Published in final edited form as:

Methods. 2009 October ; 49(2): 136–141. doi:10.1016/j.ymeth.2009.06.002.

Stable Isotope Pulse-Chase Monitored by Quantitative Mass Spectrometry Applied to *E. coli* 30S Ribosome Assembly Kinetics

Anne E. Bunner and James R. Williamson

Departments of Molecular Biology and Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California, 92037

Abstract

Stable isotope mass spectrometry has become a widespread tool in quantitative biology. Pulse-chase monitored by quantitative mass spectrometry (PC/QMS) is a recently developed stable isotope approach that provides a powerful means of studying the *in vitro* self-assembly kinetics of macromolecular complexes. This method has been applied to the *E. coli* 30S ribosomal subunit, but could be applied to any stable self-assembling complex that can be reconstituted from its component parts and purified from a mixture of components and complex. The binding rates of 18 out of the 20 ribosomal proteins have been measured at several temperatures using PC/QMS. Here, PC/QMS experiments on 30S ribosomal subunit assembly are described, and the potential application of the method to other complexes is discussed. A variation on the PC/QMS experiment is introduced that enables measurement of kinetic cooperativity between proteins. In addition, several related approaches to stable isotope labeling and quantitative mass spectrometry data analysis are compared and contrasted.

Keywords

stable isotope; quantitation; mass spectrometry; ribosome assembly; pulse-chase; assembly kinetics; *Escherichia coli*; 30S ribosomal subunit; metabolic labeling

1. Introduction

Pulse-chase monitored by quantitative mass spectrometry (PC/QMS) [1,2] is a powerful technique for studying the assembly of macromolecular complexes. The goal of this article is to provide a detailed explanation of the PC/QMS method, and illustrate its application to study the assembly kinetics of the *E. coli* 30S ribosomal subunit. The 30S subunit is an important model system for understanding the assembly of macromolecular complexes, and is composed of 20 ribosomal proteins (r-proteins) and one 1500 nucleotide ribosomal RNA (16S rRNA) [3] that can self-assemble *in vitro* through a series of RNA folding and protein binding events [4]. The hierarchy of protein binding is described by the Nomura assembly map (Figure 1A), that was elucidated by reconstituting 30S particles with different combinations of proteins under equilibrium conditions [5,6]. Kinetic binding data show a 5' to 3' directionality of assembly *in vitro* [7], which is consistent with the direction of co-transcriptional assembly during ribosome biogenesis [8].

© 2009 Elsevier Inc. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The PC/QMS method was developed to measure the assembly kinetics of all 20 r-proteins binding simultaneously. These experiments are initiated by incubating 16S rRNA with a pulse of ^{15}N r-proteins for varied amounts of time. The assembly reaction is then chased with an excess of ^{14}N r-proteins and the reaction proceeds to completion, as depicted in Figure 1B. Upon purification, the complete 30S particles contain a mixture of ^{14}N and ^{15}N ribosomal proteins. For each protein, the fraction of ^{15}N represents the fraction that was bound at the time the chase was added. The fraction of ^{15}N protein is measured by subjecting the r-proteins to mass spectrometry. PC/QMS takes advantage of the fact that isotopically labeled molecules are chemically identical to their unlabeled counterparts but give rise to a shifted peak in a mass spectrometer. Although several stable isotope labeling approaches would be compatible with PC/QMS, the experiments described here use metabolically labeled ^{15}N proteins because of their ease of production in *E. coli*. One key advantage of PC/QMS is that binding of all proteins can be monitored simultaneously in a single sample [2]. However, this approach is limited to monitoring the kinetics of stable interactions, because proteins may bind transiently during the pulse and exchange during the chase. A recent kinetic RNA footprinting study [9] provides complementary kinetic data regarding unstable initial RNA-protein complexes that are formed on the millisecond timescale. Stable isotope pulse-labeling has also been used to study protein folding kinetics *in vitro* using hydrogen-deuterium exchange (HDX) experiments [10].

Stable isotope approaches in quantitative proteomics have been used to obtain information about protein levels in biological samples [11–14] and protein turnover *in vivo* [15–18]. Stable isotope labels can either be incorporated during growth, or after the sample has been harvested, using chemical or enzymatic methods. A variety of chemical labeling strategies have been developed for introducing stable isotope labels to proteins [19]. Isotope coded affinity tagging, or ICAT, is a chemical labeling approach where a stable isotope tag is attached that enables the purification of tagged peptides [14]. A more recent approach called Isobaric Tagging for Relative and Absolute Quantitation, or iTRAQ, enables the simultaneous comparison of up to four samples, as opposed to traditional pair-wise analysis [13]. An additional advantage of iTRAQ and other amine-reactive reagents [20] over ICAT is the ability to label every peptide as opposed to only those that contain cysteine residues. Stable isotope labels can also be introduced enzymatically during protein digestion [21]. One advantage of ICAT over enzymatic labeling and iTRAQ chemical labels is that differently labeled samples can be mixed before proteolysis, which ensures identical digestion conditions.

