Accelerated Article

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Mass-Balanced $^{1}H/^{2}H$ Isotope Dipeptide Tag for Simultaneous Protein Quantitation and Identification

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Mass-balanced $^{1}H/^{2}H$ isotope dipeptide tags (MBITs) are presented for simultaneous protein quantitation and identification. MBIT is derived from *N*-acetyl-Ala-Ala dipeptide and conjugated to primary amines of target peptides. $^{1}H/^{2}H$ isotopes are encoded in the methyl groups of N-acetylated dipeptide: one tag deuterated on the *N*-acetyl group and another on the C-terminal alanine. MBIT-linked peptides comigrate in reversed-phase liquid chromatography without significant $^{1}H/^{2}H$ isotope effects and provide 2-plex quantitation signals at 114 and 117 Th as well as peptide sequence information upon MS/MS analysis with MALDI TOF/TOF. MBIT shows good quantitation linearity in a concentration range of 20−250 fmol. The performance of MBIT on protein quantitation and identification is further tested with yeast heat-shock protein (Hsp82p) obtained from three different physiological states. MBIT using nanogram-scale samples produces the relative abundance ratios comparable to those obtained from optical imaging of microgram-scale samples visualized with SYPRO Ruby stain. The MBIT strategy is a simple and low-cost alternative for 2-plex quantitation of proteins and offers possibilities of tuning the 2-plex signal mass window by replacing the N-terminal alanine with other amino acid residues.

Simultaneous protein quantitation and identification are of critical importance in proteome research. A standard approach to quantitative proteomics has been one- or two-dimensional gel electrophoresis followed by imaging fluorescent dye-stained gels to quantitate proteins and by taking the mass spectra of proteins or peptides to identify proteins.[1–10] Although this gel-based proteome analysis is very useful, it is labor-intensive, difficult to automate, and hardly reproducible.[4,6,11–13] An alternative approach is based on liquid chromatography (LC) coupled with mass spectrometry (MS), which combines automated high-throughput mass analysis with multidimensional separation of complex peptide mixtures.[14–16] For protein quantitation using LC−MS, a number of stable isotope tags have been introduced as markers.[7,12,17–28] The isotope-coded affinity tag (ICAT) employing $^{1}H/^{2}H$ isotopes

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is notable for its selective quantitation of cysteine-containing peptides.[17,20–22] Although ICAT has catalyzed substantial growth in quantitative proteome analysis, its specificity, limited to cysteine and differential peptide elution profile, become hindrances to global proteome analysis. These limitations are lifted by designing amine-reactive isobaric tags, such as tandem mass tags (TMTs)[29] and isobaric tags for relative and absolute quantitation (iTRAQ).[30] As a result, those peptides differentially labeled with isobaric tags are eluted at the same time from LC with no chromatographic isotope effect, and they appear at the same mass in the mass spectra. Moreover, the tandem mass (MS/MS) spectra display all of the sequence ions as isotopomers, yet differential quantitation signals at different m/z values, thus allowing simultaneous protein quantitation and identification.

The TMT reagents are composed of Met-Met-Gly tripeptides and Met-Pro-Met-Gly tetrapeptides. Their N-terminal amines are acylated with 6-guanidinohexanoic acid, and the first and second methionines are differentially labeled with $^{1}H/^{2}H$ isotopes for 2-plex quantitation. Although the two TMTs have a common signal reporting group, their quantitation signals appear at two different mass regions: 270/273 and 287/290 Th from tripeptide and tetrapeptide, respectively.[29] On the other hand, the iTRAQ reagents containing *N*-methylpiperazine work, in principle, similarly to TMT and present 4-plex quantitation signals from 114 to 117 Th in a quiet region of the MS/MS spectra. The iTRAQ reagents are *N*-hydroxysuccinimide (NHS) esters of *N*-methylpiperazine acetic acid differentially labeled with ^{13}C, ^{15}N, and ^{18}O-isotopes.[30] Recently, an 8-plex version of iTRAQ reagents with ^{13}C and ^{15}N isotopes has been reported with signals appearing from 113 to 119 and 121 Th, but their exact chemical structures have not been disclosed.[31] Most recently, a 6-plex version of TMT reagents with ^{13}C and ^{15}N isotopes is reported with signals between 126 and 131 Th. These multiplex versions of isobaric tags carry an NHS functional group for conjugation with primary amines.[32] However, both the high cost of ^{13}C, ^{15}N, and ^{18}O isotopes and the fixed signal mass window become limiting factors that narrow the scope of isobaric tags in quantitative proteomics.

In this report, we present 2-plex isobaric tags based on dipeptide. We design the "mass-balanced $^{1}H/^{2}H$ isotope dipeptide tags" (MBITs) by following the principle of isobaric labeling but incorporating low-cost $^{1}H/^{2}H$ isotopes rather than more expensive ^{13}C, ^{15}N, and ^{18}O isotopes and allowing the possibility of tuning the signal mass window. Like other isobaric tags, MBIT consists of three parts: the quantitation signal, the mass balance, and the amine-reactive linker. Both the quantitation signal and mass balance parts are alternately labeled with low-cost $^{1}H/^{2}H$ isotopes to provide a 2-plex pair of isobaric tags. Unlike other isobaric tags, the signal mass window of MBIT can be extended to a different region of the MS/MS spectra by replacing the N-terminal amino acid with other amino acids in dipeptide synthesis. These differentially labeled isobaric dipeptide tags can be conveniently prepared in the laboratory by solid-phase peptide synthesis. Thus, the outstanding features of MBIT include low-cost $^{1}H/^{2}H$ isotopes, the possibility of shifting the signal mass window, and perhaps the convenient laboratory-scale synthesis of dipeptide tags.

We carried out proof-of-concept studies with MBIT, assessed the performance in protein quantitation and sequencing, and addressed some important issues, such as chromatographic isotope effect, quantitation linearity, and dynamic range. Angiotensin II (DRVYIHPF) was employed to study the chemical reactivity of amine-reactive linkers, the quantitation linearity, the dynamic range, and the effects of dipeptide tags on the fragmentation pattern. Bovine serum albumin (BSA) was used to examine the chromatographic isotope effects and to evaluate quantitation errors. Finally, yeast heat-shock proteins (Hsp82p) prepared in three different physiological states were quantitated by applying MBIT as well as by optical imaging of SYPRO-Ruby-stained gel for comparison. In all cases, standard peptides or tryptic peptides were differentially labeled with MBIT and combined before reversed-phase (RP) LC separation. Peptides eluted from RPLC were collected on a MALDI plate for MS/MS using a MALDI TOF/TOF mass spectrometer.

