Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry

Huilin Zhou, Jeffrey A. Ranish, Julian D. Watts, and Ruedi Aebersold*

The adaptation of sequences of chemical reactions to a solid-phase format has been essential to the automation, reproducibility, and efficiency of a number of biotechnological processes including peptide and oligonucleotide synthesis and sequencing1–4. Here we describe a method for the site-specific, stable isotope tagging and mass spectrometry by solid-phase isotope tagging reagent (Fig. 1). Laminin B was quantitatively captured onto the solid phase (compare Fig. 2A, 2B). After 1 h of photocleavage, the tagged laminin B was recovered; it showed the expected mass

We devised a method for site-specific, stable isotopic labeling of cysteinyl peptides using a solid-phase isotope tagging reagent (Fig. 1). The nitrilotriazine-based photocleavable linker was first attached to aminopropyl-coated glass beads by solid-phase peptide synthesis. Next, the isotope tag, a leucine molecule containing either seven hydrogen (d0) or seven deuterium atoms (d7), was attached to the photocleavable linker, again by solid-phase peptide synthesis. Finally, a sulfydryl-specific iodoacetyl group was attached. Cysteinyl peptides from two samples to be compared were covalently captured on the solid phase containing isotopically heavy or normal tag. The beads were then combined, washed, and exposed to UV light (360 nm, chosen to minimize any possible photocatalyzed side reactions). This resulted in photocleavage of the linker and the transfer of isotope tags from the solid phase onto the side chain of cysteine residues. Finally, recovered tagged peptides were analyzed by µLC-MS/MS to determine the sequence and relative abundance of each peptide, essentially as described previously.

To illustrate the efficiency of the capture and release reactions, we used a mixture consisting of a cysteine-containing laminin B peptide and the non-cysteine-containing phosphoangiotensin (Fig. 2). Laminin B was quantitatively captured onto the solid phase (compare Fig. 2A, 2B). After 1 h of photocleavage, the tagged laminin B was recovered; it showed the expected mass

Institute for Systems Biology, 1441 North 34th Street, Seattle, WA 98103–8904.
*Corresponding author (raebersold@systemsbiology.org).

15. Adams, M.D. et al. A solid-phase method for stable isotope tagging of peptides is com-
aminopropyl-coated glass beads by solid-phase peptide synthesis6. The
teinyl peptides using a solid-phase isotope tagging reagent (Fig. 1).
paratively simpler, more efficient, and more sensitive.

The adaptation of sequences of chemical reactions to a solid-
phase format has been essential to the automation, reproducibility,
and efficiency of a number of biotechnological processes including
peptide and oligonucleotide synthesis and sequenc-
ty, and efficiency of a number of biotechnological processes
including peptide and oligonucleotide synthesis and sequenc-
ing1–4. Here we describe a method for the site-specific, stable iso-

topic tagging and mass spectrometry by solid-phase isotope tagging reagent (Fig. 1). Laminin B was quantitatively captured onto the solid phase (compare Fig. 2A, 2B). After 1 h of photocleavage, the tagged laminin B was recovered; it showed the expected mass

We devised a method for site-specific, stable isotopic labeling of cysteinyl peptides using a solid-phase isotope tagging reagent (Fig. 1). The nitrilotriazine-based photocleavable linker was first attached to aminopropyl-coated glass beads by solid-phase peptide synthesis. Next, the isotope tag, a leucine molecule containing either seven hydrogen (d0) or seven deuterium atoms (d7), was attached to the photocleavable linker, again by solid-phase peptide synthesis. Finally, a sulfydryl-specific iodoacetyl group was attached. Cysteinyl peptides from two samples to be compared were covalently captured on the solid phase containing isotopically heavy or normal tag. The beads were then combined, washed, and exposed to UV light (360 nm, chosen to minimize any possible photocatalyzed side reactions). This resulted in photocleavage of the linker and the transfer of isotope tags from the solid phase onto the side chain of cysteine residues. Finally, recovered tagged peptides were analyzed by µLC-MS/MS to determine the sequence and relative abundance of each peptide, essentially as described previously.

