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# Copper(II)-Assisted Enantiomeric Analysis of D,L-Amino Acids Using the Kinetic Method: Chiral Recognition and Quantification in the Gas Phase

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**Abstract:** Chiral recognition of D- and L-amino acids is achieved and mixtures of enantiomers quantified in the gas phase, using the kinetics of competitive unimolecular fragmentations of trimeric Cu(II)-bound complexes. Singly charged copper(II)−amino acid cluster ions $[Cu^{II}(A)(ref^*)_2-H]^+$ (A = amino acid; ref* = chiral reference ligand, selected from among the natural α-amino acids) undergo competitive collision-induced dissociation (CID) in a quadrupole ion trap to form the dimeric complexes $[Cu^{II}(A)(ref^*)-H]^+$ and $[Cu^{II}(ref^*)_2-H]^+$. The abundance ratio of these fragment ions depends strongly on the stereochemistry of the ligands in the precursor $[Cu^{II}(A)(ref^*)_2-H]^+$ complex ion and specifically on the chirality of the analyte amino acid. The chiral selectivity, the ratio of the two fragment ion abundances for the complex containing one enantiomer of analyte expressed relative to that for the fragments of the corresponding complex containing the other enantiomer, ranges from 0.47 to 11. An energy quantity, $\Delta(\Delta Cu^{II}BDE)$, is predicted and shown to serve as a thermochemical indicator of chiral discrimination; its value is calculated from the fragment ion abundance ratios using the kinetic method of estimating thermochemical quantities from the kinetics of cluster ion dissociation. Large chiral distinctions are observed with all of the natural chiral α-amino acids, except cysteine and arginine, by appropriate choice of the reference ligand. The $\Delta(\Delta Cu^{II}BDE)$ values range from −2.2 to 6.9 kJ/mol. Amino acids with aromatic substituents display the largest chiral distinction, which is consistent with ligand exchange chromatographic results for analogous systems. The structures of the fragment Cu(II) complexes are discussed in the light of the CID behavior of related compounds. The interactions within these ions that might contribute to chiral recognition are rationalized to account for the observed chiral effects. The sensitive nature of the methodology and the linear relationship between the logarithm of the fragment ion abundance ratio and the optical purity, which is intrinsic to the kinetic method, allows mixtures to be analyzed for small enantiomeric excess (ee) by simply recording ratios of fragment ion abundances in a mass spectrum.

## Introduction

The importance of mass spectrometry in structural studies on biological systems is increasing rapidly, largely due to the emergence of electrospray ionization (ESI)[1] and matrix-assisted laser-desorption ionization (MALDI).[2] Specific intermolecular interactions are important in biological systems, for example, in the recognition of epitopes[3] and in antibody and enzymatic reactions.[4] Mass spectrometry, especially tandem mass spectrometry,[5] provides a unique method by which intrinsic interactions between molecules can be studied. This is because the interactions are studied in the absence (or using small controlled amounts)[6] of solvent and because of the intrinsic sensitivity, molecular specificity, and tolerance to impurities of the technique. Despite this obvious promise, the potential of mass spectrometry as a route to information on molecular stereochemistry is still far from being fully realized.

These considerations have led, during the past few years, to an increasingly active search for an effective methodology to characterize chiral compounds by using mass spectrometry.[7] Techniques for chiral analysis rely on differential interactions of enantiomers with a chiral reagent or different stabilities or reactivities of the diastereomers formed in such reactions. As an example, chiral analysis by NMR[8] employs chiral reagents, and chiral stationary phases are used in liquid chromatography.[9] Since the initial chemical ionization experiments of Fales and

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Wright[10] on chiral effects on the relative abundances of protonated dimers of dialkyl tartrates, many similar observations have been made.[11] Fast atom bombardment (FAB) mass spectrometry has frequently been the ionization method of choice used to examine stereoselective interactions between chiral analytes and chiral reference compounds.[11c,12] These experiments can be performed quickly, and the recognition of a chiral effect is based simply on ion abundance data. In related experiments, equilibrium ion abundances are measured for formation of a complex between the chiral analyte and a chiral auxiliary (reference compound). As an example, Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry has been used to measure equilibrium populations of clusters of chiral molecules, such as D- and L-dialkyl tartrates, after appropriate isotopic labeling to distinguish the two optical isomers. The measured equilibrium constants are directly related to the corresponding free-energy differences and are useful indicators of chiral composition.[7f,11d,13] A particularly noteworthy experiment is that of Vèkey et al.,[12c] who observed chiral effects in the dissociations of protonated trimeric cluster ions. On the basis of this observation, Yao and co-workers used ESI to generate protonated trimers and tandem mass spectrometry to distinguish enantiomers of amino acids.[14] Simultaneously, we published a preliminary communication describing the chiral resolution of amino acids using copper(II)-bound trimeric cluster ions.[15]

Although these and other earlier experiments have demonstrated the potential of mass spectrometry in chiral recognition, practical applications of chiral analysis, especially quantitative analysis, require several characteristics not previously available simultaneously. The following features are desirable: (i) a large chiral selectivity to allow quantitative analysis, (ii) there should be no requirement for isotopically labeled reagents, (iii) tandem mass spectrometry (MS/MS) should be used for its insensitivity to impurities, and (iv) the relative abundances of the adduct ions (the measure of enantioselectivity) should be independent of the relative concentrations of the analyte and the chiral reagent. All of these features are available by employing the general method proposed here in which the kinetics of the dissociation of clusters bound by transition metal ions, represented by copper(II)-bound complexes, is measured in a tandem mass spectrometry experiment. The method represents a particular application of the kinetic method,[16] an increasingly widely used method of estimating thermochemical quantities. Recognition and quantification of the chirality of the natural amino acids is the specific test case examined in this paper. In part, this interest is driven by the discovery of nonterrestrial enantiomeric compositions of meteoritic amino acids, a finding that has generated considerable excitement in the astrobiological community.[17]

To achieve chiral recognition, multiple-point interactions (the "three-point rule")[18] are required; this means that the chiral analyte and chiral reference need to be bound together in a polydentate complex, even if only transiently. Transition metal ions, for this reason, are much better choices to form such complexes than are proton or alkali metal cations, a fact which is well-known for the condensed phase.[19a] It has become increasingly evident that most intra- and intermolecular interactions in biological systems are metal-cation mediated.[19b] In particular, copper-bound proteins and amino acid complexes are of considerable interest in biological systems and have been widely studied.[20] Metal-ion−ligand interactions in the gas phase have also become a major area of research.[21] For example, kinetic energy release measurements have been used to distinguish diastereomeric oligosaccharides on the basis of differences in metal−ligand interactions.[7b,12a,22] The interactions of copper ions with biologically important peptides have been explored,[23] and the gas-phase copper(I) affinities of amino acids and diamines have been measured, either using the kinetic method[24] or using equilibrium measurements.[25]

Energy differences between diastereomeric complexes represent the fundamental basis for chiral distinction, but the effects of these differences on ion abundances might still be too small to be observed directly without special experimental techniques or methods of data evaluation.[26] Fortunately, the kinetic method[16,27] can be used to detect processes with small differences (<1 kJ/mol) in product ion stability. Competitive dissociation channels of a cluster ion, even with very small differences in critical energies for fragmentation, result in large changes in the respective rate constants, which are reflected in the fragment ion abundances. This is the result of the logarithmic relationship between relative ion abundances and energy that characterizes this method. Traditionally, the kinetic method has been used to estimate relative values of various thermochemical quantities, including proton affinity,[28] gas-phase basicity,[29]

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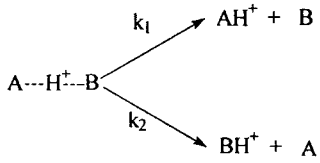
ionization energy,[30] and metal ion affinity.[21e,24,31] These measurements are based on the rates of competitive dissociations of mass-selected cluster ions. For a weakly bound cluster ion, monomer loss is often the dominant channel, as illustrated in eq 1, which describes a proton-bound dimer dissociating to give two individual protonated monomers.




