Recognition and Quantification of Binary and Ternary Mixtures of Isomeric Peptides by the Kinetic Method: Metal Ion and Ligand Effects on the Dissociation of Metal-Bound Complexes

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The kinetic method is applied to differentiate and quantify mixtures of isomeric tripeptides based on the competitive dissociations of divalent metal ion-bound clusters in an ion trap mass spectrometer. This methodology is extended further to determine compositions of ternary mixtures of the isomers Gly-Gly-Ala (GGA), Ala-Gly-Gly (AGG), and Gly-Ala-Gly (GAG). This procedure also allows to perform chiral quantification of a ternary mixture of optical isomers. The divalent metal ion Ca^{II} is particularly appropriate for isomeric distinction and quantification of the isobaric tripeptides Gly-Gly-Leu/Gly-Gly-Ile (GGL/GGI). Among the first-row transition metal ions, Cu^{II} yields remarkably effective isomeric differentiation for both the isobaric tripeptides, GGI/GGL using GAG as the reference ligand, and the positional isomers GAG/GGA using GGI as the reference ligand. This is probably due to agostic bonding: α-agostic bonding occurs between Cu^{II} and GAG and β-agostic bonding between Cu^{II} and GGI, each produces large but different steric effects on the stability of the Cu^{II}-bound dimeric clusters. These data form the basis for possible future quantitative analyses of mixtures of larger peptides such as are generated, for example, in combinatorial synthesis of peptides and peptide mimics. (J Am Soc Mass Spectrom 2003, 14, 152-160) © 2003 American Society for Mass Spectrometry

It is well known that divalent metal ions play significant roles in biological systems [1]. Some metal-lopeptides recognize and interact selectively with the DNA minor groove as a function of the identity, chirality, and positioning of amino acid side chains within the peptide-ligand framework [2]. The conformational changes induced by metal-ion binding can be immense and remarkable [3]. The effective positioning of functional groups or appropriate subunits within the peptides allows them to trigger new functions [4]. In addition, the modular synthetic strategies used in polypeptides are prone to combinatorial selection. As a result of all these considerations, peptide research as it relates to drug discovery and design has become an important field with the potential to generate new drugs [5]. Direct tests of the purity of combinatorial mixtures, especially those containing isomeric peptides that are not always easily delineated, can provide information about the reliability of synthetic protocols [6]. Therefore, simple, fast and sensitive methods are highly desirable to determine isomeric peptide contamination [7]. This paper reports on steps to develop mass spectrometric procedures for this purpose.

The emergence of soft ionization techniques such as electrospray ionization (ESI) [8] and matrix-assisted laser desorption ionization (MALDI) [9] has made mass spectrometry a major method for peptide characterization [10]. Distinction between leucine and isoleucine residues can be achieved by collision-induced dissociation (CID) [11], for example in the case of the isomeric tripeptides Gly-XXX-Arg, by studying side-chain radical losses from radical cations in a tandem mass spectrometric experiment [12]. Gas-phase calcium ion binding (including sites and affinities) in various peptides has been studied using both low-energy and high-energy CID. These experiments have been used to model the calcium ion binding site III of rabbit skeletal troponin C [13, 14]. In addition, CID or surface-induced dissociation (SID) [15] of peptides can provide important information about backbone fragment ions [16], an example being the identification of zinc-binding sites in engineered hemoglobin [17]. A novel tandem quadrupole mass spectrometer has been recently introduced

allowing both CID and SID to be performed in the same instrument for peptide sequencing [18]. In addition, correlations have been observed between CID and biological Gln-Gly cleavage [19]. However, a general limitation of these methods is the inability to quantify isomeric contamination in a direct fashion. Although quantitation of a mixture of C-terminal leucine and isoleucine peptides could be achieved by measuring the ratio of two characteristic peaks in the third-stage of an MS^n experiment in an ion trap mass spectrometer, this method gave a low correlation coefficient ($r^2 = 0.9595$) and does not appear to represent a general procedure for quantification of isomers [17].

In an attempt to overcome these problems, we explore the applicability of the kinetic method [20–22], a procedure for thermochemical determinations which has seen growing use for chiral recognition [23–25] and some use in isomeric differentiation [26]. In view of observations made in the present study we also note that the kinetic method has been of value as a source of information on auxiliary bonding, including cation-π interactions [27], salt-bridging [28], and especially agostic bonding [29] (two-electron, three-center bonds where a hydrogen atom bridges two heavier elements, often carbon and a metal) [30].

