatography from wt and SPS mutant strains and analyzed by reducing SDS-PAGE. By this approach, TBP2 was found to be more abundant and to be in various high molecular mass forms in the SPS mutants (ΔubaA, Δjamm1/2, Δjamm2, Δpan1/2, Δpan2, and Δcdc48c) compared with wt (Figure 3-6A). Of particular note was a 48 kDa form of TBP2 that was absent in wt, yet abundant in all of the SPS mutants examined (Figure 3-6A and 3-6B). We speculate that this form of TBP2 is a dimer that forms when TBP2 is at high concentration, since this form was detected in the E1-like mutant strain (ΔubaA) which does not synthesize Ubl-bonds (188, 192). Other high molecular mass forms of TBP2 were observed in the Δjamm1/2 and Δpan1/2 strains that were not detected in the ΔubaA mutant or wt cells (Figure 3-6A). Often these forms of TBP2 were found to be...
recalcitrant to separation by reducing SDS-PAGE and retained in the stacking gel (e.g., >250 kDa in Figure 3-6B) similarly to Ub-modified proteins [e.g., (300)]. These results suggested that sampylated forms of TBP2 were increased in abundance by deletion of proteasomal ATPase (Δpan1/2) and desampylase (Δjamm1/2) genes.

To further understand the covalently modified forms of TBP2 that accumulated in the SPS mutants with dysfunctional proteasomal machineries, the SAMP2 modified proteins were isolated from the TBP2-StrepII enriched fractions by Flag immunoprecipitation from the SPS mutant Δpan1/2. The ΔubaA mutant and wt were included for comparison, since the E1-like UbaA is required for sampylation (192). By this approach, SAMP2-TBP2 conjugates were purified and found to migrate as a single band of 37 kDa by reducing SDS-PAGE. Compared with wt, the SAMP2-TBP2 conjugate was found to be dramatically accumulated in the Δpan1/2 mutant and was not detected in the ΔubaA control (Figure 3-6C). The ~17 kDa increase in migration of TBP2 due to the SAMP2 linkage suggested TBP2 was di- or mono-sampylated. By contrast, TBP2 species that migrated >50 kDa were identified in the Strep-pull down samples of Δjamm2, Δjamm1/2, Δpan2, Δpan1/2 and Δcdc48c strains that were not detected in Δpan1/2 or wt strains (Figure 3-6A). We expect that (1) additional moieties of SAMP were attached to TBP2 that are encoded from the genomic copies of samp1-3 without tag, (2) the N-terminal Flag-tag inhibited the formation of polymeric SAMP chains, and/or (3) polysampylated forms of TBP2 were not stable during the tandem purification. Interestingly, in Mycobacterium tuberculosis, at least one substrate of the Pup proteasome system migrates as multiple covalent forms by SDS-PAGE including large molecular mass species that are not detected as pupylated by anti-Pup IB (200).
The low abundance of the TBP2-SAMP2 conjugates in the wt cell was likely due, at least in part, to protein instability mediated by energy-dependent proteolysis via the Rpt-like PANs and 20S proteasomes. This possibility would explain why the level of SAMP2-TBP2 species was significantly higher in the SPS (Δpan1/2) mutant compared with wt. Overall, our analysis of the tandemly purified TBP2-SAMP2 conjugates suggests that Ub-like SAMP2 modification serves as a signal for targeting TBP2 to the proteasomes for destruction.

**Two Apparent Forms of “Unsampylated” TBP2**

By optimizing the conditions of reducing SDS-PAGE to separate proteins in the 20-25 kDa range, unsampylated TBP2 was found to be shifted to a slower migrating form when its abundance was increased by SPS mutation. Compared with wt, SPS mutations (ΔubaA, Δjamm2 and Δpan2) were found to slow the migration of TBP2 in these gels (Figure 3-7). Deletion of pan1 had only a partial effect on TBP2 migration (Figure 3-7), which could have been due to altered function of PAN2, which forms a heterooligomer with PAN1 in the cells (25, 301). We suspected that the slow migrating form of TBP2 had increased acidity. Covalent attachment of small acidic (e.g., phosphoryl) groups alters SDS coating and retards migration by SDS-PAGE of proteins that have a high content of acidic residues near the post-translational modification site (302). In eukaryotes, phosphorylation and other types of covalent modification (e.g., N-terminal Met cleavage, acetylation, and glycosylation) regulate the ubiquitylation and degradation of target proteins by UPS (154, 303). The retarded migration of the unsampylated form of TBP2 in the SPS mutants suggested that TBP2 was subjected to an additional type of co-/post-translational modification that was distinct from sampylation and that was correlated with the regulated turnover of TBP2 by SPS.
TBP2 is a Mixture of N-terminal Met1 Minus and Intact Forms

In order to determine the type of co-/post-translational modification that could precede and regulate sampylation, the composition of unsampylated TBP2 was investigated by mass spectrometry (MS). TBP2 was purified by affinity chromatography and gel filtration from wt and Δpan1/2 mutant strains. For initial investigation of the post-translational modification of TBP2 that appeared to precede sampylation, the accurate mass of purified TBP2 was determined at high resolution by direct-infusion ESI-TOF mass spectrometry analysis. Two isoforms of TBP2 were detected by this approach including Met1-minus and intact forms (Figure 3-8). The Met1-minus form was presumed to be cleaved by the native methionine aminopeptidase (MetAP) (i.e., HVO_2600 homolog). MetAPs specifically remove N-terminal Met1 from the target protein if the amino acid residue at the second position is non-bulky and uncharged (e.g., Gly, Ala, Ser, Cys, Pro, Thr, and Val) (304). The deduced N-terminus of TBP2 is Met1Ser2- with Ser2 likely promoting the removal of Met1 by MetAP. Based on shotgun MS analysis of haloarchaeal proteomes, proteins with N-terminal Met1Ser2- are commonly detected as a mixture of Met1-cleaved and intact forms (305-307). Thus, our finding that TBP2 was in Met1-minus and intact forms was not unique to this transcription factor. In fact, the mixture of these two forms of TBP2 was at similar ratio in wt compared to Δpan1/2 mutant strains (Figure 3-8) and, thus, could not be attributed to the observed differences in TBP2 levels between these strains. In line with this finding, Met residues do not alter the charge of a protein and are of a low molecular mass (131 Da) that is not readily detected by migration on reducing SDS-PAGE gel. Together, these findings revealed that a portion of TBP2 was in a Met1- minus form (presumably
due to cleavage by MetAP); however, Met1 cleavage alone did not appear responsible for generating the SPS-dependent TBP2 isoforms that were observed by SDS-PAGE.

