Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS

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A novel MS/MS-based analysis strategy using isotopomer labels, referred to as “tandem mass tags” (TMTs), for the accurate quantification of peptides and proteins is described. The new tags are designed to ensure that identical peptides labeled with different TMTs exactly comigrate in all separations. The tags require novel methods of quantification analysis using tandem mass spectrometry. The new tags and analysis methods allow peptides from different samples to be identified by their relative abundance with greater ease and accuracy than other methods. The new TMTs permit simultaneous determination of both the identity and relative abundances of peptide pairs using a collision induced dissociation (CID)-based analysis method. Relative abundance measurements made in the MS/MS mode using the new tags are accurate and sensitive. Compared to MS-mode measurements, a very high signal-to-noise ratio is achieved with MS/MS based detection. The new tags should be applicable to a wide variety of peptide isolation methods.

The mainstay of protein expression analysis is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for the separation of complex protein mixtures, followed by identification of those separated components by mass spectrometry using (M S)-based peptide mass fingerprinting techniques¹–³ or tandem mass spectrometry (M S/M S)-based peptide sequencing techniques.⁴ While it is an effective tool, 2D-PAGE/M S is laborious and difficult to automate. More significantly, experiments show poor reproducibility and limited dynamic range,³ and the proteins, after electrophoretic separation, are not immediately compatible with mass spectrometry. As a result of these limitations, it is difficult to perform quantitative comparisons between samples on different gels. Attempts, with the 2D-DIGE (difference gel electrophoresis) method, for example, to overcome this deficiency by running multiple fluorescently labeled samples on the same gel⁶ have improved this, but at the cost of increasing the complexity of the gels; the same protein labeled with different fluorescent dyes does not comigrate with itself and multiple labeling products are produced for each protein. In addition, 2D-PAGE lacks functional range, because it poorly represents specific classes of proteins, such as very large or very small proteins, extremely acidic or basic proteins, and hydrophobic proteins.⁷–¹⁰ Despite the difficulties inherent in 2D-PAGE, this technique is being used for the analysis of whole proteomes,¹¹ but a method for quantitative global protein expression analysis that avoids the shortcomings of 2D-PAGE is greatly sought after.

Dispensing with 2D-PAGE has, fortunately, become possible with the advent of advanced instrumentation for in-line liquid chromatography electrospray ionization mass spectrometry. However, to date, M S- and M S/M S-based protein identification has generally been restricted to smaller peptides, and as a consequence, novel protein analysis techniques compatible with the limitations of currently available instrumentation are required to allow proteins in complex mixtures to be identified. One approach is to sample peptides from the proteins in a complex mixture in such a way that the peptide sample accurately represents the constituents of the original mixture. A number of such peptide sampling techniques have been published recently, all based on selectively reacting specific functional groups in proteins, such as cysteine residues in the isotope coded affinity tags (ICAT) procedure³ and phosphate groups.¹³–¹⁵ The ICAT procedure, in particular, isolates a small number of peptides from each protein, thus representing almost all proteins with at least one peptide.

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Multidimensional protein identification technology (MudPIT) is another related approach to automated and integrated whole proteome expression analysis exploiting mass spectrometry and its ability to identify peptides. In this approach, whole proteomes or fractions are digested and separated using a dual-phase liquid chromatography separation process followed by in-line analysis by electrospray ionization mass spectrometry. This approach differs from other approaches in that no sampling of peptides is performed. MudPIT relies on high-resolution multidimensional chromatography to resolve all the peptide components of a complex mixture. This technique has the advantage of complete representation of proteins in the original mixture, but at the cost of high redundancy, which results in high sample complexity and limits throughput.