(1)

For example, the rates of competitive dissociation, expressed as the relative abundances of the individual protonated monomers, can be used to determine the proton affinity difference between the two compounds. When the entropy changes for the two channels are equal, the relationship takes the form

$$\ln \frac{k_1}{k_2} = \ln \frac{[\mathrm{AH}]^+}{[\mathrm{BH}]^+} = \frac{\Delta PA}{RT_{\mathrm{eff}}} \qquad (2)$$

where $[AH]^+$ and $[BH]^+$ are the abundances of the two protonated monomers, $\Delta PA$ is the proton affinity difference between the two molecules A and B, and $T_{\mathrm{eff}}$ is the effective temperature of the activated proton-bound dimer. The derivation of this equation, discussion of effective temperature, and the conditions of its validity were presented elsewhere.[27] Given the sensitivity of cluster ion dissociation kinetics to small differences in ion stability, it is not surprising that the kinetic method allows enantiomeric differentiation.[12c,32] Earlier observations, some from this laboratory, however, have not been converted into a general method of recognizing and, particularly, of quantifying chirality. This is now done in this paper through a careful consideration of the structures and energetics of the metal ion−chiral ligand system. The treatment given here is in terms of energies, but a parallel treatment in terms of free energies is also possible. The kinetic method has been presented both ways.[16]

## Experimental Section

Most experiments were performed using a commercial LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA), equipped with an ESI source. Operating conditions for the ESI source were as follows: spray voltage, 5.00 kV; capillary voltage, 3 V; heated capillary temperature, 150 °C; tube lens offset voltage, 20 V; sheath gas ($N_2$) flow rate, 30 units (roughly 0.75 L/min). The experiments were conducted in the positive ion mode. Spectra shown represent the average of about 60 scans, each requiring 0.2 s. The sample was infused via a syringe pump at a flow rate of 1−2 $\mu$L/min. In the full-scan $MS^2$ and $MS^3$ modes, the parent ion of interest was first isolated by applying an appropriate waveform across the endcap electrodes of the ion trap to resonantly eject all trapped ions except those of the *m*/*z* ratio of interest. The isolated ions were then subjected to a supplementary ac signal to resonantly excite them and cause CID. The Mathieu $q_z$ values for resonance excitation and ejection were 0.25 and 0.83, respectively. The ion excitation time for CID was 30 ms with the amplitude of the excitation AC being optimized in each experiment and being kept the same for the measurements of D and L enantiomers (values range 0−100% relative collision energy corresponding to 0−2.5 V zero-to-peak resonant excitation potential). Mass/charge ratios (*m*/*z*) are reported using the Thomson unit (1 Th = 1 atomic mass per unit positive charge).[33]

Gas-phase Cu(II)−amino acid complexes were generated by electrospraying 50:50 water:methanol solutions containing a mixture of optically pure amino acids, 10 $\mu$M each, and 2.5 $\mu$M copper(II) chloride. D- and L-amino acids and cupric chloride dihydrate were obtained from Sigma Chemical (Milwaukee, WI) and methanol from Fisher (Pittsburgh, PA).

The FT-ICR experiments were performed using a 7-T Bruker BioApex Fourier transform mass spectrometer equipped with an Analytica ESI source and containing an intermediate hexapole ion storage region. Alanine cluster ions were formed by electrospraying alanine in a 50:49:1 water:methanol:acetic acid solution containing 5 $\mu$M $Cu(NO_3)_2$. The ESI capillary was held at room temperature, and low ambient gas flow rates and low capillary-skimmer voltage settings were used to minimize cluster ion fragmentation prior to storage in the hexapole trap. Protonated alanine monomers, dimers, and trimers, as well as singly charged Cu(II) dimers and trimers and low abundance of tetramers, were observed under these conditions. Storage in the hexapole for varying times led to partial or complete dissociation of higher oligomers. Isolation of alanine trimers and dimers in the ICR cell was performed individually to study the unimolecular dissociation of the relevant species.

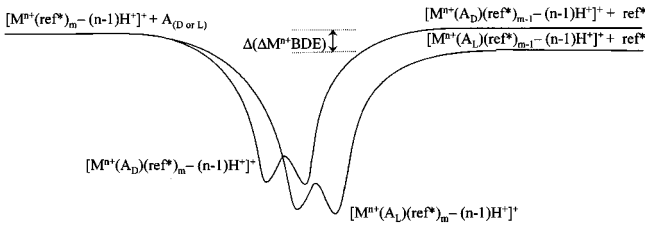



**Figure 1.** Energy diagram for dissociation of metal ion-bound cluster ions showing competitive dissociations of two deprotonated trimeric cluster ions that differ in the chirality of one ligand. The two cluster ions yield identical products by one fragmentation route and diastereomeric product ions with energies which differ by $\Delta(\Delta M^{n+}\mathrm{BDE})$ by the competitive ligand loss fragmentation.

## Results and Discussion

**Concept Underlying Transition Metal Ion-assisted Kinetic Resolution.** One enantiomer ($A_D$ or $A_L$; the (*R*), (*S*) convention is more appropriate for general chiral compounds, but the D, L configuration is so common for chiral amino acids that this terminology is used here) of a chiral analyte and a chiral reference compound (ref*) are complexed with a transition metal ion ($M^{n+}$) to form the $M^{n+}$-bound cluster ions $[M^{n+}(A_D)(\mathrm{ref}^*)_m]^{n+}$ or $[M^{n+}(A_L)(\mathrm{ref}^*)_m]^{n+}$, or in this study analogous forms such as the singly deprotonated ions $[M^{n+}(A_D)(\mathrm{ref}^*)_m - (n-1)H^+]^+$ and $[M^{n+}(A_L)(\mathrm{ref}^*)_m - (n-1)H^+]^+$.[34] Dissociation of the complex $[M^{n+}(A_D)(\mathrm{ref}^*)_m - (n-1)H^+]^+$ by competitive ligand losses produces dimeric ions $[M^{n+}(A_D)(\mathrm{ref}^*)_{m-1} - (n-1)H^+]^+$ and $[M^{n+}(\mathrm{ref}^*)_m - (n-1)H^+]^+$, while dissociation of $[M^{n+}(A_L)(\mathrm{ref}^*)_m - (n-1)H^+]^+$ generates $[M^{n+}(A_L)(\mathrm{ref}^*)_{m-1} - (n-1)H^+]^+$ and $[M^{n+}(\mathrm{ref}^*)_m - (n-1)H^+]^+$. The fragment ions from these complexes occur in abundance ratios

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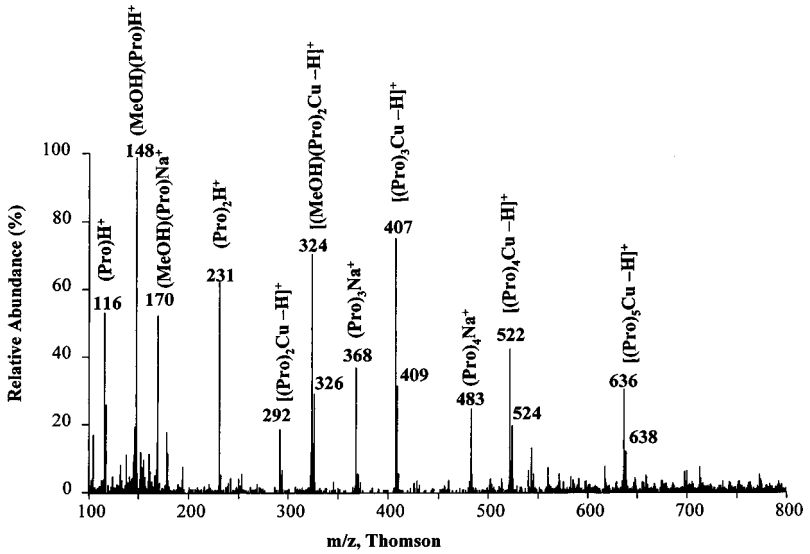


**Figure 2.** ESI mass spectrum of copper(II)−proline solution. Major cluster ions are indicated. Note the series of ions $[(Pro)_nCu^{II} - H]^+$ in which $n = 2-5$. The spectrum was recorded for a 50:50 water:methanol solution of 10 $\mu$M proline and 2.5 $\mu$M $CuCl_2 \cdot 2H_2O$.