**Ser2 of TBP2 is Phosphorylated**

Our detection of phosphorylation using an ESI-TOF approach was likely hindered by the selective suppression of the ionization of phosphoproteins in the presence of their unmodified state (308); thus, TBP2 modification was further characterized by reverse-phase liquid chromatography tandem mass spectrometry (LC-MS/MS). Free TBP2 purified from the Δpan1/2 mutant was in-gel digested with chymotrypsin and phosphopeptides were enriched by metal oxide affinity chromatography (MOAC). By this strategy, a nearly complete b and y ion series was detected by MS/MS fragmentation of a phosphopeptide that mapped to the N-terminus of TBP2, with the fragmentation pattern revealing a Met1-minus form of TBP2 was phosphorylated at Ser2 (Figure 3-9A). Chymotrypsin hydrolyzes amide bonds in peptides at slow rates, particularly those with Met at the P1 position (309). Thus, the absence of Met1 in the Ser2(p) containing peptide fragment of TBP2 could have been due to the chymotrypsin treatment. MetAPs disfavor acidic residues at the P1' position of the P1↓P1' bond that is cleaved (310); thus, phosphorylation of Ser2 [Ser2(p)] would likely prevent the removal of Met1 from proteins due to the added negative charge at the P2' position. In order to answer this next question, trypsin and Glu-C were utilized instead of chymotrypsin to generate appropriate N-terminal peptides for LC-MS/MS analysis. The only Ser2(p) form of TBP2 that was detected was when the Met1 was intact (Figure 3-9B). In fact, with 94% coverage of TBP2 by LC-MS/MS analysis, Ser2 phosphorylation appeared to be the only post-translational modification on unsampylated TBP2, besides Met1
cleavage, the latter of which did not occur in the Ser2 phosphorylated form. Thus, TBP2 was found modified by Ser2 phosphorylation but only when Met1 was intact.

**TBP2 Migration and Abundance are Altered by Substitution of Ser2**

In order to study whether phosphorylation was responsible for the SDS-PAGE migration differences of TBP2 observed in wt and SPS mutants, a site-directed mutagenesis approach was used. TBP2 Ser2 was altered to: 1) Ala to prevent phosphorylation, 2) Glu to mimic phosphorylation, and 3) Leu to prevent phosphorylation as well as Met1 cleavage. Based on reducing SDS-PAGE analysis, the S2A and S2L substitutions did not alter the migration of TBP2, while the S2E exchange shifted TBP2 to a slower migrating form (Figure 3-10A). Thus, the substitution of Ser2 to Glu caused TBP2 migration shift that was observed in the SPS mutant strains (Figure 3-7). Next, the steady-state levels of TBP2 wt and variant (S2A, S2E, and S2L) proteins in wt and SPS mutant strains were analyzed. Interestingly, the S2A substitution abolished the accumulation effect otherwise observed for the wt form of TBP2 in the SPS mutants (Figure 3-10B), suggesting TBP2 S2A turnover was no longer regulated by SPS. Furthermore, the S2E exchange, which mimicked the Ser2 phosphorylated form of TBP2, was found to dramatically decrease TBP2 protein but not transcript level in wt cells (Figure 3-10B and 3-10C). Consistent with our model, the protein level of TBP2 S2E was found to be higher (at a statistical significance of p<0.05) in the ΔubaA and Δpan1/2 mutant strains compared with wt (Figure 3-10B). However, the level of this difference was modest suggesting the S2E phosphomimic, which is not susceptible to dephosphorylation, may be more prone to degradation by SPS independent mechanisms than the S2(p) form. Sampylation may, thus, be used to drive the proteolysis pathway forward and minimize reversal by phosphatases. Taken together,
these data suggest phosphorylation of TBP2 Ser2 is linked to TBP2 stability which is regulated by SPS.

**Conclusion**

This study demonstrated that TBP2, a TATA-binding protein modified by Ubl isopeptide bonds in *Hfx. volcanii*, is phosphorylated and targeted for SPS mediated degradation (Figure 3-11). Steady-state levels of protein (but not transcript) levels of TBP2 were notably increased by deletion of archaeal SPS components including UbaA (the E1/MoeB/ThiF homolog of archaea), AAA ATPases (Cdc48/p97- and Rpt-type), a type-2 JAMM/MPN+ metalloprotease homolog (JAMM2) and 20S proteasomes. Besides, treatment of SPS specific inhibitors and genetic depletion of 20S proteasomes were found to increase the protein level of TPB2. TBP2 proteins were found to be more stable in SPS deletion strains compared with wt cells based on the chase assay. SAMP2 was proposed to target TBP2 to proteasomes for degradation via a recycling mechanism based on the findings that: 1) SAMP-TBP2 conjugates were accumulated in SPS deletion mutants (except ΔubaA strain) compared with wt cells; 2) the Ubl protein modifier (SAMP2) stimulated degradation of TBP2, but SAMP2 itself was not degraded.