In addition, labeling techniques to allow relative abundances of proteins in different samples to be determined by mass spectrometry are also needed. A number of such labeling techniques, notably ICAT, have also been published recently. These techniques are all based on standard procedures for quantification by mass spectrometry in which an analyte is quantified by comparison with an introduced isotopomer of the analyte that acts as an internal standard. Typically, the internal standard is differentiated from the analyte by incorporation of deuterium, but $^{13}$C and $^{15}$N are also used. It is assumed in these techniques that the relative signal intensities of analyte and standard are directly proportional to their relative concentrations, and since the quantity of the standard is known, this means that the quantity of the analyte may be determined from the ratios of their peak intensities. These standard methods of quantification have been adapted for the purpose of protein expression analysis by the introduction of "heavy" and "light" isotope tags that are used to label peptides from corresponding proteins in pairs of samples under comparison. The isotope taggging procedures produce pairs of labeled peptide isotopomers that are mass-differentiated and can act as mutual internal standards. The ICAT procedure, in particular, combines a method of sampling a complex protein mixture with a method of determining relative abundances by using pairs of isotopomer-differentiated cysteine-reactive affinity tags and may be regarded as the most advanced alternative to 2D-PAGE for whole proteome analysis to date. The MudPIT procedure is also compatible with the isotope labeling techniques that have been developed recently.

The currently published "heavy/light" isotope labeling techniques fall into two general categories: in vivo and in vitro labeling. The former approach requires the introduction of isotopically labeled nutrients that will be incorporated into proteins into the growth medium of a living organism. Clearly, this approach is limited to the analysis of organisms whose growth media can be controlled. Moreover, in this approach, the precise amount of incorporation of heavy and light isotopes cannot really be predicted. In vivo approaches are typified by the complex spectra of the mass modified peptides. While these patterns can be useful for the identification of some peptides, the complexities and limitations of this approach make it laborious and, thus, unattractive. In addition, the organism must be grown on minimal media, which has metabolic implications for the experimental organism; i.e., the protein expression patterns on minimal media will be a specific response to the medium, and these patterns will bias the response behavior of the model organism. Consequently, minimal media cannot permit the full range of protein expression to be explored. In contrast, in vitro labeling allows labeling of virtually any protein sample, it allows control over the degree of isotope labeling of each peptide, and the conditions under which the sample proteins are produced do not affect the labeling. In vitro labeling procedures are, thus, more appealing than in vivo procedures as long as robust labeling protocols can be developed.

Despite some successes with the isotope labeling techniques discussed above, all of the published approaches that depend on deuterium labeling suffer from a number of problems. The most significant is that, although the mass modification that results from isotope labeling is small, there is still a detectable shift in the mobility of deuterium differentiated peptides in size/mass-dependent separation procedures, such as reversed-phase high performance liquid chromatography (HPLC). Typically, the heavy peptide migrates more rapidly than the light peptide, often eluting as a separate fraction. This means that in order to accurately determine the quantities of each heavy/light peptide pair, it is necessary to allow both peptides to completely elute to allow integration of the ion current for each peptide. As a consequence, the determination of the peptide identity by sequencing using MS/MS techniques cannot easily be reconciled with the need for accurate abundance measurements. Moreover, since each peptide pair does not coelute, the isotope-tagged peptides do not act as true standards for each other, reducing confidence in the accuracy of relative quantification. In particular, it is possible that one peptide of a pair, but not the other, may coelute with another peptide that suppresses its ionization.

Another problem for quantitative analysis of peptides labeled with conventional isotope labels using LC/MS arises from the different charge states of the labeled peptides that are produced by electrospray ionization. This means that the mass difference between corresponding peptide tags labeled with conventional isotope tags varies with the charge state of the peptide. Similarly, the number of tags incorporated into a peptide will alter the mass difference between each corresponding peptide from a paired sample.

Improved labels can solve some of the problems with the above techniques, for example, the novel $^{13}$C reagent for ICAT described by ABI avoids the retention time shift.

This paper describes a novel class of reagents termed tandem mass spectrometry tags (TMTs), where the term "tandem" refers to...
to the use of MS/MS for the analysis of these tags. The TMTs overcome a number of the above issues for quantitative proteomic analyses while offering other advantages, too.