MBIT Concept and Design. The MBIT reagents are based on *N*-acyl-dipeptide as shown in Scheme 1. The quantitation signal is encoded in the *N*-acyl group (R_SCO) with $^{1}H/^{2}H$ isotopes. The isobaric mass balance is achieved by encoding the side chain (R_B) of the C-terminal amino acid with $^{1}H/^{2}H$ isotopes. The quantitation signals can be tuned by varying the N-terminal amino acid. The amine-reactive linker in Scheme 1b could be either *N*-succinimidyl ester (OSu) or benzotriazol-1-yl ester (OBt). The carbonyl group of the *N*-acyl moiety promotes amide-bond cleavage between the first and second amino acid residues to yield the quantitation signals, $^{L}b_s$ and $^{H}b_s$ (Scheme 1c). Amide-bond cleavage at the MBIT-peptide linkage gives rise to the b_0 ion as a signature of tagging. A series of isotopomeric b-type ions and unmodified y-type ions provide sequence information. MBIT-linked parent peptides (both ^{L}MBIT- and ^{H}MBIT-peptides) appear at the same mass, and their MS/MS spectra show both the signature and sequence ions as single peaks, but 2-plex quantitation signals at discrete masses (Scheme 1d).

The first generation of MBIT reagents based on the *N*-acetyl-Ala-Ala dipeptide with either an OBt or an OSu linker is shown in Scheme 2. In this case, the quantitation signals appear at 114 and 117 Th. In theory, this region is free of immonium, y-type, and

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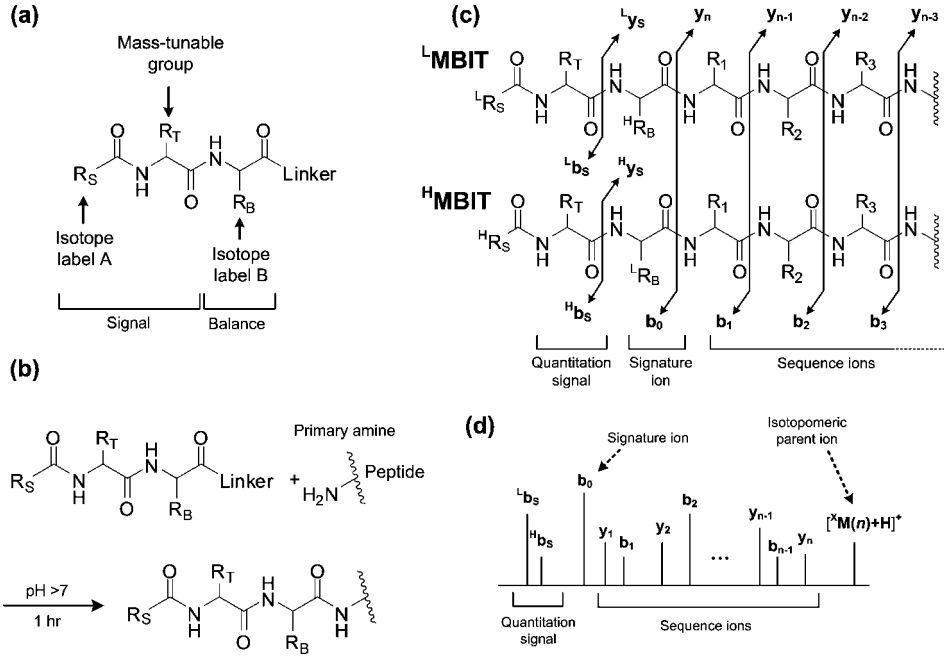
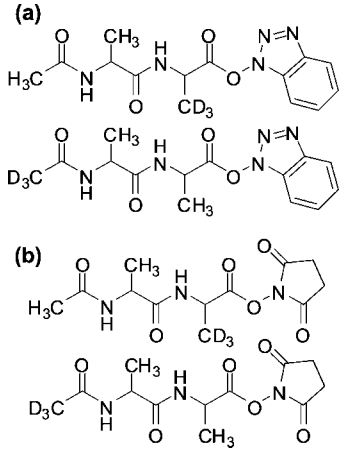
Scheme 1. Basic Concept of MBIT: (a) Structure of MBIT Reagent, (b) Amine-Specific Coupling of MBIT, (c) Expected Fragmentation of MBIT-Linked Peptides, and (d) MS/MS Spectra of MBIT-Linked Peptides



Scheme 2. Two-Plex *N*-Acetyl-Ala-Ala Dipeptide MBITs: (a) Benzotriazol-1-yl Esters and (b) *N*-Hydroxysuccinimidyl Esters



other internal ions. Scheme 3 depicts the possible signal mass window between 100 and 220 Th. Serine and histidine residues in place of the N-terminal alanine afford a pair of 2-plex signals at 130/133 and 180/183 Th, respectively, also in a quiet region of the MS/MS spectra. Valine, glutamine, phenylalanine, arginine, and tyrosine offer a pair of 2-plex signals at 142/145, 171/174, 191/194, 202/205, and 207/210 Th, respectively, in a pseudoquiet region presumably overlapping with internal ions.

EXPERIMENTAL SECTION

Materials. Angiotensin II acetate (human, 88%), BSA, and sequencing-grade modified porcine trypsin were purchased from Sigma-Aldrich (St. Louis, MO), Merck (Darmstadt, Germany), and Promega (Madison, WI), respectively. An acid form of MBIT reagents (^{X}MBIT-OH, X = L or H) was synthesized in the laboratory following the standard solid-phase procedure.[33] They were also separately prepared from the solution-phase synthesis (see Supporting Information (SI) for details). (Benzotriazol-1-yloxyl)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and NHS were from Sigma-Aldrich. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) was from Pierce (Rockford, Il). Acetonitrile (ACN, ≥ 99.8%), anhydrous *N*,*N*-dimethylformamide (DMF, ≥ 99.8%), anhydrous diisopropylethylamine (DIPEA), hydroxylamine hydrochloride, trifluoroacetic acid (TFA, HPLC grade, ≥ 99.8%), formic acid (FA, 88% in H_2O), and α-cyano-4-hydroxycinnamic acid (HCCA) were from Sigma-Aldrich.

Yeast Hsp82p Preparation. An N-terminal hemagglutinin (HA)-tagged Hsp82 protein was expressed from the endogenous *HSP82* promoter (strain YHY240HAN1) or overexpressed from the strong *PGK1* promoter (strain YHY240PGK1HAN2) (see SI). For mass spectrometry, HA-Hsp82 fusion proteins were affinity-isolated from cell lysates using anti-HA agarose beads (clone 3F10, Roche). Bound proteins were eluted, separated on a SDS-polyacrylamide gel, and then visualized by SYPRO Ruby staining (Molecular Probes, Eugene, OR). After quantifying HA-Hsp82p levels using a VersaDoc 5000 MP gel imaging system (Bio-Rad, Hercules, CA), protein bands were excised from the gel and kept at 0 °C for subsequent in-gel tryptic digestion. The gel piece obtained from YHY240HAN1 under either normal (growth at 30 °C) or heat-shock condition (growth at 39 °C) was named **30** or **39**, and that from YHY240PGK1HAN2 under overexpression condition at 30 °C was named **pgk**.