In addition, MS analysis of TBP2 fractions not modified by Ubl linkages disclosed TBP2 had multiple N-termini, including Met1Ser2-, Ser2- and Met1Ser2(p)- [where (p) represents phosphorylation]. The Met1-cleaved and intact forms of TBP2 in the wt cells were at a ratio similar to that in the Δpan1Δpan2 double mutant strain. Site-directed mutagenesis study provided evidence that TBP2 phosphorylation on Ser2 affects its migration on SDS-PAGE gel and protein abundance regulated by SPS.
Figure 3-1. TBP2 abundance is increased by mutation of the SAMP-proteasome system. TBP2 protein levels in wild type (wt) and SPS deletion strains determined by anti-StrepII immunoblotting. TBP2 was fused to a C-terminal StrepII tag, co-expressed with Flag-SAMP2 and monitored by anti-StrepII IB. Equal loading was confirmed by Coomassie Blue (CB) staining. Relative levels of TBP2 protein in SPS deletion strains versus wt are indicated below gel. Data were quantified by ImageJ. Data represent mean ± SEM of three independent experiments. (*, p<0.01; **, p<0.001). The p-values were determined by two-tailed, unpaired Student’s t-test. See methods for details.
Figure 3-2. Increased TBP2 abundance by mutation of the SAMP-proteasome system is not due to differences in transcript levels. Histogram showing relative TBP2-StrepII transcript levels by qRT-PCR. The mRNA levels were normalized to internal standard ribL. Data represent mean ± SEM of three independent experiments. (*, p<0.05; **, p < 0.01). The p-values were determined by two-tailed, unpaired Student’s t-test. See methods for details.
Figure 3-3. TBP2 abundance is increased by chemical inhibition of 20S proteasomes and Cdc48. (A) TBP2 abundance in wild type (wt) cells treated with an inhibitor of proteasomal CPs (bortezomib, +) or a mock control (dimethylformamide or DMF, -). (Right) Quantification of relative TBP2 protein levels expressed as fold change from time zero, which was set at 1.0. (B) TBP2 abundance in wild type (wt) cells treated with an inhibitor of Cdc48 (14 μM DBeQ, +) or a mock control (ethanol, -) after 24 hour. Quantification of relative TBP2 protein levels are indicated below gel. TBP2 level in mock control was set at 1.0. TBP2-StrepII was co-expressed with Flag-SAMP2 and monitored by anti-StrepII IB. Equal loading was confirmed by CB staining. Experiments were performed in at least biological duplicate with representative images shown. Data were quantified by ImageJ. See methods for details.
Figure 3-4. TBP2 abundance is increased by genetic depletion of 20S proteasomes. TBP2 abundance in GZ138 \((P_{\text{tnaA}-\text{psmB})}\) cells grown in medium containing or lacking tryptophan as well as wt cells grown in medium lacking tryptophan for the indicated times. (Lower panel) Quantification of relative TBP2 protein levels expressed as fold change from time zero, which was set at 1.0. TBP2-StrepII was co-expressed with Flag-SAMP2 and monitored by anti-StrepII IB. Equal loading was confirmed by CB staining. Experiments were performed in at least biological duplicate with representative images shown. Data were quantified by ImageJ. See methods for details.
Figure 3-5. TBP2 is stabilized by mutation of the SAMP-proteasome system. (A and B) Chase assays in wild type (wt) and SPS gene deletion strains expressing the Flag-SAMP2 and TBP2-StrepII. Log-phase cells were treated with 20 μg·ml⁻¹ actinomycin D and 50 μg·ml⁻¹ anisomycin for the indicated times and collected. TBP2 and SAMP2 protein levels were determined by anti-StrepII and anti-Flag IB, respectively. Equal loading was confirmed by CB staining. (Lower panels) Quantification of TBP2 and SAMP2 intensity in chase assays. Results are expressed as the percent change from time zero, which was set at 100%. Data represent mean ± SEM of three independent experiments. (C) TBP2 protein levels in wild type (wt) and ΔubaA strains determined by anti-StrepII immunoblotting. TBP2-strepII was expressed alone (left) and co-expressed with Flag-SAMP2 (right). Relative levels of TBP2 protein in ΔubaA versus wt are indicated below. Equal loading was confirmed by Coomassie Blue (CB) staining. Experiments were performed in triplicate with representative images shown. Data were quantified by ImageJ. See methods for details.
Figure 3-5. Continued
Figure 3-6. Detection of TBP2 conjugates modified by SAMPs. TBP2 enriched from wild type (wt) and SPS deletion strains by StrepTactin (anti-StrepII) (A-B) and tandem affinity purification (C) which included a subsequent immunoprecipitation by anti-Flag. TBP2-StreplI was ectopically expressed with Flag-SAMP2 in the wt and SPS mutant strains compared an empty vector control. Equivalent amount of cell lysate as determined by BCA assay was applied to the StrepTactin resin. TBP2, TBP2 conjugates (including the Ubl-modified form, SAMP2-TBP2), and anti-StreplI signal likely to be a TBP2 dimer (*) are indicated. See methods for details.
Figure 3-6. Continued.
Figure 3-7. Mobility shift of TBP2 in SPS deletion strains compared to wt. TBP2 was detected by anti-StrepII IB of cell lysate separated by reducing 14% SDS-PAGE. Equal loading was confirmed by Coomassie Blue (CB) staining. Experiments were performed in three independent experiments with representative images shown. See methods for details.
Figure 3-8. TBP2 is a mixture of N-terminal Met1 minus and intact forms based on ESI-MS analysis. TBP2-StrepII was purified from wt and Δpan1/2 strains as indicated. M1 (Met1 cleaved TBP2) and M2 (intact TBP2) represent the observed and theoretical average masses for (C) and observed and theoretical monoisotopic masses for (D).
Figure 3-9. TBP2 is phosphorylated at its N-terminal Ser2. Representative tandem mass (MS/MS) spectra of N-terminal peptides derived by collision-induced dissociation of the doubly charged precursor after chymotrypsin (A) and Glu-C and trypsin (B) digestion of TBP2. High mass accuracy MS/MS unambiguously confirmed Ser2 phosphorylation of TBP2 based on the near complete matches of b- (colored red) and y-type ions (colored blue).
Figure 3-10. TBP2 migration and abundance are altered by substitution of Ser2. (A) The mobility shift of wild type (wt) TBP2 and protein variant (S2A, S2E, and S2L) expressed in wt cells and detected by anti-StrepII immunoblotting (IB) of cell lysate separated by reducing 14% SDS-PAGE. Equal loading confirmed by Coomassie Blue (CB) staining. (B) TBP2 (wt, S2A, and S2E) protein levels in wt and SPS deletion strains determined by anti-StrepII IB. TBP2 was monitored by anti-StrepII IB. Relative levels of TBP2 proteins in wt and SPS deletion strains are indicated below gel. The level of wt TBP2 protein in wt strain was set at 1.0. Protein intensity was obtained by ImageJ. Quantification data represent mean ± SEM of five independent experiments for TBP2 S2E variant protein and three independent experiments for TBP2 S2A variant protein. (C) Histogram showing transcript levels of TBP2 (wt, S2A, and S2E) in wt and SPS mutant strains revealed by qRT-PCR. The mRNA levels are normalized to internal standard ribL. Data represent one experiment for TBP2 S2A and three experiments for TBP2 S2E. TBP2-StrepII was co-expressed with Flag-SAMP2 in trans in all conditions. Statistical analysis of relative TBP2 protein and mRNA intensity compared with WT: *, P<0.05; **p < 0.01; ns, not significant (two-tailed, unpaired Student’s t-test).
Figure 3-11. Model of regulated turnover of TBP2 by the archaeal SAMP-proteasome system (SPS). In this model, the phosphorylation status of TBP2 Ser2 is an important factor that regulates TBP2 turnover by the archaeal SPS. The kinase and phosphatase enzymes that control phosphorylation of TBP2 Ser2 and the methionine aminopeptidase that cleave TBP2 Met1 have yet to be identified. However, evidence suggests that the addition of a phosphoryl group to Ser2 inhibits the ability of methionine aminopeptidase to remove Met1 from TBP2. The Met1-Ser2(p) form of TBP2 is thought to be susceptible to sampylation by the E1-like UbaA and destruction by the archaeal SPS. The proteasomal AAA ATPases (Rpt-like PAN2 and Cdc48c) are used to recognize, unfold and translocate TBP2 into the proteolytic chamber of the CPs for destruction by the proteasome system, while the JAMM2 would remove and recycle the SAMP2 moiety from the TBP2 substrate during this process. JAMM1 is a desampylase that independently cleaves SAMPs from target proteins, and, thus, may serve as a proofreading enzyme to ensure proper substrate recognition by the archaeal SPS (311).
CHAPTER 4
METHIONINE SULFOXIDE REDUCTASE ESSENTIAL FOR MILD OXIDANT INDUCED UBIQUITIN-LIKE MODIFICATIONS

Introduction

Adaptive responses to protein oxidation are crucial for cell survival against reactive oxygen species (ROS), desiccation, ionizing radiation, and other harmful factors (220). Metal centers and sulfur-containing amino acid (methionine and cysteine) residues are primary targets of protein oxidation (223). Modest changes in the conformation and redox chemistry of proteins due to oxidation can be repaired by chaperone assisted refolding and specialized systems such as the methionine sulfoxide (MSO) reductases (222). MsrA/B are the most widespread types of MSO reductases (found in all domains of life). MsrA-type enzymes reduce free and protein-based methionine-S-sulfoxide, while MsrB-type enzymes reduce protein based methionine-R-sulfoxide, with enzyme reductant often provided by thioredoxin at the expense of NADPH (312). Redox controlled proteolysis serves as a last resort to ensure the timely destruction of proteins damaged beyond repair (226).