The kinetic method, as used for isomeric differentiation, is based simply on the kinetics of competitive unimolecular dissociation of metal ion-bound clusters. The competitive reactions illustrated in eq 1 are studied.

$$\text{An} \xrightarrow[\text{ESI}]{\text{M}^{\text{II}}, \text{ref*}} [\text{M}^{\text{II}}(\text{ref*})_2(\text{An}) - \text{H}]^+ \begin{array}{c} \nearrow^{k_1} \\ \searrow_{k_2} \end{array}$$

$$[\text{M}^{\text{II}}(\text{ref*})(\text{An}) - \text{H}]^+ + \text{ref*}$$

$$[\text{M}^{\text{II}}(\text{ref*})_2 - \text{H}]^+ + \text{An} \qquad (1)$$

The relative branching ratio R (eq 2) for the two competitive dissociation channels is given by:

$$R = [\text{M}^{\text{II}}(\text{ref*})(\text{An}) - \text{H}]^+ / [\text{M}^{\text{II}}(\text{ref*})_2 - \text{H}]^+ \qquad (2)$$

The relative branching ratios for the pure isomeric forms M and N of the analytes (An) are R_M and R_N, while R_{iso}, a measure of isomeric selectivity, is given by the ratio of R_M and R_N (eq 3):

$$R_{iso} = \frac{R_M}{R_N}$$
$$= \frac{[\text{M}^{\text{II}}(\text{ref*})(\text{An}_M) - \text{H}]^+ / [\text{M}^{\text{II}}(\text{ref*})_2 - \text{H}]^+}{[\text{M}^{\text{II}}(\text{ref*})(\text{An}_N) - \text{H}]^+ / [\text{M}^{\text{II}}(\text{ref*})_2 - \text{H}]^+}$$
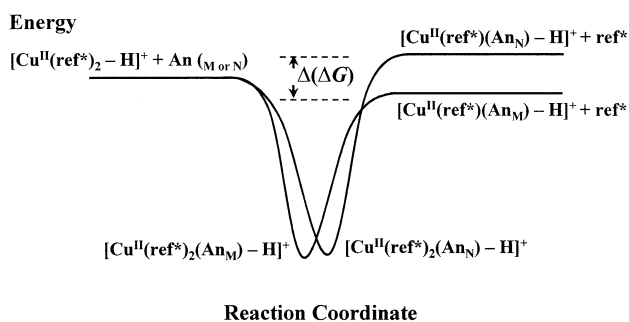$$(3)$$

Figure 1. Potential surface diagram for the dissociation of metal ion-bound cluster ions showing competitive dissociations of two singly-charged trimeric clusters for differentiation of isomers.

The difference in energy required to generate the two forms of the fragment ion $[\text{M}^{\text{II}}(\text{ref*})(\text{An}) - \text{H}]^+$ associated with the two forms of the analyte An, is referenced to that for the common fragment $[\text{M}^{\text{II}}(\text{ref*})_2 - \text{H}]^+$, generated from each of the cluster ions, as illustrated in Figure 1. These competitive processes determine the degree of isomeric distinction (R_{iso}) achievable. The more different the R_{iso} value is from unity, the higher the degree of isomeric recognition.

The main advantages of this approach are: (1) large isomeric differentiation results from the logarithmic relationship between the branching ratio and the difference in free energy between the two isomers of the product; (2) insensitivity to impurities is achieved using tandem mass spectrometry (MS/MS); (3) there is no requirement for isotopic labeling; (4) the measured abundances ratios are independent of the relative concentrations of the analyte and the reference ligand [26]. This study aims to examine the applicability of this methodology for isomeric distinction of tripeptides, to apply the method to the determination of compositions of a ternary mixture of tripeptides and to investigate metal ion and the reference ligand effects on isomer differentiation.

Experimental

All mass spectrometry (MS) experiments were performed using a commercial LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA), equipped with an ESI source and operated in the positive ion mode under the following conditions: spray voltage, 5.00 kV; capillary voltage, 20 V; heated capillary temperature, 150 °C; tube lens offset voltage, 20 V; sheath gas (N_2) flow rate, 30 u (roughly 0.45 L/min). For the ion trap mass analyzer, the automatic gain control (AGC) setting was 5×10^7 counts for a full-scan mass spectrum and 2×10^7 counts for a full product ion mass spectrum with a maximum ion injection time of 200 ms. In the full-scan MS/MS mode, the parent ion of interest was isolated by applying multiple waveforms to remove undesired ions through broadband excitation. The isolated ions were then subjected to a supplementary AC potential to resonantly excite them and so

cause CID. The Mathieu q_z values chosen for resonance excitation and resonance ejection were 0.25 and 0.90, respectively. The excitation time used was 30 ms, and the excitation amplitude was varied from 0 to 2.5 V zero-to-peak resonant excitation potential. This value was optimized in each experiment, but kept constant for the measurement of the isomers. Spectra shown represent the average of ~50 scans, where each scan is the average of five individual microscans. Mass/charge ratios (m/z) are reported using the Thomson unit (1 Th = 1 atomic mass per positive charge) [31].