One disadvantage shared by all post-growth labeling approaches is that labeling reactions can be inefficient, introducing additional errors into the quantitation, and making the analysis of low abundance proteins more challenging. Fortunately, labeling approaches that incorporate stable isotopes during the growth period avoid this problem, although labeled growth media can be prohibitively expensive, depending on the organism. Cells and organisms can be labeled with nutrients such as amino acids [22], ammonium salts [12], or labeled prey organisms such as *E. coli* fed to *C. elegans* [23], for the relative quantitation of proteins and peptides. Growth labeled samples can be combined before or just after cell lysis, minimizing errors associated with losses during sample workup. For many organisms, full metabolic incorporation of stable isotopes is not practical, but partial labeling can give comparable quantitation accuracy [11]. Both *in vivo* and *in vitro* stable isotope labeling have been used to quantitate post-translational modifications such as phosphorylation [24] and glycosylation [25]. Importantly, *in vivo* pulse-chase experiments can provide quantitative information about protein turnover. Stable isotope mass spectrometry has been used to measure protein turnover in yeast [26], rats [27], birds [16], and humans [17]. Before the advent of proteomic mass spectrometry, both protein turnover [28] and ribosome assembly [29] were studied using radiolabel pulse-chase experiments that are similar to these modern MS-based turnover studies and PC/QMS, respectively. Mass spectrometry as a readout for pulse-chase experiments offers the advantages that both labeled and unlabeled species are directly detected, and, in the case of peptide MS,

the independent observation of multiple peptides per protein gives an estimate of experimental error for the extent of labeling.

Stable isotope experiments require a means of extracting quantitative information from MS data. The PC/QMS approach uses a Least-Squares Fourier Transform Convolution (LS-FTC) method [30] that fits the MS data to a theoretical isotope distribution to find the amplitude of the labeled and unlabeled peaks, using an implementation of the Rockwood algorithm [31]. Figure 2 shows an example peak from a PC/QMS experiment with a fit calculated using LS-FTC. Quantitation of MS data using LS-FTC can be applied to a wide range of labeling schemes [30] such as amino acid labeling and partial metabolic labeling. An example peak from a mixture of unlabeled and partially metabolically labeled peptide is shown in Figure 3 with a fit calculated using LS-FTC. The fitting of theoretical isotope distributions to data for quantitation is not common for the analysis of fully labeled peptides, but has been used to quantitate partially labeled samples. The efficiency of ^{15}N and ^{13}C incorporation into archeal proteins was quantitated using an application of the Yerger algorithm [32] in combination with least-squares fitting [33,34]. Complex isotope distributions resulting from differential metabolic labeling in plants were quantitated using a fitting method similar to LS-FTC, but which only used a limited number of data points, as opposed to all the data in the distribution [35]. A more simplistic approach to quantitation of partial labeling is the pre-calculation of various spectra based on possible labeling proportions, followed by the comparison of these spectra to data in order to approximate the proportion of label in the sample [11,36]. An important advantage of this method over EIC-based quantitation methods (discussed below) is that the intensity of the peak over the entire isotope distribution is summed for quantitation.

One limitation of LS-FTC and related methods of evaluating isotope distributions is that they require medium to high resolution data, which is available from TOF-MS instruments, but which cannot be obtained on many MS/MS capable instruments, such as ion traps. A far more widespread quantitation approach that does not require such high resolution data relies on monoisotopic peak areas to generate extracted ion chromatograms (EICs) from LCMS and LC-MS/MS data. For a given peptide, a chromatogram is calculated for the monoisotopic m/z value that shows the intensity of that mass over the length of the LC-MS run, as illustrated in Figure 4. This is done for both the labeled and unlabeled m/z values, and relative quantities can be calculated based on the area under the peaks in these EICs [37], or by a more sophisticated linear analysis that gives a measure of the quality of the quantitation [11,38]. One advantage of this approach over those that use mass spectra from a fixed time window is that the intensity over the entire width of the peak in the time dimension is quantitated. Since EIC quantitation typically uses the monoisotopic peaks of the labeled and unlabeled peptides, its application to partially labeled peptides would not be straightforward.

Stable isotope labeling can be used for relative quantitation, as in PC/QMS, or for absolute quantitation, using isotope-labeled standards to find the amount of a given molecule in a sample [39]. Two highly cited methods, AQUA [40] (for absolute quantitation) and iTRAQ [13], monitor the intensity of specific MS/MS peaks for quantitation. These experiments often require expensive synthetic standards and significant optimization. Relative quantitation provides more flexibility in labeling and quantitation approaches while still giving accurate comparative data, which is adequate for many experiments.