$R_D$ and $R_L$, respectively, (eq 3 and 4), which are determined by the nature of the system and the CID conditions.

$$R_D = [M^{n+}(A_D)(ref^*)_{m-1} - (n-1)H^+]^+ / [M^{n+}(ref^*)_m - (n-1)H^+]^+ \quad (3)$$

$$R_L = [M^{n+}(A_L)(ref^*)_{m-1} - (n-1)H^+]^+ / [M^{n+}(ref^*)_m - (n-1)H^+]^+ \quad (4)$$

An energy diagram for the system is shown in Figure 1. (Figure 1 shows only the $[M^{n+}(A)(ref^*)_m - (n-1)H^+]^+$ system; the energy diagram for the alternative cluster system $[M^{n+}(A)_m(ref^*) - (n-1)H^+]^+$, which is discussed below, is analogous.) Note that the ions $[M^{n+}(A_D)(ref^*)_m - (n-1)H^+]^+$ and $[M^{n+}(A_L)(ref^*)_m - (n-1)H^+]^+$ need to be at least trimeric ($m \geq 2$) to dissociate to form diastereomeric fragment ions, $[M^{n+}(A_D)(ref^*)_{m-1} - (n-1)H^+]^+$ and $[M^{n+}(A_L)(ref^*)_{m-1} - (n-1)H^+]^+$, respectively. Note, too, that the results in this paper are restricted to the case of $m = 2$, $n = 2$, that is, to singly charged trimeric cluster ions. To simplify the interpretation of the kinetic data, it is desirable that losses of A and ref* from the precursor trimeric ion be competitive and also be the only fragmentation channels.

Under the circumstances just described, one can measure chiral selectivity $R_{chiral}$, which is the ratio of the individual ratios of fragment ion abundances, that is,

$$R_{chiral} = R_D/R_L = \frac{[M^{n+}(A_D)(ref^*)_{m-1} - (n-1)H^+]^+ / [M^{n+}(ref^*)_m - (n-1)H^+]^+}{[M^{n+}(A_L)(ref^*)_{m-1} - (n-1)H^+]^+ / [M^{n+}(ref^*)_m - (n-1)H^+]^+} \quad (5)$$

It is convenient to define the cluster ions as "homo" when the analyte A and the chiral reference, ref*, have the same chiral configuration, and "hetero" for opposite chiral configurations. Therefore, assuming ref* has the L configuration, a degree of chiral recognition, $R_{chiral} > 1$, means that the heterochiral fragment ion is more stable than the homochiral one and vice versa. The more different the $R_{chiral}$ value is from unity, the higher the degree of chiral recognition observed. $R_{chiral} = 1$ indicates no chiral discrimination, which means that the particular combination of transition metal ion and reference ligand fails to create stereochemically dependent interactions with the enantiomers under the observation conditions employed.

An alternative to the double measurement required to determine $R_{chiral}$ (eq 5) is to measure the single ratio $R$ of the rates of competitive fragmentations for the mixed system $[M^{n+}(A)(ref^*)_m - (n-1)H^+]^+$, where A is an analyte molecule of unspecified chirality selected randomly from a mixture of $A_D$ and $A_L$. The value in quantitative analysis of $R$, defined in eq 6, is shown later.

$$R = \frac{[M^{n+}(A)(ref^*)_{m-1} - (n-1)H^+]^+}{[M_{n+}(ref^*)_m - (n-1)H^+]^+} \quad (6)$$

Copper(II) complexes of the natural D- and L-amino acids were chosen to study the intrinsic contributions to chiral recognition. These are most simply evaluated if the effects examined are due only to the energy differences associated with chiral substitution, that is, due only to the energy term $\Delta(\Delta M^{n+}\text{-BDE})$ indicated in Figure 1 and defined below. Differences in the entropy requirements for trimer dissociation would complicate the interpretation of the kinetic data here, as in other cluster ion dissociation studies.[35] For this reason, it is common to evaluate such kinetic method results on the assumption that entropy effects on fragmentation are negligible.[16,27] This criterion is best satisfied for ligands of similar size and functionality. Therefore, optically active amino acids were selected as the reference ligands. The results will show that the assumption of negligible differences in the dissociation entropies is justified.

**Cluster Ion Formation and Dissociation.** Cu(II) complexes can be generated efficiently in the gas phase by electrospray ionization of aqueous methanol solutions of Cu(II).[36] The ESI spectra of Cu (II)−amino acid mixtures show singly charged cluster ions ($[Cu^{II}(A)_n - H]^+$ ($A$ = amino acid, $n = 2, 3, 4, 5$), typified by the case of a Cu(II) salt:proline mixture, the ESI mass spectrum of which is illustrated in Figure 2. Other ions, including proton and sodium-based cluster ions, as well as their

(35) (a) Cooks, R. G.; Koskinen, J. T.; Thomas, P. D. *J. Mass Spectrom.* **1999**, *34*, 85. (b) Drahos, L.; Vèkey, K. *J. Mass Spectrom.* **1999**, *34*, 79. (c) Armentrout, P. B. *J. Mass Spectrom.* **1999**, *34*, 74.

(36) (a) Gatlin, C. L.; Turecek, F.; Vaisar, T. *J. Am. Chem. Soc.* **1995**, *117*, 3637. (b) Lavanant, H.; Virelizier, H.; Hoppilliard, Y. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1217.

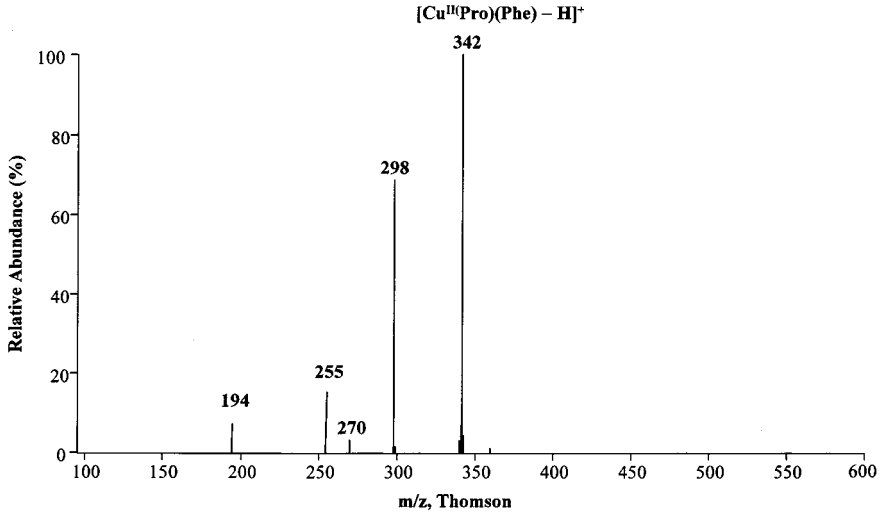


**Figure 3.** Tandem mass spectrometry ($MS^2$) product ion spectrum of cluster ion [$^{63}Cu^{II}$(L-Pro)(L-Phe) − H]$^{+\cdot}$. CID activation level was chosen as 12%, corresponding to approximately 300 mV AC.

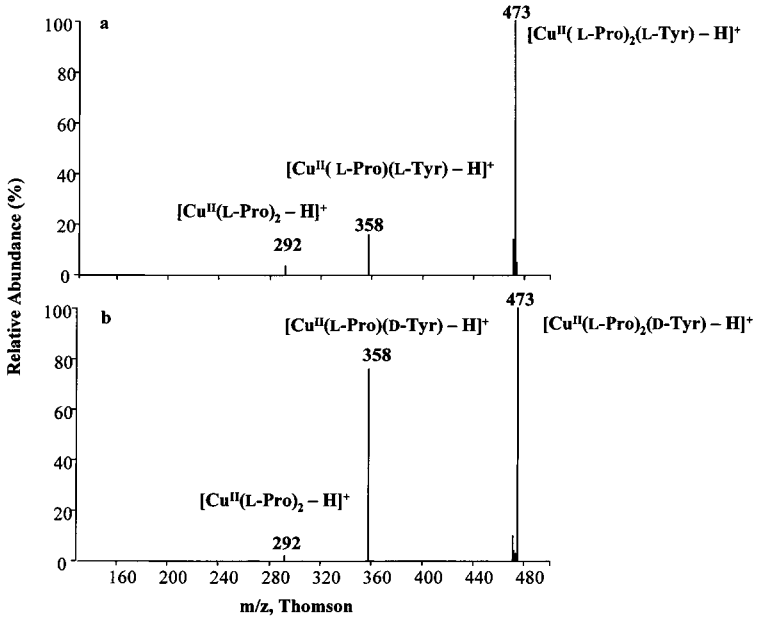


**Figure 4.** $MS^2$ product ion spectra of (a) [$^{63}Cu^{II}$(L-Pro)$_2$(L-Tyr) − H]$^{+\cdot}$(*m*/*z* 473); (b) [$^{63}Cu^{II}$(L-Pro)$_2$(D-Tyr) − H]$^{+\cdot}$ (*m*/*z* 473). CID activation level was chosen as 10%, corresponding to approximately 250 mV AC.

solvated forms, also occur. It appears that the relative abundances of copper(II)-bound amino acid cluster ions depend on the individual amino acids studied. For example, the Cu(II)-bound proline pentameric cluster ion ([$Cu^{II}(Pro)_5$ - H]$^+$) occurs in fairly high abundance in the spectrum of the Cu(II)−proline mixture (Figure 2), while even the trimeric Cu(II)-bound histidine cluster ion ([$Cu^{II}(His)_3$ − H]$^+$) is barely observed under similar conditions. The low abundance of the Cu(II)-bound histidine trimeric cluster ion is probably due to the bulky side chain of histidine and the saturation of Cu(II) coordination by its nitrogen atoms. No Cu(II)-bound monomers are observed from any of the copper(II)−amino acid solutions studied and few Cu(I)−amino acid ions are seen in the spectra.