**Tandem Mass Tag Concept and Design.** Two pairs of TMT reagents are shown in Figure 1. The reagents are peptides comprising one “tag” amino acid linked to a sensitization group, which is a guanidino functionality; one “mass normalization” amino acid; and in the second pair of tags, a cleavage enhancement group, which is proline in this case. These tags are designed so that on analysis by collision-induced dissociation (CID), the TMT fragment is released to give rise to an ion with a specific mass-to-charge ratio. The N terminal methionine and guanidino group comprises the TMT fragment and is distinguished from the second methionine, which comprises the mass normalization group. Each tag can also bear a reactive functionality. In the figure, the reactive functionality is not specified, but could be an N-hydroxysuccinimide ester, for example, which allows for the specific labeling of amino groups. Clearly, this reactive functionality can be easily varied to allow different biological nucleophiles to be labeled. In addition, the tag design can be readily modified to accommodate an affinity ligand, such as biotin. Furthermore, it should be clear that more than two tags can be generated, allowing for comparison of additional samples or for the introduction of labeled standards.

The TMT approach is similar in principle to other peptide isotope labeling techniques and enjoys the same features as these other approaches, with some additional advantages. Pairs of TMT-tagged peptides are chemically identical, like the isotope tags used in other methods, but unlike other isotope tags, the TMTs also have the same overall mass and comigrate in chromatographic separations and, thus, will act as more precise reciprocal internal standards, which leads to more accurate quantification. In addition, the TMT-labeled species means that the MS signal for each peptide pair is not split into two peaks, as in conventional isotope labeling, improving sensitivity in the MS mode. As in other approaches, a short sequence of contiguous amino acids from a large protein is often sufficient to uniquely identify the protein, and the TMT reagents are applicable to any peptide isolation procedure that other in vitro labeling techniques can be used for. The novel TMT strategy relies on a CID-based technique for quantification of tagged analytes, and this feature improves signal-to-noise ratios by operation of the MS instrumentation entirely in the MS/MS mode. This allows untagged material to be ignored, greatly improving data quality.

**EXPERIMENTAL SECTION**

**Syntheses of TMT-Labeled Peptides.** Peptides were synthesized using conventional automated Fmoc synthesizer techniques (both starting from commercially available Fmoc-Gly-Trt-PS resin from Rapp Polymere, Germany). Deuterated methionine (Met-d3) is available from ISOTEC Inc, Miamisburg, Ohio. An Fmoc-Met-d3 reagent for use in a peptide synthesizer must, however, be synthesized manually from the unprotected deuterated methionine. The guanidino sensitization enhancement group was synthesized as an N-hydroxysuccinimide ester (NHS-ester) and added to deprotected α-amino groups of synthetic peptides by conventional methods during automated peptide synthesis.

After cleavage from the solid-phase synthesis resins, the products were purified by HPLC. The identity of each of the peptides was confirmed by mass spectrometry.

**Synthesis of the Guanidino-NHS Ester.** The synthesis of the guanidino-active ester linker (6-[bis(tert-butyloxycarbonyl)-guanidino]hexanoic acid N-hydroxysuccinimide ester) was as follows:

1. **Synthesis of Aminooiminomethane Sulfonic Acid.** A 50-mL portion of acetic anhydride and 2 drops of concentrated sulfuric acid were added to 45 g (397 mmol) of 30%aqueous ice-cooled hydrogen peroxide. After 30 min, 100 mL (1157 mmol) of acetic anhydride was added to the solution at 10–12 °C once again. The reaction mixture was stirred overnight and reached room temperature (RT) in that time. After adding 150 mL of methanol, the solution made from 10 g (131 mmol) thiourea in 500 mL methanol was dropped slowly into the reaction at 15–20 °C. The reaction was stirred at RT for 48 h. After filtration, the solution was condensed to 60 mL. The obtained product was filtered and washed with ethanol and purified by crystallization from acetic acid (~1 L). Yield: 37%

2. **Synthesis of 6-Guanidinohexanoic Acid.** A 6.5-g (50 mmol) portion of 6-aminohexanoic acid and 6.9 g (50 mmol) sodium carbonate were dissolved in 50 mL of water. A 6.2-g (50 mmol) portion of aminooiminomethane sulfonic acid was added to the solution with stirring. After 20 h, the product was filtered and washed with acetic acid, methanol, and then ether. Yield: 76%