In order to attribute quantitative changes to particular proteins, peptides must first be identified in MS data. The quantitation method described here uses one-dimensional MS data, which contain no sequence information from fragmentation. Although isotope labels can assist with peptide identification [2,34], very complex samples such as cell lysate cannot be analyzed without MS/MS due to an intractable proportion of ambiguous identities. MS/MS data that provides confident identities can also be used for quantitation, although the datasets are more

complex since they contain both MS and MS/MS scans. In iTRAQ analysis, quantitation is based on fragment ion intensities [41]. The AQUA approach uses an isotopically labeled internal standard and quantitates peptides based on EICs of fragment ions [40,42]. Since MS/MS scans analyze only one precursor ion at a time, approaches that optimize signal-to-noise by focusing on a few peptides of interest limit the number of peptides that can be quantitated. The PC/QMS method described here uses one-dimensional MS for quantitation. The peaks are identified using the monoisotopic mass, measured to ± 20 ppm accuracy, and the nitrogen content that is deduced from the mass difference in the ^{14}N - ^{15}N peak pair. Including the nitrogen content in the identification process reduces ambiguity significantly [34,43] and enables confident identification of most peptides in mixtures of 30S ribosomal proteins without the use of MS/MS [2].

2. Methods

The experimental scheme for the PC/QMS experiment is outlined in Figure 1B. Assembly is initiated using ^{15}N -labeled r-proteins, and after an assembly period, a chase of excess ^{14}N r-proteins is added. The assembly is allowed to proceed to completion, and the 30S subunits are isolated using sucrose gradient ultracentrifugation. The r-proteins are extracted from the RNA, digested with trypsin, and the resulting peptides are analyzed by LC-MS. Quantitation of the fraction of ^{15}N for hundreds of peptides permits measuring the fraction ^{15}N for each protein and an experimental error. Kinetic curves for each protein are derived by performing a series of experiments with increasing pulse-times. Typical PC/QMS experiments were performed using 180 pmol of 16S rRNA, 270 pmol of r-proteins in the pulse, and 1.35 nmol of r-proteins in the chase. This experimental scale usually provides sufficient protein after sample processing to give robust signal on a capillary flow LC-MS.

2.1 Preparation of the 16S rRNA

Aliquots of 16S rRNA, prepared as described previously, are stored under ethanol. [1,2] The 16S rRNA is prepared for reconstitutions first by centrifugation at 16,000g for 10 min at room temperature. The pellet is washed with 70% ethanol and centrifuged again for 5 min at 16,000 g. The rRNA is then partially resuspended to approximately 3.5 μM with TKM buffer (25 mM Tris HCl pH 7.5, 30 mM KCl, 20 mM MgCl_2) and heated to 42°C for 5 min to complete resuspension. The rRNA is then cooled slowly by dialysis against room temperature TKM in a cold (4°C) room for at least 2 hrs. After dialysis, the RNA is stored on ice and diluted to the appropriate concentration with ice-cold TKM. Typically a generous excess of 16S rRNA is prepared in this way to allow for potential losses during dialysis.

2.2 Preparation of the TP30

Aliquots of TP30 were prepared as described previously [1,2], stored in TKMD (25 mM Tris HCl pH 7.5, 1 M KCl, 20 mM MgCl_2 , 2 mM DTT) at -80°C . TP30 is thawed on ice and quantitated in a spectrophotometer, using an effective extinction coefficient at 230 nm of $1.22 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$, where 1 mol of TP30 contains ~ 1 mol of each of the 20 r-proteins. The ^{15}N TP30 for the pulse is diluted in cold TKMD. The ^{14}N TP30 for the chase is diluted into cold Reconstitution Buffer (RB, 25 mM Tris HCl pH 7.5, 330 mM KCl, 20 mM MgCl_2 , 2mM DTT).

2.3 Pulse-Chase Reconstitution

In a typical experiment, one time point requires 409 μL of 0.44 μM of 16S rRNA in TKM, 184 μL of 1.47 μM ^{15}N TP30 in TKMD for the pulse, and 225 μL of 6 μM ^{14}N TP30 in RB for the chase. Extra DTT (to 6.45 mM) is added to the pulse solution, so that upon mixing, the RNA and ^{15}N TP30 solutions produce a buffer equivalent to RB. For the 0 min time point, the ^{14}N and ^{15}N TP30 are mixed before they are added to the rRNA. Before mixing, both

the ^{15}N TP30 and the 16S rRNA are pre-heated to 40°C for 5 min. The pulse step is performed by pipetting the ^{15}N TP30 into the tube with the 16S rRNA, and pipetting up and down once or twice for mixing. When the pulse is complete, the ^{14}N TP30 chase is added with mixing and the assembly reaction is incubated for an additional 40 min at 40°C . Upon completion of the chase, the reaction is cooled on ice for at least 5 min before loading onto a sucrose gradient for ultracentrifugation and purification of the assembled 30S subunits.