To illustrate the efficiency of the capture and release reactions, we used a mixture consisting of a cysteine-containing laminin B peptide and the non-cysteine-containing phosphoangiotensin (Fig. 2). Laminin B was quantitatively captured onto the solid phase (compare Fig. 2A, 2B). After 1 h of photocleavage, the tagged laminin B was recovered; it showed the expected mass

We devised a method for site-specific, stable isotopic labeling of cysteinyl peptides using a solid-phase isotope tagging reagent (Fig. 1). The nitrilotriazine-based photocleavable linker was first attached to aminopropyl-coated glass beads by solid-phase peptide synthesis. Next, the isotope tag, a leucine molecule containing either seven hydrogen (d0) or seven deuterium atoms (d7), was attached to the photocleavable linker, again by solid-phase peptide synthesis. Finally, a sulfydryl-specific iodoacetyl group was attached. Cysteinyl peptides from two samples to be compared were covalently captured on the solid phase containing isotopically heavy or normal tag. The beads were then combined, washed, and exposed to UV light (360 nm, chosen to minimize any possible photocatalyzed side reactions). This resulted in photocleavage of the linker and the transfer of isotope tags from the solid phase onto the side chain of cysteine residues. Finally, recovered tagged peptides were analyzed by µLC-MS/MS to determine the sequence and relative abundance of each peptide, essentially as described previously.

To illustrate the efficiency of the capture and release reactions, we used a mixture consisting of a cysteine-containing laminin B peptide and the non-cysteine-containing phosphoangiotensin (Fig. 2). Laminin B was quantitatively captured onto the solid phase (compare Fig. 2A, 2B). After 1 h of photocleavage, the tagged laminin B was recovered; it showed the expected mass

Institute for Systems Biology, 1441 North 34th Street, Seattle, WA 98103–8904.
*Corresponding author (raebersold@systemsbiology.org).
modification (+170 Da) due to the addition of leucine tag to the cysteine residue (Fig. 2C), which was also confirmed by MS/MS (data not shown). The hydrophobic nature of the leucine tag increases the retention time of tagged laminin B as compared with the untagged form. The signal intensities of untagged and tagged laminin B, contrasting with the identical amounts of phosphoangiotensin, indicated specific capture and almost complete recovery of tagged laminin B. Longer photocleavage time did not affect either the yield or the quality of the tagged laminin B (data not shown), indicating that photocatalyzed side reactions did not occur to any substantial extent.

Stable isotope tagging is a general strategy for quantitative analysis of proteins by mass spectrometry, exemplified by the previously published ICAT method(3-5). To compare the performance of the solid-phase approach with that of the standard ICAT approach, we performed a side-by-side comparison in which the two methods were used to detect protein expression changes in the yeast S. cerevisiae in response to induction with galactose. Two different amounts of starting protein material were evaluated (100 µg large sample load and 10 µg small sample load of the same yeast proteins). Because our objective was to compare the relative performance of the labeling strategies, and not the peptide separation or protein identification strategies, we performed single LC-MS/MS runs on all samples for protein identification. This minimized variations in the results due to sample processing rather than labeling, but probably resulted in the identification of fewer proteins than would be obtainable with larger sample sizes and optimized peptide separations upstream of LC-MS/MS(6,7).

In both the small- and large-scale experiments, the number of proteins identified and quantified by the solid-phase method was greater than by ICAT (Fig. 3; for full lists of the proteins quantified, see Supplementary Tables 1-4 online). Indeed, the solid-phase approach was more sensitive, identifying the majority of the proteins identified by conventional ICAT in addition to many others not identified by ICAT (Fig. 3A, 3B). Quantification of the same proteins identified in multiple experiments was also consistent (see Supplementary Tables 1-4 online). Thus, protein quantification was not influenced by either the structure of the isotope tag or the capture and release chemistry of the solid-phase method.

