

Advances in protein turnover analysis at the global level and biological insights

Qingbo Li

Center for Pharmaceutical Biotechnology, College of Pharmacy, and Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60607

Email: qkli@uic.edu; Phone: 312-413-9301.

The concept of a dynamic state of body constituents, a precursor of the modern term of proteome dynamics, was conceived over a century ago. But, not until recently can we examine the dynamics of individual ‘constituents’ e.g., proteins at a truly global level. The path of advancement in our understanding of protein turnover at the global level is marked by the introduction of some key technological innovations. These methods include the isotopic tracer technique in the 1930s, the two-dimensional gel electrophoresis technique in the 1970s, the sector mass spectrometer that could analyze isotopomers of peptides in the early 1990s, the 2D gel/MALDI-TOF proteomics technology in the late 1990s, the booming liquid chromatography/mass spectrometry proteomics technology in this decade, and the recently emerging protein-tagging approaches that offer single-cell resolution for protein turnover measurements. The long-standing inquiry raised in the 1950s about the existence of a dynamic state in different organisms at different physiological conditions can now be answered with an individual ‘constituent’ resolution on a truly global scale. Now it appears that protein degradation is not necessarily an end to the protein function. Rather, it can be the start of a new function because protein degradation clears the way for the action of other proteins. Protein turnover participates in a multi-layer complex regulatory network and shares equal importance with gene transcription and protein translation. The advances in technologies for protein turnover analysis and the improved understanding of the biological role of protein turnover will likely help to solve some long-standing biomedical problems such as the tuberculosis disease that at the present day still affects one-third of the world population.

KEY WORDS: proteomics, turnover, kinetics, mycobacteria, dormancy, proteome dynamics

Table of Contents

I. INTRODUCTION.....	3
II. PROTEIN TURNOVER AND BIOLOGICAL FUNCTIONS.....	5
A. Turnover of stationary-phase sigma factor and stress-response gene expression.....	5
B. Trans-targeting of protease substrates.....	6
C. Protein turnover and aging.....	8
III. METHODOLOGICAL DESCRIPTION OF PROTEIN TURNOVER.....	9
A. Rates of protein synthesis and degradation in a cell culture.....	9
B. Relative turnover.....	13
C. Fractional synthesis rate.....	14
IV. PROTEOME DYNAMICS.....	14
A. Whole-cell protein turnover.....	15
<i>A.1 Dynamic state in growing microorganisms.....</i>	<i>16</i>
<i>A.2 Protein turnover and its regulation in non-growing microorganisms.....</i>	<i>17</i>
<i>A.3 Distinct characteristics of protein turnover in different organisms.....</i>	<i>22</i>
B. Individual protein turnover at the global level.....	22
<i>B.1 Two-dimensional gel electrophoresis.....</i>	<i>22</i>
<i>B.2 MALDI-TOF with two-dimensional gel electrophoresis.....</i>	<i>24</i>
<i>B.3 Liquid chromatography/mass spectrometry.....</i>	<i>25</i>
<i>B.4 Protein-tagging for turnover analysis.....</i>	<i>27</i>
C. Isotopomer analysis with high-resolution mass spectrometry.....	30
D. Implication of proteome dynamics in mycobacteria.....	36
V. CONCLUDING REMARKS.....	37
REFERENCES.....	39

I. INTRODUCTION

The concept of the dynamic state of body constituents was first conceived by some scientists in the 19th and early 20th centuries (Guggenheim, 1991). This dynamic concept of metabolism, however, was not demonstrated with clear experimental evidence until Schoenheimer introduced the isotopic tracer technique (Schoenheimer and Rittenberg, 1935). The introduction of the isotopic tracer technique has been regarded as a landmark in intermediary metabolism research (Bloch, 1996; Guggenheim, 1991) and as one of the several key contributions to the beginning of the era of biosynthesis in the 20th century (Kennedy, 2001).

Soon after demonstrating the isotopic tracer technique to study fatty acid and cholesterol metabolism, Schoenheimer and colleagues applied it toward amino acids and proteins (Bloch and Schoenheimer, 1941; Schoenheimer et al., 1939; Schoenheimer et al., 1938). These early studies led to the emergence of many protein turnover studies on bacterial cells (Borek et al., 1958), mammalian cells (Eagle et al., 1959), and plants (Huffaker and Peterson, 1974). The wealth of knowledge in protein turnover accumulated to date reflects the advancement in biochemistry and molecular biology. The translation of this knowledge has also led to the advancement of new medicine, as exemplified by the introduction of anticancer drugs that target the ubiquitin-proteasome pathway (Orlowski and Kuhn, 2008). It has been well-acknowledged that intracellular protein degradation and turnover are fundamental processes in all cell types and that they have important implications to biochemical regulations, genetics, physiology, and medicine (Larrabee et al., 1980; Wilkinson, 2005).

The isotopic tracer technique remains the main method for a protein turnover study. Many earlier works on protein turnover relied on radioactive isotopes for metabolic labeling (Eagle et al., 1959; Larrabee et al., 1980). The reliance on radioactive isotopes was in part due to the high detection sensitivity and specificity provided by radioactive labels. With the advent of mass spectrometry capable of accurate measurement of peptides and proteins, however, recent studies on protein synthesis, degradation, and turnover dynamics more frequently employ stable isotopes such as ²H, ¹³C, or ¹⁵N in the form of amino acids, carbon source, ammonium salts, or water (Cargile et al., 2004; Hellerstein and Neese, 1992; Pratt et al., 2002; Rao et al., 2008b). The stable isotope labeling approach enjoys the flexibility of being applicable to almost all living

organisms, including humans. This flexibility is afforded by the multiple options available to administer a stable isotope label to an organism and the universal mode of protein analysis by mass spectrometry.

Most recently, alternative approaches based on protein tagging for global protein stability profiling have also emerged with Western blot (Belle et al., 2006), flow cytometry (Yen and Elledge, 2008; Yen et al., 2008), and fluorescence microscopy (Cohen et al., 2008). These studies deliver an excellent coverage for the proteomes of genetically manipulated yeast and mammalian culture cells, and demonstrate the feasibility to measure protein turnover with single-cell resolution (Cohen et al., 2008; Yen et al., 2008). These technologies allow a truly comprehensive protein turnover analysis at the global level.

Although the limitations of proteome coverage are well known for mass spectrometry-based approaches, a recent study of *Arabidopsis* with LC/MS-based proteomics showed that these limitations could be overcome (Baerenfaller et al., 2008): with a linear ion trap mass spectrometer, a 50% proteome coverage was achieved for an organism as complex as *Arabidopsis*. A total of 13,029 proteins were identified with 1,354 linear ion trap mass spectrometry analyses of protein samples extracted from undifferentiated culture cells and six separate organs of *Arabidopsis*. The 13,029 proteins were identified with 86,456 unique peptides from a total of 790,181 MS/MS spectra. These data demonstrate that, in principle, a high coverage of a proteome is possible when adequate resources are available to support many LC/MS analyses.

Mass spectrometry continues to serve as a major approach for a protein turnover study due to its wide availability and flexibility to analyze both single-cell cultures and multi-cellular organisms. Except for the required administration of stable isotope-labeled metabolites, amino acids, or water to the study subjects, mass spectrometry-based approaches do not require any genetic manipulation of the study subjects. The avoidance of genetic manipulation of the study subjects helps to minimize any unwanted perturbation to a biological system and delivers the most physiologically relevant results. A range of methodologies has been established to measure protein turnover based on stable isotope labeling and mass spectrometry. Most of these methods have been reviewed elsewhere with comparison of their benefits and limitations in protein turnover measurements at a proteome scale (Doherty and Beynon, 2006) and will not be repeated in this review.

This review will focus on the advances in a biological understanding of protein turnover at the global level brought about by key technological advancements. It will also briefly discuss the perspectives of global protein turnover analysis for mycobacteria research.

II. PROTEIN TURNOVER AND BIOLOGICAL FUNCTIONS

Built upon the actuality established in man that there existed a dynamic steady state between protein synthesis and breakdown, Borsook and Keighley introduced the concept of 'continuing metabolism' (Borsook and Keighley, 1935). The work by Schoenheimer et al. with the isotopic tracer technique demonstrated beyond doubt that the peptide linkages were continuously breaking and reforming. This dynamic state of proteins has now been accepted as a fundamental aspect of intermediary metabolism. The molecular machinery that executes the protein breakdown process plays essential roles in cellular functions. Thus, breakdown of a protein does not necessarily mean the termination of the function of a protein. It could serve as a starting point for many new processes, which can occur only when the way has been cleared for the action of other proteins (Marfany et al., 2008). A change in enzymatic activities normally correlates with the abundance of an enzyme in the cell, which is regulated not only by transcription and translation but also by stabilization and degradation. Regulation of protein levels by protein degradation is thus an integral part of stress response in all cells (Neher et al., 2003). In the following, a few examples are provided to illustrate the critical roles that protein turnover can play in different cellular processes.

A. Turnover of stationary-phase sigma factor and stress-response gene expression

Gene transcription is a key point at which cells decide which proteins are to be produced and at what rate. In bacteria, a sigma factor (σ) is responsible to recognize the promoter region of a gene and facilitates the initiation of transcription by the bacterial RNA polymerase. There are *ca.* a dozen of known classes of sigma factors in eubacteria (Wosten, 1998). In *Escherichia coli*, the stationary phase sigma factor σ^S regulates the expression of *ca.* 50 genes in response to many stresses such as acid shock, oxidative damage, heat shock, starvation, and transition to stationary phase (Hengge-Aronis, 2000; Loewen and Hengge-Aronis, 1994).

In *E. coli*, the degradation of the σ^S protein is fast in an exponential growth phase with a half-life of *ca.* 2 min, to keep the level of σ^S low. Upon encountering stressful conditions, σ^S is stabilized with a degradation rate of *ca.* 10-fold lower. The stabilization of σ^S leads to an accumulation of σ^S and expression of stress-response genes. Consequently, regulated degradation of σ^S becomes an integral part of control over the σ^S activity.

A probable pathway of σ^S degradation has been suggested (Zhou et al., 2001). In this pathway (**Figure 1**), the protein RssB specifically targets σ^S for degradation. RssB, which stands for ‘Regulator of Sigma S’, is homologous to response regulator proteins. RssB receives a phosphoryl group from acetyl-phosphate that effects its binding to the target σ^S . Binding between RssB and σ^S enhances the affinity of the RssB- σ^S complex to ClpXP, an ATP-dependant protease. This enhanced affinity results in the degradation of σ^S but not RssB. The regulation of σ^S degradation by RssB follows the mechanism of *trans*-targeting, in which a regulatory protein promotes the degradation of a second protein. RssB is the specific and regulatable targeting protein. It plays an essential role to regulate the degradation of the physiologically important protein σ^S . With the signal transduction from the response regulator RssB, the cell regulates the expression of stress-response genes through a controlled degradation of σ^S that is responsible to initiate transcription of the stress-response genes.

In *Salmonella enterica*, the σ^S protein accumulates when the cell is grown under magnesium limitation. The accumulation of σ^S is the result of stabilization brought about by the PhoP/PhoQ two-component regulatory system that controls the turnover of σ^S (Tu et al., 2006). PhoP serves as a transcriptional activator of the *iraP* gene, which encodes the product IraP that competes with σ^S for binding to RssB. At low magnesium conditions, IraP is upregulated. A higher level of IraP promotes the binding between RssB and IraP, to thus reduce the chance of any RssB- σ^S complex formation. Consequently, less σ^S is delivered by RssB to the ClpXP protease for degradation. This reduced delivery of σ^S to ClpXP leads to the stabilization and accumulation of the σ^S protein, which in turn enhances the expression of stress-response genes under a low magnesium condition.

B. *Trans*-targeting of protease substrates

ClpXP is known to recognize and degrade a number of important regulatory proteins. ClpXP degrades different protein substrates at different times, depending upon the growth or environmental stress conditions. One mechanism to regulate degradation of ClpXP substrates is

trans-targeting. In this mechanism, a regulatory protein promotes degradation of a second protein. In addition to the stationary phase sigma factor σ^S described above, there are other examples of this targeting mechanism. These include the DNA polymerase V subunit UmuD' and the polypeptides marked with the SsrA degradation tag. Proteolysis of the UmuD' proteins is promoted by the UmuD' precursor UmuD (Gonzalez et al., 2000; Neher et al., 2003). Degradation of the polypeptides marked by SsrA is promoted by the protein SspB (Levchenko et al., 2000).

UmuD' is part of the UmuD₂C complex; i.e., the DNA polymerase V. In *E. coli*, the DNA polymerase V is the major bypass responsible for lesion bypass during DNA damage and induced mutagenesis. At normal conditions, this highly error-prone DNA polymerase is kept at a minimum level by transcriptional and translational regulations as well as by post-translational regulation via the degradation by the Lon protease (Gonzalez et al., 1998). In the event when the cells incur DNA damage, some of the UmuD molecules escape Lon-mediated degradation and are converted to the mutagenically active UmuD' protein.

Unlike UmuD, UmuD' is relatively insensitive to Lon-mediated proteolysis and much more stable *in vivo* (Frank et al., 1996). Its stability allows it to carry out DNA repair. After the lesion in DNA is repaired, the continuing survival of UmuD' could be problematic for the cell. This problem is because of the increased likelihood that the error-prone DNA polymerase V will replicate undamaged DNA to cause unwanted mutations. As a consequence, the cell employs the *trans*-targeting mechanism to remove the mutagenically active UmuD' protein by proteolysis.

In the proteolysis process, the unprocessed UmuD is the targeting protein that facilitates binding of UmuD' to ClpXP for removal. In the presence of unprocessed UmuD, UmuD' preferentially forms a heterodimer with UmuD. This heterodimer has a higher affinity for ClpXP than either of the homodimers of UmuD and UmuD'. In this *trans*-targeting reaction, the UmuD subunit provides the sequence information essential to recognize and degrade UmuD', although neither of the two subunits is degraded on its own.