3. **Synthesis of 6-[Bis(tert-butyloxycarbonyl)guanidino]hexanoic Acid N-Hydroxysuccinimide Ester.** A 9.5-g (55 mmol) portion of 6-guanidinohexanoic acid and 55 g (270 mmol) of N,O-bis-trimethylsilyl acetamide were stirred in 100 mL of dichloromethane and heated under refluxing until a clear solution was obtained (the reaction was left for ~10 h). A 46-g (210 mmol) portion of di-tert-butyl pyrocatechol was added to the solution at RT, and the reaction mixture was heated under refluxing for 3 h after having been stirred at RT for 18 h (overnight). The solution was then cooled to RT and washed with a 10% citric acid solution and a sodium chloride solution. After evaporation of the solvent, the pyrocatechol was distilled at 80–90 °C under vacuum. The viscous liquid obtained (30 g) was dissolved in 100 mL dichloromethane with 8.6 g (75 mmol) N-hydroxysuccinimide. A 15.5-g (75 mmol) portion of dicyclohexylcarbodiimide (DCC) was added in portions to the reaction mixture with stirring at RT. After 17 h, the urea was removed by filtration. The solution was washed with a 10% citric acid solution and a sodium chloride solution. After evaporation of the solvent, the pyrocatechol was distilled at 80–90 °C under vacuum. The viscous liquid obtained (30 g) was dissolved in 100 mL dichloromethane with 8.6 g (75 mmol) N-hydroxysuccinimide. A 15.5-g (75 mmol) portion of dicyclohexylcarbodiimide (DCC) was added in portions to the reaction mixture with stirring at RT. After 17 h, the urea was removed by filtration. The solution was washed with a 10% citric acid solution, and after removing the solvent, the product was purified by chromatography (silica gel; solvent, dichloromethane/ethyl acetate). The product was then crystallized from diisopropyl ether. Yield: 19% Rf: 0.77 (dichloromethane/ethyl acetate: 3/1). fp: 106–109 °C

**MS/MS Analysis of TMT-Labeled Peptides.** MS and MS/MS analyses were performed on a QTF2 mass spectrometer (Micromass, M anchester, U.K.). HPLC analysis was performed...

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The structures of two versions of the TMT markers are shown in parts 1a and 1b. The tags are modular, comprising different functional components that correspond to individual synthetic components in the automated synthesis of these reagents. Each tag comprises a sensitization group and a mass differentiated group that together comprise the TMT fragment that is actually detected. The TMT fragment is linked to a mass-normalization group that ensures that each tag in a pair of tags shares the same overall mass and atomic composition. The first and second generation tags are distinguished by the presence of an additional fragmentation-enhancing group, proline, in the second generation tag. The tags will additionally comprise a reactive functionality (R) to enable the tag to be coupled to any peptide, but in the present experiments, R is one of a number of peptide sequences. The proposed TMT fragment that results from the markers, based on current theories on protonation dependent mechanisms of backbone fragmentation, is shown in part 1c.

Figure 1a: First Generation TMT

Figure 1b: Second Generation TMT

Figure 1c: Proposed Structure of the TMT Fragment

Figure 1. The structures of two versions of the TMT markers are shown in parts 1a and 1b. The tags are modular, comprising different functional components that correspond to individual synthetic components in the automated synthesis of these reagents. Each tag comprises a sensitization group and a mass differentiated group that together comprise the TMT fragment that is actually detected. The TMT fragment is linked to a mass-normalization group that ensures that each tag in a pair of tags shares the same overall mass and atomic composition. The first and second generation tags are distinguished by the presence of an additional fragmentation-enhancing group, proline, in the second generation tag. The tags will additionally comprise a reactive functionality (R) to enable the tag to be coupled to any peptide, but in the present experiments, R is one of a number of peptide sequences. The proposed TMT fragment that results from the markers, based on current theories on protonation dependent mechanisms of backbone fragmentation, is shown in part 1c.
with a CAP-LC HPLC system (Waters Corporation, Milford, MA) (column, PepMap C18 HPLC column from Dionex with a 75μm i.d. and a length of 150 mm; solvents, 95%water to 95%acetonitrile, both with 0.2%formic acid)