Figure 3 shows the CID spectrum of a typical copper(II)-bound dimeric cluster ion, [$Cu^{II}$(Pro)(Phe) − H]$^+$. The main fragment ion arises by $CO_2$ loss in this tandem mass spectrometry experiment. Further fragmentations (loss of $C_2H_4$, $C_3H_7$•, and $C_6H_5CH{=}CH_2$) yield the ions *m*/*z* 270, 255, and 194, respectively, and are similar to those observed by Turecek et al. for singly charged Cu(II) complexes of deprotonated amino acids and 2,2′-bipyridyl ligand.[37] No Cu (II)-bound monomeric ions (i.e., fragment ions due to simple ligand loss) were observed, even at high collision energy, which suggests that the ligands are strongly bound in the copper(II) dimeric cluster ion.

By contrast with the behavior of the dimeric cluster ions, the Cu(II)-bound trimeric cluster ions fragment readily by simple ligand loss. These cluster ions appear to be tetracoordinate and to be more loosely bound, as evidenced by the $MS^2$ product ion spectra of [$Cu^{II}$(L-Tyr)(L-Pro)$_2$ − H]$^+$ (Figure 4a) and [$Cu^{II}$-(D-Tyr)(L-Pro)$_2$ − H]$^+$ (Figure 4b). In both spectra, the only fragments observed upon mild collisional activation are the two Cu(II)-bound dimeric cluster ions, [$^{63}Cu^{II}$(L-Pro)$_2$ − H]$^+$ (*m*/*z* = 292) and [$^{63}Cu^{II}$Tyr(L-Pro) − H]$^+$ (*m*/*z* = 358).

(37) (a) Gatlin, C. L.; Turecek, F.; Vaisar, T. *J. Mass Spectrom.* **1995**, *30*, 1605. (b) Gatlin, C. L.; Turecek, F.; Vaisar, T. *J. Mass Spectrom.* **1995**, *30*, 1617.

**Table 1.** Fragment Ion Abundance Ratio $[Cu^{II}(L\text{-Pro})(A) - H]^{+\cdot}/[Cu^{II}(L\text{-Pro})_2 - H]^{+\cdot}$ in the $MS^2$ Spectra of $[Cu^{II}(L\text{-Pro})_2(A) - H]^{+\cdot}$ [a,b]

| | $[Cu^{II}(L\text{-Pro})(A) - H]^{+\cdot}/[Cu^{II}(L\text{-Pro})_2 - H]^{+\cdot}$ | | | |
|---|---|---|---|---|
| A[a] | D-isomer | L-isomer | $R_{chiral}$ | α[c] |
| Tyr | 43 | 4.7 | 9.2 | 3.1 |
| Leu | 0.099 | 0.11 | 0.87 | 1.0 |
| Met | 60 | 33 | 1.8 | 1.15 |
| Phe | 16 | 2.1 | 7.4 | 2.9 |
| Thr | 0.89 | 0.88 | 1.0 | 1.75 |
| Asp | 4.5 | 3.2 | 1.4 | 1.3 |

[a] A = analyte amino acid. [b] CID activation level is chosen as 9.5%, corresponding to approximately 225 mV AC dipolar excitation. [c] Selectivity values (α) for chromatography adopted from ref 40, in which the stationary phase is L-proline-bonded silica and $\alpha = k_L'/k_D'$. See the original article for other chromatographic conditions.

**Chiral Recognition of Amino Acids Using Proline as the Chiral Reference.** The D- and L-isomers of tyrosine are easily distinguished using L-proline as a chiral auxiliary in tandem mass spectrometry experiments. This is evident from Figure 4. The most reliable method of quantifying these differences is to measure the ratios of the relative abundances of the two fragment ions. This procedure minimizes the effects of differences in experimental conditions, including the effects of differences in the internal energy distributions of the precursor ions that are being sampled, because it is based on competitive dissociations. The relative abundance ratios are 43 for the heterochiral and 4.7 for the homochiral Cu(II)-bound dimeric cluster ion. The chiral selectivity, $R_{chiral}$, namely, the ratio of the individual ratios, is 9.2.

Figure 4 shows that the heterochiral $[Cu^{II}(D\text{-Tyr})(L\text{-Pro}) - H]^+$ ion is much more stable than the homochiral $[Cu^{II}(L\text{-Tyr})(L\text{-Pro}) - H]^+$ ion, expressed relative to the $[Cu^{II}(L\text{-Pro})_2 - H]^+$ reference ion. Comparison of the precursor vs fragment ion abundances is an alternative method of quantitatively evaluating tandem mass spectra, but it is a less reliable one.[38] Fragment ion:precursor ion ratios, rather than the kinetic method treatment, were used by Yao et al. in their study of protonated trimers.[14] However, this measurement is more subject to internal energy effects and is less structurally diagnostic than is the branching ratio of fragment ion abundances.[38] A branching ratio measurement is a necessary aspect of the kinetic method procedure, but it is not the only important feature. As discussed below, the kinetic method procedure involves a logarithmic relationship between the branching ratio and the energy difference and, hence, the enantiomeric purity.

We selected six amino acids of four different types to evaluate the magnitude of the gas-phase chiral effects that are observed when proline is used as the chiral reference. This choice facilitates comparisons with the results observed in ligand exchange chromatography (LEC),[39] in which the Cu(II)-amino acid system is also commonly used. When L-proline was chosen as the chiral reference to form Cu(II)-bound trimeric cluster ions with the other amino acids, the clusters of interest were $[Cu^{II}(A)(L\text{-Pro})_2 - H]^+$. Therefore, $[Cu^{II}(L\text{-Pro})_2 - H]^+$ was the reference fragment ion generated upon CID in each of these cases. The six amino acids chosen for examination had proton affinities similar to that of proline, a requirement to have fragmentation occur to yield both product ions and, hence, to allow accurate relative abundance ratios to be measured. The detailed results are listed in Table 1.

**Table 2.** Influence of Reference Ligand on Chiral Selectivity[a,b]

| | | $[Cu^{II}(ref*)(A) - H]^{+\cdot}/[Cu^{II}(ref*)_2 - H]^{+\cdot}$ | | |
|---|---|---|---|---|
| A[a] | ref* | D-isomer | L-isomer | $R_{chiral}$ |
| Leu | L-Val | 2.4 | 2.5 | 0.95 |
| | L-Pro | 0.099 | 0.11 | 0.87 |
| | L-Phe | 0.96 | 0.41 | 2.3 |
| | L-Ser | 9.5 | 10 | 0.91 |
| Tyro | L-Pro | 43 | 4.7 | 9.2 |
| | L-Trp | 0.21 | 0.020 | 11 |
| | L-Met | 2.8 | 0.90 | 3.1 |
| | L-Glu | 16 | 8.0 | 2.0 |
| Met | L-Pro | 60 | 33 | 1.8 |
| | 4-OH-L-Pro | 59 | 33 | 1.8 |
| | L-Trp | 1.8 | 0.23 | 7.6 |
| | L-Glu | 27 | 18 | 1.5 |

[a] A = analyte amino acid. [b] CID activation level was chosen as 10%, corresponding to approximately 250 mV AC dipolar excitation.

**Comparisons with Chiral Resolution by Chromatography Using Copper(II)-Bound Proline Complexes.** L-Proline is often used in LEC as the chiral chelator due to its stereorigidity. In these experiments, it is immobilized on the stationary phase, and repeated interactions of the analyte with the stationary phase magnify small differences in binding energy and, hence, the partition coefficient associated with the stereoisomers. Experimental conditions, such as the solvent and the underlying support material, have a large influence on the chiral separation. Comparisons of the mass spectrometric method reported here with the LEC results are facilitated by the fact that L-proline was also the chiral auxiliary in the experiments that are summarized in Table 1.