Thus, the DNA polymerase V is a temporally expressed system in response to DNA damage. The rapid induction of the Umu proteins involves escape from the protein degradation machinery and conversion to a mutagenically active form insensitive to proteolysis. Upon completion of DNA repair, these response proteins are no longer required. In the presence of

excess UmuD, the active form UmuD' is removed by the ClpXP protease through the *trans*-targeting mechanism.

SspB is a ribosome-associated protein in *E. coli*. It was demonstrated to be a specificity-enhancing factor for ClpXP that controls the choice of polypeptides for degradation by the SsrA peptide-tagging system (Levchenko et al., 2000). A stalling of ribosomes produces premature polypeptides. Such events activate the SsrA-tagging machinery to result in the resumption of translation and addition of an 11-residue peptide to the carboxyl terminus of the nascent polypeptide chain. The *ssrA*-encoded peptide tag marks the incomplete protein for degradation by the ClpXP protease. In this case, SspB serves as the targeting protein. SspB facilitates the degradation of SsrA-tagged premature polypeptide chains by the *trans*-targeting mechanism.

C. Protein turnover and aging

In mammals, accumulation of posttranslational modifications has been correlated with age-associated decline of protein functions. It was thought to be, at least in part, due to the diminished capacity of the cell to remove and replace those defective proteins by the turnover process. Evidence has been provided that the progressive decrease in protein turnover indeed can be the primary cause of the increase in concentrations of damaged proteins with age (Ryazanov and Nefsky, 2002). Conversely, protein damage itself is likely to contribute to a further decline in protein turnover to aggravate the situation. The protein damage and the decline of turnover could form a positive feedback loop, where the increase in protein damage decreases the protein turnover to lead to a further accumulation of damaged proteins. “The establishment of such a feedback loop should result in an exponential increase in the amount of protein damage—a protein damage catastrophe—that could be the basis of the general deterioration observed in senescent organisms” (Ryazanov and Nefsky, 2002).

Ferrington et al. examined the possibility that an altered protein turnover could be responsible for the diminished skeletal muscle contractile properties observed with aging (Ferrington et al., 1998). Some sarcoplasmic reticulum proteins such as the Ca-ATPase and the ryanodine receptor (calcium release channel; RyR) attribute to the age-related weakening of skeletal muscle contractile properties. Ca-ATPase and RyR are calcium regulatory proteins. The turnover of these two proteins was slowed down by 25-27% in aged muscles compared to that turnover in young muscles. Another sarcoplasmic reticulum protein, 53-kDa glycoprotein which is a putative regulator of the Ca-ATPase, had 25% faster turnover in aged muscles. Whereas the

turnover of the Ca-ATPase and the ryanodine receptor changed with age, the average turnover of all the sarcoplasmic reticulum proteins showed no age-related changes. The study showed the importance of measuring the turnover of individual proteins as opposed to the average turnover of a mixture of multiple proteins, because the turnover was not necessarily homogenous among the proteins in the mixture. Regulatory proteins are more prone to faster turnover compared to the majority of other proteins. Because regulatory proteins typically exist at a lower abundance, the faster turnover of the regulatory proteins can easily be masked by the average turnover of the protein mixture.

Although the examples provided above are not meant to be comprehensive, they clearly support the concept that protein turnover is a fundamental process in all cell types that has important implications in protein regulation and physiology. Protein turnover is the overall process of protein renewal that involves synthesis of new proteins and degradation of old proteins. There is no doubt that protein synthesis and protein degradation share equal importance for our understanding of the complex biological functions of many proteins, and the biological networks that integrate them. Although regulation of protein synthesis is much better understood by studying transcription and translation at a genome level, our understanding of protein degradation at the global level is still lacking.

III. METHODOLOGICAL DESCRIPTION OF PROTEIN TURNOVER

A. Rates of protein synthesis and degradation in a cell culture

Protein turnover is the balance between synthesis and degradation, and reflects how fast a protein is lost and regenerated. In a steady-state, the rates of synthesis are equal to the rates of degradation so that the protein levels stay constant. However, such a steady-state only represents a limited and special instance of a biological system. Under a more general condition, the rates of synthesis and the rates of degradation differ and lead to a net change in protein levels.

Protein degradation is catalyzed by proteolytic enzymes. Not surprisingly, the rate of degradation for most proteins follows first-order kinetics (Arias et al., 1969; Belle et al., 2006; Segal and Kim, 1963). On the other hand, the rate of synthesis depends on multiple factors, which possibly include transcription, translation, and precursor abundance. Very often, these variables can not be readily factored; hence, they are all included under the general notation of the rate of synthesis. Although the rate of synthesis is sometimes assumed to follow zeroth-order

kinetics, especially at a protein steady state, extrapolation of such simplification to more general conditions requires caution (Ramakrishnan, 2007).

Under the assumption that protein synthesis follows zeroth-order kinetics, and that protein degradation follows first-order kinetics, the abundance of a protein at any given time t , A_t , can be expressed by a simplified formula (Arias et al., 1969)

$$\frac{dA_t}{dt} = K_s - k_d A_t \quad (1)$$

where K_s is the zeroth-order rate constant of protein synthesis, and k_d is the first-order rate constant of protein degradation. At a steady state, $dA_t / dt = 0$. Then, the protein abundance at the steady state, A_0 , is determined by the rate of synthesis, K_s , and the rate of degradation, k_d .

$$A_0 = \frac{K_s}{k_d} \quad (2)$$

A change in either K_s or k_d will lead to a new level of protein abundance. When the cell receives a stimulation, the cell transits to a new equilibrium. At the new equilibrium, the system has the new protein abundance A_e , and the new rates of synthesis and degradation K'_s and k'_d .

$$A_e = \frac{K'_s}{k'_d} \quad (3)$$

At any time during the increase of protein level from A_0 to A_e , the protein abundance A_t can be calculated from the equation (Segal and Kim, 1963)

$$\ln(A_t - A_0) = \ln(A_e - A_0) - k_d t \quad (4)$$

Rearranging Equation 4 results in

$$A_t = A_0 + (A_e - A_0)e^{-k_d t} \quad (5)$$

Similarly, when returning from steady state A_e to A_0 , A_t follows

$$A_t = A_e - (A_e - A_0)e^{-k_d t} \quad (6)$$

Equations 5 and 6 show that the protein abundance A depends upon the rate of degradation, even though its steady state is determined by the rate of synthesis and the rate of degradation. Because protein degradation follows first-order kinetics, the rate of degradation usually means the rate constant of degradation instead of the absolute rate of degradation. The absolute rate of degradation depends upon the protein abundance level A , whereas the rate constant of degradation does not. Therefore, the rate of degradation is often expressed in terms of a half-life (Arias et al., 1969) which is

$$t_{1/2} = \frac{0.693}{k_d} \quad (7)$$

For an exponentially growing cell culture, the abundances of new proteins increase with an apparent first-order rate constant of doubling time k_{dt} . The old proteins along with a portion of the new proteins are degraded with an apparent first-order rate constant of protein degradation, k_d . At any given time t , the total abundance of a protein consists of the old fraction of the protein $A_{old,t}$, and the new fraction of the protein $A_{new,t}$. At time-point zero, the total protein abundance is A_0 , and it is assumed that the new fraction of the protein is zero. Then, the sum of the old and new fractions of the protein at a given time t follows first-order kinetics:

$$A_{old,t} + A_{new,t} = A_0 e^{k_{dt} t} \quad (8)$$

Because the loss of the old fraction of the protein $A_{old,t}$ follows the first-order kinetics

$$A_{old,t} = A_0 e^{-k_d t} \quad (9)$$

Combining Equations 8 and 9 yields

$$A_{new,t} = A_0(e^{k_{dt}t} - e^{-k_d t}) \quad (10)$$

Dividing Equation 10 by Equation 9 and rearranging the terms affords Equation 11 (Larrabee et al., 1980).

$$\ln\left(\frac{A_{new,t}}{A_{old,t}} + 1\right) = (k_{dt} + k_d)t \quad (11)$$

The $\frac{A_{new,t}}{A_{old,t}}$ ratio could be measured with a dual radioactive labeling method (Larrabee et al., 1980). In this method, one radiolabel, ^3H , was first used to pulse label the proteins. A second radiolabel, ^{14}C , was introduced to label newly synthesized fraction of a protein. The old fraction of the protein that survived degradation remained ^3H labeled. The $\frac{A_{new,t}}{A_{old,t}}$ ratio was calculated from the disintegration rates of the two radiolabels. With modern mass spectrometry analysis, only one stable isotope is needed to label newly synthesized proteins, while the old proteins remain unlabeled, or *vice versa*. The $\frac{A_{new,t}}{A_{old,t}}$ ratio can be calculated from the unlabeled isotopologue profile intensity and the labeled isotopologue profile intensity (Cargile et al., 2004; Doherty et al., 2009; Rao et al., 2008a; Rao et al., 2008b).

Based on the temporal profile of $\ln\left(\frac{A_{new,t}}{A_{old,t}} + 1\right)$ vs t , the rate constant $(k_{dt} + k_d)$ can be deduced for a protein in a logarithmically growing culture. Knowing the rate constant of culture doubling time k_{dt} , the rate of degradation k_d for individual proteins can be calculated (Larrabee et al., 1980). The calculation shown here is appropriate for a cell culture in which a doubling time can be readily measured. For a whole organism where a doubling time measurement is not practical, a more sophisticated scheme for stable isotope labeling and protein turnover

calculation would be necessary (Doherty et al., 2005). The related topic of various stable isotope labeling methods and experimental strategies to calculate a protein turnover has been extensively covered by other reviews (Beynon and Pratt, 2005; Doherty and Beynon, 2006).

B. Relative turnover

To answer the question of heterogeneity of protein degradation rates in endoplasmic reticulum (ER), Arias et al. introduced a relative turnover measurement to discern the rates of degradation for different fractions of ER proteins in rat liver (Arias et al., 1969). In that relative turnover measurement approach, animals were initially labeled with ^{14}C . Incorporation and decay of ^{14}C was allowed to proceed for a specified time, and the second label ^3H was introduced. After a limited period of time, the animals were sacrificed, and the ER proteins were fractionated. The ratio of the ^3H and ^{14}C disintegration rates was measured for the protein fractions, and was used to compare the degradation rates among the fractions. In this case, the ratio of the ^3H and ^{14}C disintegration rates represented relative turnover, which corresponds to the $\frac{A_{new,t}}{A_{old,t}}$ ratio described in Equation 11. The relative turnover reveals the difference in degradation rates among the proteins irrespective of the absolute abundance of the proteins. This $\frac{A_{new,t}}{A_{old,t}}$ ratio provides a convenient assessment of the magnitude of a relative turnover without the reliance on a particular assumption of the kinetic model of protein turnover. But, if one uses the $\frac{A_{new,t}}{A_{old,t}}$ ratio to calculate protein turnover kinetic parameters e.g., K_s or k_d , a zeroth- or a 1st-order system would be assumed (Arias et al., 1969; Larrabee et al., 1980).

This relative turnover approach was extended by Cargile et al. with modern mass spectrometry and stable isotope labeling (Cargile et al., 2004). In that work, the authors described the approach as the synthesis/degradation mass spectrometry (SDMS) method to determine ‘relative dynamic protein turnover rates’. As in (Arias et al., 1969), the SDMS method required only a single sampling time point. The SDMS method utilizes only one stable isotope label because the unlabeled form of the proteins serves as the other ‘label’. The SDMS analysis is compatible with typical quantitative proteomics experiments that use stable isotope labeling for relative abundance measurements (Beynon and Pratt, 2005; Rao et al., 2008b). With regard to

the use of the $\frac{A_{new,t}}{A_{old,t}}$ ratio measurement to derive kinetic parameters, the relative turnover

calculation is not fundamentally different from a classic pulse or constant infusion incorporation paradigm (Doherty et al., 2009; Larrabee et al., 1980; Pratt et al., 2002).

C. Fractional synthesis rate

For eukaryotic systems, protein synthesis is measured by monitoring the incorporation of a specific isotope labeled amino acid. The protein labeling takes two steps. First, in a specific labeling period, extracellular labeled amino acids are imported into cells. The intracellular labeled amino acids accumulate to a certain level. Second, these labeled amino acids are used as precursors for de novo synthesis of labeled proteins. The fraction of the labeled protein synthesized per unit time during the labeling period is termed ‘fractional synthesis rate’ and is usually taken as a measurement for protein turnover. The fractional synthesis rate is useful for taking into account limited transport and compartmentation of precursors.

The fractional synthesis rate is calculated by the tracer incorporation method (Wolfe, 1992) with the general equation

$$FSR = \frac{E_{t_2} - E_{t_1}}{E_p \times (t_2 - t_1)} \quad (12)$$

where $(E_{t_2} - E_{t_1})$ is the product enrichment of the label during the time period from t_1 to t_2 . E_p is the mean precursor enrichment between t_1 and t_2 . E_{t_1} , E_{t_2} , and E_p are typically expressed as mole percent excess (Zhang et al., 2004).

IV. PROTEOME DYNAMICS

It has been well-documented that protein turnover varies among species and even among strains. For a specific cell line of an organism, protein turnover also varies under different growth conditions, on different nutrients, and at different stages of the growth or cell cycle. These variations manifest the dynamics of the proteome.

Proteome dynamics is a natural pathway for an organism to adapt to the environments for survival and proliferation. Such proteome dynamics is controlled by the synchronization between protein synthesis and degradation. Although protein synthesis is mostly controlled at the levels of transcription and translation, protein degradation is realized via proteolysis carried out by proteasomes. Although the mechanisms of transcription and translation regulation are better understood, regulation of protein turnover remains relatively unclear. This limited understanding of the regulation of protein turnover is in part due to the lack of appropriate methodologies to study protein turnover on a global scale. In contrast, global expression analysis of mRNA transcripts and proteins has become a routine that generates ample datasets to advance the understanding of the transcription and translation regulation networks. Most recent advancement in proteomic technologies has enabled the acquisition of a similar scale of data on protein turnover (Belle et al., 2006; Cohen et al., 2008; Doherty et al., 2009; Rao et al., 2008a; Yen et al., 2008). The emergence of these modern proteomic technologies is poised to bring about a significant advancement in our understanding of the protein turnover process and its regulation at the global level.