Experiments are performed in sets of twelve samples with a range of pulse lengths. The pulse times are spaced evenly in log time between the minimum time possible for hand pipetting (10 sec) and the maximum desired pulse time. The maximum pulse time is chosen as a time at which the binding of the slowest protein is expected to be 75–95% complete, based on preliminary experiments and kinetic data obtained through other methods [7]. For each experimental time point, there are four steps that must be performed at precise times in order to maximize reproducibility: (1) beginning the pre-heating of the 16S rRNA and ^{15}N TP30, (2) mixing the 16S rRNA and the ^{15}N TP30 pulse, (3) adding the ^{14}N TP30 chase, and (4) transferring the sample to ice after incubation at 40°C . The ^{14}N TP30 chase can also be preheated, although it is generally not necessary.

2.4 30S subunit purification

After the assembly reaction is complete, the ribosomal subunits are purified on a linear 10–40% sucrose gradient by ultracentrifugation. The gradient buffers are prepared using a 60% sucrose stock solution which is made by dissolving 771.9 g of sucrose to a final volume of 1L. Gradients are prepared either top-to-bottom or bottom-to-top using a gradient maker in 14×89 mm tubes with a buffer containing RB plus 0.5 M NH_4Cl . All six gradients are balanced before samples are loaded, and then the gradients are balanced pair-wise after loading the samples. Gradients are ultracentrifuged in a Beckman SW-41 rotor at 35000 rpm for 9 hours at 4°C . Fractionation of the gradients is performed at 1 mL/min, using a peristaltic pump, monitoring absorbance at 254 nm. The 30S peak is collected (1.3–1.8 mL in volume is typical) and the proteins are precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 13% v/v.

2.5 Preparation and digestion for LC-MS analysis

TCA precipitation of the proteins is carried out overnight on ice in two separate 1.5 mL microcentrifuge tubes. The precipitated proteins are collected by step-wise centrifugation for 25 min at 14,000 g at 4°C . The first of two tubes is centrifuged, and the supernatant is carefully removed. The liquid from the second tube is transferred into the first tube, and the sample is centrifuged again. The pellet is washed with cold 10% TCA to remove excess salt and sucrose, and centrifuged for 10 min at 14,000 g at 4°C . The supernatant is carefully removed and the pellet is washed with cold acetone and centrifuged again. After the acetone is removed, the pellet is sometimes visible and is frequently coated on the side of the tube. The pellet is dried in a 37°C oven for 10–30 min, then 10 μL of 5% acetonitrile, 100 mM NH_4HCO_3 is added. The pellet is partially resuspended by scraping the side of the tube with a pipet tip and heating to 65°C for 2 min, followed by sonication in a room temperature water bath for 10 min.

The proteins are then prepared for protease digestion by adding 1.1 μL of 100 mM DTT and reducing for 1 hr at 37°C . The cysteine residues are modified by adding iodoacetamide to 40 mM and incubating for 1 hr at 37°C in the dark. Unreacted iodoacetamide is quenched by adding DTT to 40 mM. Sequencing grade porcine trypsin (Promega) is added to 20 ng/ μL , and after digestion overnight at 37°C , the reaction is stopped by addition of formic acid to 0.1%. The final volume of the sample is less than 20 μL , and typically there are undissolved particles remaining even after overnight digestion. Addition of urea facilitated resuspension of the

precipitated proteins, but tended to produce poor signal on the LC-MS. The undissolved particles are removed by centrifugation at 16,000 g for 10 min before LC-MS analysis.

2.6 Mass spectrometry

LC-MS analysis is performed using an Agilent ESI-TOF mass spectrometer coupled to an Agilent 1100 Series HPLC. The buffers used were: Buffer A (water, 0.1% formic acid), and Buffer B (acetonitrile, 0.1% formic acid). Using an autosampler, 5–8 μ L of sample is injected onto a 0.5×150 mm Agilent Zorbax SB-C18 capillary column (part no. 5064–8256) under a 7 μ L/min flow of 5% Buffer B. The elution consisted of four steps: 5% to 15% B in 10 min, 15% to 47% B in 48 min, 47% to 95% in 4 min, 95% B for 2 min.