Ion abundance ratios were determined by summation and smoothing of spectra for each peptide, as it was ionized in the electrospray source, followed by determination of peak intensities of the TMT fragments. Corrections were applied if TMT fragment peaks overlapped with isotope peaks from other ions. As long as the parent peak of a non-TMT fragment ion does not directly overlap with the TMT fragment peak, it is possible to correct for isotope peaks that overlap with the TMT fragment by determining the intensities of isotope peaks as a portion of the monoisotopic peak and subtracting this from the TMT fragment peak. These can be calculated for all of the possible atomic compositions of the monoisotopic peaks of ions that are 1 or 2 m/z units from the TMT fragment to determine an average composition, assuming the overlapping ions are peptides.29 Thus, in our experiments, an ion at 289 has a M + 1 isotope peak that overlaps with the 290 tag fragment m/z peak. The average intensity of this sort of peak is ~15% of the 289 peak intensity. Thus, 15% of the 289 peak intensity can be subtracted from the 290 peak to obtain the correct TMT fragment intensity.

RESULTS AND DISCUSSION

In the following examples, peptides have been synthesized as if they have been completely labeled on the α-amino group with the above tags; i.e., the tag was preincorporated during the synthesis to test the performance of the tags independently of the labeling reactions (see Table 1). The tagged peptides were analyzed by ESI-M S/M S and LC/ESI-M S/M S.

Comparison of First and Second Generation TMT Tags.

To demonstrate the advantages of a tag designed with a fragmentation-enhancing group, two different TMT designs were explored. The tags differ by the inclusion of proline in the second generation tags (Figure 1). Proline is known to enhance cleavage of the amide bond on its N-terminal side.4

Initial experiments on the fragmentation of the first generation TMT in the QTOF instrument showed that the intensity of the desired TMT fragments was very dependent on the amino acid sequence of the peptide, and at low collision energies (around 30 V), the TMT fragments did not accurately reflect the abundances of the tagged peptides. As shown in Figure 1c, the expected TMT fragments have an m/z of 287 or 290, but in the first generation tags, a second more intense pair of ions with mass-to-charge ratios of 270 or 273 is observed. These fragments are thought to result from the loss of ammonia from the expected tag fragments. At lower collision energies, the intensities of these two TMT fragment ions varied with the sequence of the attached peptide, but at higher CID energies (70 V), the 270/273 fragments are observed almost exclusively. At these higher collision energies, the 270/273 TMT fragments accurately reflect the abundances of the peptide pairs. This means that to get consistent behavior from the first generation tag, M S/ M S analysis had to take place at high collision energies.

In the QTOF instrument, however, at higher energies of collision, the series of b or y ion fragments that provide sequence information are further fragmented to give smaller species so that almost no sequence information can be obtained from the peptide (Figure 2c). As a result of the need for high-energy CID to guarantee the release of the TMT fragments and to obtain accurate quantification, the first generation TMT units cannot be used for identification of the peptide in the same scan; a second lower energy scan to determine sequence information would be required. This will also be true of other serial M S/ M S instruments. Although ion excitation is more selective in ion traps, it is somewhat limited in its use with TMTs, because it is not possible to detect small CID fragmentation products of larger precursors with this type of instrument. In addition, the benefit of consecutive fragmentation to produce the TMT fragment is lost in the ion trap.

These results lead to the development of a second generation TMT, which has a proline residue in the TMT unit to enhance the fragmentation.13 To demonstrate the effect of the proline, a 50:50 mixture of a peptide labeled with the first and second generation tags, respectively, was analyzed by M S/M S. The two resultant peptides (Figure 2) had ions corresponding to the [M + 3H]3+ species at mass-to-charge ratios of ~897 and 929 for the first and second generation tags, respectively. A collision profile for both peptide species was recorded automatically in one analysis in the QTOF, with the quadrupole set to alternatively select ions at m/z 897 and 929, with stepwise increases of the collision energy. This procedure ensured the same experimental conditions for both precursors.