Ub and proteasome systems function together to remove proteins damaged by oxidation in eukaryotes (226). Oxidized proteins can be tagged with poly-Ub chains for proteasomes-mediated destruction via a cascade of enzymes (E1, E2s, and E3s) (313). Generally, the E1 readies the Ub C-terminus for covalent linkage, while E2s and E3s guide Ub to its protein target (72). The E3s are diverse and numerous and impart substrate specificity by either catalyzing through a thioester intermediate or assisting in Ub transfer from E2 to its protein target (314). The 26S proteasome recognizes, unfolds and destroys the Ub-tagged proteins using ATP to drive the process (4). As the oxidative insults become extreme, protein unfolding increases, ubiquitylation appears
inhibited and severely damaged proteins are destroyed by 20S proteasomes (315).
Although evidence suggests that UPS degrades oxidatively damaged proteins beyond repair, the molecular mechanisms used to control oxidative stress responses via Ub/Ubl protein modification remain poorly understood, especially in the mild oxidative condition when protein destruction may not be necessary.

Similar to eukaryotes, archaea have Ubl (SAMP) tagging systems (170) that target proteins for destruction by proteasomes (22, 311) and other processes (e.g., control active site residue availability) (195). MSO reductases that repair oxidatively damaged proteins and Ubl protein modification appear closely tied in archaea. In particular, MsrA/B-type MSO reductases are Ubl-modified in archaeal cells exposed to the mild oxidant DMSO (189).

In this study, *Hfx. volcanii ΔmsrA/B* mutants were generated for studying the role of archaeal MsrA/B in Ubl-bond formation induced by mild oxidative stress *in vivo*. In addition, *Hfx. volcanii* MsrA was purified and tested for its activity as MSO reductase and its role in DMSO induced SAMP-conjugates formation via an *in vitro* reconstitution assay. The physiological roles of MsrA/B were also studied.

**Results and Discussion**

**MsrA is Essential for SAMP-conjugates Induced by DMSO in vivo**

*Hfx. volcanii ΔmsrA/B* mutants were constructed and compared to wild type (wt) for the formation of Ubl conjugates in the presence of DMSO which can stimulate the isopeptide linkage of the Ubl SAMP1-3 to protein targets in this archaeon (188, 189). DMSO is a mild oxidant that can oxidize various compounds by acting as a nucleophile depending on environmental conditions (316). For example, DMSO can oxidize methionine to MSO in the presence of strong acid like hydrochloric acid, and this
reaction can be accelerated at 100 °C (317). To our surprise, the levels of SAMP1-3 conjugation were dramatically reduced in ΔmsrA mutants when compared to wt, while deletion of msrB gene had no impact on the Ubl conjugate formation (Figure 4-1). Conjugation of MoaE (the large subunit of molybdopterin synthase) by SAMP1 at 50 kDa (188) was found to be MsrA-independent irrespective of growth condition (Figure 4-1A). All SAMP-conjugates were restored by trans-expression of wt MsrA in the ΔmsrA mutant (Figure 4-1), suggesting that MsrA is required for the Ubl-linkages induced by DMSO. DMSO is a competitive inhibitor of MsrA and a non-competitive inhibitor of MsrB in the reduction of MSO (318), and stimulates oxidative stress such as elevated levels of hydrogen peroxide-mediated cell death in yeast, potentially through inhibition of MsrA-mediated MSO reductase activity (319, 320). Thus, we propose that MsrA senses and responses mild oxidative stress via formation of SAMP-conjugates in Hfx. volcanii.

In order to further study the role of important MsrA residues in Ubl bond formation, site-directed mutagenesis was utilized to construct MsrA variant proteins based on biochemical and structural studies of eukaryotic and bacterial counterparts (244, 284). Conserved residues of MsrA chosen in this study included active site cysteine (C13) that acts as the nucleophile in attack of MSO, the glutamic acid residue (E56) thought to bind the oxygen atom of the sulfoxide group of MSO, and the cysteine residues (C16, C48 and C162) that may recycle the enzyme during MSO reduction. The formation of SAMP2 conjugation was abolished by C13S and E56A mutations of MsrA, while the MsrA C16S, C48S, and C162S variant proteins still functioned in the Ubl bond formation when all the MrsA variant proteins were readily expressed ΔmsrA mutant (Figure 4-2). Thus, we propose that Hfx. volcanii MsrA C13 and E56 in the active site
are important for stimulation of SAMP conjugation by recognizing the mild oxidant DMSO. Interestingly, the MsrA C13S variant protein was in higher abundance than wt MsrA (Figure 4-1 and 4-2), which was detected in apparent Ubl modified forms in the presence of DMSO (Figure 4-1), revealing DMSO-induced auto-modification of MsrA by SAMPs.

**MsrA is Essential for SAMP-conjugates Induced by MSO in vivo**

Next, the effect of the mild oxidant MSO on SAMP conjugation was tested. Similar to DMSO, MSO was also found to induce the level of all SAMP conjugates, while the impact on SAMP1 conjugation appeared less pronounced in the MSO vs. DMSO condition (Figure 4-3A). In addition, MSO stimulated Ubl conjugates were also found to require MsrA based on the fact that ΔmsrA mutants were severely impaired in the formation of SAMP conjugation which could be restored by trans-expression of wt MsrA (Figure 4-3B). Furthermore, MsrA C13 appeared important for Ubl conjugates stimulated by MSO as MsrA C13S could not restore SAMP conjugation (Figure 4-3C).

**Differential Effect of Chemicals on Stimulating SAMP-conjugates in vivo**

In addition to mild oxidants, effects of the potent oxidant NaOCl (a sodium salt of the reactive chlorine species hypochlorous acid) and the proteasome inhibitor bortezomib on SAMP conjugation were examined. Hypochlorite is the strongest oxidizing agent of the chlorine oxyanions with standard electrode potential at 1.63 V which can oxidize various biomolecules including DNA, protein and lipids at the physiological condition (321). Both NaOCl and the bortezomib were found to enhance the levels of Ubl SAMP2 conjugates, but MsrA (and MsrB) were not required for this activity (Figure 4-4A and 4-4B). The DMSO-related compounds dimethyl sulfide (DMS) and dimethyl sulfone (DMSO₂) had no detectable influence on the formation of Ubl
conjugates in all genomic backgrounds tested (Figure 4-4C), suggesting that the oxidation state of DMSO is important for stimulating the Ubl modifications. Accordingly, we propose that MsrA-dependent SAMP-conjugates are induced specifically to mild oxidative stress.