Preparation of Active Esters of MBIT Reagents. ^{X}MBIT-OBt (X = L or H) was prepared by mixing BOP into 5-fold molar excess of ^{X}MBIT-OH in DMF, to a final concentration of 6.0 (^{X}MBIT-OH) and 2.5 (BOP) mg mL^{-1}, respectively, with mild

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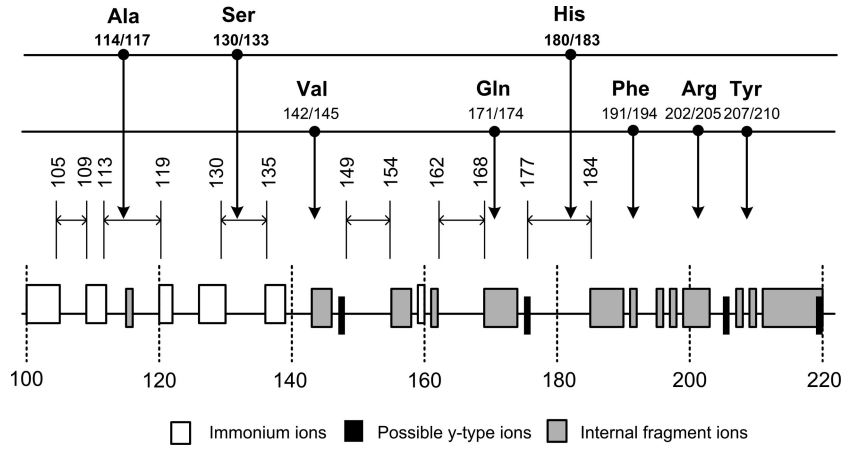
Scheme 3. Tunable Mass Window of Ala-, Ser-, and His-Containing Quantitation Signals in a Quiet Region, and Val-, Gln-, Phe-, Arg-, and Tyr-Containing Quantitation Signals in a Pseudoquiet Region Partially Overlapping with Internal Ions in the MS/MS Spectra



stirring at room temperature for 20 min. ^{X}MBIT-OSu (X = L or H) was prepared similarly to ^{X}MBIT-OBt. The 1:1 mixture of NHS/EDC was added into ~4-fold excess of ^{X}MBIT-OH in DMF, to a final concentration of 8.0 (^{X}MBIT-OH), 0.75 (NHS), and 1.25 (EDC) mg mL^{-1}, respectively, with mild stirring at room temperature for 45 min. Excess ^{X}MBIT-OH reagents ensured the complete consumption of all BOP or EDC, which could unexpectedly make active esters of target peptides during MBIT−peptide conjugation. ^{X}MBIT-OBt or ^{X}MBIT-OSu was immediately used as prepared.

Coupling of MBIT-OBt with Angiotensin II. An ^{X}MBIT-OBt solution (200 μL) was made basic at 0 °C by adding DIPEA (5 μL). Angiotensin II dissolved in DMF (1.0 mg mL^{-1}, 100 μL each) was mixed with either basic ^{H}MBIT-OBt or ^{L}MBIT-OBt solution (50 μL) at room temperature for 2 h. The reaction was terminated with 10% TFA in H_2O (v/v).

Coupling of MBIT-OSu with Angiotensin II. Angiotensin II dissolved in sodium phosphate buffer (pH 7.3) (1.0 mg mL^{-1}, 100 μL each) was mixed with either ^{L}MBIT-OSu or ^{H}MBIT-OSu solution (50 μL) at room temperature for 1 h. Hydroxylamine (100 mM, 20 μL) was used to reverse side reactions and to inactivate excess MBIT-OSu reagents. The reaction was terminated with 10% TFA.

Conjugation of MBITs to Tryptic Peptides of BSA. BSA dissolved in sodium phosphate buffer (pH 7.8) (0.6 mg mL^{-1}, 90 μL) was mixed with modified trypsin dissolved in 0.1% acetic acid (0.1 μg μL^{-1}, 10 μL) and incubated at 38 °C for 16 h. Tryptic peptides were divided into two aliquots and mixed with either ^{L}MBIT-OSu or ^{H}MBIT-OSu solution (35 μL). Resulting ^{X}MBIT-linked (X = L or H) tryptic peptides were dried with Speed Vac (SPD111V, Thermo Savant, Holbrook, NY), reconstituted in 100 μL of 0.1% TFA in H_2O, and analyzed by LC-MALDI-MS.

In-Gel Digestion and Conjugation of MBITs to Tryptic Peptides of Hsp82p. As-prepared Hsp82p was in-gel digested by following the procedure given by Shevchenko et al.[34] Briefly, each excised gel piece (**30**, **pgk**, and **39**) was digested in a 2:1 mixture of sodium phosphate buffer (pH 7.8) and trypsin solution (0.1 μg μL^{-1}). Tryptic peptides were extracted four times by swelling gel pieces with 40−100 μL of H_2O/ACN/FA (45/50/5, v/v) and collecting the supernatants. All the extracted peptides were dried using Speed Vac, reconstituted in 60 μL of sodium phosphate buffer (pH 7.8), and mixed with a freshly prepared ^{X}MBIT-OSu (X = L or H) solution (40 μL): **30** with ^{H}MBIT, and both **pgk** and **39** with ^{L}MBIT. The MBIT-linked peptides were lyophilized with Speed Vac and reconstituted in 100 μL of 0.1% TFA. An aliquot (10 μL) of ^{H}MBIT-**30** was mixed 1:1 with an aliquot (10 μL) of either ^{L}MBIT-**pgk** or ^{L}MBIT-**39**. One third of the mixture was loaded on LC.

MALDI Sample Preparation of MBIT-Angiotensin II. A solution of ^{X}MBIT-linked angiotensin II was diluted 1000 times in 0.1% TFA for MS analysis. ^{L}MBIT- and ^{H}MBIT-angiotensin II were mixed in various ratios: [H]/[L] = 9.25/0.75, 9/1, 8/2, 7/3, 6/4, 5/5, 4/6, 3/7, 2/8, 1/9, and 0.75/9.25. Each sample was diluted again in 0.1% TFA to a final concentration of 1 pmol/μL and mixed with matrix (7 mg mL^{-1} HCCA in 50/50/0.1 H_2O/ACN/TFA) in a 1:1 ratio. The sample/matrix mixture (0.5 μL) was loaded on a MALDI target plate (the total amount of angiotensin II per spot was 250 fmol).