At collision energies of 20 V or less, no fragmentation was observed for either type of TMT. At a collision energy of 35 V-40 V, it is possible to see the expected TMT fragment ions at m/z of 290 in the CID spectrum for the peptide with the second generation tag (Figure 2c), but no fragment ions m/z of 273 can

Table 1. Synthetic TMT-Labeled Peptide Pairs Used for MS and MS/MS Analysis

<table>
<thead>
<tr>
<th>Peptide sequences</th>
<th>generation 1</th>
<th>generation 2</th>
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<tr>
<td></td>
<td>M mono</td>
<td>ion at m/z (z)</td>
</tr>
<tr>
<td>1A TMT2-GVATVSLPR</td>
<td>1319.7</td>
<td>660.9 (2+)</td>
</tr>
<tr>
<td>1B TMT2-GVATVSLPR</td>
<td>1319.7</td>
<td>660.9 (2+)</td>
</tr>
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<td>897.1 (3+)</td>
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<tr>
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<td>897.1 (3+)</td>
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<tr>
<td>3A TMT2-GNKPGVYTK</td>
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<td>3B TMT2-GNKPGVYTK</td>
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<td>462.2 (3+)</td>
</tr>
<tr>
<td>4A TMT2-GDPAALKRANTEAARRSRARKLQRMKGGGC</td>
<td>3874.6</td>
<td>969.7 (4+)</td>
</tr>
<tr>
<td>4B TMT2-GDPAALKRANTEAARRSRARKLQRMKGGGC</td>
<td>3874.6</td>
<td>969.7 (4+)</td>
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</table>

be seen in the spectrum for the peptide with the first generation tag at the same energy (Figure 2c). The TMT fragment for the peptide containing the first generation TMT is not detected with optimal sensitivity until a collision energy of 70 V is used (Figure 2c). Comparison of the CID spectra from peptides labeled with TMTs containing proline with peptides labeled with TMTs without proline shows clearly that the introduction of the proline residue as a fragmentation enhancer leads to fragmentation in favor of the expected TMT tag fragment without resorting to very high collision energies. In addition, the identification of the peptide via its b- and y-series ions can also be performed at these lower collision energies. Smaller peptides labeled with the first generation TMT gave rise to the TMT fragment at lower energies, but higher collision energies were required to release the TMT fragment from larger peptides. The size dependence of the peptide on the energy needed to release the TMT fragment was much smaller for the second generation TMT.

It is worth noting that the charge state of the TMT-labeled peptide selected for MS/MS does not affect the appearance of the TMT fragments in the CID spectra of the labeled peptides. The results in Figure 2 were obtained from [M + 3H]^3+ ions. Sequence and tag abundance data have also been obtained from 4+ and 5+ ions (not shown). This is advantageous, because it means that scanning of the spectrum can take place without complex adjustments of the scanning software to compensate for the charge state of each peptide. In other isotope tagging procedures, such as ICAT, the charge state alters the mass difference between each tagged ion pair such that for doubly charged ions, the mass difference is halved; for triply charged ions, the mass difference is one-third of that for the singly charged
ions; etc. Software to scan for peptide pairs using conventional isotope labeling techniques, such as ICAT, must therefore compensate for these sorts of problems by allowing for the different possible mass differences or by ignoring certain classes of ions, which either increases the chance of erroneous identification of peptide pairs or misses out on potential ion pairs that could offer useful information.

The improved behavior of the second generation TMT can be seen in Figure 2b (see also 6c), which shows a typical CID spectrum of a peptide labeled with these tags. The TMT fragments revealing the abundance ratios are easily seen at the expected m/z values of 287 and 290. In addition, it is possible to see both b-series and y-series ions, allowing the sequence of the peptide to be determined.

ESI-MS/MS analyses of mixtures of the peptides shown in Table 1 were carried out to assess the ability of the two types of tag to determine the relative abundances of the mixtures of peptide pairs labeled with the tags. The peptide mixtures with the expected and measured abundance ratios for both the first and second generation tags are shown in Figure 3. It can be seen that both generations of TMT provide accurate representation of abundance ratios of the peptides in the mixtures and that the tags show linear behavior over the entire range of peptide ratios tested. In addition, the lower collision energy needed for the second generation tag allows simultaneous sequence determination.

