Distinguishing and Quantifying Peptides and Proteins Containing d-Amino Acids by Tandem Mass Spectrometry

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Tandem mass spectrometry (MS/MS) utilizing both electron capture dissociation (ECD) and collisionally activated dissociation (CAD) was used to develop a qualitative and quantitative analytical method for chiral analysis of individual amino acid residues in polypeptides. ECD produced a more distinct chiral recognition than CAD, which is attributed to the smaller degree of vibrational excitation in ECD. Several peptide and protein model systems were used in this study, including the smallest known protein, tryptophan cage, a lactoferrin peptide, and the biologically relevant opioid peptide, dermorphin. An adaptation of the kinetic method was used to quantify the degree of separation in peptides and proteins, broadening the application area for tandem mass spectrometry.

Though eukaryotic systems use almost exclusively L-amino acids in protein synthesis, d-forms as well as unnatural amino acids are known to occur in some animal peptides.4 The origin of chiral selectivity within biological systems still remains unclear, and it is only recently that experimental data have been obtained showing that RNA aminoacylation acts as a chiral driving force during protein synthesis, favoring L-amino acids.2 Upstream from protein synthesis and especially in the world of prebiotic chemistry, we are left with an enigma of life’s chiral rules.3

The conversion from L- to d-form within peptides and proteins is thought to be the most subtle of all posttranslational modifications, which nonetheless may invoke profound biological implications.4 One of the most elegant displays of protein chirality and its relation to functionality was provided by Kent et al., who showed through total chemical synthesis that the d-HIV protease retained activity, but its specificity was limited to substrates and inhibitors of a chirality opposite to the natural l-form.5 In another example, the d-Ala2 variant of the opioid peptide dermorphin is recognized by the human µ receptor. The chirality inversion of one amino acid residue within this peptide causes a slight structural shift and an analgesic effect 1000 times more potent than morphine, while L-Ala2 (native) exhibits no such effect.6

The importance of amino acid substitution is thought to play a considerable role in the progression of many disease states. Studies within the amyloidose family, which includes proteins playing critical roles in both Alzheimer’s and Parkinson’s diseases, have also highlighted the importance of amino acid chirality.7,8

Fibril formation by the amyloid β-peptide (Aβ) relevant to Alzheimer’s disease has been studied extensively by many research groups. Tomiyama et al. showed the importance of L-Asp in positions 7 and particularly 23. The effect of d-Asp substitution on fibril formation, as measured by both turbidity and electron microscopy, was dramatic.9 Shapira et al. have as well attributed Aβ fibril formation to the racemization of aged proteins, finding 5% d-Asp content within amyloid proteins of Alzheimer’s patients.10 The Amyloid-ß and α-synuclein are proteins associated with Parkinson’s syndrome that form fibrils better known as Lewy bodies. The importance of aspartic acid racemization within the ß-protein was explored by Kennesey et al., who revealed, in line with Shapira’s findings, that brains of neuropathologically inflicted patients had on average 4.9% d-Asp content.11

As peptide and protein therapeutics continue to advance, d-amino acid position and content is apt to become a subject of routine analytical measurement. Today, within the pharmaceutical industry, a routine task is the elucidation of the chirality of drug candidates in pure as well as mixed forms.32 But the L/d-amino acid conversion in a polypeptide is likely to go unnoticed by most


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modern bioanalytical techniques. Analysts are currently employing high-performance liquid chromatography (HPLC), capillary electrophoresis, circular dichroism, nuclear magnetic resonance, and enzymatic techniques for chiral recognition. The choice of stationary phase, internal standard, and data interpretation influences the outcome and causes much variability. These procedures are demanding in both sample size and analysis time. Most critically, many of these analysis techniques use acid hydrolysis or Edman degradation to release free amino acids before final separation, and cleavage of the peptide bond by chemical methods usually involves racemization at a 3–15% level. Since this racemization level often exceeds the expected D-amino acid content as a result of the disease state, several quantitative studies have failed to arrive at definitive conclusions. A fast and sensitive analysis that does not require chemical hydrolysis and possesses a detection limit below 1–3% would be of significant analytical utility.