2.7 Data analysis

The extraction of protein binding kinetics from the raw LC-MS data occurs in several steps, that are described in detail elsewhere [2]. Briefly, a list of experimental peaks containing mass/charge (m/z), charge state, and retention time information for every peak is exported using the Agilent programs Mass Hunter and Mass Profiler. The $^{14}\text{N}/^{15}\text{N}$ isotopomers have near-identical retention times, identical charge states, and m/z values that differ by an amount proportional to their nitrogen content. The peak pairs are identified by comparing the experimental m/z values, charge states, and nitrogen content to a theoretical digest of the 20 r-proteins. After the peak pairs have been identified, their amplitudes must be quantitated. The raw MS data are exported from the Agilent files and used to create a subspectrum centered around each of the identified $^{14}\text{N}/^{15}\text{N}$ peak pairs. These subspectra combine data from a ~ 0.2 min window from the LC-MS run, which is the width of most peaks in the gradient. The ^{14}N and ^{15}N peptide peaks in these subspectra are quantitated using LS-FTC, which fits the data to a theoretical isotope distribution (Figure 2) [30]. The amplitudes of the ^{14}N and ^{15}N peaks are given by the LS-FTC fit parameters and are used to calculate the fraction ^{15}N , $f_L = A_L/(A_L + A_U)$, where A_L is the amplitude of the labeled ^{15}N peak, and A_U is the amplitude of the unlabeled ^{14}N peak. The average and standard deviation of the fraction ^{15}N is calculated for every protein from the entire set of observed peak pairs belonging to peptides from that protein. These averaged relative quantities of ^{14}N and ^{15}N peptides from various time points are used to construct protein binding progress curves. The binding rate constants are calculated by fitting single or double exponential curves to the data, using the calculated standard deviations for each protein to weight each point.

2.8 Other applications

The PC/QMS assay can also be applied to other macromolecular complexes that meet certain criteria. The complex must self-assemble *in vitro*, and the components of the assembled complex must have a sufficiently low off-rate to prevent exchange with unlabeled proteins during the chase. In addition, the PC/QMS method requires a means of purifying the completed complex from excess component parts. The 30S ribosomal subunits are conveniently purified by ultracentrifugation, but other methods such as gel filtration are also tractable. In the purification step, it is important to exclude incomplete sub-particles, because they could alter the observed kinetics. The use of affinity tags for the purification of complexes might risk contaminating the preparation with incomplete sub-complexes that contain the tagged protein, or free tagged protein itself. Any stable isotope label that allows resolution of labeled and unlabeled peptides by mass spectrometry would be compatible with PC/QMS, such as partial metabolic labels [33], amino acid labels [16], or chemical modification [14].

Complexes that contain one copy of each protein component, such as the 30S subunit, enable the separate measurement of the kinetics of each protein, but PC/QMS could be applied to a complex containing several copies of some proteins. In that case, observed kinetics would represent the overall binding rate of all monomers. In the PC/QMS experiments with 30S

ribosomal subunits, the rRNA serves as the nucleating component that is present in limiting amounts and provides a nucleation site for assembly. A nucleating component is necessary for the PC/QMS reaction scheme, however, it could be a protein in experiments with non-RNA containing complexes. For example, if the 26S proteasome could be reconstituted *in vitro*, PC/QMS experiments that determine the kinetics of assembly might use α subunits as the nucleating component [44].

3. Discussion

The PC/QMS method has been used to investigate the mechanism of *in vitro* 30S ribosome assembly. The thermodynamic dependencies shown in the Nomura assembly map (Figure 1A) suggest an order of protein binding that is consistent with kinetic data where proteins that bind directly to the RNA (primary binding proteins) tend to bind fastest, followed by secondary binding proteins (which depend on at least one primary binding protein), followed by the slowest group, tertiary binding proteins (which require at least one primary and one secondary binding protein) [1,7]. The structure of the 30S subunit is divided into the structurally discrete [45] 5' domain, central domain, and 3' domain, each of which can be reconstituted independently [46–48].

Assembly at low temperature results in accumulation of an intermediate lacking some r-proteins and containing others in reduced amounts, that bears similarities to intermediates isolated from cold-sensitive mutants [49]. These related intermediates support the hypothesis that *in vitro* reconstitutions proceed through similar pathways as pre-30S particles *in vivo*, and validate the study of *in vitro* assembly. Also, the *in vivo* order of protein addition has been approximated using pulse-label experiments, and the pattern of protein-binding is similar to that seen *in vitro* [29]. This reconstitution intermediate (RI) is thought to undergo a conformational change upon heating that alters its sedimentation coefficient, and the resulting activated intermediate (RI*) is competent to bind the remaining r-proteins without further heating. If this transition were the single rate-limiting step in the assembly process, or the step with the greatest activation energy, kinetic data would be expected to show a populated intermediate with a subset of proteins bound. However, kinetic data from low-temperature reconstitutions reveal a wide distribution of binding rates with no single well-populated intermediate [1]. In addition, the temperature dependence of protein binding rates indicates that there are many slow steps during *in vitro* 30S assembly with similar activation energies [1]. These data imply that RI does not represent an obligate assembly intermediate through which all 30S particles must pass. The conformational change observed upon heating RI particles could be a stable kinetic trap that is refolded through several local conformational changes in the central and 3' domains, rather than a single global rearrangement.