Demonstration of Identical Chromatographic Behavior of TMT Tags in LC/MS. A mixture of pairs of synthetic second generation TMT-labeled peptides was prepared at a ratio of 60:40. The sequences, theoretical monoisotopic masses, and the observed ion mass-to-charge ratios are shown in Table 1. The peptides were loaded independently onto a C-18 reversed-phase HPLC column and chromatographed. The purpose of this experiment was to demonstrate the exact coelution of corresponding pairs of peptides with different TMT tags without any other complications. The expected ratios of the peptide pairs were observed by MS/MS and found to be consistent over the entire elution time for each peptide pair.

Figure 4 shows the elution profiles of two example peptides monitored at both of the mass-to-charge ratios of the b2 ions from the TMT fragments. It can be clearly seen that the peptide pairs elute as a single fraction. In MS/MS mode, monitoring of the tag fragment ions produces virtually identical results in each case. For each peptide pair, the observed ratios closely matched the expected ratios.

Since the tagged peptides exactly coelute, the ratios of the peptide pairs are conserved throughout the elution profile, which means that it is not necessary to integrate the total ion current for the eluting ions to determine the relative abundance of each peptide pair. In addition, quantification of the peptide pairs could be performed with simultaneous sequence determination (data not shown).

Analysis of the Sensitivity and Robustness of the TMT Technology. To test the dynamic range of the system and to show that the properties of the TMT labels are consistent over the entire dynamic range, the conservation of peptide ratios was examined at a range of different concentrations of one of the tagged synthetic peptides (peptides 3A and 3B). As can be seen from Figure 5, over a serial dilution of peptides 3A and 3B mixed in a ratio of 40:60 from 100 pmoles to 100 fmoles, the ratios were reliably conserved, with a deviation between 5 and 10% in most cases from the expected ratio. These and other results (not shown) indicate that the TMT labels do not reduce the intrinsic sensitivity with which a peptide is detected in the MS/MS mode; i.e., the analysis of TMT-labeled peptides by CID has at least the same sensitivity as the MS/MS of untagged peptides. The sensitivity with which it is possible to determine the sequence of tagged peptides also does not seem to be significantly changed in any of the peptides tested so far when compared with the unlabeled peptide. Meaningful differences in the ratios of the peptides can be detected over the entire range of concentrations tested.

In a further experiment, the ability to detect labeled peptides in a complex mixture was examined. The peptide pair 3A and 3B bearing the second generation TMT was spiked into an excess of a tryptic digest of an unfractionated yeast protein extract. To
simulate a global proteomics analysis, the peptide mixture was analyzed by LC/MS/MS, and the five most intense ions from each elution scan were subjected to CID to identify the peptides. The TMT-labeled peptides were detected, and the region of spectrum corresponding to the TMT fragments was analyzed to determine the abundance ratio of the detected peptides. Analysis by CID (collision energy of 30 V), provides the spectrum shown in Figure 6c. The ratio of peptides 3A and 3B was found to be 39.3% to 60.7%. The expected ratio was 40% 3A to 60% 3B. The quality of the MS/MS spectrum obtained (Figure 6b and 6c) at the low collision energy used allows a clear identification of the peptide sequence by database searching. This experiment clearly shows that a complex mixture of tryptic peptides does not hinder the analysis of peptide pairs labeled with the second generation TMT labels, and the TMTs can help to overcome noise in the sample. In addition, there do not seem to be any suppression problems; ratios of peptides present in low concentrations can still be determined in the presence of other peptides that are in high concentrations.

CONCLUSIONS AND PERSPECTIVES

The above results clearly demonstrate the advantages of the TMT labels for the simultaneous identification and quantification of the components of complex protein mixtures. The TMT labels overcome a number of problems associated with previously described isotope labeling techniques. Conventional deuterium labeling suffers from the problem that identical peptides labeled with different deuterium-isotope tags do not coelute during chromatographic separations. Although this issue can be solved...
using $^{13}\text{C}$, as in the new ICAT reagent, there is also a problem with different charge states of isotope-labeled peptides, because the mass difference between corresponding peptides with conventional isotope tags varies with the charge state of the peptide. Both of these problems create difficulties for the accurate quantification and identification of peptide pairs, and these problems restrict the throughput of tagged peptides that can be identified for any given instrument.