Galactose is known to strongly induce expression of several genes involved in galactose utilization, including galactokinase (GAL1), galactose permease (GAL2), galactotransferase (GAL7), and UDP-glucose-4-epimerase (GALX)(8). After induction with galactose, we identified and quantified multiple peptides from proteins including GAL1, GAL2, GAL7, and GALX by the solid-phase method (Supplementary Tables 1-4 online). In contrast, using the ICAT method we identified only one peptide from the GAL1 protein in both the small- and large-sample-load experiments, and found a lower signal-to-noise ratio than with the solid-phase method (data not shown). These data confirmed the superior sample recovery and sensitivity of the solid-phase over the ICAT approach. In addition, the reproducibility of the solid-phase method was demonstrated by the substantial overlap in proteins identified by the small- and large-scale experiments (Fig. 3C).

The data presented here show that the solid-phase method is simple, reproducible, efficient, and sensitive for quantitative protein analysis. Indeed, as compared with the ICAT method, it has several advantages. First, both the isolation of cysteine-containing peptides and the stable incorporation of isotopes are achieved essentially in a single step. Thus, the solid-phase method is faster and simpler, requiring less manual input than the ICAT approach. Second, the covalent capture of peptides to a solid phase permits the use of stringent wash conditions to remove non-covalently associated molecules. Indeed, the experiments presented here resulted in the recovery of almost exclusively cysteinyl peptides. Third, this procedure is unaffected by the presence of proteolytic enzymes such as trypsin or of strong denaturants and detergents such as urea and SDS. There is therefore no need for additional steps to remove such molecules. Because of the minimal sample handling, this solid-phase method is more sensitive than the ICAT method. As many biologically interesting events involve relatively low-abundance regulatory proteins, the solid-phase method should be useful for the analysis of induced changes to such proteins. Fourth, the standard solid-phase peptide chemistry involved in the coupling process allows the use of a range of natural or unnatural amino acids as the isotopic mass tag in place of the d0-leucine used here. This could facilitate the synthesis of beads with a range of mass tags for analysis of multiple samples (more than two) in a single experiment. Fifth, the mass tag on the cysteine used here weighs 170 Da for the d0-leucine tag. Because of the small size and the chemical nature of the tag, the observed peptide fragmentation in the MS/MS mode was not complicated by undesirable fragmentation of the label itself (data not shown), in contrast to the situation with ICAT-labeled peptides(9). Finally, before photocleavage, the covalently immobilized peptides provide ideal substrates for additional chemical and enzymatic reactions if desired(10).

One significant way that the solid-phase method differs from the ICAT method is that the solid-phase reagent labels peptides after proteolysis, whereas in ICAT, proteins are labeled before proteolysis. Therefore, the ICAT approach is preferred in cases where separation of labeled proteins is required, such as gel electrophoresis. The solid-phase method presented here should, however, provide a tool suitable for general application to quantitative proteomics and amenable
Figure 3. Summary of the number of proteins identified and quantified by the solid-phase and ICAT methods. (A) Number of proteins identified from large-scale experiment (L), in which 100 µg total protein sample was labeled and 20 µg was analyzed by µLC-MS/MS. We quantified 82 proteins by the solid-phase method and 33 proteins by the ICAT method, with 25 proteins in common. (B) Number of proteins identified from small-scale experiment (S), in which 10 µg of total sample was labeled and 5 µg analyzed. We quantified 57 proteins by the solid-phase method and 18 proteins by the ICAT method, with 13 proteins in common. (C) Number of proteins identified by the solid-phase method in large-scale (L) and small-scale (S) experiment. Numbers in segments of Venn diagrams indicate the numbers of proteins quantified.

to automated implementation. It thus represents a step forward towards much wider applications of stable isotope tagging for quantitative protein analysis by mass spectrometry.

Experimental protocol
Synthesis of the solid-phase isotope labeling beads. Unless otherwise noted, chemicals were purchased from Aldrich (Milwaukee, WI). First, 0.5 g ofaminopropyl-coated controlled-pore glass beads (amine content ~400 µmol/g; Sigma, St. Louis, MO) were washed with anhydrous dimethylformamide (DMF). Then 600 µmol each of 1-hydroxybenzotriazole (HOBt; Nova Biochem, Laufelfingen, Switzerland), Fmoc–aminoethyl photolinker (Nova Biochem), and diisopropylcarbodiimide (DIC) were mixed for 30 min at room temperature. Five milligrams of the beads prepared as described above, and then 50% of the peptide mixture recovered (representing 5 µg of combined protein extracts) was analyzed by the same µLC-M/MS method.