Gas-phase peptide-metal ion complexes were generated simply by electrospraying 50/50 water/methanol solutions containing a mixture of the analyte of peptide(s), a reference of peptide, each at a concentration of 100 μM, and 25 μM of the metal ion. Peptides and metal chloride salts were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Methanol (HPLC grade) was obtained from Fisher Co. (Pittsburgh, PA). The standard error of the mean was calculated based on triplicate measurements made on separate occasions.
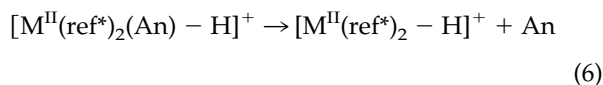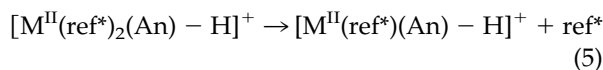
Results and Discussion

Method for Quantification of Binary Isomeric Peptide Mixtures

For a metal ion-bound cluster containing two reference ligands and one analyte ligand, the relationship between the relative branching ratio (R) and the molar fraction (α) of one isomer is given by the kinetic method expression (eq 4) [20, 22].

$$\ln R = \frac{\Delta(\Delta G)}{\mathbf{R}T_{\text{eff}}} \tag{4}$$

Here $\mathbf{R}$ is the gas constant, T_{eff} is the effective temperature of the activated trimeric cluster (related to the average internal energy of the two activated complexes for the competitive dissociation channels [32]), and $\Delta(\Delta G)$ is defined as the difference in free energies for the reactions 5 and 6 whose reverse barriers are considered negligible (see Figure 1).

$$[M^{II}(\text{ref*})_2(An) - H]^+ \rightarrow [M^{II}(\text{ref*})(An) - H]^+ + \text{ref*} \tag{5}$$

$$[M^{II}(\text{ref*})_2(An) - H]^+ \rightarrow [M^{II}(\text{ref*})_2 - H]^+ + An \tag{6}$$

When the analyte consists of a pure isomer, viz. M or N, $\Delta(\Delta G)$ becomes $\Delta(\Delta G)_M$ and $\Delta(\Delta G)_N$, and eq 4 takes the forms of eq 7 and eq 8:

$$\ln R_M = \frac{\Delta(\Delta G)_N}{\mathbf{R}T_{\text{eff}}} \tag{7}$$

$$\ln R_N = \frac{\Delta(\Delta G)_N}{\mathbf{R}T_{\text{eff}}} \tag{8}$$

For a binary mixture with an isomeric molar fraction of M given by α, one can write:

$$\Delta(\Delta G) = \alpha \cdot \Delta(\Delta G)_M + (1 - \alpha) \cdot \Delta(\Delta G)_N$$
$$= \Delta(\Delta G)_N + [\Delta(\Delta G)_M - \Delta(\Delta G)_N] \cdot \alpha \tag{9}$$

hence, the relationship between R and α can be expressed by combining eqs 3, 4, 7, 8, and 9 to obtain eq 10:

$$\ln R = \frac{\Delta(\Delta G)_N}{\mathbf{R}T_{\text{eff}}} + \frac{\Delta(\Delta G)_M - \Delta(\Delta G)_N}{\mathbf{R}T_{\text{eff}}} \cdot \alpha$$
$$= \ln(R_N) + \ln(R_{\text{iso}}) \cdot \alpha \tag{10}$$

Eq 10 predicts a linear relationship between the isomeric composition α and the natural logarithm of the branching ratio R. Such a linear relationship has been confirmed in the case of binary mixtures of isomeric dipeptides [26]. It has also been demonstrated for chiral analysis of amino acids [23, 33, 34], α-hydroxy acids [25, 35], chiral peptides [36], sugars [37], an antiviral nucleoside drug [24], and oxazolidinones (in this last case, by using a novel variant of the kinetic method that uses fixed ligands [38]). The kinetic method has also been applied to chiral quantification of amino acids in mixtures [39]. It is worthwhile pointing out that eq 10 also gives physical meaning to the calibration curves: The slope is equal to the natural logarithm of the isomeric selectivity and the intercept is the natural logarithm of the branching ratio when the analyte is pure N. It is clear that the larger the isomeric selectivity, the larger is the contribution to the measured ratio R upon unit change in isomeric molar fraction, and the higher accuracy that will be obtained, provided the abundance of two fragments can be accurately measured simultaneously.

The Formation and Dissociation of Singly-Charged Ca(II) and Fe(II) Cluster Ions

Isomeric distinction using the kinetic method is achieved by the formation of singly-charged cluster ions, which are mass-selected and then collisionally dissociated. If weakly bound, such cluster ions will dissociate simply by competitive ligand loss, the ratio of fragment ion abundance being characteristic of the analyte and reference ligand composing the cluster ions. Recent experimental evidence [40] shows that doubly-charged cluster ions are often generated when electrospraying solutions of carbohydrates containing Ca^{II} salts. Peptides, which sometimes show multiple deprotonated sites when coordinated to transition metal ions, yield singly-charged Ca^{II}-bound clusters, as
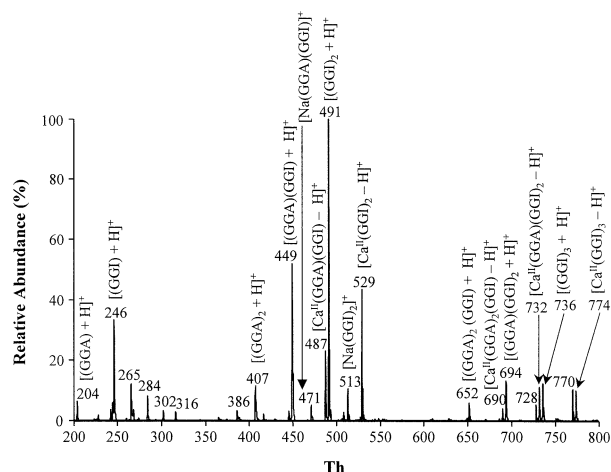
Figure 2. ESI mass spectrum of a sample containing GGI (100 μM), GGA (100 μM), and calcium chloride (25 μM) in a 1:1 methanol/water solution. Major cluster ions are designated.