LC-MALDI Sample Preparation of MBIT-Linked Tryptic Peptides of BSA and Hsp82p. An aliquot (6.4 μL) of MBIT-linked tryptic peptides of either BSA (~24 pmol) or Hsp82p (~0.9−1.1 pmol) was injected into a RP Nano-LC system (LC Packings, Sunnyvale, CA) equipped with a PepMap column (100-Å pore, 3-μm particle diameter, 75-μm i.d., 150-mm length). LC was run for 60 min with the flow rate of ~0.3 μL min^{-1} using a two-solvent gradient: H_2O/ACN/TFA 95%/5%/0.1% (solvent A) and H_2O/ACN/TFA 20%/80%/0.1% (solvent B). The [A]/[B] gradient was started from 100/0, changed to 40/60 between 0 and 40 min and to 10/90 for 40−41 min, maintained at 10/90 between 41 and 47 min, and immediately dropped to and kept at 100/0 between 47 and 60 min. The eluted peptides were collected in every 25 s on a single MALDI spot with HCCA using a Probot micro fraction collector (total 144 MALDI spots in 60 min). Each tryptic peptide was eluted over 8 spots on average; thus, each spot contained ~3 pmol of peptides for BSA and ~110−150 fmol of peptides for Hsp82p.

MALDI-MS and MS/MS. A 4700 Proteomic Analyzer (Applied Biosystems, Foster City, CA) was employed in a positive mode at the mass range of 500−2500 Th. At each MALDI spot, the TOF mass spectra were obtained by accumulating 1000 single

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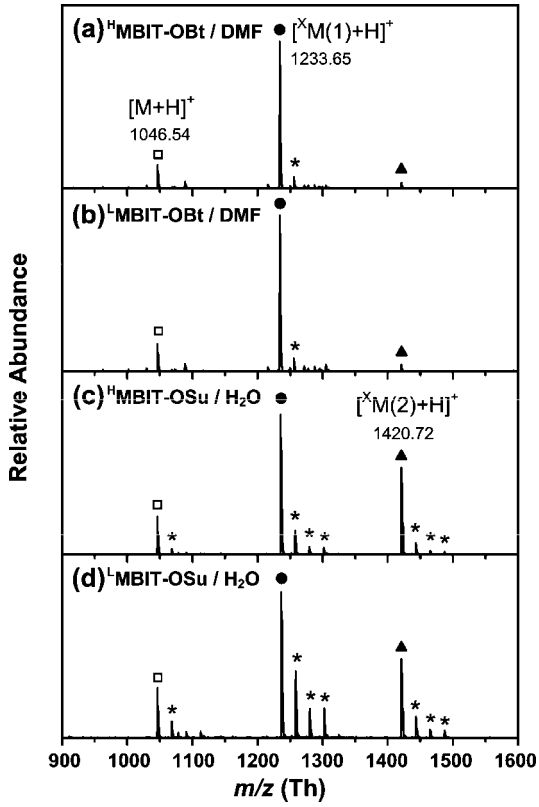

Figure 1. MALDI-TOF mass spectra of angiotensin II after reaction with (a) ^{H}MBIT-OBt in DMF, (b) ^{L}MBIT-OBt in DMF, (c) ^{H}MBIT-OSu in sodium phosphate buffer (pH 7.3), and (d) ^{L}MBIT-OSu in sodium phosphate buffer (pH 7.3). The number of MBITs attached to angiotensin II is denoted in parentheses. Sodium adducts are marked with asterisks.

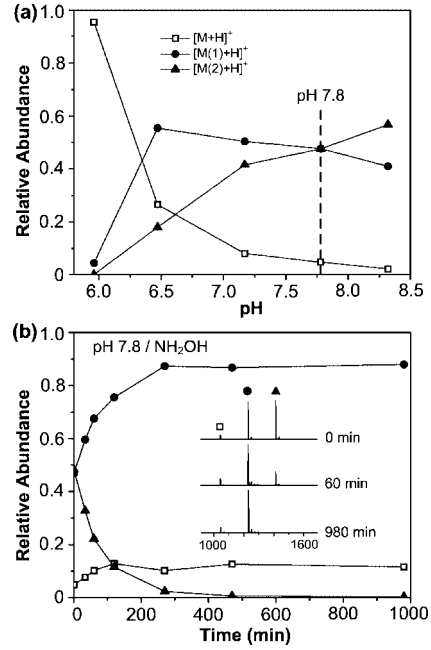

Figure 2. (a) pH dependence of the coupling between MBIT-OSu and angiotensin II in buffered aqueous solution. (b) Temporal variations of unmodified $[M + H]^+$, singly labeled $[M(1) + H]^+$, and doubly labeled $[M(2) + H]^+$ peaks obtained at pH 7.8 after incubating with excess hydroxylamine. Inset shows the mass spectra of the reaction mixtures after 0-, 60-. and 980-min incubation.

laser-shot spectra. For MALDI-TOF/TOF analysis, collision-induced dissociation (CID) was performed under 1.3×10^{-6} Torr of air. The CID spectra were obtained by summing 2000 single laser-shot spectra. For ^{X}MBIT-angiotensin II, the precursor ion appeared at 1233.6 Th. For LC-separated ^{X}MBIT-linked peptides from either BSA or Hsp82p, the precursor ions were selected with the aid of database searching program. After acquiring the mass spectra from all of the 144 MALDI spots, the Mascot program (Matrix Science) was used to identify protein and MBIT-linked peptides from the peptide mass fingerprint data.[35] CID was performed on those peaks identified as MBIT-linked peptides with the signal-to-noise (S/N) ratio of >20. The baseline of the CID mass spectra was corrected using ABI-4700 DataExplore software. After baseline correction, the heights of the signal ions (m/z at 114 and 117 Th) were used for relative quantitation. Each CID spectrum was analyzed using PEAKS 4.5 (Bioinformatics Solutions Inc., Canada) to perform de novo sequencing.[36]

RESULTS AND DISCUSSION

Reactivity of ^{X}MBIT-OBt and ^{X}MBIT-OSu (X = L and H). The reactivity of ^{X}MBIT reagents toward the model peptide angiotensin II was checked by mass spectrometry. Figure 1 shows the mass spectra of the reaction mixtures after ^{X}MBIT-OBt and ^{X}MBIT-OSu coupling. Unmodified, singly labeled, and doubly labeled peptides yielded $[M + H]^+$ at 1046.54 Th, $[^{X}M(1) + H]^+$ at 1233.65 Th, and $[^{X}M(2) + H]^+$ at 1420.72 Th, respectively. The two isobaric reagents (^{L}MBIT and ^{H}MBIT) showed almost identical reactivity with greater than 85% yield from both coupling schemes, indicating no significant deuterium isotope effects on the reactivity. Reactions with ^{X}MBIT-OBt in DMF mostly resulted in singly labeled peptides (Figure 1a,b), whereas those with ^{X}MBIT-OSu in buffered aqueous solution yielded significant amounts of doubly labeled peptides (Figure 1c,d). The MS/MS analysis of the doubly labeled peak revealed that tyrosine was another reactive moiety (data not shown). Although the OSu linker is known to be amine-specific, the labeling of tyrosine in angiotensin II (DRVYIHPF) suggests that unwanted side reactions occur with the hydroxyl groups,[37] complicating the MS/MS analysis. Thus, we optimized the reaction condition for MBIT-OSu coupling in water.