In contrast, the CID analysis of TMT labels gives rise to dependable TMT fragment ions that reflect the relative abundances of the peptides from which they are derived. With MS/MS-based scanning of the ions eluting from a chromatographic separation, TMTs make it possible to easily identify tagged species, allowing untagged material to be ignored. The tags show consistent properties over a wide dynamic range, and labeled peptides can be detected with a sensitivity that is comparable to other labeling procedures. In addition, the charge state of TMT labeled peptides does not affect the ability to detect the abundance ratios of peptide pairs.

Since the tagged peptides exactly coelute, the ratios of the peptide pairs are conserved throughout the elution profile, which means that it is not necessary to integrate the total ion current for the eluting ions to determine the abundance of peptide pairs, as is necessary for ICAT.\textsuperscript{22} In the QTOF instrument, CID of the peptide pairs allows differences in ion abundance of the peptide pairs to be detected while simultaneously producing the CID spectrum necessary to determine the sequence of these peptide pairs. This feature allows TMT-labeled peptides to be quantified and identified using the automated selection of the most intense ions in the MS-mode spectra. This has advantages for global analysis of protein samples, because this should allow more proteins to be identified in a single analysis in the same time as other techniques while using conventional instrumentation. Alternatively, an include mass list can be specified for the quantification of peptides with ions of known mass-to-charge ratios.

The use of proline containing TMT labels allows low collision energies to be used to obtain the TMT fragments that represent the ratios of the tagged peptides in their source samples. So far, the preferential cleavage of the tag fragment has not noticeably diminished our ability to sequence the rest of the tagged peptide. It should be noted that two of the peptides tested in the experiments in this paper contain proline, while a third contained an aspartic acid/proline (DP) linkage, and no problems were encountered with these peptides. The DP peptide probably cleaves at the DP linkage before the TMT label can fragment, but consecutive fragmentation in the QTOF produces the desired tag fragments. Consequently, we are optimistic that this will be a robust tag design for wider use.

Furthermore, the TMT reagents are modular, and variants are easily produced. Modifications that could be developed are changes in the fragmentation enhancer, the inclusion of biotin into the tag to allow TMTs to be used as affinity ligands, and modification of the reaction specificity of the tags to allow the tags to be linked to other reactive functionalities in proteins and other biomolecules. The inclusion of a biotin moiety into a peptide
sequence, which can be achieved with standard peptide synthesis techniques, does not interfere with the quality of the peptide identification. Different tag and mass normalization functionalities can be used to allow larger sets of tags, with the same mass to be synthesized for multiplexing. This is an advantage of TMTs that is not readily available to conventional isotope labeling procedures. In approaches using conventional isotope tags, labeling each sample or set of standards with a different isotope tag results in an additional peak in the mass spectrum for each peptide in each sample: if two samples are analyzed together, there will be twice as many peaks in the spectrum, and similarly, with three samples, the spectrum will be three times more complex than for one sample alone. The TMT labels do not increase the complexity in the mass spectrum at all.

Because there is no particular restriction on the reactive functionality that might be used with these tags, it is anticipated that the TMT labeling should be applicable to both global peptide isolation procedures, such as MudPIT or ICAT, and to specific procedures, such as phosphopeptide and glycopeptide isolation. Therefore, TMT labeling should allow peptide-based protein characterization procedures to be fully automated for quantitative, comparative, functional proteome analysis. The TMT strategy, when applied, will therefore enjoy the same advantages as other in vitro peptide isolation and labeling techniques in that it will be applicable to proteins that are problematic for 2-D gel electrophoresis, such as small proteins, highly basic or acidic proteins, and hydrophobic proteins.

The use of MS/MS-based tag detection produces high-quality data with good sensitivity, excellent signal-to-noise ratios, and a broad dynamic range. These are all features essential to the development of truly meaningful proteome analysis techniques. In addition, the effective exploitation of the intrinsic properties of MS instrumentation to maximize throughput and quality of peptide identification is crucial to achieving the goal of genuine global analysis of cell and tissue samples. Some of the abilities of TMT labeling to achieve these goals have been demonstrated here, and further advantages will become available with newer reagents. Thus, TMT labeling provides a new benchmark for automated quantitative proteome analysis.

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