Figure 3. MS/MS product ion spectra of $[Ca^{II}(GGA)_2(An) - H]^+$ (690 Th). The analyte, An, is (**a**) GGI (An_M) and (**b**) GGL (An_N), respectively. The CID activation level was chosen as 10.5%, corresponding to ca. 263 mV zero-to-peak AC excitation amplitude.

is clearly shown by the data given in Figure 2. There are various kinds of ions in the typical ESI-MS spectrum shown in Figure 2, including abundant protonated, sodiated, and Ca^{II}-bound clusters, involving each of the ligands in various combinations. The most interesting ions are the singly-charged Ca^{II}-bound trimers and dimers, formed by deprotonation of one of the constituent ligands. The trimers are relatively weakly bound, for example, the isomeric Ca^{II}-bound trimeric cluster ion at 690 Th undergoes CID to yield two dimers via loss of one or other intact ligand, either the analyte or the reference (Figure 3). Since the cluster ions are loosely bound [23], the kinetic method can be applied to their dissociation. The branching ratio for the two competitive fragmentations of the trimeric complex ions depends strongly on the isomeric configurations and can be used to characterize the composition of an isomeric mixture.

Quantification of the positional isomeric peptides GAG/GGA can also be achieved readily using Fe^{II} as the central metal ion and GGL as the reference ligand. Using An to represent pure GAG (An_M), the branching ratio R_M is 2.09 (Figure 4a), whereas the corresponding branching ratio R_N is 0.989 for pure GGA (An_N) (Figure 4b). The isomeric selectivity, R_{iso}, is 2.11 in this case. For quantitative analysis of these two isomeric tripeptides, a calibration curve was constructed, using GGL as the reference and mixing it with the analyte in various proportions. The individual R values were found to give a correlation coefficient (r^2) of 0.9993 (Figure 5a), clearly showing the existence of a log-linear relationship between the branching ratio and the molar fraction (α) of each isomer. This dependence is intrinsic to the kinetic method and is a consequence of the relationship between rate constant and free energy in unimolecular kinetics.

Although isobaric Leu/Ile containing peptides such as GGL/GGI with Leu or Ile at the carboxylic acid-terminus are more difficult to distinguish and hence to

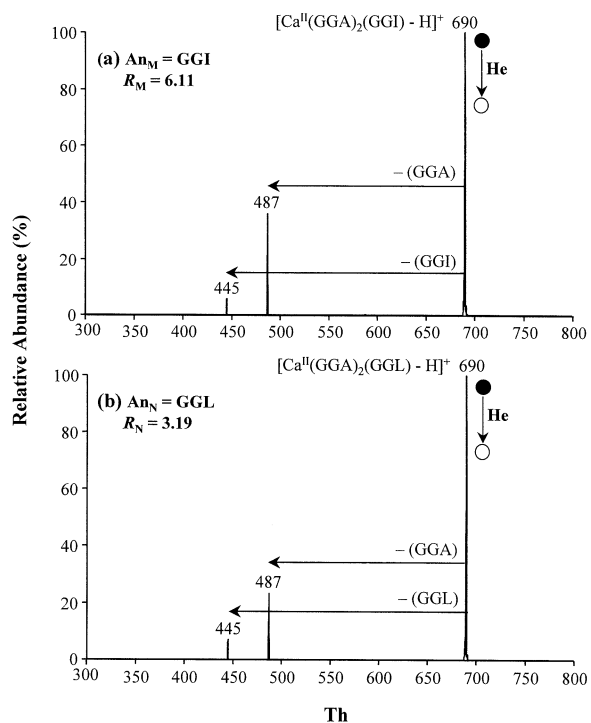quantify than positional isomeric peptides, the differentiation of this pair of tripeptides has been achieved using Ca^{II} as the central metal ion and GGA as the reference ligand. For this particular case, when An is pure GGI (An_M), the branching ratio R_M is 6.11 (Figure 3a), whereas R_N is 3.19 for pure GGL (An_N) (Figure 3b). The isomeric selectivity, R_{iso}, is therefore 1.92. The same procedure was applied to quantifying these two isobaric tripeptides, and the calibration curve has a correlation coefficient (r^2) of 0.9990 (Figure 5b). An "unknown" solution was prepared to test this calibration curve. The experimental results for binary mixtures of isomers (Table 1) show that as little as 1% isomeric fraction of GGI (or GGL) can be measured.