**Schema of MsrA-dependent Ubl-protein Modification**

Several reasons could account for our finding that MsrA was required for SAMP-conjugates stimulated by mild oxidative stress. First, among 50 protein targets known to be modified by the SAMPs through MS analysis (176, 188, 189), only MsrA is modified by SAMPs detected by immunoblotting. If so, the ΔmsrA mutation may have simply disrupted MsrA as a substrate of SAMP modification [as five lysine residues of MsrA are isopeptide linked to SAMP1 (189)]. However, counter to this argument, the pattern of SAMP conjugates: i) was not restored by MsrA C13S or E56 variants, both of which had all lysine residues intact, and ii) did not resemble the MsrA pattern based on comparison of anti-StrepII and anti-Flag IBs (Figure 4-1). An alternative explanation could be that MsrA controls the redox status of methionine residue(s) on signaling proteins that regulate sampylation pathway. Much like phosphorylation, methionine oxidation can serve as a reversible mechanism to regulate the activity of proteins [e.g., the HypT transcription factor (251) and Mge1 co-chaperone (322)]. If so, reductant like DTT would be important for MSO reductase activity in vitro. Another possibility could be that MsrA helps select and guide protein substrates for SAMP modification during mild oxidative stress (Figure 4-5A). Interestingly, the thalidomide binding domain of cereblon is a structural homolog of MsrB (273, 274) (Figure 4-5B). Cereblon functions in ubiquitylation as a flexible substrate presenting subunit of the many diverse DCAFs (DDB1 and CUL4-associated factors) that function with E3 cullin 4-RING ligase CRL4
complexes (273, 274). MsrA and MsrB, while not related in primary amino acid sequence, have mirror-image active sites and generally bind diverse protein substrates (242). Thus, MsrA could in theory function as a protein substrate receptor for guiding the formation of SAMP-conjugates during mild oxidative stress.

**MsrA is Essential to Form SAMP-conjugates in vitro**

To further understand the molecular basis of our findings, MsrA was purified to homogeneity and characterized for its ability to reconstitute Ubl protein modification *in vitro*. Substrate proteins and other components of the Ubl-pathway (e.g., E1-like UbaA and SAMPs) were made available by use of the cell lysate of ΔmsrA strains. The conserved active site cysteine variant (MsrA C13S) was included for comparison. By this approach, MsrA was shown to catalyze Ubl-bond formation and to require the conserved active site cysteine (C13) for this activity (Figure 4-6A). MsrA-mediated reconstitution of Ubl-bond formation occurred irrespective of the SAMP-type (Figure 4-6A and 4-6B). The Ubl-linked products were found to be hydrolyzed by HvJAMM1, a JAMM/MPN+ metalloprotease that specifically cleaves the Ubl-bond (Figure 4-6C). In addition, MsrA was functional when purified from either recombinant *E. coli* or *Hfx. volcanii*, revealing archaeal proteins which could potentially copurify with MsrA were not responsible for the *in vitro* activity. Signaling mechanisms that require synthesis of new transcript/protein, intact cell membranes, and other related functions would have been disrupted during the preparation and dialysis of the ΔmsrA cell lysate. Thus, we propose MsrA functions as a protein factor required for mediating Ubl protein modification during mild oxidative stress.
DTT is not Needed to Form MsrA-dependent SAMP-conjugates \textit{in vitro}

In order to determine whether MsrA-mediated MSO reductase activity is responsible for the formation of Ubl-conjugates, the \textit{in vitro} reconstitution assay was performed in the presence and absence of DTT, an efficient reductant commonly used for the MSO reductase assay. The C13 of MsrA was required for the formation of the 50- and 75-kDa SAMP-conjugates by the \textit{in vitro} reconstitution assay (Figure 4-7A). Similar level of MsrA-dependent SAMP-conjugates were detected by the reconstitution assay with and without DTT when the cell lysate after dialysis was used as a resource of substrate proteins (Figure 4-7A); by contrast, the MSO reductase activity of MsrA from the cell lysate was significantly impaired when DTT was excluded from the assay (Figure 4-7B). Thus, a signaling mechanism that would require MsrA-mediated MSO reductase activity was not needed to generate the MsrA-dependent Ubl modifications, which further supports our hypothesis that MsrA is an integral component of the Ubl protein modification pathway.

ATP and DMSO are Essential to Form MsrA-dependent SAMP-conjugates \textit{in vitro}

In order to further characterize the role of MsrA in Ubl-bond formation \textit{in vitro}, the impact of different nucleotide ligands and effectors on MsrA-dependent reconstitution of Ubl protein modification was tested. The Ubl-conjugates were formed \textit{in vitro} only when both ATP and DMSO were present in the assay, while removal of ATP or DMSO could abolish the MsrA-dependent Ubl-conjugates (Figure 4-8A). Thus, both ATP and DMSO are required for MsrA-dependent Ubl protein modification \textit{in vitro}. Of all the nucleotides tested, only ATP was required for the formation of Ubl-conjugates, while ADP, AMP, and AMP-PNP (a non-hydrolyzable ATP analog) were found to have no effect to produce Ubl-conjugates in the reconstitution assay (Figure 4-8B), consistent with our
finding that ATP hydrolysis is required for the E1-like enzyme UbaA to form a thioester intermediate with all three SAMP types and to release PPI during the process of Ubl modification (323). Furthermore, the DMSO related compounds DMS and DMSO₂ could not substitute for DMSO in this reaction (Figure 4-8C), suggesting that the oxidation state of DMSO is important for inducing MsrA-dependent Ubl protein modification.

**Robust MsrA-mediated SAMP-conjugates is Reconstituted *in vitro***

To further understand the cellular components that are required for Ubl-bond formation under DMSO condition, the MsrA, UbaA and SAMP proteins were purified to homogeneity and characterized for their ability to reconstitute Ubl protein modification. All three proteins were found to be required for high level of SAMP2-conjugates that were formed *in vitro* under the DMSO specific condition when using the cell lysate of ΔmsrA ΔubaA Δsamp1/2/3 strains (Figure 4-9A). By contrast, the 50- and 75-kDa Ubl conjugates that did not require MsrA and were apparent auto-modified forms of UbaA, based on our detection of these proteins by anti-His and anti-Flag IB (Figure 4-9A) and the migration of these proteins similarly to ‘autosampylated’ UbaA, as recently described (197). Addition of only SAMP2 and MsrA proteins to ΔmsrA ΔubaA Δsamp1/2/3 cell lysate did not restore the formation of Ubl-bonds via this *in vitro* assay (Figure 4-9A). Furthermore, the treatment time needed to trigger DMSO stimulated Ubl protein modification by MsrA in the *in vitro* assay was found to closely correlate with that observed *in vivo* (Figure 4-9A and 4-9B). Thus, robust MsrA-mediated Ubl-conjugates could be reconstituted *in vitro* by adding sampylation components including SAMP, MsrA, and UbaA proteins with ATP and DMSO.
DMSO Stimulates MsrA-dependent SAMP-conjugates in a Dose Dependent Manner in vivo and in vitro

Since a robust MsrA-dependent sampylation assay was established after optimization, I next studied the Ubl-bonds formed by MsrA in the presence of increasing concentration of the mild oxidant DMSO in vitro and in vivo. The MsrA-dependent Ubl-conjugates were generated at 2.5 mM DMSO by in vivo and in vitro assays (Figure 4-10A and 4-10B). The level of MsrA-dependent SAMP-conjugates was increased by DMSO in a dose dependent manner in vivo and in vitro (Figure 4-10A and 4-10B). Thus, we provided evidence that DMSO can stimulate the activity of MsrA for the Ubl-bond formation.