A. Whole-cell protein turnover

Prior to the advent of the two-dimensional gel electrophoresis technique (O'Farrell, 1975), most protein turnover studies were on whole-cell proteins instead of individual proteins due to the difficulty to resolve many individual proteins simultaneously from the proteome. Most of these studies employed radioactive tracers to label proteins. Double tracers were often used, one to measure degradation of old proteins, and the other to measure synthesis of new proteins. Double tracers also provided a means to calculate a relative turnover that could be represented conveniently as the isotope ratio of the two tracers. Even though those early works were on whole-cell protein turnover, they provided valuable insights into the protein turnover process in different species at different conditions. Those elegant early works reflect important advancements in our understanding of the protein turnover process, and might continue to serve as worthy references for designing new experiments for protein turnover studies using modern technologies.

Schoenheimer's classic experiments revealed that labeled amino acids were rapidly incorporated into tissue proteins in animals, and confirmed the continuous incorporation and subsequent release of amino acids from proteins in animals (Schoenheimer, 1942). Considerable

later evidence further established that proteins were unstable in animal tissues and cells due to apparent protein turnover.

Tumors showed different protein turnover dynamics, however. Mider et al. referred to tumor tissues as a 'nitrogen trap' due to their tendency to take amino acids from the body pool without returning them (Mider, 1951). From a current perspective, this lack of release of amino acids back to the body pool means that the protein degradation rate in tumor tissues is much smaller than that in normal tissues, or that the protein synthesis rate is much higher than the protein degradation rate in tumor tissues. At the time, this discrepancy raised the question about the extent of protein degradation in other rapidly growing systems (Koch and Levy, 1955). The question was whether the dynamic state originally observed in animals and mammalian cells should be absent from microorganisms.

A.1 Dynamic state in growing microorganisms

Following the experimental confirmation of the continuous incorporation and subsequent release of amino acids from proteins in mammals (Schoenheimer, 1942), Podolsky studied protein degradation in *E. coli* (Podolsky, 1953). This early experiment measured the loss of incorporated ¹⁴C labeled amino acids from bacterial proteins. Aerobic *E. coli* cultures were grown in synthetic media with lactate and/or amino acids as the carbon source. The rate of protein degradation for total cell protein was 8±2% per 30-hour period. Amino acid-supplemented media stimulated a faster cell growth as well as a faster protein degradation compared to the media with only lactate as the carbon source. The protein degradation rate in the amino acid-supplemented media was 5-7 times that in the synthetic medium without amino acids. From the experiments, it appeared that conditions favorable to rapid synthesis of proteins were also conducive to the rapid degradation of proteins. The results provided evidence that the protein constituents and proteome dynamics depended upon the medium in which the cells grew. The observed extent of protein degradation, however, was not related to the extent of net bacterial growth. The cell culture in the quasi-stationary phase was not metabolically inactive. This study provided evidence on the response of the bacterial proteome that involved protein turnover.

Koch and Levy measured protein turnover in growing *E. coli* cultures by monitoring the release of purines from radiolabeled cells (Koch and Levy, 1955). This approach to use the release of purines as a measurement of protein turnover was based on the assumption that the

increase of labeling found in the purines reflected the rate of protein degradation and the probability of protein degradation products used for purine synthesis. *E. coli* cultures were grown in M-9 media in two stages. First, the cells were grown in the presence of a ^{14}C -labeled substance such as carbohydrates or glycine and unlabeled purines. The unlabeled purines suppressed the de novo synthesis of purines. The ^{14}C -labeled substances provided precursors to label proteins. Second, the labeled cells were washed and resuspended in unlabeled media without purines. If protein were degraded to precursors, then some of these precursors would be used for purine synthesis in the absence of exogenous purines. Thus, the appearance of radioactivity in the purine fraction of the culture was a measure of protein degradation. The protein degradation rate was determined to be *ca.* 10^{-3} per hour, which corresponded to a 30-day half-life. The authors concluded that degradation in the growing bacterial cell occurred at a very small rate, 'if it occurred at all'.

Using an auxotrophic strain of *E. coli* that required leucine and threonine, Mandelstam determined the rate of protein degradation in growing cells by monitoring the loss of incorporated ^{14}C -labeled leucine (Mandelstam, 1957; Mandelstam, 1958). The growing cells showed <4% loss of label. Protein degradation was non-linear in the growing cells; most of the loss of label occurred in the first 30 min of lag phase. After the initial lag phase, proteins tended to be very stable. This protein stability after the lag phase concurred with the observation by Koch and Levy that protein degradation occurred at a very small rate in growing *E. coli* cells (Koch and Levy, 1955).

A.2 Protein turnover and its regulation in non-growing microorganisms

Mandelstam further measured the protein synthesis and degradation rates in the non-growing cells of the *E. coli* strain auxotrophic for leucine and threonine (Mandelstam, 1958). The cells were first grown in the presence of labeled leucine. The bacteria were washed and suspended in media without threonine and ammonium so that growth could not occur due to nitrogen starvation. Labeled glycine was added into the nitrogen-starved cultures. The rate of protein synthesis was measured by incorporation of labeled glycine, and the rate of protein degradation was measured by release of labeled leucine. Incorporation of glycine and release of leucine were linear for the 3-4 hour experiment. The synthesis and degradation rates were both *ca.* equal, and were *ca.* 4.5% per hour in the non-growing cells. This evidence showed that protein synthesis continued even when there was no net cell growth. The protein degradation rate

in the non-growing cells, however, was much higher than that in growing cells. On the other hand, the protein synthetic rate in the non-growing cells was much smaller than that in the growing cells.

Protein degradation was shown to be inhibited by metabolites (Mandelstam, 1958). Addition of an ammonium salt into a nitrogen-starved culture inhibited protein degradation. The presence of threonine accelerated the development of the inhibition. The effect of an ammonium salt or threonine on protein degradation was quite similar to that of chloramphenicol, an inhibitor of protein synthesis. The effect was also similar to that of azide, an inhibitor of the respiratory chain enzyme cytochrome oxidase. These results provided an alternative explanation for the action of azide and chloramphenicol on protein degradation. Azide and chloramphenicol might not be a direct inhibitor of protein degradation. Rather, their effect might be indirectly linked to the accumulation of metabolites (Mandelstam, 1958). In other words, reduced protein degradation could be due to an accumulation of certain inhibitory metabolites.

In biochemical studies of bacterial sporulation and germination, Spudich and Kornberg provided additional insight into the regulation of protein turnover process (Spudich and Kornberg, 1968). For an exponentially growing *Bacillus subtilis* culture, the rate of protein turnover was <1% per hour. There was no increased protein turnover until the cells entered sporulation. When the cells entered sporulation, the rate of protein turnover proceeded at 18% per hour. In the 4-hour sporulation period, the high protein turnover rate resulted in a total loss of 49% of radioisotope pre-incorporated into the cells. Proteins degraded during this period provided precursors for synthesis of sporulation proteins. If chloramphenicol were added at the late log phase, then protein turnover was inhibited during sporulation. If it was added in the stationary phase just prior to sporulation, then protein turnover was not inhibited. Those observations suggested that proteins synthesized early in the stationary phase were necessary for protein turnover. Proteins synthesized during sporulation were not needed for protein turnover during sporulation.

It was likely that a protease or proteases necessary for protein degradation was/were synthesized during the early stationary phase. Alternatively, the proteins synthesized during the early stationary phase were required to depress the level of a protease inhibitor so that proteases could be activated during sporulation. Due to the high rate of turnover during sporulation, it was suggested that multiple turnovers occurred during the period (Spudich and Kornberg, 1968).

Throughout the sporulation period, the total protein level remained constant, whereas proteins turned over by 49%. Extensive degradation of the proteins in vegetative precursor cells provided precursors for new synthesis during sporulation. Thus, protein turnover played a crucial role in sporulation.

A high rate of protein turnover was a striking aspect of sporulation in *Bacilli*. Because many *Bacilli* do not contain storage materials, it was assumed that monomers for energy conservation and biosynthesis in sporulation were made available to the cell mainly by protein turnover. Under normal growth conditions, sporulation is prevented by a catabolite repression. Upon exhaustion of nutrients, catabolite repression is relieved. The relief of catabolite repression leads to an endogenous modification of cellular metabolism to allow the expression of the sporulation phenomenon, which includes a high rate of protein turnover. At the end of the exponential growth phase, *B. licheniformis* induced the expression of several intracellular proteases that could degrade the intracellular protein content into free amino acids at an appreciable high rate (Bernlohr, 1967). When the expression of these proteases was blocked by the addition of chloramphenicol at the end of the exponential growth phase, protein turnover was inhibited (Spudich and Kornberg, 1968).

Bernlohr argued that classical methods for turnover measurement, represented by the use of radioactively labeled amino acids as tracers, were not satisfactory to study protein turnover in the sporulation system (Bernlohr, 1972). This argument was based on the observation that some of the amino acids could not be purged out of the cells due to the limitation in exit transport systems (Bernlohr and Clark, 1971). The limitation in the exit transport of amino acids could lead to an underestimation of protein turnover due to the intracellular recycling of labeled amino acids released by protein degradation.

Bernlohr devised an alternative to circumvent this problem; he used ^{18}O labeled water instead of isotopically labeled amino acids. During proteolysis, ^{18}O was incorporated into the carboxyl groups of released amino acids. When these labeled amino acids were incorporated into newly synthesized proteins during biosynthesis, the newly synthesized proteins became labeled at the carbonyl oxygen for the amino acid that was labeled during proteolysis. Based on the first-order kinetics, the number of turnovers of an average amino residue was expressed as $-2.3 \log(1-F)$, where F equaled the fraction of the oxygen that was replaced. The F -values were

calculated by measuring the atomic percent excess of carbonyl oxygen in proteins (Bernlohr, 1972).

The validity of using the ^{18}O -labeled water method to measure turnover depended upon the assumption that the equilibrium of water between the extracellular medium and intracellular space was not limited. It was known that permeability constants of water transport through cell membranes were greater than any known solutes, and were in the range of one microbial cell diameter per min (Mazur, 1965). Given that experiments were typically on the time scale of tens of minutes to hours, the fast equilibrium of extracellular and intracellular water contents would not impose a limitation in the transport of solutes through cell membranes. With the use of labeled water, transport of other solutes through the cell membranes was not needed. In addition, turnover values $>100\%$ could be measured by this approach (Bernlohr, 1972).

Using the ^{18}O labeled water method, Bernlohr observed turnover rate of 20 to 24% per hour for the first several hours of sporulation. This turnover rate was *ca.* 20% higher than the 18% turnover rate reported by Spudich and Kornberg (Spudich and Kornberg, 1968). The higher turnover rate observed by Bernlohr was consistent with the original expectation that the ^{18}O -labeled water method would avoid underestimation of turnover rate due to the limitation in the exit transport systems for amino acids released from degraded proteins. In addition, the use of the ^{18}O -labeled water method allowed the measurement of a turnover $>200\%$ upon completion of sporulation after *ca.* 18 hours. This method, however, was not without limitation. One issue was that the validity of the method also depended on whether the net synthesis of amino acids during sporulation was minimal if not prevented. This requirement of the absence of a net amino acid synthesis was because net amino acid syntheses incorporated ^{18}O and could lead to an overestimation of turnover. The experimental conditions were thus carefully controlled to meet this requirement in order to measure the actual turnover rate during sporulation (Bernlohr, 1972).

Borek et al. provided an example of the use of doubly labeled water to determine the rate of protein turnover in *E. coli* (Borek et al., 1958). The purpose of the work was to answer the question whether the dynamic state originally observed in animals and mammalian cells should be absent from microorganisms. In that work, protein turnover was measured for non-growing cultures of an *E. coli* strain auxotrophic of methionine. Prior to that study, multiple investigators already attacked this question by more or less indirect methods in which turnover was determined with the measure of the liberation of labeled amino acids from pre-labeled proteins

(Hogness et al., 1955; Koch and Levy, 1955; Mandelstam, 1957; Podolsky, 1953). With deuterium- and ^{18}O -labeled water, Borek et al. studied the question with a 'direct procedure'.

In the 'direct procedure', protein turnover was measured by monitoring the release of amino acids from degraded proteins, and subsequent incorporation of these amino acids into re-synthesized proteins. In the presence of $^2\text{H}_2^{18}\text{O}$, amino acids released by proteolysis incorporated ^{18}O from water. Because of resonance, both oxygen atoms in the carboxyl group in an amino acid were equally labeled and contained half the isotope concentrations of water in the medium. Reformation of the peptide bond would remove one of the two oxygen atoms, and leave half the ^{18}O concentration of the water in the peptide bond. With the presence of $^2\text{H}_2^{18}\text{O}$, free amino acids also underwent a transamination reaction that rapidly incorporated deuterium into the α -position by the action of the enzyme transaminase. Re-incorporation of the amino acids into proteins resulted in the appearance of deuterium in the proteins. Because transamination occurred at a much more rapid rate than proteolysis and protein synthesis, the appearance of deuterium in proteins would be a measure of the protein turnover.

Under methionine starvation, the *E. coli* strain auxotrophic of methionine did not grow. Borek et al. found that incorporation of ^2H and ^{18}O into proteins, expressed as atomic percent excess of ^2H or ^{18}O , was linear at least within the first 2 hours after methionine starvation. The incorporation of the isotopes was aborted by the addition of dinitrophenol, to confirm that the appearance of the isotopes in proteins was indeed due to protein synthesis that required energy. From the curves of isotope incorporation, a protein turnover rate was calculated at *ca.* 6% per hour with either ^2H or ^{18}O . The results were in agreement with the work of Mandelstam in which the turnover rate was determined to be 4.5% per hour for a leucineless mutant of *E. coli* under a non-growth condition (Mandelstam, 1957).

