Iodoacetyl Tandem Mass Tags for Cysteine Peptide Modification, Enrichment and Quantitation

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Overview

Purpose: To develop an iodoacetyl Tandem Mass Tag (iodoTMT) reagent for irreversible cysteine peptide labeling, enrichment and multiplexed quantitation.

Methods: Reduced sulfhydryls of protein cysteines were labeled with iodoTMTzero and/or iodoTMTsixplex reagents. Labeled peptides were enriched using an immobilized anti-TMT antibody resin before mass spectrometry (MS) analysis.

Results: We developed an iodoTMT reagent set to perform duplex isotopic or sixplex isobaric mass spectrometry (MS) quantitation of cysteine-containing peptides. IodoTMT reagents showed efficient and specific labeling of peptide cysteine residues with reactivity similar to iodoacetamide. Using an anti-TMT antibody, we characterized the immunoaffinity enrichment of peptides labeled with iodoTMT reagents from complex protein cell lysates and for detection of S-nitrosylated cysteines.

Introduction

Thermo Scientific Tandem Mass Tag (TMT) Reagents enable concurrent identification and multiplexed quantitation of proteins in different samples using tandem mass spectrometry.[1] Previously, we described a cysteine-reactive, isobaric Tandem Mass Tag (cysTMT™) reagent that utilized a dithiopyridine reactive group to selectively label cysteine sulfhydryls. Although this labeling chemistry is highly specific and efficient, it results in a reversible di-sulfide linkage between the peptide and isobaric tag. Here, we report the development of an irreversible, cysteine-reactive TMT™ reagent containing an iodoacetyl reactive group (iodoTMT™). Due to the irreversible labeling of the iodoTMT reagent, it can be used for quantifying cysteine modifications such as S-nitrosylation, oxidation and disulfide bridges.

Methods

Sample Preparation

Preparation of iodoTMT-labeled proteins: Proteins and/or cell lysates were solubilized at 2 mg/mL in 50 mM HEPES pH 8.0, 0.1% SDS and reduced with 5 mM TCEP for 1 hour at 50˚C. Reduced proteins were labeled with 5-10 mM iodoTMT reagent (~10 molar excess) for 1 hr at 37 ˚C protected from light. Excess iodoTMT reagent was removed by acetone precipitation of samples at -20 ˚C for 4-20 hrs. Proteins were enzymatically digested at 37 ˚C for 4 hrs and desalted before liquid chromatography (LC)-MS/MS analysis or enrichment.

Selective labeling of S-nitrosylated proteins: Proteins and cell lysates were solubilized at 2 mg/mL in modified HENS buffer (100 mM HEPES pH 8.0, 1 mM EDTA, 0.1 mM neocuporine, 1% SDS). Free sulfhydryls were blocked with methyl methane thiosufate (MMTS) for 20 min at room temperature and desalted to remove excess blocking reagent. S-nitrosylated cysteine sulfhydryls were selectively labeled using 0.4 mM iodoTMT reagent in the presence of 20 mM sodium ascorbate. Excess iodoTMT reagent was removed by acetone precipitation of samples at -20˚C for 4-20 hrs. Un-labeled cysteines were reduced and alkylated with 20 mM iodoacetamide. Proteins were enzymatically digested at 37 ˚C for 4 hrs and desalted before LC-MS/MS analysis or enrichment.

Enrichment of iodoTMT-labeled peptides: Labeled peptides (25–100 µg) were resuspended in TBS at 0.5 µg/µL and incubated with an immobilized anti-TMT antibody resin (20–100 µL) overnight with end-over-end shaking at 4 ˚C. After collection of the unbound sample, the resin was washed 4X with 4 M Urea/TBS, 4X with TBS, and 4X with water. Peptides were eluted 3X with 50% acetonitrile/0.4% TFA, frozen, and then dried under vacuum before LC-MS/MS analysis.