Low temperature (15°C) and standard condition (40°C) protein binding rates obtained using PC/QMS are compared in Figure 5. Although the 15°C experiment had a slightly different reaction scheme than the 40°C experiment, comparing the two experiments still demonstrates how the rates change at low temperatures. At 15°C, S8 has large error bars because it binds with multiphasic kinetics and hence cannot be fit well with a single exponential. The binding rates of most proteins are reduced by at least 20-fold at 15°C, with the exception of the four fastest proteins S4, S17, S20, and S16, which are reduced by less than 9-fold. The temperature dependence of S7 binding kinetics, which has not been previously reported, is consistent with that of other proteins with similar rates at 40°C.

In order to characterize the slow steps in assembly at 15°C, a variation on PC/QMS was developed. These pre-binding experiments involve an additional step where proteins or small molecules are pre-incubated with the rRNA before the pulse (Figure 6A). If pre-binding an upstream protein can rescue efficient binding of downstream proteins, that suggests that the

rate-limiting step is the binding of the upstream protein, or an RNA folding event associated with its binding. A pre-binding experiment in which purified recombinant S7 was pre-bound to rRNA is shown in Figure 6B and C, compared to a buffer-only control. As expected, ^{15}N S7 bound only to a small extent, because the S7 binding sites were already occupied with ^{14}N pre-binding protein when the pulse began. Pre-binding of S7 had a significant effect on the kinetics of S9 binding (Figure 6C) and this kinetic cooperativity is consistent with the known thermodynamic dependency of S9 on prior S7 binding [5]. Therefore, it is likely that the binding of S9 is limited by an RNA folding event associated with the binding of S7. Experiments such as these elucidate the kinetic relationships between the proteins and help describe the rate-limiting steps in the 30S assembly landscape.

PC/QMS could be applied to biological questions regarding 30S biogenesis *in vivo*. The role of ribosomal assembly factors has been investigated using this *in vitro* kinetic method (A.E. Bunner, S. Nord, P.M. Wikström, J.R. Williamson, unpublished work). In addition, ^{15}N pulses during the growth of *E. coli* could be used to investigate the size of free ribosomal protein pools or the rate of ribosome turnover in cells.

4. Conclusion

The PC/QMS assay is a powerful new method to measure macromolecular assembly kinetics that has a potentially large number of applications. Experiments on 30S ribosome assembly have laid significant foundation for the understanding of assembly kinetics, and many more such experiments will be required to fully understand the mechanism. The pre-binding variation on PC/QMS has the potential to further elucidate the assembly landscape by revealing the kinetic cooperativity between pairs of proteins. The PC/QMS technique adds the dimension of time to the arsenal of quantitative proteomic tools that use ratiometric analysis and stable isotope labeling. The LS-FTC method for quantitative analysis of arbitrarily complex isotope distributions resulting from metabolic or post-growth labeling strategies should also be widely applicable. The quantitative MS methods outlined here should be readily applicable to the growing inventory of multicomponent regulatory complexes and macromolecular machines that are accessible to biochemical and biophysical investigation.

Acknowledgements

This work was supported by NIH Grant R37-GM53757 to J.R.W. The authors thank Dr. Michael T. Sykes, Dr. Zahra Shajani, and William Ridgeway for critical comments on the manuscript.

References

1. Talkington MWT, Siuzdak G, Williamson JR. *Nature* 2005;438:628–632. [PubMed: 16319883]
2. Bunner AE, Trauger SA, Siuzdak G, Williamson JR. *Anal. Chem* 2008;80:9379–9386. [PubMed: 19007188]
3. Hardy SJS, Kurland CG, Voynow P, Mora G. *Biochemistry* 1969;8:2897–2905. [PubMed: 4897206]
4. Traub P, Nomura M. *Proc. Natl. Acad. Sci. USA* 1968;59:777–784. [PubMed: 4868216]
5. Held WA, Ballou B, Mizushima S, Nomura M. *J. Biol. Chem* 1974;249:3103–3111. [PubMed: 4598121]
6. Grondek JF, Culver GM. *RNA* 2004;10:1861–1866. [PubMed: 15525707]
7. Powers T, Daubresse G, Noller HF. *J. Mol. Biol* 1993;232:362–374. [PubMed: 8345517]
8. Kaczanowska M, Rydén-Aulin M. *Microbiol. Mol. Biol. R* 2007;71:477–494.
9. Adilakshmi T, Bellur DL, Woodson SA. *Nature* 2008;455:1268–1272. [PubMed: 18784650]
10. Konermann L, Simmons DA. *Mass Spectrom Rev* 2003;22:1–26. [PubMed: 12768602]
11. Huttlin EL, Hegeman AD, Harms AC, Sussman MR. *Mol. Cell. Proteomics* 2007;6:860–881. [PubMed: 17293592]