MsrA/B are Active MSO Reductases

DMSO is a known competitive inhibitor of yeast MsrA in the reduction of protein-bound MSO (318, 319), yet this small molecule ligand is required for triggering MsrA-dependent archaeal Ubl protein modification. To further understand this difference, cell lysate of Hfx. volcanii was prepared by me and examined for MSO reductase activity by Jackob Moskovitz using DTT as the reductant and a racemic mixture of dabsyl-MSO as the substrate to mimic protein-bound MSO (Figure 4-11A). Cell lysates of ΔmsrA/B single and double mutants were found to be significantly reduced in MSO-peptide reductase activity when compared to in wt (Figure 4-11B). Ectopic expression of wt MsrA/B in ΔmsrA/B mutant strains was found to increase the MSO peptide reductase activity to levels 3- to 3.5- fold higher than wt; whereas, MsrA C13S (while expressed) had no effect (Figure 4-11B). Treatment of cells with DMSO was found to significantly reduce the level of MsrA-mediated MSO-peptide reductase activity, while having little if any influence on MsrB activity. This finding was particularly apparent in the ΔmsrA
mutant expressing MsrA \textit{in trans}, which had an over 2-fold reduction in MSO-peptide reductase activity under DMSO conditions compared to the no treatment control (Figure 4-11B). MsrA required DMSO for mediating Ubl protein modifications and conversely inhibited in its MSO peptide reductase activity by DMSO. Thus, we propose that DMSO can trigger the conformational change of \textit{Hfx. volcanii} MsrA for its switch from MSO reductase to a protein factor required for sarkylation induced by the mild oxidative stress. In fact, hundreds of moonlighting proteins are identified that undergo structural changes that result in two or more distinct and physiologically relevant biochemical or biophysical functions (324).

Further analysis of \textit{Hfx. volcanii} MsrA in its purified form was found to readily reduce dabsyl-MSO by a mechanism that was stimulated by high ionic strength buffer and required reductant (DTT) and the conserved active site cysteine (C13) and glutamic acid residues (E56) for activity (Figure 4-11C), as previously observed for yeast (284, 325), bovine (231) and \textit{E. coli} (326) MsrA. The MSO-peptide reductase activity of the purified \textit{Hfx. volcanii} MsrA was inhibited by the mild oxidants DMSO and MSO in a dose dependent manner; whereas, methionine had no effect on this activity (Figure 4-11C). DMSO/MSO inhibition was presumably through a mechanism of competitive active site inhibition similarly to yeast MsrA (318, 319). Thus, we conclude that \textit{Hfx. volcanii} MsrA/B are catalytically active MSO reductases and that the conserved active site cysteine (C13) and glutamic acid (E56) residues of MsrA are required for its activity in reducing MSO-peptides as well as guiding proteins for Ubl tagging. The mild oxidants (DMSO and MSO) control MsrA at multiple levels including inhibiting its MSO-peptide
reductase activity, stimulating its E3 ligase function, and regulating its protein levels in the cell.

**MsrA/B are not Essential for MSO Assimilation**

In order to understand the physiological importance of MsrA/B in *Hfx. volcanii*, I next analyzed the sensitivity of the ΔmsrA/B single and double mutants to oxidative stress including mild (DMSO and MSO) and potent (H₂O₂ and NaOCl) oxidants. The ΔmsrA/B mutants were found to be no more sensitive than wt cells to any of the oxidative stress conditions examined (data not shown). As MsrA/B are commonly needed for oxidative stress resistance among different organisms (225), our results suggested that additional MSO reductases not predicted by genome sequence compensated for loss of MsrA/B in *Hfx. volcanii* cells exposed to different oxidants. To address this possibility, methionine synthase (*metE1* and *metE2*) gene homologs were deleted from *Hfx. volcanii*, and the resulting ΔmetE1/2 mutant was analyzed for growth in the absence of methionine. The ΔmetE1/2 mutant was found to be a methionine auxotroph because this strain could not grow in the media without methionine while addition of methionine or MSO could restore the cell growth similar to that of wt cells (Figure 4-12). Further analysis revealed that msrA/B were not required for the ΔmetE1/2 mutant to grow on MSO as the sole source of methionine (Figure 4-12), suggesting a novel MSO reductase likely accounts for the ability of *Hfx. volcanii* ΔmsrA/B mutants to overcome mild and severe oxidative stress.

**Conclusion**

This study demonstrated that *Hfx. volcanii* MsrA is essential for DMSO/MSO induced Ubl-conjugates formation *in vivo*. Deletion of the *msrA* gene abolished the attachment of SAMP1/2/3 onto protein targets induced by DMSO and SAMP2/3-
conjugates stimulated by MSO, while deletion of the msrB gene had no effect on the level of SAMP-conjugates stimulated by DMSO or MSO treatment. All these SAMP-conjugates could be restored by trans-expression of wt MsrA but not MsrA variant proteins (C13S and E56A) in the ΔmsrA mutant. On the contrary, SAMP2-conjugates induced by the potent oxidant NaOCl and the proteasome inhibitor bortezomib did not require MsrA (and MsrB).

*Hfx. volcanii* MsrA was also determined to be essential for DMSO stimulated Ubl-conjugates *in vitro*. ATP and DMSO were required for the Ubl-bond modification by MsrA in the *in vitro* assay, which could not be substituted by other nucleotide ligands (ADP, AMP, AMP-PNP) and DMSO related components (DMS and DMSO₂), respectively. Robust MsrA-mediated SAMP-conjugates could be reconstituted *in vitro* by addition of purified SAMP, MsrA, and UbaA proteins to cell lysate of ΔmsrA ΔubaA Δsamp1/2/3 strains with ATP and DMSO. E1 homolog UbaA was found to be required for MsrA-dependent conjugation *in vitro*. Consistent with this finding, hydrolyzable ATP was needed for MsrA-dependent Ubl modification *in vitro* in the presence of UbaA. Furthermore, DMSO was found to stimulate Ubl-bond modification in a dose dependent manner both *in vivo* and *in vitro* assays. The SAMP-conjugates generated *in vitro* could be cleaved by HvJAMM1, a JAMM/MPN+ metalloprotease specific for Ub/Ubl tagged proteins. Although reductant DTT was needed for MSO reductase activity, Ubl bond formation mediated by MsrA *in vitro* was independent of DTT.