Because the reactivity of MBIT-OSu toward the hydroxyl groups and primary amines should depend on pH, we optimized pH first. We measured the relative abundances of unmodified and labeled peptides after running the MBIT-OSu coupling reaction as a function of pH between 5.9 and 8.3 for 1 h (Figure 2a). Below pH 5.9, no product was observed. Above pH 5.9, both singly and doubly labeled peptides were detected. Above pH 7.8, doubly labeled peptides were more abundant than singly labeled peptides. Because the hydroxyl group became increasingly reactive with increasing pH, adjusting pH alone could not prevent unwanted side reactions. We then turned our attention to the reversal of the ester-forming side reaction with hydroxylamine.[37] Figure 2b shows how the product pattern from the 1-h-long MBIT-OSu

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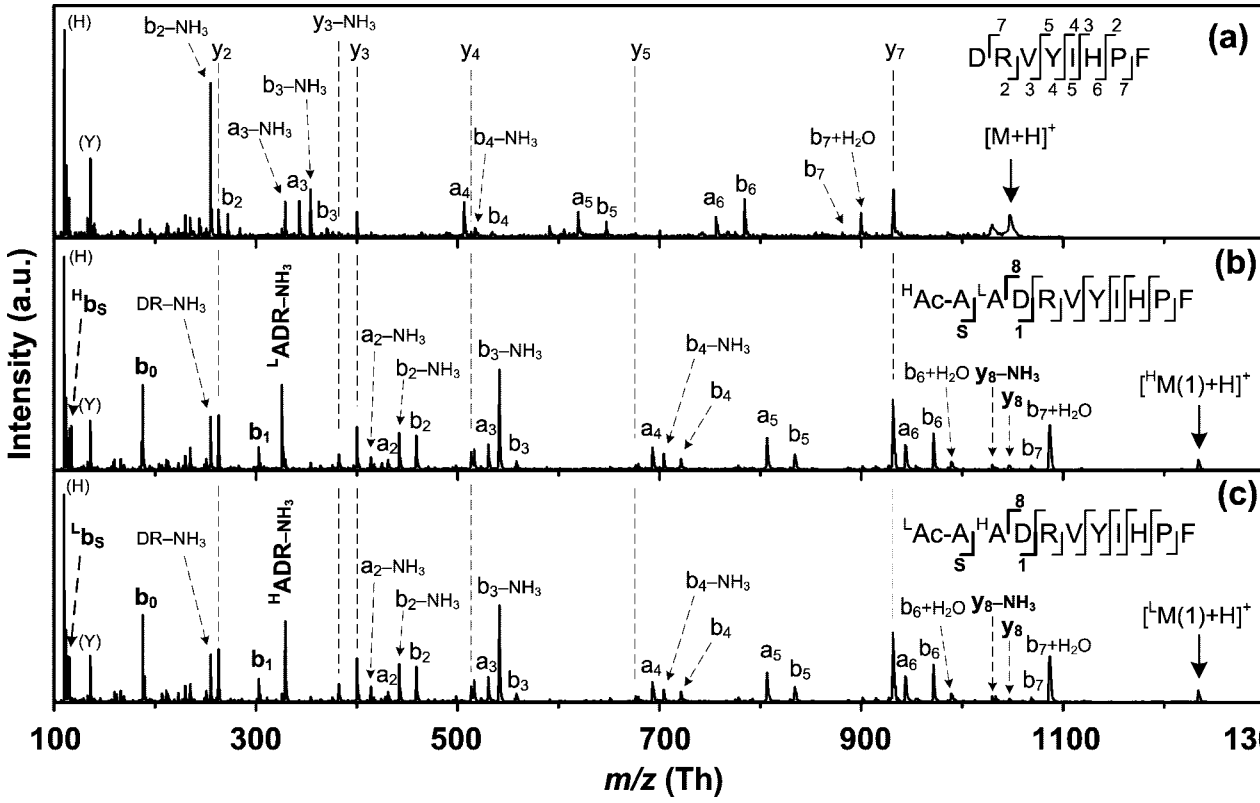

Figure 3. MS/MS spectra of (a) unmodified $[M + H]^+$, (b) ^{H}MBIT-linked $[^{H}M(1) + H]^+$, and (c) ^{L}MBIT-linked $[^{L}M(1) + H]^+$ peaks from angiotensin II. Immonium ions are denoted in parentheses.

coupling reaction at pH 7.8 changes with time after hydroxylamine addition. Of importance, the ester-forming side reactions were fully reversed with the pseudo-first-order rate constant of $(2.0 \pm 0.07) \times 10^{-4}$ s^{-1}. Thus, the MBIT-OSu coupling can be made amine-specific by running the reaction in aqueous solution at pH 7.8 for 1 h and incubating the reaction mixture with excess hydroxylamine at room temperature for 7 h. All of the subsequent amine-reactive coupling reactions were run under these conditions, unless noted otherwise.

Performance of MBITs. The CID mass spectra of MBIT-angiotensin II were compared with those of unmodified peptide to see the effect of N-acetylated dipeptide tagging on the CID fragmentation patterns. Figure 3 shows the MS/MS spectra of (a) unmodified, (b) ^{H}MBIT-, and (c) ^{L}MBIT-angiotensin II. The conjugation of MBIT on the N-terminal amine of angiotensin II shifted the precursor ion by +187 Th. Both ^{L}MBIT- and ^{H}MBIT-linked peptides yielded almost identical CID fragmentation patterns. All of the isotopomeric a- and b-type ions containing ^{X}MBIT at the N-terminus were shifted by +187 Th, whereas all of the y-type ions remained unchanged. Of the fragments, the immonium ion of histidine at 110 Th showed the highest intensity, and those of arginine and tyrosine at 112 and 136 Th, respectively, appeared strong. The relative intensities of y- and b-type sequence ions reached 5−70% of the highest peak and changed slightly by MBIT conjugation. More importantly, a number of new peaks appeared in the spectra as anticipated, including $^{X}b_S$ (X = L or H), b_0, b_1, and y_8 ions. The quantitation signals, $^{L}b_S$ and $^{H}b_S$, appeared at 114 and 117 Th, respectively. The b_0 ion at 188 Th served as the signature for MBIT conjugation, and the concomitant appearance of the complementary y_8 ion confirmed the conjugation again. The b_1 ion augmented sequence information for peptide identification. The internal fragment ions, ^{H}ADR-NH_3 and ^{L}ADR-NH_3 at 329 and 326 Th, respectively, showed up as another quantitation signals by chance. The relative intensities of $^{X}b_S$ and ^{X}ADR-NH_3 pairs reached ~20 and ~50% of the highest peak, respectively. Thus, MBIT offers the quantitation signals without losing sequence information.