12. Snijders AP, Koning Bde, Wright PC. *J. Proteome Res* 2007;6:97–104. [PubMed: 17203953]
13. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlett-Jones M, He F, Jacobson A, Pappin DJ. *Mol. Cell. Proteomics* 2004;3:1154–1169. [PubMed: 15385600]
14. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. *Nat. Biotechnol* 1999;17:994–999. [PubMed: 10504701]
15. Milner E, Barnea E, Beer I, Admon A. *Mol. Cell. Proteomics* 2006;5:357–365. [PubMed: 16272561]
16. Doherty MK, Whitehead C, McCormack H, Gaskell SJ, Beynon RJ. *Proteomics* 2005;5:522–533. [PubMed: 15627957]
17. Bateman RJ, Munsell LY, Chen XH, Holtzman DM, Yarasheski KE. *J. Am. Soc. Mass Spectr* 2007;18:997–1006.
18. Cargile BJ, Bundy JL, Grunden AM, Stephenson JL. *Anal. Chem* 2004;76:86–97. [PubMed: 14697036]
19. Ong SE, Mann M. *Nat. Chem. Biol* 2005;1:252–262. [PubMed: 16408053]
20. Schmidt A. *Proteomics* 2005;5:826–826.
21. Mirgorodskaya OA, Kozmin YP, Titov MI, Korner R, Sonksen CP, Roepstorff P. *Rapid Commun. Mass Spectrom* 2000;14:1226–1232. [PubMed: 10918372]
22. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. *Mol. Cell. Proteomics* 2002;1:376–386. [PubMed: 12118079]
23. Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RHA, Heck AJR. *Nat. Biotechnol* 2003;21:927–931. [PubMed: 12858183]
24. Jensen ON. *Curr. Opin. Chem. Biol* 2004;8:33–41. [PubMed: 15036154]
25. Zhang H, Li XJ, Martin DB, Aebersold R. *Nat. Biotechnol* 2003;21:660–666. [PubMed: 12754519]
26. Pratt JM, Petty J, Riba-Garcia I, Robertson DH, Gaskell SJ, Oliver SG, Beynon RJ. *Mol. Cell. Proteomics* 2002;1:579–591. [PubMed: 12376573]
27. Wu CC, MacCoss MJ, Howell KE, Matthews DE, Yates JR. *Anal. Chem* 2004;76:4951–4959. [PubMed: 15373428]
28. Kaplan G, Unkeless JC, Cohn ZA. *Proc. Natl. Acad. Sci. USA* 1979;76:3824–3828. [PubMed: 386350]
29. Pichon J, Marvaldi J, Marchis-Mouren G. *J. Mol. Biol* 1975;96:125–137. [PubMed: 1099210]
30. Sperling E, Bunner AE, Sykes MT, Williamson JR. *Anal. Chem* 2008;80:4906–4917. [PubMed: 18522437]
31. Rockwood AL, Van Orden SL, Smith RD. *Anal. Chem* 1995;67:2699–2704.
32. Yergey JA. *Int. J. Mass Spectrom. Ion Phys* 1983;52:337–349.
33. Snijders APL, de Koning B, Wright PC. *J. Proteome Res* 2005;4:2185–2191. [PubMed: 16335965]
34. Snijders AP, de Vos MG, Wright PC. *J. Proteome Res* 2005;4:578–585. [PubMed: 15822937]
35. Palmblad M, Mills DJ, Bindschedler LV. *J. Proteome Res* 2008;7:780–785. [PubMed: 18189342]
36. Maccoss MJ, Wu CC, Matthews DE, Yates JR. *Anal. Chem* 2005;77:7646–7653. [PubMed: 16316172]
37. Stemmann O, Zou H, Gerber SA, Gygi SP, Kirschner MW. *Cell* 2001;107:715–726. [PubMed: 11747808]
38. Maccoss MJ, Wu CC, Liu HB, Sadygov R, Yates JR. *Anal. Chem* 2003;75:6912–6921. [PubMed: 14670053]
39. Kito K, Ito T. *Curr Genomics* 2008;9:263–274. [PubMed: 19452043]
40. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. *Proc. Natl. Acad. Sci. USA* 2003;100:6940–6945. [PubMed: 12771378]
41. Aggarwal K, Choe LH, Lee KH. *Proteomics* 2005;5:2297–2308. [PubMed: 15887184]
42. Kirkpatrick DS, Gerber SA, Gygi SP. *Methods* 2005;35:265–273. [PubMed: 15722223]
43. Nelson CJ, Huttlin EL, Hegeman AD, Harms AC, Sussman MR. *Proteomics* 2007;7:1279–1292. [PubMed: 17443642]
44. Kusmierczyk AR, Hochstrasser M. *Biol. Chem* 2008;389:1143–1151. [PubMed: 18713001]