LC-MS/MS Analysis

A nanoflow high-pressure liquid chromatography system with a Thermo Scientific PepMap C18 column (75 µm ID x 20 cm) was used to separate peptides using a 5%-40% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min over 60 min. A Thermo Scientific LTQ Orbitrap XL ETD linear ion trap mass spectrometer was used to detect peptides using a top-3 CID, -3 HCD experiment for peptide identification and reporter ion quantitation.

Data Analysis

MS spectra were searched using Thermo Scientific Proteome Discoverer software v1.3 with SEQUEST® and Mascot™ against a mammalian Swiss-Prot® database. Modifications included carbamidomethyl, iodoTMTzero™ (324.42 Da) and iodoTMTsixplex™ reagent (329.38 Da) for cysteines and oxidation for methionine.

FIGURE 1. iodoTMT reagents and labeling reaction. A) Mechanism of iodoTMT reagent reaction with cysteine-containing proteins or peptides. B) Structure of iodoTMTsixplex reagents for cysteine labeling, enrichment, and isobaric MS quantitation.

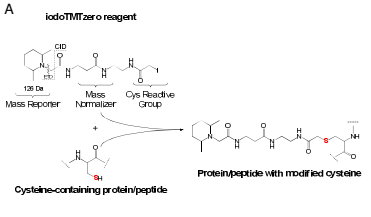


FIGURE 2. Schematic of iodoTMTsixplex reagent workflow. Six different sample conditions can be prepared for iodoTMT reagent labeling. Labeled proteins are combined before iodoTMT peptide enrichment using immobilized anti-TMT antibody resin and subsequent LC-MS/MS analysis of isobaric reporter ions.

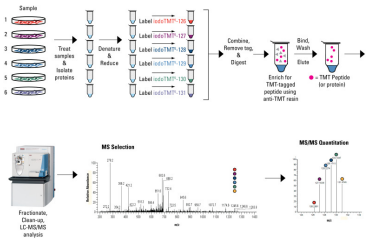


FIGURE 3. iodoTMT reagent labeling specificity and efficiency. A) Reduced BSA (100 µg) was labeled with increasing concentrations of iodoTMT reagent. IodoTMT reagent labeling efficiency was determined by peptide signal (XIC) of modified of cysteines compared to total cysteine-containing peptides signal. B) Reduced BSA (100 µg) was labeled with 10 mM iodoTMT reagent. IodoTMT reagent labeling specificity was determined by comparing modified peptide signal to total peptide signal for different amino acids. Labeling specificity and efficiency were also assessed by peptide spectral counting which gave similar results (data not shown).

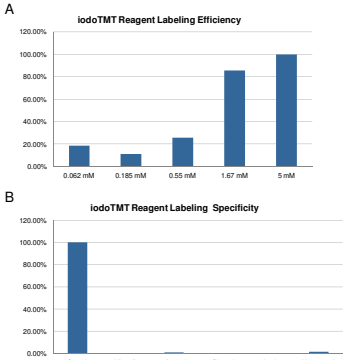


FIGURE 4. iodoTMTsixplex reagent relative quantitation of BSA peptides. Reduced BSA (100 µg) was labeled with 10 mM iodoTMTsixplex reagent and combined in fixed ratios (126:127:128:129:130:131 = 10:3:1:1:3:10 & 1:10:1:10:1:10) before protein digestion. Graph of peptide relative quantitation for all quantified peptides. Signals are normalized to the 126 quantitation channel.

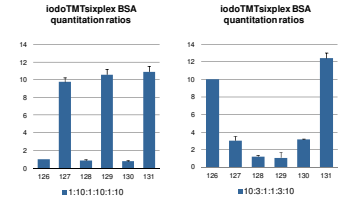


FIGURE 5. Anti-TMT enrichment of iodoTMT-labeled peptides. A) Percent of iodoTMT-labeled peptide modifications identified before and after enrichment. B) Comparison of unique proteins identified from unique peptides from A549 cell lysates before and after anti-TMT antibody resin enrichment.