Those works demonstrated that proteins were continuously being degraded and synthesized in non-growing bacteria. The rate, however, was faster than that found in mammalian tissues. In contrast to growing bacteria and yeast, turnover occurred at about the same rate in growing and resting mammalian cell cultures with the rate of *ca.* 1% per hour (Eagle et al., 1957).

Eagle et al. demonstrated that the observed rate of turnover *ca.* 1% per hour in both growing and resting human cell cultures was largely an intracellular turnover process rather than cell replacement or protein secretion (Eagle et al., 1959). This conclusion was validated by

mechanically coupling pre-labeled and unlabeled cultures. The combined cultures were located in two separate flasks that were joined and overlaid with a common unlabeled medium under gentle shaking. Labeled and unlabeled amino acids released by extracellular turnover would be mixed, diluted, and incorporated by both cultures. If the turnover were extracellular, then the rate of loss of label from the labeled culture would be equal to the gain of label in the unlabeled culture, and the rate would be half of that measured in either of the two cultures alone. That hypothesis was not supported by the experimental data. Eagle et al. thus concluded that the protein turnover process observed in growing and resting cultures was intracellular. Extracellular turnover, if it occurred at all, was only a small part of the overall protein renewal process.

A.3 Distinct characteristics of protein turnover in different organisms

The above studies on whole cell protein turnover showed that there was a profound difference among mammal, yeast, and bacteria. The turnover rate was >20-times in non-growing cells compared to exponentially growing cells of bacteria and yeast, whereas the turnover rate was essentially the same in growing and resting mammalian cells. The rate of turnover in non-growing bacteria was substantially higher than that in mammalian cells by at least several-fold. However, those studies could not conclude whether the dynamic state described in mammal and non-growing bacteria existed in exponentially growing bacteria. The loss of label from intact cells, due to the slow degradation of the whole cell protein, would be difficult to distinguish from the loss of label from a small number of proteins that had a more rapid turnover. Without direct experimental evidence, Borek et al. suggested that the existence of a dynamic state in growing microorganisms should not be dismissed (Borek et al., 1958). That question would better be answered by a study of the turnover of individual proteins in a proteome.

B. Individual protein turnover at the global level

B.1 Two-dimensional gel electrophoresis

Using 2D gel electrophoresis, Larrabee et al. performed the first global protein turnover study of growing *E. coli* cultures (Larrabee et al., 1980). The intent of the work was to identify classes of proteins that might have different turnover rates during exponential growth of the cell culture. The high-resolution 2D gel electrophoresis technique (O'Farrell, 1975) permitted a direct measurement of the rates of synthesis and degradation of a large number of proteins. To carry out the analysis, cells were pre-labeled with a long-term exposure to ³H-labeled leucine. The pre-

labeled cells were centrifuged, resuspended in a medium that contained ^{14}C -labeled leucine, and allowed for a short growth time. The loss of the ^3H -label from a protein reflected degradation, and the incorporation of a ^{14}C -label into a protein reflected synthesis. After individual proteins were resolved on a 2D gel, individual protein spots were excised and measured for the ratio of ^{14}C to ^3H . Such ratios represented the relative rate of protein synthesis over degradation. The ratios were mathematically defined as a function of the apparent rate constant of exponential growth, the first-order kinetic rate constant of degradation, and the specific radioactivity incorporated into the proteins (Equation 11). Based on the temporal profile of the isotope ratio of ^{14}C to ^3H , and on the apparent rate constant of culture doubling, the rate of degradation for individual proteins was calculated (Larrabee et al., 1980). If there were no protein turnover, then the isotope ratios for all the proteins would cluster around the mean. The results showed otherwise. Although the majority of the 220-360 proteins detected in several experiments had isotope ratios around the mean, three proteins had isotope ratios much higher than the mean and appeared to follow a turnover process analogous to turnover in mammalian tissues in all the experiments. Earlier experiments showed that *E. coli* contained a mechanism for selective degradation of abnormal proteins, and that such degradation proceeded at similar rates in non-growing and growing cells (Goldberg, 1972). Combined together, that research showed that a dynamic state for individual proteins existed in non-growing and growing bacterial cells.

Although Larrabee et al. performed the first protein turnover study at the global level (Larrabee et al., 1980), the lack of sensitive methods for protein identification did not allow them to further elucidate the exact proteins that underwent a turnover process analogous to that in mammalian cells. The existence of the small number of proteins found to have dynamic state in the growing bacterial cells was not enough to prove that the majority of the proteins lacked that dynamic state. It is possible that many proteins have first-order kinetics of degradation in growing bacterial cells. However, there were no sensitive methods to identify and confirm that they undergo a mammalian cell-like protein-degradation process in growing bacterial cells. This lack of sensitivity to study larger number of proteins, especially low-abundance ones, remained as a major limitation in protein turnover study for another two decades (Futcher et al., 1999; Gerner et al., 2002; Grunfelder et al., 2001; Hellerstein and Neese, 1999).

B.2 MALDI-TOF with two-dimensional gel electrophoresis

With the advent of MALDI-TOF mass spectrometry, individual proteins could be identified from gel spots separated on 2D gels. With the global proteomic approach that combined 2D gel separation and MALDI-TOF peptide fingerprinting, Grunenfelder et al. analyzed the timing of protein synthesis and degradation during the *Caulobacter crescentus* cell cycle (Grunenfelder et al., 2001). A total of 979 protein spots were analyzed. The expression of 234 spots was found to oscillate significantly during the progression of synchronized cultures. Ninety-one of these proteins were identified with MALDI-TOF or immunodetection. The concentration of several important modulators of the *Caulobacter* cell cycle progression fluctuated as a consequence of timed synthesis and degradation during the cell cycle, and included the transcriptional regulator CtrA, the cell-division protein FtsZ, and the DNA methyltransferase CcrM. The identification of those important modulator proteins and the determination of their oscillation due to timed synthesis and degradation provided the evidence for a global role of protein turnover in bacterial cell cycle control (Grunenfelder et al., 2001).

Pratt et al. studied yeast protein turnover at the global level by combining 2D gel separation and MALDI-TOF identification of individual proteins, and advocated that protein turnover is ‘a missing dimension of proteomics’ (Pratt et al., 2002). In this study, a yeast culture auxotrophic of leucine was maintained in a chemostat. The cells were initially grown in the presence of ^2H -labeled leucine. After the cells were fully and uniformly labeled, a cold-chase period was started by adding an excess amount of unlabeled leucine and switching the feedstock to an unlabeled form. Samples were taken at multiple time points during the chasing period. Protein extracts from each time point were separated with 2D gel electrophoresis. Gel spots were excised and processed for protein identification. Relative isotope abundance (RIA) was calculated from the MS spectra of the identified peptides. The RIA of a protein was expressed as the fraction of old protein that remained in the total amount of the protein. It was calculated as the quotient of the labeled fraction of a protein to the sum of the unlabeled and labeled fractions of the protein. The rate constant of the loss of label from pre-labeled proteins, k_{loss} , was calculated based on the steady-state theory and first-order kinetics:

$$RIA_t - RIA_\infty = (RIA_0 - RIA_\infty)e^{-k_{\text{loss}}t} \quad (13)$$

where RIA_0 and RIA_∞ were the RIA for the fully labeled and the fully unlabeled forms of a protein, and RIA_t was the RIA at time t . With a single time point or multiple time point RIA_t values, k_{loss} was calculated from Equation 12. The true protein degradation rate k_{deg} was then calculated by subtracting the cell dilution factor in the chemostat from k_{loss} (Pratt et al., 2002). The k_{deg} values of *ca.* 50 abundant proteins were calculated and shown to be remarkably heterogeneous. That work provided direct evidence that protein turnover in growing yeast cells could be large for individual proteins, and suggested the existence of a dynamic state in growing yeast cells.

B.3 Liquid chromatography/mass spectrometry

Recent advancement in high-precision mass spectrometry such as the liquid chromatography/linear ion trap-Fourier transform mass spectrometry (LC/LTQ-FTMS) system provides unprecedented automation, mass resolution, and dynamic range for protein turnover studies at the global level as exemplified in the protein turnover study of mycobacteria (Rao et al., 2008a; Rao et al., 2008b).

With one-dimensional SDS/PAGE fractionation and subsequent nanoLC/LTQ-FTMS analysis, Rao et al. determined the global turnover profiles of *Mycobacterium smegmatis* under acid-shock and iron-limitation conditions (Rao et al., 2008b). A dynamic range of 3-orders of magnitude was demonstrated for relative turnover measurements. The study provided direct evidence that relative turnover in growing mycobacterium cells, with or without stress, was highly heterogeneous. The results obtained in that study addressed the long-standing question whether a ‘dynamic state’ exists in growing bacteria (Borek et al., 1958), and illustrated the benefits and needs to study protein turnover at the global level with the most advanced mass spectrometry technology.

In another study, Rao et al. used nanoLC/LTQ-FTMS to perform protein-turnover and abundance measurements (Rao et al., 2008a). The approach employed many large-scale quantitative proteomics techniques to make it readily accessible for protein turnover studies at the global level. The concomitant measurement of protein turnover and abundance was previously shown by Gerner et al. with 2D gel electrophoresis for separation and fluorography and autoradiography for quantitation of individual gel spots without any protein quantification (Gerner et al., 2002). With the advanced nanoLC/LTQ-FTMS system and a label-free

quantitation approach, Rao et al. demonstrated that protein abundance and turnover could both be measured with a dynamic range of at least 3-orders or magnitude in a fully automated fashion (Rao et al., 2008a). The study compared the sensitivity of relative turnover and relative abundance measurements to detect a dynamic response of *Mycobacterium tuberculosis* when the cell culture was shifted from an iron-starved to iron-sufficient condition. The results indicated that a relative turnover measurement was much more sensitive to monitor the dynamic response of the *M. tuberculosis* cells. Meanwhile, a combination of turnover and abundance measurements provided insight into the correlation of protein synthesis, degradation, and secretion. A further principal component analysis of the *M. tuberculosis* proteome dynamics (Rao and Li, 2009) reveals that protein relative turnover properties are orthogonal to protein relative abundance properties (**Figure 2**). Thus, a study of protein turnover at the global level would likely bring forward new findings that can be missed with a protein abundance analysis alone.

In a recent study, Doherty et al. employed a nanoLC/LTQ system and one-dimensional SDS/PAGE separation to study the turnover of the proteome of a human A549 lung adenocarcinoma cell line with SILAC strategy (Doherty et al., 2009). Nearly 600 proteins were measured for their k_{deg} , the degradation rate constant, which ranged from 2×10^{-5} to 5.4 h^{-1} ; a 5-orders of magnitude range. This range corresponded to half lives from many tens of hours to 6 min. Some putative features of the proteins were explored for their potential to dispose a protein to a high or low rate of turnover. The putative features included protein molecular weight, isoelectric point, sequence motif and N-terminal amino acid, and protein disorder. Most of these features showed weak or no correlation with the rate constants of degradation. This outcome can be understood because protein turnover is governed by more sophisticated mechanisms than just the physical or chemical properties of a protein (see Section II. Protein turnover and biological functions).

The authors also compared the k_{deg} values of 27 subunits of the 40S and 60S ribosomes. The stability of these subunits differed as much as 2-orders of magnitude in k_{deg} values, even though they belonged to the same protein complex. This finding was consistent with that of an earlier study of turnover and exchange of ribosomal subunits in rat liver (Dice and Schimke, 1972). Dice et al. concluded that ‘rat liver ribosomes are not synthesized and degraded as functional units but are in a continual state of flux in which most protein constituents of the ribosome rapidly exchange with similar ribosomal proteins in the cytoplasm’. The conclusion

drawn by Dice et al. was based on two types of evidence. First, the relative degradation rates of ribosomal proteins were heterogeneous. Second, ribosomal proteins in the cytoplasm appeared to exchange easily with the constituents of the ribosome complexes to likely lead to an equilibrium between the free and assembled ribosomal proteins in cytoplasm and ribosome complexes. Protein-assembly dynamics have also been observed for the chemoreceptor clusters in *E. coli* (Schulmeister et al., 2008), for the *E. coli* 30S ribosomal subunit (Talkington et al., 2005), and for FisZ, the homolog of eukaryotic tubulins, in *M. tuberculosis* (Chen et al., 2007).

B.4 Protein-tagging for turnover analysis

Recently, alternative approaches have emerged for global protein stability profiling based on protein-tagging. Large-scale protein stability measurements used Western blot (Belle et al., 2006), DNA microarray technology coupled with flow cytometry (Yen and Elledge, 2008; Yen et al., 2008), and fluorescence microscopy (Cohen et al., 2008). These studies use a similar strategy in that individual proteins are tagged by a common epitope or fluorescent protein for immunodetection or fluorescence detection. Each cell clone or strain contains one tagged protein. By analyzing individual clones or strains in a massively parallel fashion, these studies deliver excellent coverage of the proteomes for protein turnover profiling.

Belle et al. measured the half-life of more than 3,750 tandem affinity purification (TAP)-tagged proteins in the budding yeast proteome (Belle et al., 2006). In the protein half-life experiments, the TAP-tagged strains were grown to the log phase, and cycloheximide was added to halt protein synthesis. Equal numbers of cells were collected at different time-points for protein extraction. The TAP tag served as a common epitope to quantify the tagged protein in each strain with a Western blot after the cell extracts were separated with SDS/PAGE. The half-life of the tagged proteins was derived from the first-order kinetics of protein degradation.

The half-lives of a total of 3,751 proteins were measured, with a mean and median half-life of *ca.* 43 min. The half-life of these proteins followed an approximate log-normal distribution. The 161 proteins with a half-life of less than 4 min suggested that protein degradation might play a key role in determining the abundance of these proteins.