Next, we evaluated the performance of MBIT on the relative quantitation. Of the mixtures of ^{L}MBIT- and ^{H}MBIT-angiotensin II prepared in various ratios, 250 fmol was loaded on a MALDI spot for CID. Figure 4a exhibits variations of the quantitation signals $^{X}b_S$ (114 and 117 Th) and the fortuitous quantitation signals ^{X}ADR-NH_3 (326 and 329 Th) as a function of the premixing ratio from 9.25/0.75 to 0.75/9.25. As the relative amount of ^{H}MBIT-linked angiotensin II increased, the relative intensities of $^{H}b_S$ and ^{L}ADR-NH_3 linearly increased. Surprisingly, a peak at 115 Th, which originated from the unexpected fragmentation of unmodified peptides, appeared in all mixing ratios, indicating that 115 Th is not a good quantitation marker. Note that both 4-plex and 8-plex versions of iTRAQ include 115 Th as one of the quantitation signals.[30,31] The peak at 114 Th also derived from unmodified peptides, although its intensity was much weaker than that of 115 Th. When the relative yield of $^{X}b_S$ was not so great, this peak at 114 Th interfered strongly with the $^{L}b_S$ signal. Unlike the $^{X}b_S$ pair, the unexpected quantitation signals ^{X}ADR-NH_3 showed no overlap with chemical noise. The peak at 329 Th, which was ascribed to a_3-NH_3 of unmodified angiotensin II, was shifted by +187 Th after MBIT conjugation. The measured ratios of [114]/[117] from the $^{X}b_S$ pair and [329]/[326] from the ^{X}ADR-NH_3 pair are compared with the expected ratios of premixing in Figure 4b. The data were fit to eq 4, where α denotes the fraction of unexpected chemical noise at 114 or 329 Th.

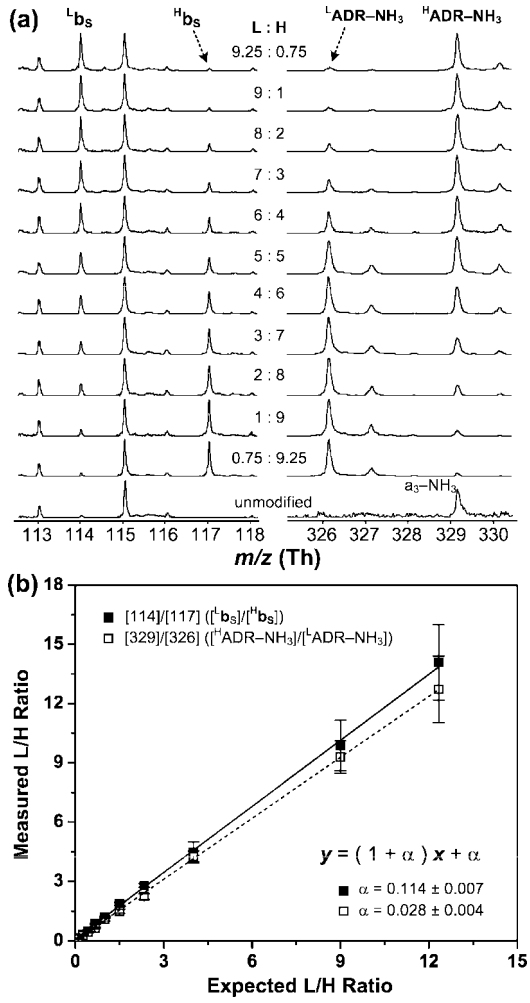

Figure 4. MS/MS spectra of ^{L}MBIT- and ^{H}MBIT-linked angiotensin II premixed in various ratios. (a) The region of quantitation signals ($[^{L}b_S]/[^{H}b_S]$, [114]/[117]) and fortuitous quantitation signals ($[^{H}ADR\text{-}NH_3]/[^{L}ADR\text{-}NH_3]$, [329]/[326]); (b) The plot of measured vs expected L/H ratios (L/H = $[^{L}b_S]/[^{H}b_S]$ or $[^{H}ADR\text{-}NH_3]/[^{L}ADR\text{-}NH_3]$). The fitting function is given in eq 4. Filled squares with a solid line represent $[^{L}b_S]/[^{H}b_S]$, whereas empty squares with a dashed line denote $[^{H}ADR\text{-}NH_3]/[^{L}ADR\text{-}NH_3]$. Each data point is an average of 8 measurements. Error bars stand for the 95% confidence interval.

$$y = (1+\alpha)x + \alpha$$

$$y = \frac{[114]_{\text{measured}}}{[117]_{\text{measured}}};\ x = \frac{[114]_{\text{expected}}}{[117]_{\text{expected}}};$$

$$\alpha = \frac{[\text{chemical noise at}114]_{\text{unexpected}}}{[114]_{\text{expected}} + [117]_{\text{expected}}}$$

or

$$y = \frac{[329]_{\text{measured}}}{[326]_{\text{measured}}};\ x = \frac{[329]_{\text{expected}}}{[326]_{\text{expected}}};$$

$$\alpha = \frac{[\text{chemical noise at}329]_{\text{unexpected}}}{[326]_{\text{expected}} + [329]_{\text{expected}}}$$

The value of α was 0.114 ± 0.007 and 0.028 ± 0.004 for $^{X}b_S$ and ^{X}ADR-NH$_3$ pair, respectively. The linearity of 1.11 and 1.03 was obtained from $^{X}b_S$ and ^{X}ADR-NH$_3$ pair, respectively, over the entire range of premixing ratios from 0.08 to 12. The ^{X}ADR-NH$_3$ pair showed a better agreement with the expected value than does the $^{X}b_S$ pair. The $^{X}b_S$ pair overestimated the expected ratio by 11% due to chemical noise at 114 Th. Despite chemical noise, MBIT worked well in the peptide concentration range of 20−200 fmol with good quantitation linearity, and without losing sequencing information.