Determination of Compositions of Ternary Mixtures of Isomeric Tripeptides

Although quantification of binary mixtures of tripeptides has been successfully achieved, as shown in the previous section, three or more isomeric forms might need to be considered in the cases of tri- or higher peptides. It is worthwhile, therefore, to extend the method to investigate the possibility of determining the compositions of ternary mixtures of peptides.

With a linear relationship as shown above, a two-point calibration allows quantitative isomeric analysis
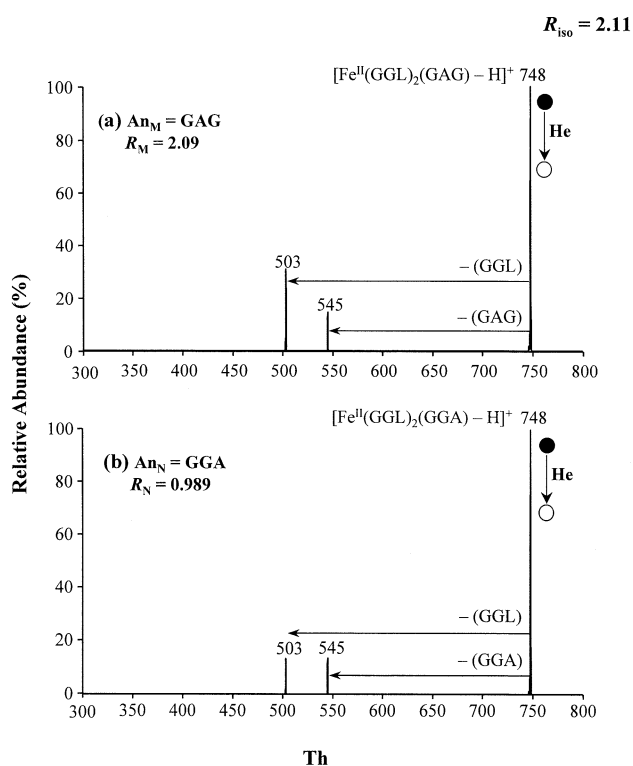
Figure 4. MS/MS product ion spectra of $[Fe^{II}(GGL)_2(An) - H]^+$ ($m/z = 748$). The analyte, An, is (**a**) GAG (An_M) and (**b**) GGA (An_N). The CID activation level was chosen as 12%, corresponding to ca. 300 mV zero-to-peak AC excitation amplitude.

in the binary mixture case. However, if a third isomer is present, it will also contribute to the branching ratio. Based on the extensive property of free energy change, for a ternary mixture with isomeric fractions of M, N,
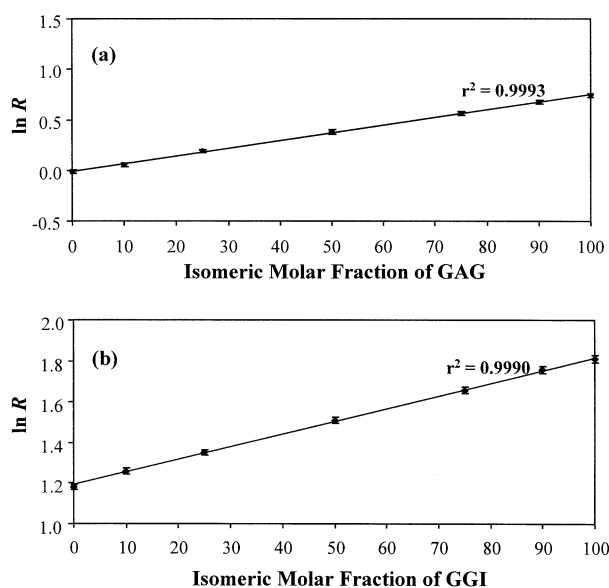

Figure 5. Calibration curve for isomeric analysis of (**a**) GAG/GGA using Fe^{II} as the central metal ion and GGL as the reference; and (**b**) GGI/GGL using Ca^{II} as the central metal ion and GGA as the reference.

Table 1. Quantification of a binary isomeric mixture GGI (M)/GGL (N)[a]

Sample		Actual (%)	Experimental (%)			Mean (uncertainty[b])
				Isomeric Fraction		
I	α_M	99	97.9	99.8	99.3	99.0 (1.0)
	α_N	1				
II	α_M	66	66.7	65.8	65.6	66.1 (0.5)
	α_N	34				
III	α_M	32	31.3	32.5	32.8	32.2 (0.8)
	α_N	68				
IV	α_M	1	1.92	0.462	0.511	0.964 (0.828)
	α_N	99				

[a]Using Ca^{II} as the central metal ion and GGA as the reference ligand.
[b]Uncertainty obtained based on triplicate measurements on separate occasions within 95% confidence level.

and O given by α_M, α_N, and α_O, respectively, one can write:

$$\Delta(\Delta G) = \alpha_M \cdot \Delta(\Delta G)_M + \alpha_N \cdot \Delta(\Delta G)_N$$
$$+ (1 - \alpha_M - \alpha_N) \cdot \Delta(\Delta G)_O \quad (11)$$

With this additional contribution, eq 10 takes the form shown as eq 12:

$$\ln R = \alpha_M \cdot \ln R_M + \alpha_N \cdot \ln R_N +$$
$$(1 - \alpha_M - \alpha_N) \cdot \ln R_O \quad (12)$$

If the contribution from the third isomeric peptide (O) is constant, it is expected that the two-point calibration procedure will be applicable. This situation might sometimes be encountered for chiral molecules having three possible forms (R, S, and meso).