Mass spectrometry is a versatile and sensitive analytical method, meeting the qualifications of specificity, accuracy, sensitivity, and speed matched by few other techniques. Being the workhorse of today’s proteomics field, tandem MS (MS/MS) provides rapid and sensitive identification of proteins by database search, as well as the analysis of posttranslational modifications. In the analysis of constitutional isomers, MS/MS using high-energy collisions with neutrals, or energetic (~10 eV) electrons provide distinction between Leu and Ile residues. Recently, tandem MS using electron capture dissociation (ECD) could reliably distinguish Asp and iso-Asp residues in peptides. However, chiral measurements present a far greater challenge for mass spectrometry as fragment masses are the same for all stereoisomers and no characteristic losses are found in the mass spectra. Thus, distinction of D-and L-amino acid residues by tandem MS must rely on the difference in fragment ion abundances. These abundances are subject to experimental conditions, such as the internal temperature of ions and the details of ion excitation. As these parameters are not easy to control, there have been very few successful attempts to determine the chirality of individual amino acid residues by traditional fragmentation techniques. One of the successful approaches involved collisionally activated dissociation (CAD) of stereoselective systems consisting of noncovalent complexes of a metal ion or stereoisomer of a peptide or antibiotic. The relative abundances of fragments have been used to determine the so-called chiral recognition factor Rchiral, usually ranging between 1.1 and 3, which characterizes the ability to distinguish stereoisomers. By calibrating the instrument carefully and using controlled MS/MS conditions with known mixtures of stereoisomers, one creates a calibration curve that is then used to deduce the stereoisomer composition in a sample mixture. The accuracy of this approach depends on the magnitude of Rchiral and is usually a few percent. Importantly, this approach has so far been confined to small molecules up to tetrapeptides, which limits its application toward biologically interesting polypeptides. In the study by Roepstorff et al., CAD provided differentiation between stereoisomers of a tripeptide via probing the strength of their noncovalent binding to the antibiotic vancomycin. The degree of molecular recognition in the gas phase has been related to the stability of the noncovalent complexes in solution.

The alternative to CAD fragmentation, ECD is found to exhibit surprising reproducibility and repeatability of fragment abundances. Recently, we have shown that the ECD mass spectrum of a 20-residue protein, Trp-cage, significantly changed when one of the amino acids (Tyr) responsible for tight tertiary structure in solution is replaced by the D-form. The effect was attributed to the preservation of the main features of the tertiary structure in the gas phase. The obtained value of Rchiral = 8.6 raised expectations that quantitative measurements of the relative D-amino acid content in proteins were possible. Here we report the results of such a quantification study. We start with the Tyr substitution and sequentially test other D-substitutions within the same molecule. Furthermore, we extend this technique to shorter peptides that are unlikely to possess distinct tertiary gas-phase structure. Additionally, with the biologically important peptide dermorphin, we demonstrate that at carefully controlled conditions CAD can also provide chiral recognition, albeit with a lower recognition factor than ECD. Finally, a combination of on-line reversed-phase LC–MS/MS with CAD or ECD is suggested as a technique for quantification of complex peptide mixtures of varying chirality.

**EXPERIMENTAL SECTION**

**Protein and Peptide Synthesis.** Peptides and the tryptophan cage protein, including the all-L form as well as the D-substituted variants, were synthesized in-house using solid-phase Fmoc chemistry with an Applied Biosystems 431A (Foster City, CA) and Intavis AG ResPep (Gladbach, Germany) peptide synthesizers. D-Isomer amino acids were purchased from Novabiochem (Lauffen, Switzerland) and Cambridge Research Biochemicals Ltd. (Cleveland, U.K.). Peptides were further purified by reversed-phase HPLC using a Vydac C18 column (Hesperia, CA). Final purity was tested by Fourier transform mass spectrometry.