Isolation and isotope labeling of tryptic digest of yeast proteins by the solid-phase capture-release method. For the larger-scale experiment, 50 µg of each protein extract (100 µg combined) from yeast cells grown with or without galactose was prepared in 100 µl of 200 mM Tris (pH 8.0), 5 mM EDTA. Each protein extract was digested by 5 µl trypsin for 3 h at 37°C and released with 5 µl TCEP, and cysteinyl peptides were then captured by beads with either d0- or d7-leucine tag for 15 min as described above. The beads were combined and washed, and labeled peptides were released by 2 h of light illumination. The released peptides were loaded on an M CX column (Waters, Milford, MA) and washed sequentially with 4 ml 0.1% trifluoroacetic acid (TFA), 4 ml 80% acetonitrile/0.1% TFA, and water (to neutralize). Peptides were eluted by 1 ml of a mixture of 9 volumes methanol and 1 volume 28% ammonia, and dried under reduced pressure. Dried peptides were resuspended in water for µLC-M/MS analysis. Next, 20% of the recovered peptides (representing the sum of the combined proteins) were analyzed by µLC-M/MS to identify the peptides from rat liver or rat brain. For the small-scale experiment, 5 µg of each protein extract from cells grown with or without galactose was digested by 0.5 µg trypsin and processed as described above, and then 50% of the peptide mixture recovered (representing 5 µg of combined protein extracts) was analyzed by the same µLC-M/MS method.

Isotope labeling of yeast proteins and isolation of peptides by ICAT. As starting material, 100 µg of each protein extract from cells grown with or without galactose was prepared in labeling buffer containing 200 mM Tris (pH 8.0), 0.5 mM EDTA, 6 M urea, and 0.05% SDS. Proteins were reduced with 5 mM TCEP and labeled with 500 µl of d0- and d8-ICAT was added to the protein extracts from raifinnan- or galactose-grown cells, respectively. After 90 min of labeling at room temperature, the reactions were quenched by addition of β-mercaptoethanol to 10 mM, and combined. Each sample was diluted 10-fold with 20 mM Tris (pH 8.3), 0.01% SDS. Trypsin (10 µg) was added to digest proteins for 3 h at 37°C. The sample was digested with a mixture of trypsin (5 µg/mM KH2PO4 (pH 3), 25% CH3CN) and the pH was adjusted to 3 with dilute TFA. Either 100 µg (large-scale experiment) or 10 µg (small-scale experiment) of the combined protein digest was applied to a cation-exchange cartridge (Applied Biosystems, Foster City, CA) equilibrated in buffer A. The cartridge was washed with 2 ml buffer A, followed by 2 ml of buffer A + 40 mM KCl. Bound peptides were eluted with 600 µl buffer A + 600 mM KCl. Sample volume was reduced to 300 µl under reduced pressure, and 500 µl of PBS and 12 µl 1 M NH4HCO3 were added. Samples were then filtered over a monomeric avidin cartridge (Applied Biosystems) and washed with 2 ml PBS, 1 ml x4 PBS, and 1 ml 50 mM NH4HCO3 with 20% methanol. Labeled peptides were eluted with 1 ml 0.4% TFA with 30% acetonitrile, dried under reduced pressure, and resuspended in 10 µl of 0.4% acetic acid with 5% acetonitrile. For either the large- or small-scale experiment, the same amount of sample was analyzed by the same µLC-M/MS method as used in the solid-phase method.

Note: Supplementary information is available on the Nature Biotechnology website.
Acknowledgments
This work was supported in part by the US National Cancer Institute grant (CA84698), National Institutes of Health (NIH) Research Resource Center (RR11823), NIH grant (GM 41109) to R.A., and NIH postdoctoral fellowship (GM 19884) to J.A.R.

Competing interests statement
The authors declare that they have no competing financial interests.

Received 12 October 2001; accepted 14 February 2002