It was noted that ribosomal proteins and enzymes were generally stable. Transcription factors and cell-cycle proteins were more rapidly degraded than the average. The proteins involved in the degradation machinery were more stable than the average, and the proteins involved in a cell wall were less stable than the average. The study also showed that proteins

with similar protein production rates can have very different degradation rate constants that lead to differences in protein abundances. The difference in degradation rate constants for the proteins with similar production rates underscored the contribution of degradation to steady-state protein abundance. To buffer differences in half-life of proteins, cells repress and activate the genes that code for stable proteins more strongly than the genes that code for rapidly degrading ones.

Yen et al. developed a high-throughput approach for proteome-scale protein-turnover analysis in mammalian cells, called global protein stability (GPS) analysis (Yen et al., 2008). In this cell-based system to measure GPS, a retroviral reporter construct was built. The expression cassette contained a single promoter that, with an internal ribosome entry site (IRES), permitted the translation of two fluorescent proteins from one mRNA transcript. In the expression cassette, the first fluorescent protein, *Discosoma* sp. red fluorescent protein (DsRed), served as an internal control. The second, enhanced green fluorescent protein (EGFP), was expressed as a fusion with the protein of interest (X). When the cassette was integrated into the genome of cells, DsRed and EGFP-X proteins were produced at a constant ratio because they were derived from the same mRNA expressed from a single promoter. The EGFP/DsRed ratio of cells represents the stability of protein X in this system, and can be quantified with fluorescence-activated cell sorting (FACS). Events that selectively affect the protein stability of EGFP-X e.g., turnover of X are expected to change the abundance of EGFP-X, but not DsRed, to thus lead to an altered EGFP/DsRed ratio.

To apply GPS analysis at the global level, Yen et al. designed a multiplexing strategy by integrating the power of fluorescence-based protein stability quantitation with that of DNA microarray technology for reporter cell identification. A reporter cell library was created that consisted of retroviral reporter constructs for ~8,000 unique human proteins. The library was fractionated into seven subpopulations based on the increase in the EGFP/DsRed ratio. The EGFP-fused ORF sequences served as cell identifiers, and were PCR-amplified. The PCR products from the subpopulations were screened on a DNA microarray to identify the composition of each subpopulation of the library. In this way, an identified reporter cell was assigned with the stability of the protein X that it carried, as represented by the EGFP/DsRed ratio. A bimodal distribution of protein half-lives was observed to center around 0.5 and 2 hours. The GPS technology was used to screen for substrates of the Skp1–cullin–F-box ubiquitin ligase in human cells (Yen and Elledge, 2008).

Cohen et al. presented a dynamic proteomics approach that measured the levels and locations of nearly 1000 different endogenously tagged proteins in individual living cells at high temporal resolution (Cohen et al., 2008). A retrovirus-based approach, called 'CD tagging', in human H1299 lung carcinoma cells was used to construct a library of over 1,200 cell clones. Each clone expressed a different fluorescently tagged, full-length protein from its endogenous chromosomal location. Time-lapse fluorescence microscopy was used to obtain movies of the proteins over several days of growth. The movies were analyzed with image analysis software to quantify the temporal changes of tagged proteins, and to follow the spatial distribution of the tagged proteins. The proteomic response of the cells to drug treatment was assayed by this approach. The study provided a view of the response to a drug in space and time for *ca.* 1,000 proteins in individual cells. Whereas most proteins showed a uniform behavior in different cells, a small subset of proteins showed cell-to-cell variability that increased sharply after the drug was added. These proteins might play a functional role for some cells to escape from the drug action.

The protein-tagging approaches overcome some limitations in mass spectrometry, which include the inability to detect low-abundance proteins and the inability to monitor living cells with single-cell resolution in real time. However, the gain in the protein-tagging methods comes with a compromise as well. These protein-tagging approaches require genetic manipulation of the subject of study that limits its application to culture cells that are amenable to genetic manipulation at the global level.

A genome-wide scale genetic manipulation is not yet possible for some important organisms, such as *M. tuberculosis*, which is very difficult to work with. It grows slowly, tends to form clumps in culture, and has a waxy cell wall. The availability of genetic manipulation techniques for *M. tuberculosis* is still lagging far behind many other organisms. The concept of measuring protein turnover with single-cell resolution appears very attractive (Cohen et al., 2008), especially to study a drug-resistant subpopulation in *M. tuberculosis*. Large-scale transposon mutagenesis has been employed to mutate virtually every non-essential gene in *M. tuberculosis* (Sasseti et al., 2003; Sasseti and Rubin, 2003). However, the possibility to create a fusion protein for each gene within *M. tuberculosis* on a large scale (Belle et al., 2006; Cohen et al., 2008; Yen et al., 2008) awaits testing.

C. Isotopomer analysis with high-resolution mass spectrometry

Hellerstein pioneered the isotopomer analysis of biopolymers with mass spectrometry (Hellerstein, 1991), and introduced the mass isotopomer distribution analysis (MIDA) technique to measure the biosynthesis and turnover of biopolymers (Hellerstein and Neese, 1992). With a magnetic sector mass spectrometer and a quadrupole mass spectrometer, Hellerstein and colleagues demonstrated the feasibility of MIDA to measure protein synthesis and precursor pool enrichments (Papageorgopoulos et al., 1999).

In the MIDA technique, a stable isotope-labeled amino acid was administered to allow incorporation into proteins. The labeled and unlabeled amino acids were both incorporated into proteins to generate a combinatorial pattern that could be described by a binomial or multinomial expansion (Hellerstein and Neese, 1992). The incorporation of a labeled amino acid into a protein perturbed the mass isotopomer pattern of an enzyme-digested peptide of the protein. The perturbed mass isotopomer pattern of a peptide from a labeled protein is compared to the pattern from the unlabeled protein (natural abundance). Enrichment of a labeled amino acid in the amino acid precursor pool leads to an alteration the isotopomer pattern of the peptides from a protein. The altered isotopomer pattern of a peptide is unique to the enrichment of a labeled amino acid in the precursor pool. These distinctive patterns reflect the unique combination of labeled and unlabeled precursors at different precursor enrichment. With mass spectrometry, a mass isotopomer pattern can be measured and compared to theoretical patterns calculated for the peptides of a protein over a range of precursor enrichments. A match between the experimental and theoretical combinatorial patterns for the peptides of the protein of interest uniquely establishes the isotopic enrichment in the protein. In a sampled pool at time t , proteins contain unlabeled (old) and labeled (new) proteins. At $t = \infty$, the enrichment reaches a plateau at which all the proteins are newly synthesized, and the enrichment in proteins and precursors are in an equilibrium. Thus, fractional synthesis is represented by the quotient of the enrichment in the sampled pool at time t to the enrichment in newly synthesized proteins at $t = \infty$. Papageorgopoulos et al. assumed first-order kinetics for the increase of fractional synthesis. Based on the first-order kinetics equations, the fractional synthetic rate constant was derived by measuring the fractional synthesis for individual mass isotopomers in timed physiologic experiments (Papageorgopoulos et al., 1999).

Although the principle of MIDA to measure protein synthesis kinetics was well-established (Hellerstein and Neese, 1992), the application of MIDA to proteins and peptides was limited by the mass resolution and mass range of the mass spectrometers available at the time (Papageorgopoulos et al., 1999). A magnetic sector mass spectrometer could only provide required resolution for peptides up to 1000 Da with a single charge, and was not widely available. A quadrupole mass spectrometer did not provide baseline resolution of the mass isotopomers, thus the ability to deconvolute mass isotopomer signals was limited. The need for higher resolution mass spectrometers for MIDA in protein applications was stressed (Hellerstein and Neese, 1999).

Global protein turnover studies were accelerated by the advent of the 2D gel/MALDI-TOF technology (Grunenfelder et al., 2001; Pratt et al., 2002). Two-dimensional gel electrophoresis provided a high-resolution separation of individual proteins at the global level, and MALDI-TOF made it possible to identify individual proteins from gel spots. With high mass-resolution and a large mass-range, MALDI-TOF recorded peptide isotopologue profiles that were important to accurately quantify protein synthesis and degradation. The 2D gel/MALDI-TOF approach continues to be used for protein turnover studies (Xiao et al., 2008; Zhao et al., 2008).

The large mass-range of MALDI-TOF also allowed one to quantify intact proteins in an *in vitro* study of protein-binding kinetics in the 30S ribosomal subunit of *E. coli* (Talkington et al., 2005). In that study, the *E. coli* 30S subunit was formed by incubating the *E. coli* 16S rRNA with ¹⁵N-labeled 30S ribosomal proteins. At various time points during this process, the ¹⁵N-labeled proteins were chased with an excess of ¹⁴N-labeled proteins. After purification of the fully assembled 30S subunits, the ¹⁵N/¹⁴N ratios of the proteins were determined with MALDI-TOF. The majority of the proteins in the assembly were observed in a single scan. The ¹⁵N/¹⁴N ratios of the proteins at various time points were used to determine the protein-binding rates in the 30S subunit, and to construct the assembly landscape for the 30S subunit. In that study, the capability of MALDI-TOF was well-demonstrated to quantify isotopic ratios of large polypeptides in the analysis of protein complex dynamics.

An electrospray ionization-time-of-flight (ESI-TOF) mass spectrometer provided high resolution spectra for a sophisticated isotope distribution analysis of partially-labeled proteins with a fractional isotope enrichment (Sperling et al., 2008). For higher organisms, it is often

difficult to achieve a high isotope enrichment. When the isotope enrichment is fractional i.e., lower than unity, it can be difficult to perform an automated quantitative analysis of the resulting complex isotopologue profiles to calculate precursor enrichment and fractional synthesis. Sperling et al. developed a general and flexible analytical treatment of the complex isotope distributions that arise in the experiments with only a fractional labeling of proteins. Fourier transform convolution was used to calculate the theoretical labeled isotope distributions, and least-squares were employed for quantitative comparison with experimental peaks. The degree of fractional labeling was determined from experimental peaks after the least-square fitting. At the same time, the integrated intensity of all of the isotopomers in the isotope distribution was also obtained. The fractional synthesis was calculated. The Fourier transform convolution approach provides a means to calculate theoretical isotope distribution for large polymers such as peptides without any truncation of values. The least-squares Fourier transform convolution approach facilitates the quantitative interpretation of complex isotope distributions of peptides from high-resolution mass spectrometers (Sperling et al., 2008).

With a combination of 2D gel electrophoresis and micro-liquid chromatography/hybrid linear ion trap-Fourier transform mass spectrometry (μ LC/LTQ-FTMS), MacCoss et al. demonstrated a high-throughput and robust approach to measure protein isotope enrichment from high-resolution mass spectra of peptides (MacCoss et al., 2005). Protein samples were first resolved with 2D gel electrophoresis. Gel spots were excised for in-gel enzyme digestion. Peptide extracts from the 2D gel spots were analyzed with μ LC/LTQ-FTMS. During data acquisition, a single high-resolution FTMS spectrum was acquired in the ion-cyclotron resonance cell in parallel with five low-resolution MS/MS spectra in the linear ion trap. The low-resolution MS/MS spectra were used for peptide identification, and the high-resolution FTMS spectra were used for quantitative analysis of isotope enrichment. The isotope distribution was predicted iteratively for all isotope enrichments. The predicted isotope distributions were correlated against the experimental isotope distribution to find the best match. The correlation coefficient between the predicted distribution and the measured distribution was used to determine the isotope enrichment of the peptide. The accuracy and precision of the method was validated with proteins of known enrichment and with selected ion monitoring/gas chromatography mass spectrometry measurements of the amino acids from the total acid hydrolysis of the protein mixtures. In different studies, the high-resolution and highly sensitive FTMS has also been used to accurately

measure the isotopomer pattern of amino acids and metabolites in a metabolic flux analysis and pathway confirmation (Pingitore et al., 2007; Tang et al., 2007).

Rao et al. utilized nanoLC/LTQ-FTMS to study protein turnover in mycobacteria at the global level (Rao et al., 2008a; Rao et al., 2008b). In those studies, old and new proteins were distinguished by $^{15}\text{N}/^{14}\text{N}$ isotope labeling. Old proteins in a preexisting condition were fully labeled with a nitrogen source at unity enrichment of either the ^{15}N or ^{14}N isotope. After a change in the stress condition, the newly synthesized proteins were labeled with the other isotope. Three experiments were described in those two studies. In the acid-stress experiment for *M. smegmatis* (Rao et al., 2008b), a ^{14}N -labeled culture grown at pH 7 was doped with a ^{15}N isotope with a concomitant change of pH to 5. The proteins newly synthesized after the pH drop were labeled with a 50% enrichment of the ^{15}N -isotope. In the iron-limitation experiment for *M. smegmatis*, fully ^{15}N -labeled cells were centrifuged from a culture grown with high iron and were washed off the old medium. The fully ^{15}N -labeled cells were resuspended in a fresh unlabeled medium with low iron. The newly synthesized proteins at the low-iron condition were labeled with ^{14}N at near natural enrichment (Rao et al., 2008b). In the iron-starvation experiment (Rao et al., 2008a), *M. tuberculosis* cells were first grown in an unlabeled low-iron medium. The low-iron culture was diluted in a ^{15}N -labeled fresh low-iron and high-iron medium, respectively. The proteins that were newly synthesized in the fresh media were labeled with the ^{15}N -isotope at ca. 75% enrichment.