MsrA/B enzymes were determined to be active MSO reductases. Cell lysates of ΔmsrA/B single and double mutants were found to be significantly reduced in MSO-peptide reductase activity compared to the wt. Ectopic expression of wt MsrA/B in
ΔmsrA/B mutant strains was found to increase the MSO peptide reductase activity; whereas, MsrA C13S (while expressed) had no effect. Treatment of cells with DMSO was found to significantly reduce the level of MsrA-mediated MSO-peptide reductase activity, while having little if any influence on MsrB activity. Purified MsrA from Hfx. volcanii was found to readily reduce dabsyl-MSO by a mechanism that was stimulated by high ionic strength buffer and required reductant (DTT) and the conserved active site cysteine (C13) and glutamic acid residues (E56) for activity. DMSO and MSO were found to inhibit the MSO-peptide reductase activity of purified Hfx. volcanii MsrA enzyme in a dose dependent manner; whereas, methionine had no effect on this activity.

MsrA/B were shown to be not essential for MSO assimilation. Deletion of methionine synthase (metE1 and metE2) gene homologs of Hfx. volcanii led to a methionine auxotroph strain that could not grow in the media without Met while addition of Met or MSO could restore the cell growth. Both MsrA and MsrB were not required for the ΔmetE1/2 mutant to assimilate MSO as the sole source of Met for cell growth.
Figure 4-1. MsrA is required for the Ubl-protein conjugates induced by DMSO. *Hfx. volcanii* strains expressing the Flag-SAMPs and MsrA-StrepII (wt and C13S variant) were grown to stationary phase in the presence of the mild oxidant DMSO. Flag-SAMP1 (A), Flag-SAMP2 (B), and Flag-SAMP3 (C) are indicated. Cell proteins were analyzed by Coomassie blue (CB) staining and immunoblotting (IB) with anti-Flag and anti-StrepII antibodies. See methods for details. wt, wild type. MsrA*, covalently modified forms of MsrA.
Figure 4-1. Continued.
Figure 4-2. MsrA active site residues are required for the Ubl-protein conjugates induced by DMSO. *Hfx. volcanii* strains expressing the Flag-SAMP2 and MsrA-StrepII (wt and variant proteins) were grown to stationary phase under mild oxidizing (DMSO) conditions as indicated. Cell proteins were analyzed by Coomassie blue (CB) staining and immunoblotting (IB) with anti-Flag and anti-StrepII antibodies. wt, wild type. See methods for details.
Figure 4-3. MsrA is required for the Ubl-protein conjugates induced by MSO. *Hfx. volcanii* strains expressing the Flag-SAMPs and MsrA-StrepII (wt and C13S variant) were grown to stationary phase in the presence of MSO. Cell proteins were analyzed by Coomassie blue (CB) staining and immunoblotting (IB) with anti-Flag and anti-StrepII antibodies. See methods for details. wt, wild type. MsrA*, covalently modified forms of MsrA.
Figure 4-4. Differential effect of chemical agents on stimulating Ubl-conjugate levels in *Hfx. volcanii*. (A) The potent oxidative stress agent sodium hypochlorite (NaOCl) and (B) proteasome inhibitor bortezomib were found to stimulate SAMP2 Ubl-conjugate levels by an MsrA-independent mechanism. (C) Dimethyl sulfide (DMS) and dimethyl sulfone (DMSO₂) had no effect on the Ubl-conjugates formed in the cell. *Hfx. volcanii* strains expressing the Flag-SAMPs were grown to stationary phase with the effector treatment as indicated. Cell proteins were analyzed by Coomassie blue staining (CB) and immunoblotting (IB) with anti-Flag antibodies. wt, wild type. See methods for details.
**In vivo DMS and DMSO₂ treatment**

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Figure 4-4. Continued.
Figure 4-5. Model of MsrA-dependent sampylation. (A) Schematic representation of the proposed function of MsrA in Ubl-bond formation induced by mild oxidant. In this pathway, the E1-like UbaA adenylates and forms a thioester intermediate with the SAMP that is essential for Ubl-bond formation. HvPPA is an inorganic pyrophosphatase. MsrA undergoes a DMSO/MSO-induced switch from an MSO reductase to a protein factor required for mediating Ubl protein modification during mild oxidative stress. (B) The Cereblon-DDB1 E3 ligase thalidomide-binding domain is a structural homolog of MsrB and Lon domains (PDB: 4C11).
Figure 4-6. *In vitro* reconstitution of Ubl-bond formation with MsrA. (A) *In vitro* reconstitution of SAMP1-conjugates with MsrA wt but not MsrA C13S variant purified from *E. coli*. (B) *In vitro* reconstitution of SAMP2-conjugates with wt MsrA purified from *Hfx. volcanii*. (C) HvJAMM1 desampylase cleaves the SAMP1-conjugates formed by *in vitro* assay. IB analysis included detection of SAMP-conjugates (anti-Flag) and MsrA (anti-StrepII) as indicated. See methods for details.
In contrast to MSO reductase activity, DTT was not needed for MsrA-dependent \textit{in vitro} reconstitution of Ubl-bond formation. (A) Reconstitution of SAMP2-conjugates by adding purified MsrA (wt and C13S variant proteins) and SAMP2 to lysate of \textit{ΔmsrAΔsamp1/2/3} cell in the presence and absence of DTT. IB analysis included detection of SAMP-conjugates (anti-Flag) and MsrA (anti-StrepII) as indicated. (B) MsrA mediated MSO reductase activity. Data represent mean ± S.D. (n=3) for MSO reductase activity. (**, p<0.001). The p-values determined by two-tailed, unpaired Student’s t-test.
Figure 4-8. Hydrolyzable ATP and DMSO were required for MsrA-dependent Ubl-conjugates via in vitro reconstitution. (A) The effect of ATP and DMSO on MsrA-dependent SAMP1-conjugates by in vitro reconstitution. (B) The effect of different nucleotide on MsrA-dependent SAMP1-conjugates by in vitro reconstitution. (C) The effect of DMSO related chemicals on MsrA-dependent SAMP1-conjugates by in vitro reconstitution. IB analysis included detection of SAMP-conjugates (anti-Flag) and MsrA (anti-StrepII) as indicated. See methods for details.
Figure 4-8. Continued.
Figure 4-9. *In vitro* reconstitution of robust SAMP-conjugates by MsrA. (A) *In vitro* reconstitution of SAMP2-conjugates along with time by addition of purified SAMP2, UbaA, and MsrA to cell lysate of ΔubaAΔmsrAΔsamp1/2/3 strain with ATP and DMSO. (B) MsrA-dependent SAMP2-conjugates formed *in vivo* along with time. *Hfx. volcanii* strains expressing the SAMP2 and MsrA were grown to stationary phase and then treated with DMSO for certain amount of time as indicated. IB analysis included detection of SAMP2-conjugates (anti-Flag), MsrA (anti-StrepII) and UbaA (anti-N-terminal His) as indicated. See methods for details.
Figure 4-10. DMSO stimulated MsrA-dependent Ubl-conjugates in a dose dependent manner *in vitro* and *in vivo*. (A) *In vitro* reconstitution of SAMP2-conjugates by addition of purified SAMP2 and UbaA with/without MsrA to cell lysate of ΔubaAΔmsrAΔsamp1/2/3 strain with increasing concentration of DMSO. (B) MsrA-dependent SAMP2-conjugates formed *in vivo* induced by increasing concentration of DMSO. *Hfx. volcanii* strains expressing the SAMP2 and MsrA were grown to stationary phase with different concentration of DMSO as indicated. IB analysis included detection of SAMP2-conjugates (anti-Flag), MsrA (anti-StrepII) and UbaA (anti-N-terminal His) as indicated. See methods for details.
Figure 4-11. MsrA/B are catalytically active MSO reductases. (A) Schematic representation of MsrA/B- type enzyme activities in vivo compared to in vitro assay. X, represents reductant such as glutaredoxin, thioredoxin, thionein or a selenocompound. (B) Comparison of MSO reductase activities in cell lysate of *Hfx. volcanii* strains grown in 0 and 100 mM DMSO as indicated. Data represent mean ± S.D. (*, p<0.05; **, p<0.001; n.s., not significant). The p-value was determined by two-tailed, unpaired Student’s t-test. (C) MSO reductase activity of purified MsrA. Left panel, MsrA assay buffer was modified to include NaCl (0.25 to 2 M) and 0 to 20 mM effector [(DMSO, MSO and methionine (Met)] as indicated. Right panel, MsrA wt was compared to C13S and E56S for enzyme activity in assay buffer modified to include 2M NaCl. DTT was excluded from assay buffer where indicated. Error bars represent S.D.; U, units of activity defined as nmoles dabsyl-MSO per min. See methods for details.
Figure 4-11. Continued.
Figure 4-12. MsrA/B are not required for MSO assimilation. *Hfx. volcanii* cell growth on minimal medium supplemented with 5 mM methionine (Met) or methionine sulfoxide (MSO) as monitored by OD\(_{600}\). Statistical analysis relative to wt: *, P < 0.05; **, P < 0.01; n.s., not significant (two-tailed, unpaired Student’s t test). Error bars represent the mean results ± S.D. from three biological replicates. See methods for details.
CHAPTER 5
SUMMARY AND FUTURE PERSPECTIVES