A ternary mixture containing three isomeric tripeptides AGG (M), GGA (N), and GAG (O) was chosen for examination. Another tripeptide, GGI, was chosen as the reference ligand and Ni^{II} as the central metal ion. R values were measured for these ternary mixtures with various compositions of each tripeptide, as summarized in Table 2. Two-point calibration curves (Figure 6a) for $\ln R$ versus the molar fraction of the tripeptide AGG were constructed for cases in which GAG made up 0%, 25%, 50%, 75%, and in the extreme case, 100% of the sample as shown in Table 2. The slopes for lines **i**, **ii**, **iii**, and **iv** are 0.0140 ($\alpha_{GAG} = 0\%$), 0.0139 ($\alpha_{GAG} = 25\%$), 0.0140 ($\alpha_{GAG} = 50\%$), and 0.0139 ($\alpha_{GAG} = 75\%$), respectively. Within the margin of error these calibration curves are parallel to each other. The only significant difference is found in the intercepts, which shift up or down because of the varying contribution from the third isomeric peptide. The calibration curve (as illustrated in Figure 6b) for the binary system of GAG/GGA was also constructed using the values in Table 2. Although this curve represents an extreme case of a ternary mixture in which there is no AGG, it can be

Table 2. Measured branching ratios (R values) for ternary mixtures of AGG(An_M)/GGA(An_N)/GAG(An_O) using Ni^{II} as the central metal ion and GGI as the reference[a]

Isomeric Fraction (%)				
α_M	α_N	α_O	R (uncertainty[b])	ln R (uncertainty[b])
0	100	0	1.64 (0.03)	0.495 (0.019)
100	0	0	6.68 (0.13)	1.90 (0.02)
0	75	25	2.65 (0.05)	0.975 (0.018)
75	0	25	7.51 (0.15)	2.02 (0.02)
0	50	50	4.16 (0.08)	1.43 (0.02)
50	0	50	8.38 (0.08)	2.13 (0.01)
0	25	75	6.86 (0.14)	1.93 (0.02)
25	0	75	9.68 (0.09)	2.27 (0.01)
0	0	100	10.9 (0.11)	2.39 (0.01)

[a]Trimeric cluster ion at m/z = 750 was isolated with an isolation window width of 4.0 and further dissociated at the relative collision energy of 12% which corresponds ca. 300 mV AC excitation amplitude.
[b]Uncertainty obtained based on triplicate measurements of four samples using separate occasions within 95% confidence level.

applied to any case where the ratio of GGA/AGG varies, due to the parallel character of the calibration curves of Figure 6a. The values obtained from the binary curve in fact represent the intercepts of each calibration curves, **i**, **ii**, **iii**, **iv**, in Figure 6a, and these
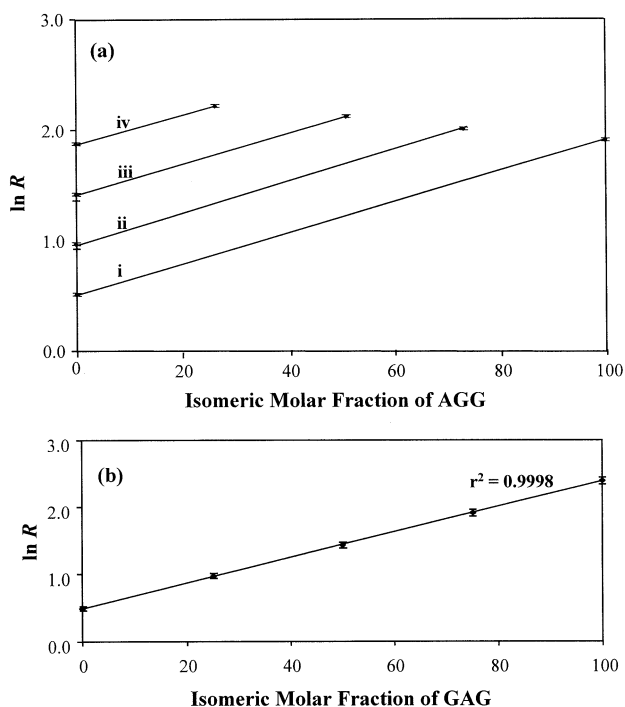

Figure 6. (**a**) Two-point calibration curves (constructed based on the data shown in Table 2) for isomeric analysis of GGA/AGG in a ternary mixture of GGA/AGG/GAG using Ni^{II} as the central metal ion and GGI as the reference: **i** (slope = 0.0140), **ii** (slope = 0.0139), **iii** (slope = 0.0140), and **iv** (slope = 0.0138) were obtained using a mixture containing GAG of 0%, 25%, 50%, and 75%, respectively; (**b**) calibration curve for ln R versus the molar fraction of GAG in the isomeric mixture of GGA/GAG/AGG (when α_{AGG} =0%) using Ni^{II} as the central metal ion and GGI as the reference.