**Mass Spectrometry.** Mass spectrometry was performed using a 7-T LTQ FT mass spectrometer (Thermo, Bremen, Germany) as well as an LTQ linear ion trap instrument (Thermo, San Jose, CA).
CA). Peptides were dissolved in electrospray solvent consisting of water, methanol, and acetic acid in proportions 49:49:2 (v/v/v), to a concentration of \(~5\) pmol/mL. Peptides were electrosprayed by direct infusion using a Proxeon nanosource (Odense, Denmark) with applied voltages ranging from 700 to 1200 V. Thermo instruments utilize automated gain control (AGC), in which the same preset number of ions are accumulated in each scan. The AGC setting of the linear ion trap ranged between \(2 \times 10^4\) and \(10^5\) ions in MS mode for CAD spectra generated. ECD with LTQ FT was done as previously described. Briefly, electrons were produced using a commercially available indirectly heated cathode provided by Thermo, with an irradiation time of \(~70\) ms with an electron energy less than 1 eV. For LC–MS/MS, data-dependent acquisition was used, in which the full MS scan was followed by a high-resolution "zoom scan", followed by ECD and finally CAD.

**Liquid Chromatography.** Proxeon fused-silica capillaries (Odense, Denmark), 150 mm \(\times\) 75 \(\mu\)m inner diameter, were self-packed with Dr. Maish, ReproSil-Pur 120 C18 material 3 \(\mu\)m (Ammerbuch, Germany). Agilent 1100 nano LC (PaloAlto, CA) was used for liquid chromatography at a flow of 200 nL/min with standard mobile phase A (water with 0.5% acetic acid) and mobile phase B (89.5% acetonitrile, 10% water, and 0.5% acetic acid). A 22-min gradient from 5% B to 45% B was used for peptide separation.

**RESULTS AND DISCUSSION**

**Introduction to Chiral Quantification.** The kinetic method, as developed by Cooks et al., is applicable to studying a broad range of gas-phase phenomena, including gas-phase amino acid basicities, metal ion affinities, heterolytic bond dissociation energies, ion structural features, structures of metal complexes of biological molecules, and finally stereoelectronic effects on cation affinities. The method utilizes differences in fragment ion abundances and from them extracts the differences in fundamental parameters, such as free energy and entropy. In the case of fragmentation of chiral molecules or clusters, the fragment ion branching ratios are \(R_D\) and \(R_L\) for the species with \(\alpha\) and \(\beta\)-chiral forms, respectively. A quantitative measure, namely, the chiral recognition factor \(R_{\text{chiral}}\), is then introduced:

\[
R_{\text{chiral}} = R_D/R_L
\]

We have used this approach in ref 29 for chiral recognition in Trp-cage, where \(R_D\) and \(R_L\) were ratios of the abundances of a pair of ECD ionic fragments that by preliminary investigation were found to change with \(L \to \beta\) substitution. The exact mechanism inducing the change in branching ratios upon chiral substitution remains at the moment unclear, as the exact ECD mechanism of peptide fragmentation is still being debated. It is likely, however, that the changes in branching ratios are related to the differences in the secondary and tertiary gas-phase structures. These, in turn, are determined by such thermodynamic parameters as proton affinities, internal temperature, and structure-related entropy changes, that is to say, the same parameters that give rise to the kinetic method. This justifies the empirical application of the equations derived in the kinetic method for quantification of chiral recognition in tandem MS, which showed good agreement with the experimental data (vide infra).

In the kinetic method,\(^{14,23}\)

\[
\ln R_{\text{chiral}} = \Delta(\Delta G)/RT_{\text{eff}}
\]

where \(\Delta(\Delta G)\) is the difference in the free energy change during fragmentation of the \(L\)-forms and the \(D\)-forms of the precursor ion, respectively, \(R\) is the gas law constant, and \(T_{\text{eff}}\) is the effective temperature. For pure \(L\) and \(D\)-forms, \(\Delta(\Delta G)\) are \(\Delta(\Delta G)_L\) and \(\Delta(\Delta G)_D\). For a mixture containing both \(D\) and \(L\)-forms with the molar fraction of \(D\)-form \(\alpha\), it can be written,\(^{14,23}\)

\[
\Delta(\Delta G)_M = \alpha \Delta(\Delta G)_D + (1 - \alpha) \Delta(\Delta G)_L = \Delta(\Delta G)_L + (\Delta(\Delta G)_D - \Delta(\Delta G)_L) \alpha
\]