Chromatographic Behavior of MBIT-Linked Peptides. The 1:1 mixture of ^{L}MBIT- and ^{H}MBIT-linked tryptic peptides of BSA (~24 pmol) was separated by RPLC followed by MS analysis. Figure 5a shows a two-dimensional intensity plot of LC elution time versus *m/z*. Mascot identified BSA from the peptide mass fingerprint data with the highest MOWSE score (see Table S1 in SI). PEAKS 4.5 correctly identified the sequences of MBIT-linked BSA peptides by de novo sequencing with highest confidence in most cases (see Table S5 in SI). The MS/MS spectra of MBIT-linked BSA peptides are displayed in Figures S2 and S3 (SI). The b_1 ion from MBIT-linked peptides was useful in N-terminal sequencing. Knowing that the peptide was labeled or not prior to de novo sequencing was critical. Fortunately, this information was conveniently provided by the signature ion b_0 in the MS/MS spectra. For example, if the CID spectra of three MBIT-linked peptides (C_1, D_1, G_1) were compared with those of unmodified ones (C_0, D_0, G_0), only the labeled peptides showed the signature ion b_0 at 188 Th (Figure 5b). A pair of quantitation signals, $^{L}b_S$ and $^{H}b_S$, appeared concomitantly with the b_0 peak in the MS/MS spectra. On the contrary, unmodified peptides lacked both the signature ion and quantitation signals. In practice, we searched the database or carried out de novo sequencing under the condition of a fixed number of MBIT modification, when the b_0 ion appeared in the MS/MS spectra.

The effect of MBIT on the chromatographic resolution of peptides was checked by comparing the quantitation signals, $^{L}b_S$ and $^{H}b_S$, as a function of elution time. Because *N*-acetyl-Ala-Ala dipeptide tags were hydrophobic, the elution of MBIT-linked peptides was retarded in RPLC. The retention time increased with increasing number of labeling. The total number of MBIT in each peptide was identical to the total number of primary amines in the target peptide. No hydroxyl groups were labeled because the ester-forming side reaction was reversed with hydroxylamine. Figure 5c shows the variations of the $[^{L}b_S]/[^{H}b_S]$ ratio as a function of LC elution time for three different peptides: C_1 ($MBIT_1$-SEIAHR) at 899.43 Th, D_1 ($MBIT_1$-YLYEIAR) at 1114.50 Th, and G_1 ($MBIT_1$-DAFLGSFLYEYSR) at 1754.71 Th. Although the $[^{L}b_S]/[^{H}b_S]$ ratio fluctuated spot to spot, it remained almost constant over the entire elution time for all mass range, confirming the coelution of ^{L}MBIT- and ^{H}MBIT-linked peptides with no significant $^{1}H/^{2}H$ isotope effect. In comparison with MBIT, ICAT consists of a pair of deuterated and unmodified reagents. The deuterated ICAT is 8 Th heavier than the unmodified ICAT. They are simply not isobaric. The deuterated ICAT shows a pronounced isotope effect due to eight deuterium atoms.[38] On the other hand, MBITs consist of a pair of isobaric reagents (^{L}MBIT and ^{H}MBIT) having both CH_3 and CD_3. The $^{1}H/^{2}H$ isotope effect on LC elution profile is primarily determined by the number of deuterium atoms and the type of alkyl group.[38] Since a pair of MBITs contains the same number of deuterium atoms in the same type of alkyl group, the $^{1}H/^{2}H$ isotope effect is expected to be identical, so the peptides differentially labeled with a pair of MBIT reagents are not easily resolvable in LC separation. The average $[^{L}b_S]/[^{H}b_S]$ ratio slightly

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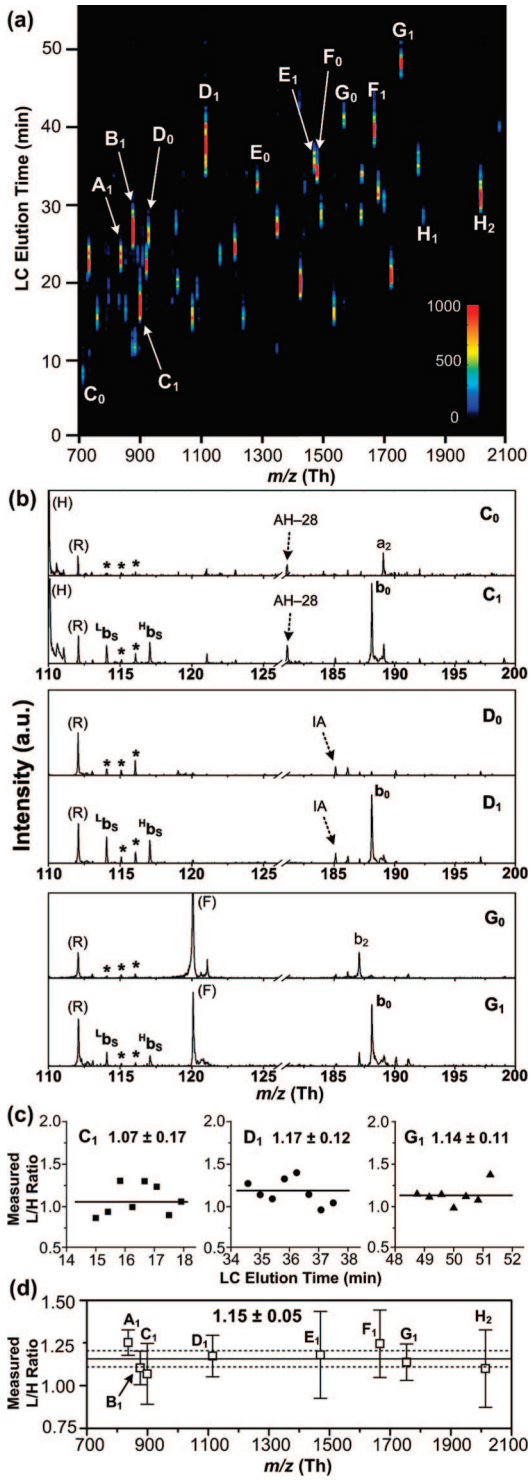

Figure 5. Quantitation of the 1:1 mixture of ^{H}MBIT- and ^{L}MBIT-linked tryptic peptides from BSA. (a) A two-dimensional intensity plot of LC elution time vs *m/z*. The color bar shows the intensity in arbitrary units; (b) the low-mass region of the MS/MS spectra obtained from the 1:1 mixture of ^{H}MBIT- and ^{L}MBIT-linked tryptic peptides (C_1, D_1, G_1) and unmodified peptides (C_0, D_0, G_0) of BSA; C_0 and C_1 (SEIAHR), D_0 and D_1 (YLYEIAR), and G_0 and G_1 (DAFLGSFLYEYSR). Immonium ions are denoted in parentheses; (c) the LC elution profile of three different isotopomeric peptides, C_1, D_1, and G_1; (d) the measured L/H ratio (L/H = $[^{L}b_S]/[^{H}b_S]$). Alphabetical labels represent the peptides subjected to MS/MS; A (IETMR), B (AWSVAR), C (SEIAHR), D (YLYEIAR), E (HPEYAVSVLLR), F (LGEYGFQNALIVR), G (DAFLGSFLYEYSR), and H (KVPQVSTPTLVEVSR). The subscript denotes the number of MBITs attached to each peptide. The reported L/H ratio is the average over values measured from 5 to 11 different spots. The standard error is reported at the 95% confidence interval.