Table 3. Quantification of a ternary mixture GGA (An_M)/GAG (An_N)/AGG (An_O)[a]

Sample		Isomeric Fraction				
		Actual (%)	Experimental (%)			Mean (uncertainty[b])
I	α_M	90	96.6	94.8	91.5	94.3 (2.6)
	α_N	8	7.2	5.6	2.9	5.23 (2.2)
	α_O	2				
II	α_M	8	10.2	9.81	12.6	10.9 (1.5)
	α_N	90	83.1	88.9	86.3	86.1 (2.9)
	α_O	2				
III	α_M	2	8.55	4.38	2.16	5.03 (3.24)
	α_n	2	0.23	0.502	0.184	0.305 (0.172)
	α_O	96				
IV	α_M	20	21.5	24.6	23.8	23.3 (1.6)
	α_N	30	25.7	27.1	26.9	27.2 (1.7)
	α_O	50				

[a]Using eqs 13 and 14 based on measurements on two separate systems (1) Ni^{II} as the central metal ion and GGI as the reference ligand; and (2) Co^{II} as the central metal ion and GGI as the reference ligand.
[b]Uncertainty obtained based on triplicate measurements on separate occasions within 95% confidence level.

values can be used to measure the isomeric fraction of AGG. In addition, the correlation coefficient in Figure 6b reflects the intrinsic relationship between ln R and the mole fraction of GAG, and it can be used to further check the accuracy of two-point calibration curves in Figure 6a.

Based only on eq 12 it is not possible to determine a ternary mixture of isomeric tripeptides, since only one measurement $R_{(Ni)}$ (or ln $R_{(Ni)}$) is made. In one such case (AGG, GGA, and GAG), the measured branching ratios of $R_{M(Ni)}$, $R_{N(Ni)}$, and $R_{O(Ni)}$ were equal to 1.90, 0.495, and 2.39, respectively. However, if the same analyte is also measured under *different* conditions, a second set of equations will be obtained and the system of two unknowns can be solved. Note that small changes in conditions are less useful than changes that include both the nature of the reference and the choice of metal ion. Such an orthogonal system using Co^{II} as the central metal ion and GGI as the reference was found to have $R_{M(Co)}$, $R_{N(Co)}$, and $R_{O(Co)}$ equal to 3.51, 1.26, and 5.00, respectively. In this example, eq 12 changes into eqs 13 and 14.

$$\ln R_{(Ni)} = 0.642 \cdot \alpha_M + 0.703 \cdot \alpha_N + 0.871$$
$$\cdot (1 - \alpha_M - \alpha_N) \qquad (13)$$

$$\ln R_{(Co)} = 1.26 \cdot \alpha_M + 0.231 \cdot \alpha_N + 1.61$$
$$\cdot (1 - \alpha_M - \alpha_N) \qquad (14)$$

This allows quantification of ternary mixtures comprised of several representative compositions of AGG (M), GGA (N), and GAG (O), with the results being summarized in Table 3. In comparison with a binary mixture, larger errors are associated with quantitation of a ternary mixture. The variances associated with
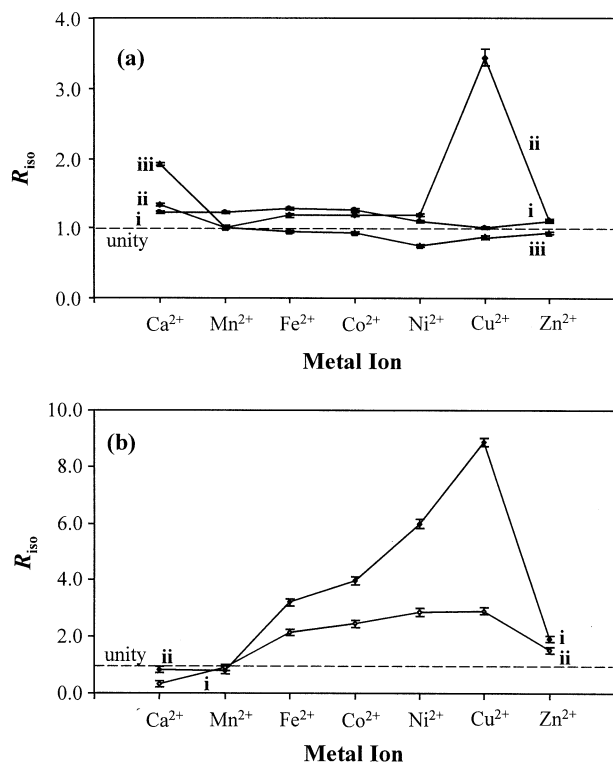
Figure 7. Metal effects on isomeric differentiation of (**a**) GGL/GGI using AGG (**i**), GAG (**ii**), and GGA (**iii**) as the reference ligands; and (**b**) GAG/GGA using GGI (**i**) and GGL (**ii**) as the reference ligands.

these measured isomeric compositions depend on the magnitude of the differences in the values of R_M, R_N, and R_O. It is expected that they will decrease by choosing a suitable (orthogonal) system, although detailed exploration of this point was not undertaken. A closely related treatment to that shown here has been used to quantify chiral compounds consisting of three possible optical isomers, as in the case of R-, S- and meso-tartaric acid [41].