In the three experiments of mycobacterial stress response to acid shock and iron limitation (Rao et al., 2008a; Rao et al., 2008b), the isotopologue profiles of the old and new proteins were well-separated in m/z space without any overlap. Therefore, no deconvolution of the isotopologue profiles was performed. The intensities of the mass isotopomers were individually integrated. The peptide intensities were determined by summing the individual isotopomer intensities under the isotopologue profiles that corresponded to the old and new proteins, respectively. The LTQ-FTMS system provides state-of-the-art mass resolution and accuracy in a FTMS scan (Syka et al., 2004). The high-resolution FTMS spectra allowed a more reliable integration of the intensities of individual mass isotopomers than other lower-resolution MS spectra. In the FTMS spectra, integration of the intensity of a mass isotopomer was limited to the width of the mass isotopomer. This process avoided the interfering signals that might exist between two adjacent mass isotopomer peaks to reduce contamination from unrelated

compounds. The peptide intensities of old and new proteins were used to calculate protein relative turnover; i.e., the synthesis-over-degradation ratio (Arias et al., 1969; Cargile et al., 2004; Larrabee et al., 1980). The relative turnover quantitation procedures were fully compatible with established methodologies for large-scale quantitative proteomics with stable-isotope labeling. It also allowed integration of relative turnover and abundance measurements to study protein dynamics at the global level (Rao et al., 2008a). More importantly, the relative-turnover measurement does not assume a specific kinetic model to make it suitable for stress-response analysis in which the cells might not follow a simple zeroth or first-order kinetic model. Modeling of kinetics in stressed mycobacteria needs further work. In the meantime, relative turnover is a measurement that can be applied to all the protein turnover studies in a mycobacterial stress response before a kinetic model can be established. Avoidance of isotopologue profile deconvolution further simplifies the analysis of relative turnover in the studies (Rao et al., 2008a; Rao et al., 2008b).

With lower-resolution mass spectrometers, peptide isotopomer analysis and quantitation of proteins can be prone to large errors due to the effects of contamination from unrelated compounds. To circumvent this limitation in low-resolution mass spectrometers, Bateman et al. introduced the method of ‘stable isotope labeling tandem mass spectrometry’ (SILT) (Bateman et al., 2007). SILT was achieved with MS/MS ion intensities instead of extracted precursor-ion intensities for quantitation. The software package *SILTmass* was developed to automate protein identification and quantification with the SILT method (Elbert et al., 2008). In addition to relative and absolute protein quantitation, *SILTmass* can analyze the kinetics of protein turnover. Instead of extracting precursor ion intensity chromatograms to find elution peaks, *SILTmass* uses only MS/MS scans in which a peptide is identified and that meet an ion intensity threshold. Using only MS/MS scans of identified peptides, the accuracy and precision of SILT was shown to be superior to the methods that used precursor-ion intensities. The SILT method is particularly useful at high or low dilutions of the isotope-labeled compounds, or with low-abundant proteins, where noise interference is more prominent.

The SILT method is specific for SILAC labeling. It has not been shown to work for other labeling strategies. In the SILT experiments with a nanoLC/LTQ instrument (Elbert et al., 2008), data were acquired in a data-dependant mode with mass tags enabled. The mass tag corresponded to the m/z difference between the labeled amino acid and its unlabeled counterpart.

Enabling the mass tags in a LC/MS run allowed the acquisition of MS/MS spectra for the pair of unlabeled and labeled peptides. The fractional labeling of a protein was calculated as the quotient of the sum of the *b*- and *y*-series ion intensities in the MS/MS spectrum of a labeled peptide to the sum of the *b*- and *y*-series ion intensities in MS/MS spectra of the labeled and unlabeled peptides. The calculated fractional labeling of a protein allowed an analysis of the protein turnover kinetics. The combination of peptide identification with SILT facilitates the quantitation of protein fractional labeling without any precursor ion intensity chromatographic peak detection.

The demonstrated SILT method is conceptually applicable to a global turnover kinetics study that uses the SILAC labeling strategy with a nanoLC/LTQ system, as shown by Doherty et al. (Doherty et al., 2009). SILT improves the accuracy and precision on the low-resolution LTQ mass spectrometer. It remains to be assessed whether SILT will benefit protein-turnover measurements using higher resolution mass spectrometers such as the LTQ-FT system.

In **Figure 3**, the mass spectrometry spectra obtained with a nanoLC/LTQ-FT system are compared with those obtained with a nanoLC/LTQ system for a tryptic peptide from a superoxide dismutase in *M. smegmatis*. The MS₂-scan spectra from the two mass spectrometry systems have a nearly identical pattern and a similar signal-to-noise ratio, as shown in the panels a and b. The MS-scan spectra, however, show significant difference in resolution and signal-to-noise ratio. The isotopic peaks of the +2 peptide charge state in the FTMS full MS-scan spectrum are baseline-resolved (panel c) whereas those in the ITMS full MS-scan spectrum are not (panel e). Thus, a zoom scan is necessary in the nanoLC/LTQ system to further resolve the isotopic peaks to determine the charge of a precursor ion and to select a precursor ion for MS₂ analysis. Although the ITMS MS-scan spectra from the nanoLC/LTQ are noisy (panels d and e), the ITMS MS₂-scan spectrum obtained in the nanoLC/LTQ (panel b) and the ITMS MS₂-scan spectrum in the nanoLC/LTQ-FT (panel a) share an identical resolution and a similar signal-to-noise-ratio. This is not surprising because both systems use the same type of ITMS to acquire the MS₂-scan spectra. The results shown in Figure 3 illustrate that a peptide peak in a MS-scan spectrum in a LTQ can be more prone to a contamination of unrelated compounds than that in a FTMS. Thus, the rationale is well justified for the SILT method described by Bateman et al. to avoid the potential contamination with the use of MS₂- instead of MS-scan spectra for quantitation in a LTQ (Bateman et al., 2007).

The results in Figure 3 demonstrate that a FTMS full MS-scan spectrum has a superior resolution, a higher mass accuracy, and an improved signal-to-noise ratio over an ITMS MS-scan spectrum in a LTQ. As illustrated in panel c of Figure 3, the FTMS can improve a signal-to-noise ratio in a MS-scan spectrum, at least in part because it can separate the isotopic peaks of a peptide of interest from other noise peaks (panel c) whereas the LTQ alone can not (panels d and e). The ability to resolve more peaks to avoid a contamination from unrelated compounds is particularly important in a protein turnover study where an analysis with a stable isotope labeling generates a more crowded peak pattern than an analysis without labeling. Thus, a LTQ-FT mass spectrometer is more likely to deliver accurate results for a protein turnover study at the global level (Rao and Li, 2009; Rao et al., 2008a; Rao et al., 2008b).

D. Implication of proteome dynamics in mycobacteria

With the advent of high-precision and automated mass spectrometry instrumentation to support large-scale proteomic studies, protein turnover analysis at the global level potentially has an increasing importance for biomedical research. One example is the application of protein turnover analysis to study *M. tuberculosis*, especially at its non-replicating or dormant state. Whereas over a hundred research articles have been published on mycobacterial proteomes, only a few dealt with non-replicating *M. tuberculosis* (Betts et al., 2002; Cho et al., 2006; Rosenkrands et al., 2002). Information about protein turnover in non-replicating and dormant *M. tuberculosis* is scarce in the literature. With the potential importance of proteome dynamics in bacterial cell sporulation or dormancy (Bernlohr, 1967; Bernlohr, 1972; Mandelstam, 1958; Spudich and Kornberg, 1968), a study of mycobacterial protein turnover at the global level (Rao et al., 2008a; Rao et al., 2008b) will likely help to advance our understanding of the molecular basis of *M. tuberculosis* persistence.

M. tuberculosis is the causative agent of tuberculosis disease. It was one of the first pathogenic bacteria to be identified. Over the last century, a variety of control and eradication measures have been implemented against tuberculosis such as vaccination, aggressive chemotherapy, and public health surveillance. But tuberculosis still remains a major global health problem to continue to cause nearly 2 million deaths and 9 million new infection cases per year (WHO, 2007). *Ca.* one third of the world population is infected with *M. tuberculosis*. A

majority of the infected individuals remain asymptomatic whereas they carry a lifetime risk to develop an active disease. This state of infection is called latent tuberculosis infection. The state of latency represents the greatest obstacle to eradicate the tuberculosis disease.

The metabolic requirement of *M. tuberculosis* in latency is unclear and difficult to study because the bacilli presumably remain dormant in a granuloma (Hett and Rubin, 2008). The anti-tuberculosis drugs used today have their maximum effect against the growing but not the dormant bacilli. The long therapeutic regime required to treat latent tuberculosis infection is probably explained by the lack of a direct target that is specific to dormant *M. tuberculosis*. There are new drug treatment regimes and several new anti-tuberculosis drugs in a development pipeline that aim to shorten the treatment period and to overcome multi-drug resistant strains (Murphy and Brown, 2008). Most of these new drugs are still based on existing classes of antimicrobial compounds to target the conventional pathways and molecular machinery that are critical for the growth of *M. tuberculosis*. These drugs could still be countered by drug resistant strains that emerge from the non-adherence of a prolonged regime against latent tuberculosis infection. Thus, the need to discover novel drug targets, especially those against dormant bacteria, is urgent.

A ‘simple but nonetheless vexing problem’ (Murphy and Brown, 2008) in target discovery against non-replicating *M. tuberculosis* is that many methods rely on a growth-inhibition measurement to assess the effect of drug treatment. Rao et al. showed that a protein-turnover measurement was much more sensitive than a protein relative-abundance measurement alone to uncover protein synthesis activities in *M. tuberculosis* (Rao et al., 2008a); those data suggest that protein dynamics analysis with turnover and abundance measurements could potentially add a valuable alternative to the drug target discovery problem for non-replicating *M. tuberculosis*. The sensitive protein turnover analysis could also be useful to detect and validate drug treatment effects at an early phase, during which the most relevant drug effect can be isolated from other non-specific cell stress responses.

V. CONCLUDING REMARKS

Proteome dynamics involves a continuous degradation and synthesis of proteins. The steady-state of a protein depends on the kinetics of both protein synthesis and protein degradation.

Protein degradation does not necessarily mean an end to the protein function. It can be the start of a new function for other proteins after degradation clears the way. An increase in protein abundance can result from either an increased synthesis or decreased degradation, or both. Likewise, a decrease in protein abundance can result from either a decreased synthesis or increased degradation, or both. If protein synthesis and protein degradation both change in the same direction, there might not be a net change in protein abundance. However, the change in protein turnover would be detectable. Thus, a protein turnover analysis is a more-sensitive approach to detect a change in protein regulation. Protein degradation is a process separate from gene transcription and protein translation, and plays at least an equally important role in the multi-layer complex biological regulatory network.

At the global level, proteome dynamics has been better-characterized in growing cells than in non-replicating cells. Non-replication can be a highly important physiological state for an organism such as *M. tuberculosis*. Dormant *M. tuberculosis*, the reservoir of tuberculosis infection, does not replicate but remains metabolically active. The mechanism of *M. tuberculosis* dormancy is poorly understood. Information on protein turnover is scarce for mycobacteria. The area of protein turnover in mycobacteria remains to be explored. A protein turnover study at the global level for mycobacteria would very likely help to advance our understanding of the molecular basis of *M. tuberculosis* dormancy. It would also likely help the endeavor to discover drug targets specific for dormant *M. tuberculosis*, which is very urgent, in the global challenge to eradicate the tuberculosis disease.

Recent advancements in the quantitative proteomics technology has made available a set of effective protein sample separation techniques, high-resolution mass spectrometry detection methods, and high-throughput data processing tools for large scale analysis of a proteome. With multiple options available for stable isotope metabolic labeling, a very effective protein turnover study at the global level can be carried out in a highly automated fashion for many organisms.

Recently emerging protein-tagging approaches for global protein turnover analysis deliver impressive proteome coverage, and in some cases provide single-cell resolution. These technologies require a genetic manipulation of the cells under study to generate clone or strain libraries that carry epitope or fluorescent protein tags for detection. Thus, they are only applicable to the culture cells that are amenable to large-scale genetic manipulation.

Meanwhile, mass spectrometry approaches for protein turnover analysis are well-established and more universally applicable. They only require an administration of a stable isotope to the cell or organism in the form of metabolites, amino acids, or water. The stable isotope-labeling strategy is applicable to almost all living organisms but most importantly to humans. It is conceivable that the high-resolution mass spectrometry technologies will continue to play an important role to advance our understanding of the protein turnover process and its biological functions at the global level.

REFERENCES

- Arias, I. M., Doyle, D., and Schimke, R. T. (1969). Studies on the Synthesis and Degradation of Proteins of the Endoplasmic Reticulum of Rat Liver. *J Biol Chem* *244*, 3303-3315.
- Baerenfaller, K., Grossmann, J., Grobei, M. A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W., and Baginsky, S. (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. *Science* *320*, 938-941.
- Bateman, R. J., Munsell, L. Y., Chen, X., Holtzman, D. M., and Yarasheski, K. E. (2007). Stable isotope labeling tandem mass spectrometry (SILT) to quantify protein production and clearance rates. *J Am Soc Mass Spectrom* *18*, 997-1006.
- Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O'Shea, E. K. (2006). Quantification of protein half-lives in the budding yeast proteome. *Proc Natl Acad Sci U S A* *103*, 13004-13009.
- Bernlohr, R. W. (1967). Changes in amino acid permeation during sporulation. *J Bacteriol* *93*, 1031-1044.
- Bernlohr, R. W. (1972). ¹⁸Oxygen Probes of Protein Turnover, Amino Acid Transport, and Protein Synthesis in *Bacillus licheniformis*. *J Biol Chem* *247*, 4893-4899.
- Bernlohr, R. W., and Clark, V. (1971). Characterization and regulation of protease synthesis and activity in *Bacillus licheniformis*. *J Bacteriol* *105*, 276-283.

- Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., and Duncan, K. (2002). Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol Microbiol* 43, 717-731.
- Beynon, R. J., and Pratt, J. M. (2005). Metabolic Labeling of Proteins for Proteomics. *Mol Cell Proteomics* 4, 857-872.
- Bloch, K. (1996). The origins of intermediary metabolism at Columbia College of Physicians and Surgeons (P&S). *Faseb J* 10, 802-805.
- Bloch, K., and Schoenheimer, R. (1941). THE BIOLOGICAL PRECURSORS OF CREATINE. *J Biol Chem* 138, 167-194.
- Borek, E., Ponticorvo, L., and Rittenberg, D. (1958). Protein Turnover in Micro-Organisms. *Proc Natl Acad Sci U S A* 44, 369-374.
- Borsook, H., and Keighley, G. L. (1935). The "Continuing" Metabolism of Nitrogen in Animals. *Proceedings of the Royal Society of London Series B, Biological Sciences (1934-1990)* 118, 488-521.
- Cargile, B. J., Bundy, J. L., Grunden, A. M., and Stephenson, J. L., Jr. (2004). Synthesis/degradation ratio mass spectrometry for measuring relative dynamic protein turnover. *Anal Chem* 76, 86-97.
- Chen, Y., Anderson, D. E., Rajagopalan, M., and Erickson, H. P. (2007). Assembly Dynamics of Mycobacterium tuberculosis FtsZ. *J Biol Chem* 282, 27736-27743.
- Cho, S. H., Goodlett, D., and Franzblau, S. (2006). ICAT-based comparative proteomic analysis of non-replicating persistent Mycobacterium tuberculosis. *Tuberculosis (Edinb)* 86, 445-460.
- Cohen, A. A., Geva-Zatorsky, N., Eden, E., Frenkel-Morgenstern, M., Issaeva, I., Sigal, A., Milo, R., Cohen-Saidon, C., Liron, Y., Kam, Z., *et al.* (2008). Dynamic proteomics of individual cancer cells in response to a drug. *Science* 322, 1511-1516.
- Dice, J. F., and Schimke, R. T. (1972). Turnover and Exchange of Ribosomal Proteins from Rat Liver. *J Biol Chem* 247, 98-111.
- Doherty, M. K., and Beynon, R. J. (2006). Protein turnover on the scale of the proteome. *Expert Rev Proteomics* 3, 97-110.
- Doherty, M. K., Hammond, D. E., Clague, M. J., Gaskell, S. J., and Beynon, R. J. (2009). Turnover of the human proteome: determination of protein intracellular stability by dynamic SILAC. *J Proteome Res* 8, 104-112.

- Doherty, M. K., Whitehead, C., McCormack, H., Gaskell, S. J., and Beynon, R. J. (2005). Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. *Proteomics* 5, 522-533.
- Eagle, H., Piez, K. A., and Fleischman, R. (1957). The utilization of phenylalanine and tyrosine for protein synthesis by human cells in tissue culture. *J Biol Chem* 228, 847-861.
- Eagle, H., Piez, K. A., Fleischman, R., and Oyama, V. I. (1959). Protein Turnover in Mammalian Cell Cultures. *J Biol Chem* 234, 592-597.
- Elbert, D. L., Mawuenyega, K. G., Scott, E. A., Wildsmith, K. R., and Bateman, R. J. (2008). Stable isotope labeling tandem mass spectrometry (SILT): integration with peptide identification and extension to data-dependent scans. *J Proteome Res* 7, 4546-4556.
- Ferrington, D. A., Krainev, A. G., and Bigelow, D. J. (1998). Altered turnover of calcium regulatory proteins of the sarcoplasmic reticulum in aged skeletal muscle. *J Biol Chem* 273, 5885-5891.
- Frank, E. G., Ennis, D. G., Gonzalez, M., Levine, A. S., and Woodgate, R. (1996). Regulation of SOS mutagenesis by proteolysis. *Proc Natl Acad Sci U S A* 93, 10291-10296.
- Futcher, B., Latter, G. I., Monardo, P., McLaughlin, C. S., and Garrels, J. I. (1999). A sampling of the yeast proteome. *Mol Cell Biol* 19, 7357-7368.
- Gerner, C., Vejda, S., Gelbmann, D., Bayer, E., Gotzmann, J., Schulte-Hermann, R., and Mikulits, W. (2002). Concomitant determination of absolute values of cellular protein amounts, synthesis rates, and turnover rates by quantitative proteome profiling. *Mol Cell Proteomics* 1, 528-537.
- Goldberg, A. L. (1972). Degradation of abnormal proteins in *Escherichia coli* (protein breakdown-protein structure-mistranslation-amino acid analogs-puromycin). *Proc Natl Acad Sci U S A* 69, 422-426.
- Gonzalez, M., Frank, E. G., Levine, A. S., and Woodgate, R. (1998). Lon-mediated proteolysis of the *Escherichia coli* UmuD mutagenesis protein: in vitro degradation and identification of residues required for proteolysis. *Genes Dev* 12, 3889-3899.
- Gonzalez, M., Rasulova, F., Maurizi, M. R., and Woodgate, R. (2000). Subunit-specific degradation of the UmuD/D' heterodimer by the ClpXP protease: the role of trans recognition in UmuD' stability. *Embo J* 19, 5251-5258.

- Grunenfelder, B., Rummel, G., Vohradsky, J., Roder, D., Langen, H., and Jenal, U. (2001). Proteomic analysis of the bacterial cell cycle. *Proc Natl Acad Sci U S A* 98, 4681-4686.
- Guggenheim, K. Y. (1991). Rudolf Schoenheimer and the concept of the dynamic state of body constituents. *J Nutr* 121, 1701-1704.
- Hellerstein, M. K. (1991). Relationship between precursor enrichment and ratio of excess M2/excess M1 isotopomer frequencies in a secreted polymer. *J Biol Chem* 266, 10920-10924.
- Hellerstein, M. K., and Neese, R. A. (1992). Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol* 263, E988-1001.
- Hellerstein, M. K., and Neese, R. A. (1999). Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *Am J Physiol* 276, E1146-1170.
- Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*, In *Bacterial stress responses*, G. Storz, and R. Hengge-Aronis, eds. (Washington, DC: ASM Press), pp. 161-178.
- Hett, E. C., and Rubin, E. J. (2008). Bacterial growth and cell division: a mycobacterial perspective. *Microbiol Mol Biol Rev* 72, 126-156, table of contents.
- Hogness, D. S., Cohn, M., and Monod, J. (1955). Studies on the induced synthesis of beta-galactosidase in *Escherichia coli*: the kinetics and mechanism of sulfur incorporation. *Biochim Biophys Acta* 16, 99-116.
- Huffaker, R. C., and Peterson, L. W. (1974). Protein Turnover in Plants and Possible Means of Its Regulation. *Annual Review of Plant Physiology* 25, 363-392.
- Kennedy, E. P. (2001). Hitler's gift and the era of biosynthesis. *J Biol Chem* 276, 42619-42631.
- Koch, A. L., and Levy, H. R. (1955). PROTEIN TURNOVER IN GROWING CULTURES OF *ESCHERICHIA COLI*. *J Biol Chem* 217, 947-958.
- Larrabee, K. L., Phillips, J. O., Williams, G. J., and Larrabee, A. R. (1980). The relative rates of protein synthesis and degradation in a growing culture of *Escherichia coli*. *J Biol Chem* 255, 4125-4130.
- Levchenko, I., Seidel, M., Sauer, R. T., and Baker, T. A. (2000). A specificity-enhancing factor for the ClpXP degradation machine. *Science* 289, 2354-2356.
- Loewen, P. C., and Hengge-Aronis, R. (1994). The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Annu Rev Microbiol* 48, 53-80.

- MacCoss, M. J., Wu, C. C., Matthews, D. E., and Yates, J. R., 3rd (2005). Measurement of the isotope enrichment of stable isotope-labeled proteins using high-resolution mass spectra of peptides. *Anal Chem* *77*, 7646-7653.
- Mandelstam, J. (1957). Turnover of protein in starved bacteria and its relationship to the induced synthesis of enzyme. *Nature* *179*, 1179-1181.
- Mandelstam, J. (1958). Turnover of protein in growing and non-growing populations of *Escherichia coli*. *Biochem J* *69*, 110-119.
- Marfany, G., Farras, R., Salido, E., Xirodimas, D. P., and Rodriguez, M. S. (2008). Much to know about proteolysis: intricate proteolytic machineries compromise essential cellular functions. *Biochem Soc Trans* *36*, 781-785.
- Mazur, P. (1965). The role of cell membranes in the freezing of yeast and other single cells. *Ann N Y Acad Sci* *125*, 658-676.
- Mider, G. B. (1951). Some aspects of nitrogen and energy metabolism in cancerous subjects: a review. *Cancer Res* *11*, 821-829.
- Murphy, D. J., and Brown, J. R. (2008). Novel drug target strategies against *Mycobacterium tuberculosis*. *Curr Opin Microbiol* *11*, 422-427.
- Neher, S. B., Sauer, R. T., and Baker, T. A. (2003). Distinct peptide signals in the UmuD and UmuD' subunits of UmuD/D' mediate tethering and substrate processing by the ClpXP protease. *Proc Natl Acad Sci U S A* *100*, 13219-13224.
- O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* *250*, 4007-4021.
- Orlowski, R. Z., and Kuhn, D. J. (2008). Proteasome inhibitors in cancer therapy: lessons from the first decade. *Clin Cancer Res* *14*, 1649-1657.
- Papageorgopoulos, C., Caldwell, K., Shackleton, C., Schweingrubber, H., and Hellerstein, M. K. (1999). Measuring protein synthesis by mass isotopomer distribution analysis (MIDA). *Anal Biochem* *267*, 1-16.
- Pingitore, F., Tang, Y., Kruppa, G. H., and Keasling, J. D. (2007). Analysis of amino acid isotopomers using FT-ICR MS. *Anal Chem* *79*, 2483-2490.
- Podolsky, R. J. (1953). Protein degradation in bacteria. *Arch Biochem Biophys* *45*, 327-340.

- Pratt, J. M., Petty, J., Riba-Garcia, I., Robertson, D. H., Gaskell, S. J., Oliver, S. G., and Beynon, R. J. (2002). Dynamics of protein turnover, a missing dimension in proteomics. *Mol Cell Proteomics* 1, 579-591.
- Ramakrishnan, R. (2007). Alternative equations for whole-body protein synthesis and for fractional synthetic rates of proteins. *Metabolism* 56, 1550-1560.
- Rao, P. K., and Li, Q. (2009). Principal Component Analysis of Proteome Dynamics in Iron-Starved Mycobacterium Tuberculosis. *J Proteomics Bioinform* 2, 19-31.
- Rao, P. K., Rodriguez, G. M., Smith, I., and Li, Q. (2008a). Protein dynamics in iron-starved Mycobacterium tuberculosis revealed by turnover and abundance measurement using hybrid-linear ion trap-Fourier transform mass spectrometry. *Anal Chem* 80, 6860-6869.
- Rao, P. K., Roxas, B. A., and Li, Q. (2008b). Determination of global protein turnover in stressed mycobacterium cells using hybrid-linear ion trap-fourier transform mass spectrometry. *Anal Chem* 80, 396-406.
- Rosenkrands, I., Slayden, R. A., Crawford, J., Aagaard, C., Barry, C. E., 3rd, and Andersen, P. (2002). Hypoxic response of Mycobacterium tuberculosis studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. *J Bacteriol* 184, 3485-3491.
- Ryazanov, A. G., and Nefsky, B. S. (2002). Protein turnover plays a key role in aging. *Mech Ageing Dev* 123, 207-213.
- Sasseti, C. M., Boyd, D. H., and Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* 48, 77-84.
- Sasseti, C. M., and Rubin, E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* 100, 12989-12994.
- Schoenheimer, R. (1942). *The Dynamic State of Body Constituents* (Cambridge, Mass.: Harvard Univ. Prerss).
- Schoenheimer, R., Ratner, S., and Rittenberg, D. (1939). The Process of Continuous Deamination and Reamination of Amino Acids in the Proteins of Normal Animals. *Science* 89, 272-273.
- Schoenheimer, R., and Rittenberg, D. (1935). Deuterium as an Indicator in the Study of Intermediary Metabolism. *Science* 82, 156-157.

- Schoenheimer, R., Rittenberg, D., Foster, G. L., Keston, A. S., and Ratner, S. (1938). The Application of the Nitrogen Isotope N15 for the Study of Protein Metabolism. *Science* 88, 599-600.
- Schulmeister, S., Ruttorf, M., Thiem, S., Kentner, D., Lebiedz, D., and Sourjik, V. (2008). Protein exchange dynamics at chemoreceptor clusters in *Escherichia coli*. *Proc Natl Acad Sci U S A* 105, 6403-6408.
- Segal, H. L., and Kim, Y. S. (1963). Glucocorticoid Stimulation of the Biosynthesis of Glutamic-Alanine Transaminase. *Proc Natl Acad Sci U S A* 50, 912-918.
- Sperling, E., Bunner, A. E., Sykes, M. T., and Williamson, J. R. (2008). Quantitative analysis of isotope distributions in proteomic mass spectrometry using least-squares Fourier transform convolution. *Anal Chem* 80, 4906-4917.
- Spudich, J. A., and Kornberg, A. (1968). Biochemical Studies of Bacterial Sporulation and Germination. VII. PROTEIN TURNOVER DURING SPORULATION OF *BACILLUS SUBTILIS*. *J Biol Chem* 243, 4600-4605.
- Syka, J. E., Marto, J. A., Bai, D. L., Horning, S., Senko, M. W., Schwartz, J. C., Ueberheide, B., Garcia, B., Busby, S., Muratore, T., *et al.* (2004). Novel linear quadrupole ion trap/FT mass spectrometer: performance characterization and use in the comparative analysis of histone H3 post-translational modifications. *J Proteome Res* 3, 621-626.
- Talkington, M. W., Siuzdak, G., and Williamson, J. R. (2005). An assembly landscape for the 30S ribosomal subunit. *Nature* 438, 628-632.
- Tang, Y., Pingitore, F., Mukhopadhyay, A., Phan, R., Hazen, T. C., and Keasling, J. D. (2007). Pathway confirmation and flux analysis of central metabolic pathways in *Desulfovibrio vulgaris hildenborough* using gas chromatography-mass spectrometry and Fourier transform-ion cyclotron resonance mass spectrometry. *J Bacteriol* 189, 940-949.
- Tu, X., Latifi, T., Bougdour, A., Gottesman, S., and Groisman, E. A. (2006). The PhoP/PhoQ two-component system stabilizes the alternative sigma factor RpoS in *Salmonella enterica*. *Proc Natl Acad Sci U S A* 103, 13503-13508.
- WHO (2007). 2007 Tuberculosis Facts.
(http://www.who.int/tb/publications/2007/factsheet_2007.pdf).
- Wilkinson, K. D. (2005). The discovery of ubiquitin-dependent proteolysis. *Proc Natl Acad Sci U S A* 102, 15280-15282.