At the same time,

\[
\ln R_L = \Delta(\Delta G)_L/RT_{\text{eff}}
\]

\[
\ln R_D = \Delta(\Delta G)_D/RT_{\text{eff}}
\]

Combining eqs 1–5, and introducing \(R_M\), which is the experimentally measured fragment ion branching ratio for the mixture, derive\(^{32}\)

\[
\ln R_M = \Delta(\Delta G)_M/RT_{\text{eff}} = \Delta(\Delta G)_L/RT_{\text{eff}} + ((\Delta(\Delta G)_D - \Delta(\Delta G)_L)/RT_{\text{eff}}) \alpha = \ln R_L + \ln(R_{\text{chiral}}) \alpha
\]

We use eq 6 to obtain a calibration plot and then quantitatively measure the \(D\)-form peptide content in mixtures. This method requires a priori knowledge of \(R_L\) and \(R_D\) values for pure \(L\) and \(D\)-forms, which is one of its limitations.

Another limitation is that this method is only applicable as long as the kinetic method approximations and other assumptions are satisfied.\(^{14,23}\) The theoretical validation of the use of eq 6 hinges upon the development of appropriate mechanistic models, work that is currently in progress but as yet unfinished. Below we report the results of the application of the above approach to various \(\alpha\)-substituted polypeptide molecules.

**Trp-cage Protein.** The smallest known protein, Trp-cage (NLYIQWLKDGGPSSGRPPPS), forms a compact globular structure in solution.\(^{30,34}\) It has been shown that \(\alpha\)-Tyr substitution significantly alters the ECD fragmentation pattern,\(^{29}\) most noticeably the abundances of the \(z_{38}\) and \(z_{39}\) fragment ions. The chiral recognition factor based on the ratio \(z_{38}/z_{39}\) was very high (\(R_{\text{chiral}} \sim 8.6\) in ref 29) compared to the typical values reported in the literature for chiral quantification using the dissociation of chiral dimers.\(^{29}\) Not surprisingly, mixtures of all-\(L\) and \(D\)-Tyr forms of Trp-cage showed reproducible and easily quantifiable patterns (Figure 1, left). The dependence of the logarithm of \(R_M\) (the ratio

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of abundances of $z_{18}$ and $z_{19}$ fragments) upon the D-Tyr form content in the diastereomeric mixture was fit to a line in typical kinetic method fashion, with the slope $m$ representing the isomeric selectivity ($\ln R_{\text{chiral}}$). The rather large value of $m = 2.33$ obtained with the Thermo instrument reflects the large chiral recognition factor for D-Tyr substitution ($R_{\text{chiral}} \approx 9.9$). This line can now serve as a calibration plot for quantitative measurements. In the experiment composed of 14 consecutive measurements altering between the pure all-L form and 1% admixture of D-Tyr form, the all-L form showed the average value of $R_{\text{L}} = 0.300 \pm 0.014$, while 1% admixture gave $R_{\text{D}} = 0.331 \pm 0.022$, indicating the detection limit at $1\%$.

In our previous publication, we attributed the large chiral recognition factor to the preservation of the tertiary structure of Trp-cage in the gas phase and its destabilization by the D-Tyr substitution. Initial studies on this molecule were done using a 4.7-T Ion Spec mass spectrometer; the results reported here are with a 7-T hybrid instrument that has the added feature of controlling the number of ions entering both the linear ion trap and the Penning trap. Variations in the $R_{\text{chiral}}$ values reported in ref 29 and in this work (8.6 and 9.9, respectively) are attributed to the instrumental differences. Chiral recognition decreased with destabilization of the "native" tertiary structure by temperature or additional protonation of the molecule. Furthermore, chiral recognition has not been found in CAD spectra. However, additional studies on other peptides have led us to conclude that the presence of intact tertiary structure is advantageous (higher $R_{\text{chiral}}$) rather than a necessary requirement for chiral recognition and quantification.