Summary of Findings

The discovery of archaeal SPS advanced the fundamental knowledge of signal-guided proteolysis in archaea and shed light on components that are related to the UPS of eukaryotes. By using TBP2 as the model substrate, we demonstrated that the archaeal SPS consists of UbaA (the E1/MoeB/ThiF homolog of archaea), AAA ATPases (Cdc48/p97 and Rpt types), a type 2 JAB1/MPN/MOV34 metalloenzyme (JAMM/MPN+) homolog (JAMM2), and 20S proteasomes. The Ubl protein SAMP2 stimulated the degradation of TBP2, but SAMP2 itself was not degraded, suggesting SAMP2 serves as the tag to target protein substrates to proteasomes for degradation by a recycling mechanism. Furthermore, we provided evidence that the attachment of the Ubl protein is regulated by an N-terminal phosphodegron. Our determination of the effect of proteasomal gene mutations and inhibitions on degradation of TBP2 has deepened our understanding of the types of degrons and substrates targeted for proteolysis by archaeal proteasomes, which provides scientific guidance for adjusting the proteasome proteolysis pathway to optimize archaea for bioconversion processes.

In addition, our finding that archaeal MsrA serves as an integral component of the sampylation during mild oxidative stress is a significant step forward in elucidating archaeal Ubl protein modification pathway. In this study, we discovered a novel function of MsrA, a MSO reductase that is conserved in all domains of life, in guiding the Ubl modification of proteins in archaea. MsrA required the conserved active site residues C13 and E56 and the mild oxidant DMSO (or MSO) for this activity and was conversely inhibited in its MSO peptide reductase activity by DMSO. MsrA was specific for the Ubl
conjugates formed only during mild oxidative stress (not severe oxidative stress or proteasome inhibition). Thus, MsrA represents a moonlighting protein that may undergo a DMSO/MSO-induced switch from an MSO reductase to a protein factor required for mediating sampylation during mild oxidative stress. Our determination of the dual functions of MsrA provides new insights into the molecular mechanisms of Ub/Ubl protein modification in response to oxidative stress, a common cause for aging and human diseases such as Alzheimer’s and Parkinson’s diseases.

**Future Directions**

Future work will focus on deep understanding archaeal SPS-mediated proteolysis pathway. Although this study suggests SAMP2 serves as the signal to bring TBP2 to proteasomes for degradation, how archaeal proteasomes recognize the SAMP tag on protein substrates is still unclear. Further investigation of the SAMP receptor in proteasomes will be very helpful to answer this question. Moreover, it is important to investigate the proteins associated with HvJAMM2 and determine whether or not streamlined forms of 19S RP-like complexes exist in archaeal proteasomes. Capture of the entire proteasome complex will be one of the ultimate goal, which will allow us to reveal the molecular architecture of archaeal proteasomes and conduct *in vitro* protein degradation assay by using sampylated substrates.

In addition, future investigation will concentrate on reconstitution of the archaeal sampylation pathway. At this stage, E1-like UbaA and MsrA are the two known enzymes required for the Ubl bond formation induced by the mild oxidant (DMSO). Protein substrates modified by SAMPs should be enriched by pull-down and identified by MS/MS analysis in mild oxidative condition. Identification of the substrate proteins will facilitate simplification of the *in vitro* reconstitution consists of purified SAMP, UbaA,
MsrA, and substrate protein. The real 

\textit{in vitro} reconstitution will be conducted in the presence and absence of ATP and DMSO. Alternatively, identification of MsrA partner proteins by pull-down assay coupled with MS/MS would be important if additional components are required for sanylation. Finally, investigation of the conformational switch of MsrA from an MSO reductase to E3 ligase induced by DMSO at atomic level will provide new insights into the mechanism of MsrA-mediated Ubl bond formation.
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BIOGRAPHICAL SKETCH

Xian Fu was born in Xiangtan, Hunan, China. He received the Bachelor of Science degree with honor from Central South University, China in June of 2011. He worked with Dr. Xueduan Liu and Dr. Huaqun Yin at MOE Key Laboratory of Biohydrometallurgy, China from September of 2008 to May of 2012. He joined the graduate program at the Department of Microbiology and Cell Science at the University of Florida in August of 2012. After three rotations, he joined Dr. Julie Maupin-Furlow’s lab and worked with her to study the proteolytic role and conjugation mechanism of Ubl-protein SAMPs in Haloferax volcanii. Xian Fu plans to continue research training as a postdoctoral associate at the Yale University.