- Wolfe, R. R. (1992). *Radioactive and Stable Isotope Tracers in Biomedicine: Principles and Practice of Kinetics Analysis* (New York, NY: Willey-Liss).
- Wosten, M. M. (1998). Eubacterial sigma-factors. *FEMS Microbiol Rev* 22, 127-150.
- Xiao, G. G., Garg, M., Lim, S., Wong, D., Go, V. L., and Lee, W. N. (2008). Determination of protein synthesis in vivo using labeling from deuterated water and analysis of MALDI-TOF spectrum. *J Appl Physiol* 104, 828-836.
- Yen, H. C., and Elledge, S. J. (2008). Identification of SCF ubiquitin ligase substrates by global protein stability profiling. *Science* 322, 923-929.
- Yen, H. C., Xu, Q., Chou, D. M., Zhao, Z., and Elledge, S. J. (2008). Global protein stability profiling in mammalian cells. *Science* 322, 918-923.
- Zhang, X. J., Chinkes, D. L., Wu, Z., Martini, W. Z., and Wolfe, R. R. (2004). Fractional synthesis rates of DNA and protein in rabbit skin are not correlated. *J Nutr* 134, 2401-2406.
- Zhao, Y., Lee, W. N., Lim, S., Go, V. L., Xiao, J., Cao, R., Zhang, H., Recker, R. R., and Xiao, G. G. (2008). Quantitative Proteomics: Measuring Protein Synthesis Using (15)N Amino Acid Labeling in Pancreatic Cancer Cells. *Anal Chem*.
- Zhou, Y., Gottesman, S., Hoskins, J. R., Maurizi, M. R., and Wickner, S. (2001). The RssB response regulator directly targets sigma(S) for degradation by ClpXP. *Genes Dev* 15, 627-637.

[Biography]



Qingbo Li received his Ph.D. degree in Analytical Chemistry from Iowa State University in 1995. From 1996 to 2002, he was a senior scientist in a startup biotech company developing a DNA sequencing and mutation detection technology with capillary electrophoresis and laser-induced fluorescence detection. Between 2003 and 2005, he was a postdoctoral research associate in the Department of Biochemistry and Molecular Biology at Penn State University,

working on the anaerobic archaeon *Methanosarcina acetivorans*. In 2005, he joined the faculty of University of Illinois at Chicago. He is currently an assistant professor in the Department of Microbiology and Immunology and the Center for Pharmaceutical Biotechnology. His research interest is in pathogen-host interactions with a focus on the proteomics of mycobacteria.

Figure Captions

Figure 1. A model for the mechanism of action of RssB to regulate the degradation of σ^S by ClpXP. A σ^S can interact with either the core RNA polymerase or a RssB, but not with both at the same time. When a σ^S binds a phosphorylated RssB, its affinity to ClpX increases. The increased binding affinity of the σ^S -RssB complex to ClpX leads to a degradation of σ^S but not RssB. When a σ^S binds to the core RNA polymerase, it is protected from degradation by ClpXP. Reprinted from Zhou et al., [2001], with permission from Cold Spring Harbor Laboratory Press, copyright 2001.

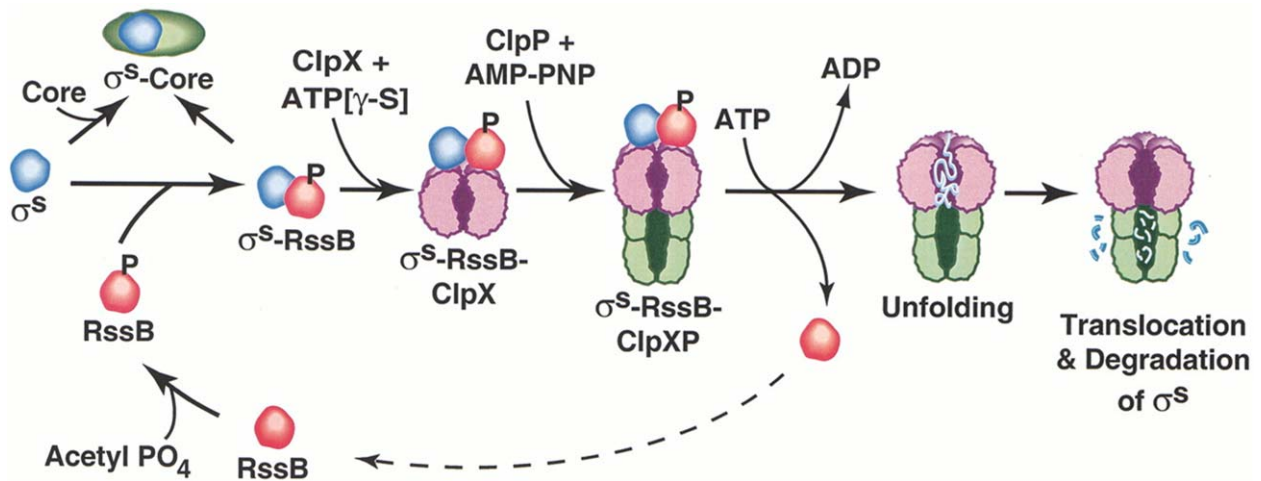


Figure 2. A spatial relationship among the protein-abundance property vectors (RA_L , RA_M , and RA_T) and the protein-turnover property vectors (SD_{LI} , SD_{HI} , and RSD) derived in a protein dynamics study of iron-starved *M. tuberculosis* cells (Rao and Li, 2009). The figure depicts a biplot based on the output from a principal component analysis of six protein-ratio categories that include: 1) a relative abundance of the old portion of a protein between the high-iron (HI) and low-iron (LI) cells, RA_L ; 2) a relative abundance of the newly synthesized portion of a protein between the HI and LI cells, RA_M ; 3) a relative abundance of a total protein, including both of its old and new portions, between the HI and LI cells, RA_T ; 4) a relative turnover of a protein in the HI cell, SD_{HI} ; 5) a relative turnover of a protein in the LI cell, SD_{LI} ; 6) a ratio of SD_{HI} over SD_{LI} . The biplot illustrates the distribution of the vectors of the six protein-ratio categories in a new space defined by the first three principal components that are indicated by a label of 'component x' on the axis's, where $x=1, 2, \text{ or } 3$. It is noteworthy that two approximately orthogonal planes are defined by the vectors of the protein-ratio categories. One is defined by the

RA_L , RA_M , and RA_T vectors, and the other is defined by the SD_{LI} and SD_{HI} vectors. The principal component analysis indicates that a protein relative turnover is a property distinct from a protein relative abundance, although both types of properties are derived from the exact same set of protein abundance data in a simple way. Reprinted from Rao and Li [2009], an open access publication from OMICS Publishing Group, 2009.

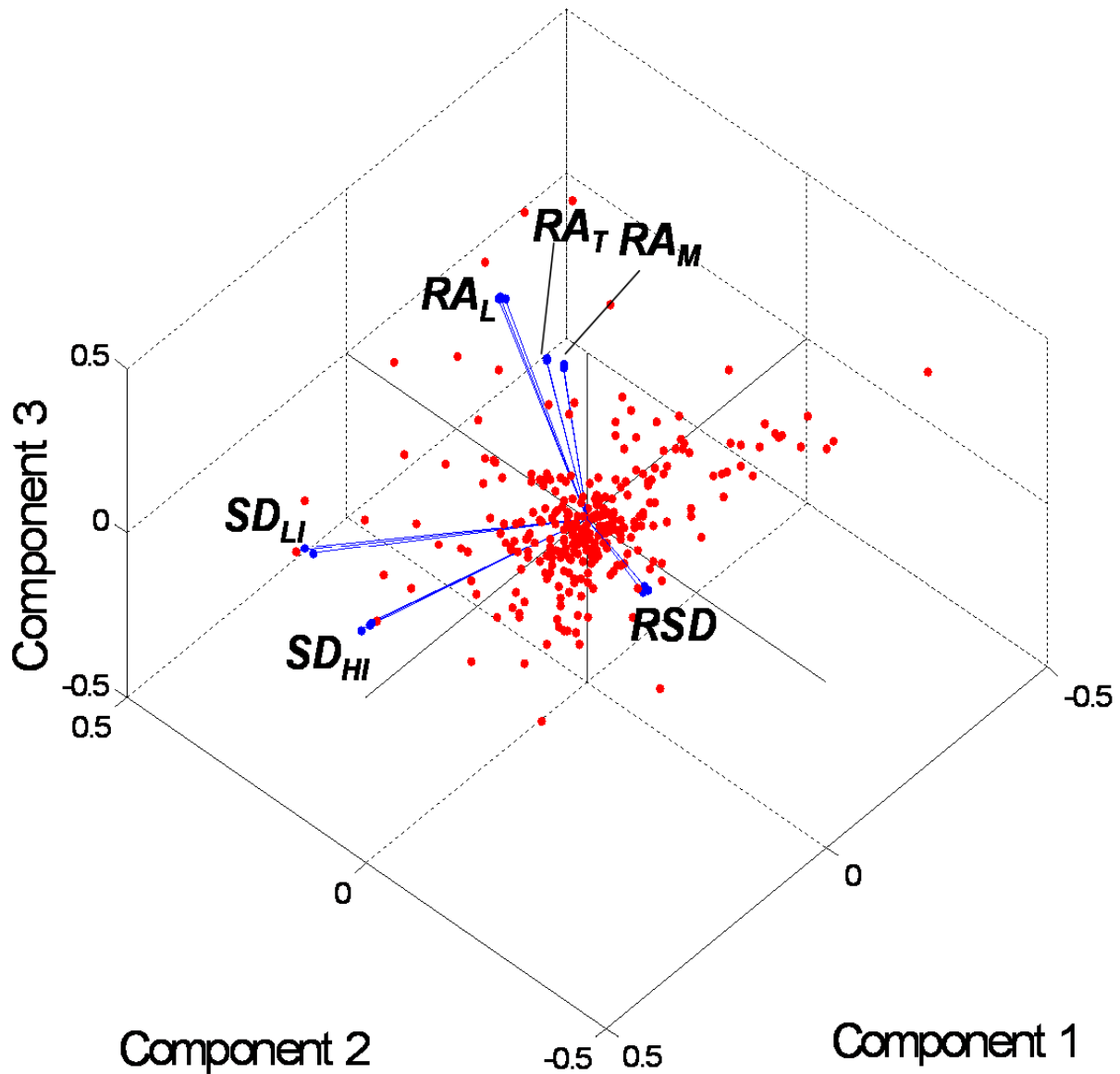
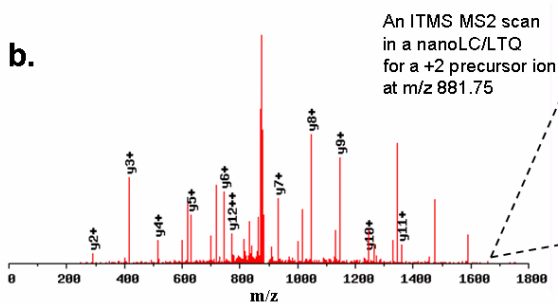
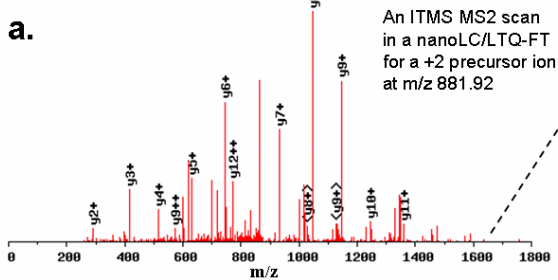
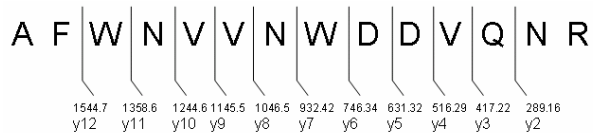


Figure 3. A resolution and a signal-to-noise ratio in MS-scan and MS2-scan spectra are compared between a nanoLC/LTQ-FT and a nanoLC/LTQ mass spectrometry systems. The spectra shown in the panels a to e are for a tryptic peptide from a superoxide dismutase in *M. smegmatis* (MSMEG_6427; Mn) with a sequence of AFWNVVNWDDVQNR. The panels a and b are the ion-trap mass spectrometry (ITMS) MS2-scan spectra for a +2 precursor ion of the peptide in the nanoLC/LTQ-FT and the nanoLC/LTQ respectively. Shown atop of panel a is the peptide sequence labeled with the detected y-series ions in the MS2-scan spectra. For clarity, only y-series ion fragments are shown with labels. The panels c to e show the MS-scan spectra obtained with a FT mass spectrometry (FTMS) scan (c), an ITMS zoom MS-scan (d), and an ITMS full MS-scan (e), respectively. Only an m/z range of 880 to 885 is shown for the +2 peptide charge state. The three short arrows in the panels c, d, and e indicate the first three isotopic peaks of the +2 peptide charge state. The asterisks in panel c indicate the isotopic peaks probably not related to the peptide. The theoretical molecular weight of the peptide is shown atop of panel c. The long dashed arrows point to the respective MS-scans from which a precursor ion is selected for the MS2-scans. Experimental conditions: a protein extract from a *M. smegmatis* cell culture was digested with trypsin, and was subject to a nanoLC/LTQ-FT or a nanoLC/LTQ analysis with methods similar to those described by Rao et al. (Rao et al., 2008a). The spectra were analyzed and annotated with the Sorcerer™ SEQUEST® (Sage-N Research, Inc.) software and the Xcalibur® (Thermo Electron Corp.) software. The mass spectrometer systems and the software are provided at the Proteomics and Informatics Services Facility in the Research Resources Center at The University of Illinois at Chicago.



Precursor ion
 MH^+ 1762.8301, m/z 881.9187 (calculated)