The condensed-phase selectivity values (α) obtained from LEC chromatography[40] are compared with the gas-phase chiral selectivity, $R_{chiral}$, in Table 1. No more than a rough correlation of the chiral selectivity $R_{chiral}$ with the α values can be expected, given the enormous differences between the experiments. Nevertheless, in both cases, amino acids with aromatic side chains show a very strong chiral effect, but aliphatic amino acids (i.e., D- and L-leucine) show little, if any, stereochemical difference. An exception to the general correlation is that although amino acids with polar groups such as hydroxyl display fair resolution in LEC, no chiral distinction was observed with D- and L-threonine in the gas-phase proline complex experiments.

**Chiral Resolution of Other Amino Acids and the Influence of the Chiral Reference Ligand.** The absence of solvent or stationary phase in mass spectrometry means that direct interactions between the ligands serve as the basis for chiral differentiation. This enhances the importance of the electronic and steric effects of the chiral reference compound that serves as chiral auxiliary. Mixtures of D- and L-leucine, tyrosine, and methionine were tested with various chiral reference ligands selected from different types of amino acids. The chiral selectivity resulting from this series of experiments is given in Table 2. L-Valine and L-serine references, like L-proline (see Table 1), give low chiral selectivity (0.95 and 0.91, respectively) for D,L-leucine as analyte. On the other hand, with L-phenylalanine as the reference ligand, high chiral selectivity of D,L-leucine is achieved and the $R_{chiral}$ value reaches 2.3. In a similar manner for the selectivity of D,L-methionine, although L-proline,

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(39) (a) Davankov, V. A.; Navratil, J. D.; Walton, H. F. *Ligand Exchange Chromatography*; CRC Press: Boca Raton, FL, 1988. (b) Davankov, V. A. *J. Chromatogr. A* **1994**, *666*, 55.

(40) Gübitz, G.; Jellenz, W.; Santi, W. *J. Chromatogr.* **1981**, *203*, 377.

**Table 3.** Degree of Chiral Recognition of Natural Chiral α-Amino Acids[a,b]

| A[a] | ref* | $[Cu^{II}(ref^*)(A) - H]^{+\cdot}/[Cu^{II}(ref^*)_2 - H]^{+\cdot}$ D-isomer | $[Cu^{II}(ref^*)(A) - H]^{+\cdot}/[Cu^{II}(ref^*)_2 - H]^{+\cdot}$ L-isomer | $R_{chiral}$ | $\Delta(\Delta Cu^{II}BDE)$[c] (kJ/mol) |
|---|---|---|---|---|---|
| Ala | L-Phe | 0.049 | 0.024 | 2.0 | 2.1 |
| Val | | 0.75 | 0.17 | 4.5 | 4.3 |
| Leu | | 0.96 | 0.41 | 2.3 | 2.5 |
| Ile | | 1.7 | 0.36 | 4.8 | 4.6 |
| Pro | | 12 | 2.2 | 5.3 | 4.9 |
| Asp | | 3.0 | 1.1 | 2.7 | 2.9 |
| Glu | | 11 | 3.7 | 3.1 | 3.3 |
| Ser | | 0.28 | 0.18 | 1.5 | 1.2 |
| Thr | | 1.4 | 0.76 | 1.8 | 1.7 |
| Cys[d] | | | | | |
| Met | L-Trp | 1.8 | 0.23 | 7.6 | 5.9 |
| Phe | | 0.11 | 0.013 | 8.3 | 6.2 |
| Tyr | | 0.21 | 0.019 | 11 | 6.9 |
| Asn | | 6.1 | 3.3 | 1.8 | 1.7 |
| Gln | | 50 | 7.3 | 6.8 | 5.6 |
| Trp | L-Asn | 6.1 | 3.3 | 1.8 | 1.7 |
| His | L-Arg | 0.022 | 0.046 | 0.47 | −2.2 |
| Lys | L-His | 0.91 | 1.6 | 0.56 | −1.7 |

[a] A = analyte amino acid. [b] CID activation level was chosen as 10%, approximately 250 mV AC dipolar excitation. [c] $\Delta(\Delta Cu^{II}BDE)$ as defined in eq 10. [d] Cysteine is oxidized to cystine.

4-hydroxyl-L-proline, and L-glutamic acid give only fair selectivity, much higher chiral selectivity is obtained when L-tryptophan is employed as the reference ligand. As shown above, excellent selectivity is achieved for D,L-tyrosine with proline as the reference ligand. However, even greater chiral selectivity was observed when L-tryptophan was used as the reference ligand, the $R_{chiral}$ value being 11. The selectivity decreased dramatically when L-methionine or L-glutamic acid was chosen as the chiral reference. The overall results clearly show that chiral amino acids with an aromatic side chain are good choices as reference ligands in the gas-phase trimeric copper(II)−amino acid system.

The promising results just described encouraged us to examine all natural chiral α-amino acids by selecting aromatic amino acids as reference ligands. The choice of appropriate chiral references among the different aromatic amino acids was based on their proton affinities, which served in lieu of the (unknown) Cu(II) affinities to judge whether the ligands were sufficiently similar. This similarity allows the complexes to form easily and it also allows accurate relative abundance ratios to be measured; otherwise, dissociation proceeds overwhelmingly to form only the more stable product. Tryptophan and basic amino acids have high proton affinities and also high affinities toward the copper ion, so the choice of reference ligands in these cases can only come from among the basic amino acids. Large chiral recognition was achieved for D,L-tryptophan, histidine, and asparagine, when appropriate choices of the reference ligands are made. As shown in Table 3, all the natural chiral α-amino acids, except cysteine and arginine, give high chiral selectivity with appropriate reference ligands. Arginine has the highest affinity to the copper ion. No suitable chiral reference ligand can be selected from the natural amino acids to give accurate relative abundance ratios, and it will require an alternative type of reference compound. Cysteine differs from the other amino acids in that it does not give the corresponding $[Cu^{II}Cys(ref^*)_m - H]^+$ ion; instead, it is oxidized to cystine by Cu(II) ions in solution. All five D- and L-aliphatic amino acids, which are usually hard to resolve by chromatography,[39a] are differentiated by a chiral selectivity $R_{chiral}$ of at least 2.0 when using L-phenylalanine as reference. Although it is hardly possible to find strict regularities governing the selectivity achieved, it is still quite obvious that enantiomeric selectivity increases with the size of α-carbon side chains, as shown in the case of amino acids with aliphatic side chains. As expected, D- and L-phenylalanine and D- and L-tyrosine with their aromatic side chains give the best selectivity ($R_{chiral}$ values are 8.3 and 11, respectively) with tryptophan as the reference ligand. By contrast, amino acids having aliphatic side-chains are resolved to a poorer extent than their analogues. Glutamine and methionine show much larger chiral distinctions, which suggests that the functional groups on their side chains may interact with the copper(II) ion. These points are discussed further below.

The reproducibility of the present method was investigated using the corresponding isomers (D-isomers) as the chiral references. The complex ions $[Cu^{II}(A_L)(ref^*_D) - H]^+$ are enantiomers of $[Cu^{II}(A_D)(ref^*_L) - H]^+$ and, therefore, the two systems should display reciprocal $[Cu^{II}(A)(ref^*) - H]^+/[Cu^{II}(ref^*)_2 - H]^+$ ratios. This is, indeed, the case within ca. 3% error; the abundance ratios from consecutive measurements of the same system were also reproducible to ca. 3%. The complex ions $[Cu^{II}(A_D)(ref^*_D) - H]^+$, likewise, exhibit behavior that is identical to their enantiomers $[Cu^{II}(A_L)(ref^*_L) - H]^+$. The identical behavior of the clusters generating two homochiral enantiomers or the two heterochiral $[Cu^{II}(A)(ref^*) - H]^+$ enantiomers justifies the use of the present method and reflects its high reproducibility. Upon the basis of these observations and by using either the D- or L-isomer of the reference compounds, the method can be applied for quantitative analysis for enantiomeric excess (ee) of the amino acids (see Figure 6b and the corresponding discussion below).