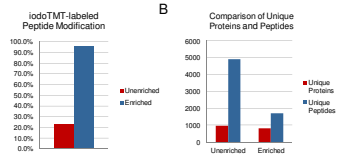
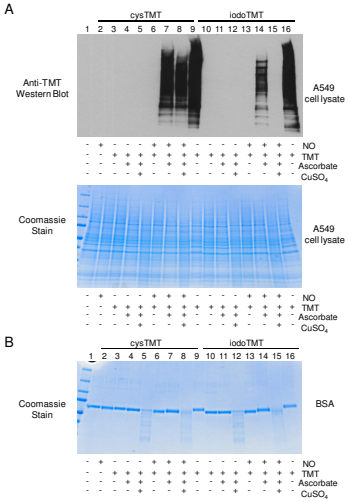


FIGURE 6. cysTMT and iodoTMT reagent labeling of S-nitrosylated proteins. A549 cell lysates (A) and BSA (B) were blocked with MMTS (lanes 1-8, 10-15) or untreated (lanes 9, 16) before 500 mM nitro-glutathione (NO) treatment and TMT reagent labeling in the presence or absence of ascorbate and copper sulfate ($CuSO_4$). Proteins were separated by SDS-PAGE and analyzed by anti-TMT antibody Western blotting or Coomassie stain.



Results

We have developed and used an iodoacetyl TMT reagent (iodoTMT) to irreversibly label sulfhydryls of cysteine-containing peptides for multiplex quantitation by LC-MS (Figure 1). Compared to the cysTMT reagent workflow, the iodoTMT reagent workflow is simpler since reducing agents are not removed from protein samples before labeling (Figure 2). The iodoTMT reagents showed efficient and specific labeling of peptide cysteine residues with reactivity similar to iodoacetamide (Figure 3A & 3B). IodoTMT reagents were also used for sixplex isobaric quantitation of cysteine-containing peptides (Figure 4). We also characterized an anti-TMT antibody developed against the reporter region of the TMT reagent for immuno-enrichment of iodoTMT-labeled peptides (Figure 5) and Western blot detection of iodoTMT-labeled proteins (Figure 6A).

We used the iodoTMT reagent as a probe for labeling S-nitrosylated cysteines in a modified S-nitro switch assay (Figure 6). IodoTMT reagents successfully labeled S-nitrosylated cysteines after selective reduction using ascorbate; however, labeling efficiency was less than cysTMT reagents. This result is consistent with different efficiencies of the sulfhyrdyl-reactive groups (dithiopyridine vs. iodoacetyl) of each reagent. In addition, we discovered that addition of 1mM copper sulfate to the switch reaction buffer inhibited iodoTMT reagent labeling but not cysTMT reagent labeling. Addition of copper sulfate is thought to facilitate S-NO bond reduction during the labeling reaction; however, in the presence of ascorbate, $Cu2+$ is readily reduced to $Cu1+$, which can generate free radicals.[2] The free radicals generated resulted in protein degradation (Figure 6B: lanes 5, 8, 12 and 15) and possible loss of iodine from the iodoacetyl reactive group.

Overall, using the combination of iodoTMT labeling with anti-TMT enrichment has several advantages over previously described cysteine-reactive workflows[3,4] for labeling, enrichment and quantitation of cysteine-containing peptides and cysteine modifications such as S-nitrosylation.

Conclusions

- Iodoacetyl Tandem Mass Tags (iodoTMT) are novel reagents for specific irreversible labeling of cysteine residues pre- or post-digestion.
- iodoTMT reagents can be used as either isotopic pairs or as an isobaric set for MS- or MS/MS-based multiplexed quantitation.
- An antibody to the TMT reagent reporter region allows specific detection, capture, and enrichment of iodoTMT-labeled proteins and peptides.
- iodoTMT reagents can be used for detection of cysteine modifications such as S-nitrosylation.

References

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