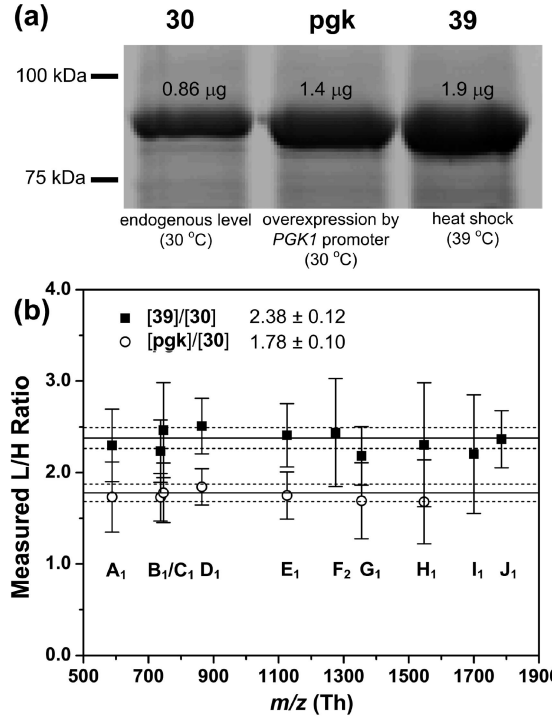

Figure 6. Quantitation of Hsp82p obtained from three different physiological states: (a) SYPRO Ruby-stained Hsp82p expressed at the endogenous level at 30 °C (**30**), overexpressed from the strong *PGK1* promoter at 30 °C (**pgk**), or expressed under heat shock at 39 °C (**39**). Optical imaging of the protein gives the relative ratio of 1:1.62:2.16 ([**30**]/[**pgk**]/[**39**]). (b) MBIT yields the relative ratio of 1:1.78:2.38 ([**30**]/[**pgk**]/[**39**]). Alphabetical labels represent the peptides subjected to MS/MS: A (LLR), B (LVYR), C (GTILR), D (EIFLR), E (LLDAPAAIR), F (TKPLWTR), G (LGVHEDTQNR), H (QLETEPDLFIR), I (GVVDSEDLPLNLSR), and J (SVDELTSLTDYVTR). The subscript denotes the number of MBITs attached to each peptide. The reported L/H ratio is the average over the values obtained from 5 to 11 spots. The standard error is reported at the 95% confidence interval.

varied with peptide, 1.07 ± 0.17 for C_1, 1.17 ± 0.12 for D_1, and 1.14 ± 0.11 for G_1, but was almost identical within error range. Figure 5d depicts the measured $[^{L}b_S]/[^{H}b_S]$ ratio from the fully labeled peptides in the mass range from 700 to 2100. The average measured ratio was 1.15 ± 0.05. As mentioned before, MBIT overestimated the relative amount of $[^{L}b_S]$ due to unknown chemical noise at 114 Th. Although the relative intensities of the quantitation signals greatly depended on the sequence, the $[^{L}b_S]/[^{H}b_S]$ ratio was quite reproducible.

Quantitation and Identification of Hsp82p Peptides. The performance of MBIT for protein quantitation was further tested with the yeast heat-shock protein, Hsp82p, obtained from three different physiological states (**30**, normal growth condition at 30 °C; **pgk**, overexpression condition mediated by the strong *PGK1* promoter; and **39**, heat-shock condition at 39 °C). The absolute amount of Hsp82p on a polyacrylamide gel shown in Figure 6a was determined from the intensity of SYPRO Ruby stain calibrated against known amounts of BSA loaded on the same gel (see SI). The amount of Hsp82p was determined to be 0.86, 1.4, and 1.9 μg for **30**, **pgk**, and **39**, respectively. Those Hsp82p were then in-gel digested, differentially labeled with ^{X}MBIT, and analyzed by LC-MALDI-MS/MS. After coupling with ^{X}MBIT, the total amount of the 1:1 mixture loaded on LC was 0.9 and 1.1 pmol for

^{L}MBIT-**pgk**/^{H}MBIT-**30**, and ^{L}MBIT-**39**/^{H}MBIT-**30**, respectively, assuming complete extraction. Each peptide was eluted over eight spots on average. Thus, ~110−150 fmol of peptides per spot were used for simultaneous quantitation and sequencing. Mascot identified the ATP-dependent molecular chaperones Hsp82p and Hsc82p with highest MOWSE scores from the peptide mass fingerprint data (see SI). Hsp82p and Hsc82p share ~97% sequence identity. PEAKS 4.5 identified 9 out of 10 MBIT-linked peptides from de novo sequencing (see SI). However, F_2 ($MBIT_2$-TKPLWTR) was not correctly assigned due to low S/N ratios for both the precursor and fragment ions. The MS/MS spectra of MBIT-linked Hsp82p peptides are displayed in Figures S4−5 (SI). The relative quantitation results are presented in Figure 6b (see Table S2 in SI for peptide sequences and quantitation ratios). The relative ratio determined by MBIT was 1.78 ± 0.10 and 2.38 ± 0.12 for [**pgk**]/[**30**] and [**39**]/[**30**], respectively. The **30**:**pgk**:**39** ratio of 1:1.78:2.38 by MBIT using nanogram-scale samples agreed well with that of 1:1.6:2.2 by optical imaging of microgram-scale samples. Again, the ^{L}MBIT signal was overestimated by ~10% due to chemical noise at 114 Th.

CONCLUSION

The isobaric ^{1}H/^{2}H isotope label in MBIT shows no resolvable isotope effects on the chromatographic elution of differentially tagged target peptides from BSA. The strong b_0 ion, which is the signature of MBIT conjugation, allows de novo sequencing of target peptides with high confidence. MBIT provides discrete quantitation signals in a quiet region of the MS/MS spectra at intensity levels comparable to the peptide sequence ions and results in accurate protein quantitation and identification. The chemical noise appearing in a supposedly quiet region of the MS/MS spectra leads to overestimation of the quantitation signal; however, the signal mass window of *N*-acetyl-Ala-Ala dipeptide tags can be tuned to other MS/MS spectral regions to avoid unexpected chemical noise by replacing N-terminal alanine with other amino acid residues. The outstanding features of MBIT include low-cost ^{1}H/^{2}H isotopes, the possibility of shifting the signal mass window, and perhaps the convenient laboratory-scale synthesis of dipeptide tags.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures for solution-phase syntheses of N-acetylated dipeptides, preparation of yeast heat-shock protein, Hsp82p, in three different physiological states, detailed sequencing and quantitation results, and the MS/MS spectra from bovine serum albumin and Hsp82p. This material is available free of charge via the Internet at http://pubs.acs.org.

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