**Isotopically Labeled Reference Compounds.** Isotopically labeled reference compounds have seen frequent use in mass spectrometric studies of chiral compounds, particularly those that use a single stage of mass analysis rather than tandem experiments.[11c,11d] As an alternative to the main methodology, we have examined the applicability of this approach to the present tandem mass spectrometry experiments because an amino acid can serve as its own chiral reference, if appropriately labeled. Although this approach involves the extra effort of the synthesis of isotopically labeled compounds, it maximizes the similarity between analyte and reference, a highly desirable situation in quantitative applications of the kinetic method. Nevertheless, this is not the most important feature to consider in the choice of reference ligand for chiral analysis, as illustrated in Figure 5, in which the method of isotopic labeling is extended to the Cu(II) trimeric cluster ion dissociation procedure. No enantioselectivity is observed when attempts are made to differentiate D- and L-alanine, either in FT-ICR experiments on $[Cu^{II}(D\text{-Ala})(L\text{-Ala-}d_3)_2 - H]^+$ (Figure 5a) or in quadrupole ion-trap experiments on the isotopomer $[Cu^{II}(D\text{-Ala})(L\text{-Ala-}d_4)_2 - H]^+$ (Figure 5b). The relative abundance ratios of the two fragment ions formed by ligand loss are statistical (2:1) in both of these experiments. In both cases, the reference ligand is alanine itself and the lack of chiral differentiation is consistent with the observations that have already been made regarding alkyl-substituted amino acids as chiral auxiliaries and is ascribed to insufficiently strong ligand interactions in the dimeric cluster ion.

In contrast, a trimeric complex composed of a single L-phenylalanine ligand as reference and having both D- and L-alanine ligands does allow differentiation of the enantiomeric alanine ligands, provided they are isotopically labeled (Figure 5c). The chiral selectivity (in this case, simply the relative

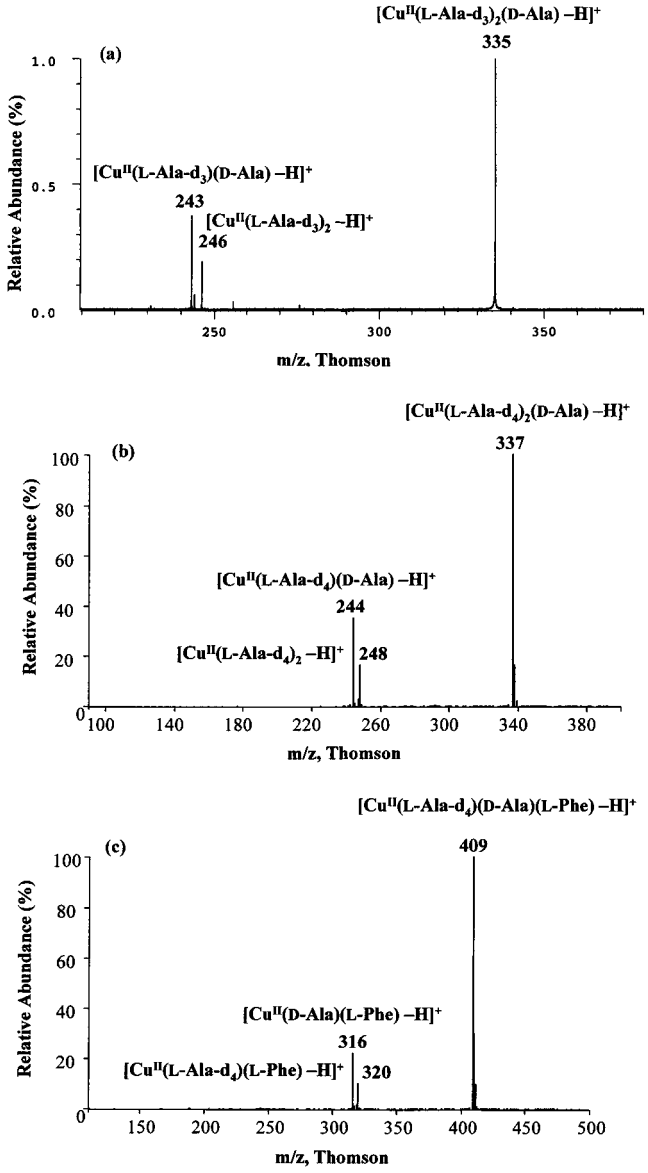



**Figure 5.** (a) MS$^2$ product ion spectrum of cluster ion [$^{63}$Cu$^{II}$(D-Ala)-(L-Ala-$d_3$)$_2$ − H]$^{+\cdot}$ after 3-s decay, performed using an FT-ICR mass spectrometer; (b) MS$^2$ product ion spectrum of cluster ion [$^{63}$Cu$^{II}$(D-Ala)(L-Ala-$d_4$)$_2$ − H]$^{+\cdot}$ performed using a quadrupole ion-trap mass spectrometer; (c) MS$^2$ product ion spectrum of cluster ion [$^{63}$Cu$^{II}$(D-Ala)(L-Ala-$d_3$)(L-Phe) − H]$^{+\cdot}$ performed using a quadrupole ion-trap mass spectrometer. CID activation level was chosen as 10%, corresponding to approximately 250 mV AC.

abundance of [Cu$^{II}$(D-Ala)(L-Phe) − H]$^+$ to [Cu$^{II}$ (L-Ala-$d_4$)(L-Phe) − H]$^+$) is not the statistical ratio of 1.0, but is 2.0, which is exactly the same as the above result obtained in two separate measurements without using labeled alanine. Figure 5c, therefore, suggests that chiral analysis might be performed by using complexes having one chiral auxiliary ligand, with the chiral purity being read directly from the relative abundance ratio of two fragment ions in one mass spectrum. This procedure requires isotopic labeling and so is less practical. As will be shown in the section on quantification, the same aim can be achieved without using isotopically labeled chiral auxiliaries or analytes.

**Thermochemical Measurement of Chiral Discrimination.** The degree of chiral discrimination can be evaluated in terms of the underlying thermochemistry using the kinetic method.[27] Dissociation of copper(II)-bound trimeric cluster ions occurs

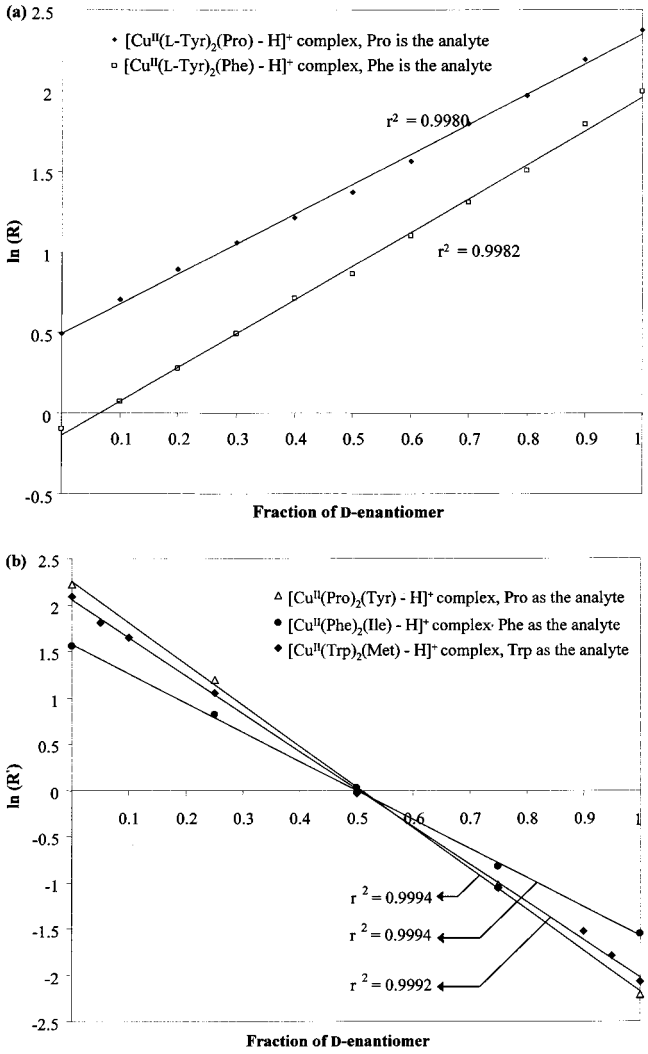
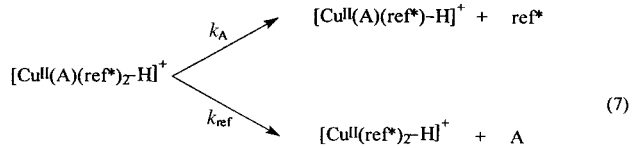



**Figure 6.** Calibration curves for chiral analysis of various amino acids using (a) single ratio method with the [Cu$^{II}$(A)(ref*)$_2$ − H]$^+$ system; (b) quotient-ratio method with the [Cu$^{II}$(A)$_2$(ref*) − H]$^+$ system (A = analyte amino acid presents as a chiral mixture, and ref* is the chiral reference).

as shown previously in Figure 1 and, more specifically, in eq 7.