Substitutions of other amino acids in the Trp-cage molecule by D-residues invariably produced a unique fragmentation pattern, in which it was always possible to identify a pair of fragments indicative of the chiral substitution. As an example, Figure 2 shows the ECD spectrum of 2+ of D-Gln substitution, for which the $z_{15}$ and $z_{16}$ fragments composed such a pair, which gave the $R_{\text{chiral}}$ value of 1.6. Table 1 summarizes the results for four substitutions.

Closer inspection of the ECD mass spectra of D-substituted molecules led to an interesting observation; namely, the $z$ fragment that was affected most by D-substitution was the one originating from the cleavage N- or C-terminal to the substituted residue. More specifically, for the bulky D-substituted residues Tyr and Trp, the $z$ fragments originating from the cleavage N-terminal to them gave by far the largest intensity enhancements. Generally, the localization of D-substituted ions within this molecule could be made within two residues by analyzing which $z$ fragment increased its abundance most. For instance, D-Gln substitution gave an increased intensity of $z_{16}$ (Figure 2), which became the peak of highest intensity among backbone fragments. To analyze this effect further, we introduced a $R_{\text{chiral global}}$ factor, which is calculated similarly to $R_{\text{chiral}}$, but the abundance of the $z$ fragment that changed most was normalized by the sum of the abundances of all $z_{5-19}$ fragment ions. The term global refers to the normalization factor. The results shown in Table 1 for four substitutions reveal that the biggest change in $R_{\text{chiral global}}$ occurred when

![Figure 1. Shift in $z_{18}$ and $z_{19}$ fragment abundances as a function of D-amino acid content (left), a property that is quantifiable as seen in the plot (right). An equation, $\ln R_{\text{chiral}} = A + m \alpha$, where $\alpha$ is the molar content of the D-form, $m = \ln R_{\text{chiral}}, R_{\text{chiral}} = \text{ratio of the abundances of } z_{18} \text{ and } z_{19} \text{ fragments}, \text{was fitted to the experimental data points.}]

![Figure 2. ECD mass spectrum of D-Gln Trp-cage 2+ ions, the chiral recognition can be seen by the enhanced abundance of $z_{16}$, which corresponds to the cleavage C-terminal to the substitution.]

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substituting Trp and Tyr residues, in concordance with the $R_{\text{chiral}}$ values.

In solution, almost any amino acid substitution in Trp-cage alters the tertiary structure, which is most stable for the all-L isomer.\(^{35}\) Likewise, the change of the fragmentation pattern due to $L \rightarrow D$ substitution should reflect the relative importance of each amino acid in the stability of the gas-phase tertiary structure. The value of $R_{\text{chiral global}}$ provides a measure of the degree of importance. As another, perhaps even more universal measure, we chose the value ($r_{DL}$)$^{-1}$ where $r_{DL}$ is the correlation factor\(^{28}\) between abundances of $z_{19}$-$z_{18}$ of all-L variant, on one hand, and of the $D$-substituted isomer, on the other hand. The results are shown in Table 1. The highest ($r_{DL}$)$^{-1}$ value was obtained for Trp$_4$ and the second highest for Tyr$_1$ substitution, consistent with the fundamental importance of these residues for intact tertiary structure.\(^{35}\)

Summarizing the Trp-cage results, chiral recognition proved sufficient for quantitative $D$-amino acid determination in mixtures. Various quantitative measures of the effect of chiral substitution demonstrate the same trend; specifically, the largest impact is obtained for residues most involved in stabilization of the tertiary structure. Not surprisingly, these also happen to be the residues with the bulkiest side chains. As a final comment, the attempts to reproduce these results quantitatively using molecular dynamics simulation have led us to question the simplified view of the ECD mechanism, according to which the fragment abundances directly reflect the charge solvation pattern.\(^{36,37}\) An alternative explanation to the ECD mechanism is under development.