Influence of Metal Ions on Differentiation of Isobaric Tripeptides

The dissociation behavior of metal-bound clusters will vary with the choice of metal ion, and hence the isomeric selectivity will vary. For the isobaric peptides GGL/GGI, there will be small differences in energy because of the difference in the C-terminus of the isobaric amino acids Leu/Ile. However, with an appropriate reference ligand, small differences in metal-bound properties of the isobaric peptides can be amplified into significant changes in $\Delta(\Delta G)$; added to this is the fact that even a small free energy change can be detected using the kinetic method. Taking GGI and GGL as An_M and An_N, respectively, different R_{iso} values are obtained using Ca^{II} and most of first-row transition metal ions and the reference ligands AGG (**i**), GAG (**ii**), and GGA (**iii**), as illustrated in Figure 7a. For

example, when using GGA (Figure 7a **iii**) as the reference ligand, Ca^{II} yields the largest isomeric distinction, while Ni^{II} gives relatively little isomeric differentiation, and none of the other metal ions studied allows obvious recognition. On the other hand, the isomeric selectivity is dramatically improved when using Cu^{II} as the central metal ion and GAG (Figure 7a **ii**) as the reference ligand. The choice of reference, AGG (Figure 7a **i**), yields no obvious discrimination for any of the divalent metal ions examined. The remarkably higher isomeric differentiation using Cu^{II} and GAG (Figure 7a **ii**) indicates that there are strong interactions between Cu^{II} and the peptide ligands in this cluster ion. This is suggested to be the result of agostic bonding [30] previously observed experimentally and confirmed by ab initio calculations in a kinetic method study of pyridine SiF_n^+ cluster ions [29]. Referring to bonding between the Cu^{II} center and the methyl group in alanine of the tripeptide GAG as α-agostic bonding, one notes that the methyl group on the β-carbon of isoleucine (I, the α-amino acid that has two chiral centers) might promote additional β-agostic bonding [42] with the central metal ion, in contrast to the case of GGL. Because agostic bonding depends on transition metal d-orbitals [30], it is believed that only when the central metal ion has specific coordination geometries is this bonding maximized. Since Cu^{II} has d^9 orbitals that are easily distorted [43], in comparison with other first-row transition metal ions, this might be the reason why Cu^{II} can more readily satisfy the directional requirements for agostic bond formation with H_2C–H, especially when alanine is the central amino acid as in the tripeptide GAG. This effect serves to further stabilize the dimeric clusters. As a result, an increase in isomeric selectivity is clearly illustrated in Figure 7a (**ii** for the differentiation of GGI/GGL using GAG as the reference ligand) and Figure 7b (**i** for the distinction of GGA/GAG using GGI as the reference ligand), when GAG and GGI are both bonded to Cu^{II}; this yields the most stable dimeric cluster ion and hence gives the largest isomeric recognition. Agostic bonding might also exist in the trimeric precursor ion. It is interesting to observe that the data plotted in Figures 7a (**ii**) and 7b (**i**) follow the Irving-Williams series [44], which is an empirical trend describing the stability of first-row transition metal-bound complexes [45], although we recognize that isomeric selectivity is directly related to the *difference* in stability of two dimeric complexes. Since isomeric distinction is the direct result of metal-ligand and ligand-ligand interactions, there is not a simple relationship between isomeric selectivity and thermochemical properties like proton affinity.

Conclusion

The kinetic method has been extended to quantitative measurements of mixtures containing three isomeric tripeptides, as demonstrated by the case of positional tripeptides GGA/GAG/AGG. This procedure can also

be applied to achieve chiral quantification of a ternary mixture of optical isomers, a topic of great interest in enantioselective synthesis. Besides the first-row transition metal ions, calcium has been used as the central metal ion for isomeric differentiation and quantification of binary and ternary peptide mixtures. When complexed with Cu^{II}, GAG can be easily distinguished from GGA, and it is also a good reference for quantifying the leucine/isoleucine containing tripeptide pair GGI/GGL. This is due to large chiral effects, probably resulting from dual independent agostic bonds (α-agostic bonding between Cu^{II} and GAG and β-agostic bonding between Cu^{II} and GGI). This bonding stabilizes Cu^{II}-bound dimeric product ions with appropriate coordination geometries. In future, the new fixed-ligand version of the kinetic method [38] will be used to improve quantitative accuracy by simplifying the dissociation kinetics and increasing the selectivity (by changing the properties such as the size and functionality of the fixed ligands). The successful analysis of tripeptides in binary and ternary mixtures in this study suggests that these procedures can be extended to more complex isomeric compounds in mixtures of greater complexity.

Acknowledgments

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