(7)

The relative rates of the two competitive dissociations ($k_A$ and $k_{ref}$) can be expressed as the relative abundance ratio [Cu$^{II}$(A)-(ref*) − H]$^+$/[Cu$^{II}$(ref*)$_2$ − H]$^+$, namely,

$$R = k_A/k_{ref} = [Cu^{II}(A)(ref^*) - H]^+/[Cu^{II}(ref^*)_2 - H]^+ \quad (8)$$

From the kinetic method, the natural logarithm of the ratio of rate constants, $R$, is proportional to the differences between the Cu(II) affinities of the two dimeric products of eq 7, that is,

$$\ln R = \frac{(\Delta Cu^{II}BDE((A)(ref^*)) - \Delta Cu^{II}BDE(ref^*)_2)}{\mathbf{R}T_{eff}} \quad (9)$$

where **R** in the denominator is the gas constant, and ΔCu$^{II}$-

BDE((A)(ref*)) is defined as the energy change for the reaction

$$A + ref^* + Cu^{2+} \rightarrow [Cu^{II}(A)(ref^*) - H]^+ + H^+ \quad (10)$$

and the designation $\Delta Cu^{II}BDE$ is used to recognize that the reaction involves both deprotonation and binding to copper.

The difference in copper(II) affinities, $\Delta(\Delta Cu^{II}BDE)$, is related to the experimentally measured ion abundance ratio $R_{chiral}$ according to eq 11

$$\Delta(\Delta Cu^{II}BDE) = \Delta Cu^{II}BDE[(A)(ref^*)_{hetero}] - \Delta Cu^{II}BDE[(A)(ref^*)_{homo}] = \mathbf{R}T_{eff}\ln(R_{chiral}) \quad (11)$$

The energy quantity, $\Delta(\Delta Cu^{II}BDE)$, reflects the stereochemical interactions in the cluster ions. The effective temperature of the trimeric cluster is estimated to be approximately 350 K from a study by Feng et al. of the lithium and sodium ion binding energies of amino acids in an ion trap.[41] The $\Delta(\Delta Cu^{II}BDE)$ values for 17 amino acids are listed in Table 3. Large positive $\Delta(\Delta Cu^{II}BDE)$ values, as observed for both tyrosine and phenylalanine, using tryptophan as the reference ligand, indicate that the heterochiral Cu(II)-bound dimeric complexes are much more stable than their homochiral analogues. Heterochiral Cu(II)-bound complexes in this table are more stable than the homochiral ones for all of the amino acids measured except two, in which, surprisingly, histidine is involved as either the analyte or the reference. In these two cases, dimeric cluster ions exhibit higher stability when the ligands are of the same chirality. Intrinsic interactions that lead to chiral distinction will be discussed further below.

**Quantitative Measurements of Enantiomeric Purities of Amino Acids.** The large chiral distinctions that are achieved and the sensitive nature of the kinetic method allow quantitative measurement of the optical purity of amino acid samples. In making such measurements using trimeric complexes of the type $[Cu^{II}(A)(B)_2 - H]^+$ and $[Cu^{II}(A)_2(B) - H]^+$, it is possible to consider either A or B as the analyte, that is, to examine $[Cu^{II}(A)(ref^*)_2 - H]^+$ or $[Cu^{II}(A)_2(ref^*) - H]^+$. For quantitative analysis, the only procedural difference is which complex is selected. A more subtle point is that a choice of a complex containing two molecules of analyte requires measurement of a quotient of two ratios ($R'$) whereas only a single ratio ($R$) needs to be measured for trimeric clusters containing one molecule of analyte. We now show that both procedures for chiral distinction (two ligands of the reference compound or the inverse, two ligands of the analyte) do, in fact, give good quantitative results.

In all of the experiments, calibration studies of the behavior of the complexes containing the enantiomerically pure chiral reference and the analyte in various states of chiral purity are performed. For complexes of the type $[Cu^{II}(A)(ref^*)_2 - H]^+$, the enantiomeric purity of the analyte was measured from the ratio of the two fragment ions in a single tandem (MS/MS) spectrum (single ratio method) after appropriate calibration. Such measurements are illustrated by choosing L-tyrosine as the reference and proline and phenylalanine as the analyte, respectively, that is, by examining the complexes $[Cu^{II}(Pro)(L\text{-}Tyr)_2 - H]^+$ and $[Cu^{II}(Phe)(L\text{-}Tyr)_2 - H]^+$ as the precursor ions. The results (Figure 6a) show a linear correlation of ln($R$) versus the molar fraction of D-enantiomer of the analytes. Such a linear correlation is expected when one considers the ln($R$) vs energy correlation of the kinetic method (eq 9). At a fixed collision energy (i.e., constant $T_{eff}$), ln($R$) is linearly proportional to the $\Delta Cu^{II}BDE$ difference between the two dimeric fragment ions. The $\Delta Cu^{II}BDE$ difference reflects the stereochemical interactions in the cluster ions, and if it is linearly proportional to the optical purity, then the observed linear relationship between ln($R$) and the optical purity of the analyte is accounted for.[42]

The singular advantage of this simple ratio method is that quantitative chiral analysis is performed by measuring a branching ratio of two fragment ions in a single spectrum after appropriate calibration. In only one case among many examples of the application of this method, that of $[Cu^{II}(Tyr)(L\text{-}Pro)_2 - H]^+$, was an unexplained nonlinear relationship observed. The measurement allows accurate determinations of optical purity of proline and phenylalanine, as demonstrated by the high correlation coefficients ($r^2$) of 0.9980 and 0.9982, respectively; measurement of ee values as small as 2% with an uncertainty of the same magnitude is clearly possible.

The quantification of amino acids can also be achieved by studying complexes of the type $[Cu^{II}(A)_2(ref^*) - H]^+$. Such measurements have been made for three representative copper(II)-bound complexes: (i) the $[Cu^{II}(Pro)_2(Tyr) - H]^+$ system, with proline as the chiral analyte (two moles) and tyrosine as the reference (1 mole); (ii) the $[Cu^{II}(Phe)_2(Ile) - H]^+$system, with phenylalanine as the analyte and isoleucine as the reference; and (iii) the $[Cu^{II}(Trp)_2(Met) - H]^+$system, with tryptophan as the analyte and methionine as the reference. In these experiments, the analytes proline, phenylalanine, and tryptophan, respectively, were used in mixtures of various optical purities with the optically pure reference amino acids (D- and L-enantiomers, examined separately) being used to generate the corresponding trimeric copper clusters, the dissociation behavior of which is summarized in Figure 6b. In this quotient ratio method, every calibration point was obtained by two consecutive measurements using optically pure D- and L-reference compounds. As expected, $R'$ was largest when pure D- or L-analyte was examined and, as also expected, the $R'$ value was equal to 1 ($\ln(R') = 0$) when the racemic analyte was examined. Linear relationships of ln($R'$) versus the molar fraction of D-isomer were obtained, and they showed correlation coefficients ($r^2$) of 0.9994 for both the Cu(II)-Pro-Tyr and Cu(II)-Phe-Ile systems, as illustrated in Figure 6b. The sensitivity to small differences in optical purity is high, as demonstrated by the Cu(II)-Trp-Met system in which the correlation coefficient $r^2$ is 0.9992, with the measured optical purity of the D- or L-isomers as low as 5% (Figure 6b). The linear correlation observed for this type of complex can again be explained on the basis of the kinetic method derivation. When the D-reference isomer is used, $R'_D = [Cu^{II}(A)(ref^*_D) - H]^+/[Cu^{II}(A)_2 - H]^+$, while $R'_L = [Cu^{II}(A)(ref^*_L) - H]^+/[Cu^{II}(A)_2 - H]^+$ for the L-reference. Therefore,

$$R' = R'_D/R'_L = \frac{[Cu^{II}(A)(ref^*_D) - H]^+/[Cu^{II}(A)_2 - H]^+}{[Cu^{II}(A)(ref^*_L) - H]^+/[Cu^{II}(A)_2 - H]^+} \quad (12)$$

where the quotient ratio $R'$ is analogous, but not identical, to $R$ as defined in eq 8 simply because of the change in the type of complex studied, that is, $[Cu^{II}(A)_2(ref^*) - H]^+$ vs $[Cu^{II}(A)$-

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(42) An alternative justification for such a semilog relationship is simply that differences in enantiomeric composition cause differences in critical energies of dissociation in an additive fashion and that these are transformed in a logarithmic fashion into differences in rates, hence, the overall semilog relationship. A detailed treatment is included in the Supporting Information.