**Lactoferrin 406-423 Peptide.** Lactoferrin is a glycoprotein belonging to the iron transporter family and is found in the secretion of mammals. Upon fermentation, amino acids in milk proteins are known to undergo racemization, with aspartic acid having the highest racemization rate.\(^{33}\) We synthesized a tryptic peptide 406-423 (GEADALNLDGGYITAGK) of the lactoferrin protein in all-L form as well as replaced Asp-409 (D$_4$ in the peptide sequence) with $D$-form aspartic acid, thereby mimicking the complete $L \rightarrow D$ conversion process in this portion of the molecule. Figure 3a displays the ECD spectra of 2+ ions of these molecules. It is easy to see that the pair $z_{19}$ and $c_{14}$ (Figure 3a, central insets)-provides chiral recognition, and the $R_{\text{chiral}}$ value of 5.1 obtained with this pair is large. Note that, as in the case of Trp-cage, $z_{14}$ originates from the cleavage C-terminal to the $D$-substituted residue. The $c_{14}$ ion was chosen for normalization of $z_{14}$ abundance because the $m/z$ values of both ions are similar.

The ECD spectra of 3+ ions of all-L peptide in Figure 3b revealed the location of two charges for 3+ molecular ions,\(^{29}\) specifically the N-terminus and the C-terminal lysine residue (the same for both stereoisomers). For 2+ ions, the position of only one charge (located at the more basic site)\(^{38}\) can be directly deduced from the ECD spectra. For both isomers, this charge was located on the central (..DG..) part of the molecule. However, the differences in the [M - $R$] region (Figure 3a, right insets), which is indicative of the charge solvation opportunities for the neutralized proton, revealed that these opportunities have changed for the $D$-Asp$_4$ variant. More specifically, the ratio of the reduced species ([M + 2H]$^+$) and the (~17 Da) loss for the $L$-form was 8.8 and for the $D$-substituted form it was 4.6. Thus, these two stereoisomers have contrasting secondary structure, albeit the exact difference is unclear.

Secondary structure of gas-phase polypeptides is known to affect the CAD fragmentation pattern.\(^{30}\) Thus, CAD of 2+ ions of both stereoisomers was attempted, and the abundances of the pair b$_{14}$ and y$_{14}$ that correspond to the ECD pair $z_{14}$ and c$_{14}$ were examined (Figure 3c). The pair showed a clear chiral recognition, albeit much weaker ($R_{\text{chiral}}$ = 1.8) than the $z_{14}, c_{14}$ pair in ECD.

The sensitivity of ECD fragment abundances (the ratio of abundances of even-electron $z_n$ and radical $z_n$ fragments from the same-bond cleavage) to chiral substitution of certain critical amino acids in short peptides has been noticed by Polfer.\(^{30}\) However, chiral recognition through secondary structure dependent fragment abundances has been considered an unlikely phenomenon,\(^{29}\) since chiral recognition requires three points of contact.\(^{14}\) Chiral recognition by CAD was thought to be even less likely, as in collisional activation the ions fragment at elevated internal temperatures at which rearrangements, including racemization, may occur at a high rate. Further experiments have nevertheless validated the chiral recognition by CAD in even smaller peptides.

**Dermorphin.** Dermorphin (YAFGYPS) and deltorphin (YAF-ENVVG) are two of several opioid peptides isolated from frog (Phyllomedusa sauvagei) skin.\(^{41}\) These peptides are good examples of the importance of stereochemistry in biology. They are both part of the frog’s defense mechanism and highly selective ligands to $\mu$- and $\delta$-opioid receptors,\(^{42}\) respectively. The activity of these peptides is inherent to $D$-Ala$_2$ forms, while all-L forms are found

<table>
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<th>amino acid substitution</th>
<th>most varied z ion from all-L form</th>
<th>$z_{19}$</th>
<th>$z_{18}$</th>
<th>$z_{17}$</th>
<th>$z_{16}$</th>
<th>$z_{15}$</th>
<th>$R_{\text{chiral}}$</th>
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<td>0.276</td>
<td>4.6</td>
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</table>