45. Schuwirth BS, Borovinskaya MA, Hau CW, Zhang W, Vila-Sanjurjo A, Holton JM, Cate JH. Science 2005;310:827–834. [PubMed: 16272117]
46. Weitzmann CJ, Cunningham PR, Nurse K, Ofengand J. FASEB J 1993;7:177–180. [PubMed: 7916699]
47. Recht MI, Williamson JR. J. Mol. Biol 2004;344:395–407. [PubMed: 15522293]
48. Samaha RR, O'Brien B, O'Brien TW, Noller HF. Proc Natl Acad Sci U S A 1994;91:7884–7888. [PubMed: 8058729]
49. Held WA, Nomura M. Biochemistry 1973;12:3273–3281. [PubMed: 4581788]
50. Sykes MT, Williamson JR. BMC Bioinformatics 2008;9:446. [PubMed: 18937869]

Fig. 1. Equilibrium assembly map of the 30S ribosomal subunit. Arrows represent protein binding dependencies at equilibrium. Colored circles refer to observed binding rates at 40°C as measured by PC/QMS [2]. Red, 9–16 min⁻¹; orange, 5.4–6.7 min⁻¹; green, 1.9–3.9 min⁻¹; blue, 0.31–0.85 min⁻¹; purple, 0.25–0.14 min⁻¹. For the most part, assembly proceeds in a 5'-to-3' direction and a primary-to-tertiary direction. B) PC/QMS reaction scheme for 40°C 30S reconstitution experiment.

Fig. 2. Example LC-MS peak pair from a PC/QMS experiment, showing unlabeled and fully labeled peptide. Data (circles) and fit calculated with LS-FTC (line) are shown. Programs that perform LS-FTC are available for free download at <http://williamson.scripps.edu/software> [30] [50].

Fig. 3. Example spectra from a mixture of unlabeled and partially labeled peptide, with data (circles) and fit (line).

Fig. 4. Quantitation with extracted ion chromatograms (EICs) is based on the intensity over time of the monoisotopic peaks of the labeled and unlabeled peptide.

Fig. 5.

Temperature dependence of r-protein binding. (A) PC/QMS reaction scheme for 40°C 30S reconstitution experiment. (B) PC/QMS reaction scheme for experiment performed at 15°C. (C) Protein binding rates from 40°C (red) [2] and 15°C (blue). Error bars are standard deviations between two replicate experiments. S8 binds with multiphasic kinetics at 15°C and cannot be fit well to a single exponential. Most proteins bind at least 20-fold slower at 15°C as compared to 40°C. S2 and S21 bind transiently and hence their kinetics cannot be measured by PC/QMS.

Fig. 6.

Schematic of pre-binding PC/QMS reconstitution experiment. The quantities of materials used are as follows: 383 μL of 0.47 μM rRNA in TKM, 172 μL of 1.57 μM S7 in TKMD for the pre-binding solution, 45 μL of 6 μM ^{15}N TP30 in RB for the pulse, and 135 μL of 10 μM ^{14}N TP30 in RB for the chase. These experiments have six steps: (1) pre-heating the rRNA and pre-binding solution to 40°C, (2) mixing the rRNA and the pre-binding solution, (3) transferring the ^{15}N TP30 and the rRNA to 15°C, (4) adding the ^{15}N TP30 to the rRNA, (5) adding the ^{14}N TP30 and transferring the reaction to 40°C, and (6) transferring the sample to ice. The chase must be performed at 40°C to ensure an optimal yield of completed 30S ribosomal subunits. (B) S7 binding kinetics at 15°C with ^{14}N S7 pre-bound (red) and without S7 pre-bound (blue). ^{15}N S7 binds to a lower extent because most S7 binding sites are occupied with pre-bound ^{14}N S7. (C) S9 curve binding kinetics at 15°C with (red) and without (blue) S7 pre-bound. S9 binds faster and with multiphasic kinetics when S7 is pre-bound.