*The fragment abundances were normalized by the sum of all $z_{19}$-$z_{18}$ fragments for that particular stereomer. $R_{\text{chiral}}, R_{\text{chiral global}},$ and ($r_{DL}$)$^{-1}$ were derived as described in the text. Note the variation in the abundance of fragments adjacent to the $D$-substitution (boldface type).
to be biologically inactive. How and where this L- to D-Ala conversion takes place is still a matter of debate. Recently, it has been suggested that autism in humans may be linked to an elevated level of endogeneous opioid peptides, including dermorphin and deltorphin (so-called opioid theory of autism). Testing this somewhat controversial hypothesis requires not only quantitative measurements in body fluids of the corresponding peptides and confirmation of their sequences but also determination of the chirality of the Ala residue. It appears likely that the endogenous peptides will exist as a mixture of all-L and D-Ala forms, with the active D-Ala form being a minor component. Thus, quantitative measurement of a mixture with a low detection limit is required. Within the current work, we address the feasibility of quantitative chiral measurements in dermorphin stereoisomer mixtures by tandem mass spectrometry.

All-L and D-Ala forms were synthesized in-house and analyzed by ESI MS/MS. The heptapeptides produced preferentially singly charged ions, with a low abundance of doubly charged species. ECD in the LTQ FT gave a few fragment peaks with a poor signal/noise ratio, and therefore, the more sensitive LTQ analyzer together with CAD MS/MS were used for quantitative measurements on 2+ ions (Figure 4a). As a chirality-reporting pair of fragments, b5 and y5 ions were chosen (Figure 4a, insets). Note again, that y5 represents the cleavage C-terminal to the substituted residue. Similarly, the b5 ion was chosen as a normalization factor because it appears in the spectrum close to the y5 ion. The results of the quantitative measurements of the b5/y5 ratios are given in Figure 4b as a function of the CAD excitation (expressed in relative units of the LTQ instrument). The chiral recognition factor Rchiral remained roughly constant when CAD excitation was varied. Since the relative errors of measurements were lower at a higher excitation level, the excitation of 50 units was used in additional experiments.

It should be noted that the b5/y5 ratios in CAD experiments, unlike corresponding fragment ratios in ECD experiments, were dependent upon the number of ions in the trap. Unlike other tandem mass spectrometers, the LTQ instrument can employ AGC, in which mode it acquires in each scan the same preset number of ions. This feature allowed for reproducible and stable quantitative measurements on a mixture of all-L and D-Ala forms, resulting in the calibration plot in Figure 4c. We can speculate that the absence of AGC doomed earlier attempts to use CAD for quantitative chiral measurements. The slope of the plot, m = 0.686, was much smaller than that in Figure 1b, reflecting a smaller chiral recognition factor in CAD compared to ECD. Based on the slope and the statistical errors of the abundance measurements, we estimated that the lowest fraction of the D-Ala form of dermorphin detected as an admixture to the dominant all-L form is 3−5%.

Since this detection limit may not be sufficient in some real-life applications, an alternative approach to mixture quantification was needed. The remedy was found with the help of reversed-phase liquid chromatography, which is known to separate stereoisomers of peptides at certain conditions. The selected ion current chromatogram in Figure 5a shows that the stereoisoforms of dermorphin mixed in 1:1 ratio are nicely separated in a 75-μm nanoflow column at a flow rate of 200 nL/min. The first peptide eluted at 32 min, and the difference in retention times was ΔRT = 36 s, while the peak duration was ~7 s. Figure 5a inset confirms

Figure 3. Lactoferrin 406−423 peptide. (a) ECD mass spectra of 2+ molecular ions of all-L form (top) and D-Asp substituted form (bottom). Chiral recognition via fragment abundances of z14 and c14 is obvious in the central panels. The [M − X] regions, indicative of charge solvation patterns, are show in right insets. (b) ECD mass spectra of 3+ molecular ions of the all-L form. The lack of 2+ fragment ions indicates the location of two charges on the terminal ends of the peptide. (c) CAD spectra for 2+ ions, Asp substituted (top) and all-L form (bottom).
Figure 4. (a) CAD mass spectra of the opioid peptide dermorphin, all-L form (top) and d-Ala$_2$ (bottom). The $b_5$ and $y_5$ fragments shown in the upper right panels were used for chiral determination, with $R_L \approx 2.3$ and $R_D \approx 4.8$ at an excitation of 50 arbitrary units. (b) The effects of CAD excitation on fragment ratios, the $R_{\text{chiral}}$ value remained relatively constant. (c) Calibration plot quantifying the admixture of d-Ala$_2$ form to all-L form of the dermorphin peptide (see Figure 1b for further explanation).
that the masses of the eluted molecules coincided within 1 ppm, suggesting that they were isomers. Figure 5b provides partial CAD MS/MS spectra of the molecules. From the comparison of the $b_5/y_5$ ratios it followed that the first-eluting molecule was the all-L form (Figure 5b, top panel), while the second molecule was the D-Ala$_2$ form (Figure 5b, bottom panel). To verify this result, the two pure stereoisomers were separately analyzed by LC/MS/MS in the same manner. Figure 6a shows that the elution time of the pure all-L form was rather well reproduced compared to the mixture (32.00 vs 32.08 min), while the measured $b_5/y_5$ ratio was only slightly lower than for the mixture (1.5 vs 1.6; see Figure 6a inset). For the D-Ala$_2$ form, both the retention time (RT = 32.55 min vs 32.68 min) and the $b_5/y_5$ ratio (3.0 vs 3.0) were well reproduced (Figure 6b). One should remember that these online MS/MS data were based on only one MS/MS scan (consisting of three integrated “microscans”), as opposed to tens of integrated scans for off-line analysis. Thus, the ion statistics in the on-line MS/MS fragment peaks was by necessity narrower, but the task for the on-line LC/MS/MS analysis was also limited. Only stereoisomer identification was required, while the abundance measurements were performed through the precursor ion abundances, as customary in LC–MS.

The stereoisomer identification by on-line reversed-phase nano-LC combined with MS/MS requires a priori knowledge of the approximate retention time of each stereoisomer as well as the relative abundances of fragment ions within its fragmentation pattern. This knowledge can only be gained by running pure synthetic isomers as standards, unlike the situation with constitutional isomers (e.g., amino acids Leu and Ile; Asp and iso-Asp or β-Asp), where specific losses or fragments are often found in MS/MS spectra acquired at certain conditions. Another complication is that, even for the short heptapeptide dermorphin,
there are several (seven) possible stereoisomers including the all-L form if a single L → D amino acid conversion is considered (glycine is the only stereosymmetric residue whose D- and L-forms are identical) and 15 more stereoisomers if double L → D substitution is allowed. This is why the retention time alone may not be sufficient for identification of all stereoisomers. The same could be true for MS/MS alone, if the short time scale of the peak elution does not provide enough time for collecting sufficient statistics. There are reasons to believe that a combination of the retention time together with the MS/MS fragmentation pattern is specific enough to separate the isomers of at least singly substituted dermorphin and other short peptides, as long as only a few are present in the mixture so overlapping retention times do not occur. This suggestion can be illustrated by a simple calculation. If chromatography provides on average five separation grades and MS/MS gives three independent separation grades, then L → D stereoisomer identification is possible for up to a 15-mer peptide. Given that the average size of tryptic peptides is 10–12 residues, this estimation gives hope that the above approach could work in most applications involving single L → D substitution.

CONCLUSIONS

We have shown that MS/MS of peptides and the smallest protein possessing tertiary or secondary structure in the gas phase can distinguish and quantitatively measure the relative content of stereoisomers, provided the stereoisomeric composition is known in advance. In all examples considered, the largest change in the abundance was in the fragments arising from the cleavage N- or C-terminal to the D-substituted residue. Further studies should show whether this observation has a general character. High-performance reversed-phase liquid chromatography is shown to afford a first dimension of chiral separation followed by chiral distinction by tandem MS. This approach gives reasonable hope that target stereoisomers within complex samples containing

Figure 6. (a) Total ion current chromatogram of pure L-form dermorphin. (inset) On-line nano-LC/CAD mass spectra of L-form. (b) Total ion current of D-Ala₂ dermorphin. (inset) On-line nano-LC CAD mass spectra of D-Ala₂ dermorphin.
slightly racemized protein or peptide can be identified and quantified.

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