**Table 4.** Fragment Ion Abundance Ratios of $[Cu^{II}(A)(ref^*) - H]^{+\cdot}/[Cu^{II}(ref^*)_2 - H]^{+\cdot}$ in the $MS^2$ Spectra of $[Cu^{II}(ref^*)_2((a) - H]^{+\cdot}$ at Different Relative Concentration Ratios of Analyte (A) and Chiral Reference (ref*)[a]

| [ref*]/[A] in the solution | 4:1 | 2:1 | 1:1 | 1:2 | 1:4 |
|---|---|---|---|---|---|
| [(L-Phe)(L-Thr)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Phe)$_2Cu^{II}$ − H]$^{+\cdot}$ | 0.77 | 0.77 | 0.76 | 0.78 | 0.75 |
| [(L-Phe)(L-Pro)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Phe)$_2Cu^{II}$ − H]$^{+\cdot}$ | 1.9 | 2.2 | 2.2 | 2.4 | 2.9 |
| [(L-Pro)(L-Tyr)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 4.2 | 4.2 | 4.2 | 4.2 | 4.3 |
| [(L-Pro)(D-Tyr)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 36 | 37 | 39 | 38 | 43 |
| [(L-Pro)(L-Phe)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 1.8 | 1.8 | 1.9 | 1.9 | 1.9 |
| [(L-Pro)(L-Leu)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 0.13 | 0.13 | 0.13 | 0.12 | 0.10 |
| [(L-Pro)(L-Met)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 23 | 27 | 26 | 28 | 34 |
| [(L-Pro)(L-Thr)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 0.87 | 0.89 | 0.87 | 0.88 | 0.85 |
| [(L-Pro)(L-Asp)$Cu^{II}$ − H]$^{+\cdot}$/[(L-Pro)$_2Cu^{II}$ − H]$^{+\cdot}$ | 2.0 | 3.0 | 2.9 | 3.0 | 3.0 |

[a] CID activation level was chosen as 10%, corresponding to approximately 250 mV AC dipolar excitation.

**Table 5.** CID Spectra of $^{63}Cu$-bound Dimeric Cluster Ions[a]

| cluster ion (*m*/*z*) | fragments *m*/*z* (dissociation pathway) |
|---|---|
| $[Cu^{II}(Pro)_2 - H]^{+\cdot}$ (292) | 248 ($-CO_2$); 220 ($-CO_2$, $-C_2H_4$); 205 ($-C_3H_7$, $-CO_2$) |
| $[Cu^{II}(Phe)_2 - H]^{+\cdot}$ (392) | 348 ($-CO_2$); 244 ($-C_6H_5CH{=}CH_2$, $-CO_2$) |
| $[Cu^{II}(Pro)(Phe) - H]^{+\cdot}$ (342) | 298 ($-CO_2$); 270 ($-CO_2$, $-C_2H_4$); 255 ($-C_3H_7$, $-CO_2$); 194 ($-C_6H_5CH{=}CH_2$, $-CO_2$) |
| $[Cu^{II}(Pro)(^{13}C_1{-}Phe) - H]^{+\cdot}$ (343) | 299 ($-CO_2$); 298($-^{13}CO_2$); 271 ($-CO_2$, $-C_2H_4$); 256 ($-C_3H_7$, $-CO_2$); 194 ($-C_6H_5CH{=}CH_2$, $-^{13}CO_2$) |
| $[Cu^{II}(Pro)(Phe\text{-}OCH_3) - H]^{+\cdot}$ (356) | 312 ($-CO_2$); 284 ($-CO_2$, $-C_2H_4$); 269 ($-C_3H_7$, $-CO_2$) |
| $[Cu^{II}(Pro\text{-}OCH_3)(Phe) - H]^{+\cdot}$ (356) | 312 ($-CO_2$); 208 ($-C_6H_5CH{=}CH_2$, $-CO_2$) |
| $[Cu^{II}(Pro\text{-}OCH_3)_n(Phe\text{-}OCH_3)_{2-n} - H]^{+\cdot}$ ($n = 0{-}2$) | not observed |
| $[Cu^{II}(Ac{-}Pro)_2 - H]^{+\cdot}$ (376) | 332 ($-CO_2$) |
| $[Cu^{II}(Ac{-}Pro)(Phe) - H]^{+\cdot}$ (384) | 340 ($-CO_2$) |
| $[Cu^{II}(Ac{-}Pro)(Pro\text{-}OCH_3) - H]^{+\cdot}$ (348) | 304 ($-CO_2$) |
| $[Cu^{II}(Ac{-}Pro)(Phe\text{-}OCH_3) - H]^{+\cdot}$ (398) | 354 ($-CO_2$) |

[a] CID activation level was chosen as 10%, approximately 250 mV AC dipolar excitation.

$(ref^*)_2 - H]^+$. According to the simple form of the kinetic method, the natural logarithm of the ratio of rate constants, ln($R'$), is proportional to the difference between the $\Delta Cu^{II}BDE$ values for formation of the two dimeric products, that is,

$$\ln(R') = \frac{\Delta Cu^{II}BDE[(A)(ref^*_D)] - \Delta Cu^{II}BDE[(A)(ref^*_L)]}{\mathbf{R}T_{eff}} \quad (13)$$

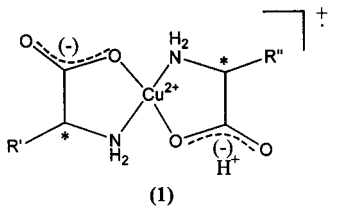
By analogy with the argument already employed for complexes of the type $[Cu^{II}(A)(ref^*)_2 - H]^+$, if the $\Delta Cu^{II}BDE$ difference in eq 13 is linearly proportional to the optical purity of analyte A, we should observe a linear relationship between ln($R'$) and analyte optical purity (a detailed derivation of this relationship is included in the Supporting Information, S2). Such a relationship is indeed observed, as illustrated in Figure 6b.

A rugged analytical method for the determination of enantiomeric excess requires that the results not depend on concentrations. A systematic study of the influence of relative solution concentration of the analyte and the reference on the value of $[Cu^{II}(A)(ref^*) - H]^+/[Cu^{II}(ref^*)_2 - H]^+$ was, therefore, carried out, and the results are summarized in Table 4. The study shows that, at least in the cases examined, the relative abundance ratios are virtually independent of the relative concentration ratio of the analyte and the reference in the solution (the larger deviation seen for $[Cu^{II}(A)(ref^*) - H]^+/[Cu^{II}(ref^*)_2 - H]^+$ at higher relative analyte to reference concentrations is due to the formation of the parent ion $[Cu^{II}(A)(ref^*)_2 - H]^+$ in low abundance and, thus, to the much weaker signal available). As a consequence of these results, it is clear that the quotient ratio method also allows mixtures with unknown concentration to be analyzed for enantiomeric excess (ee).

**Structures of the Copper(II)-Bound Complexes and Intrinsic Interactions Influencing Chiral Recognition.** Chiral selectivity of amino acids in this experiment is due to the different stabilities of the two diastereomeric, dimeric complexes that are produced by trimeric ion dissociation (Figure 1). Accordingly, the following consideration of ion structures is focused on the dimeric complexes ($[Cu^{II}(A)(ref^*) - H]^+$). The gas-phase structures of copper(II)−amino acid complexes were first studied by Turecek et al., who observed that the CID of the $[Cu^{II}(AA - H)(bpy)]^+$ (AA = amino acid, bpy = 2,2′-bipydyl) type suggests that the complexes are strongly covalently bound.[36a,37] Our CID spectra (as shown in Figure 3) indicate that this is the case for the copper(II)-bound dimeric cluster ions ($[Cu^{II}(A)_2 - H]^+$) because they do not lose an intact amino acid on dissociation.

Further structural details were uncovered by studying CID of a complex containing $^{13}C$-labeled and unlabeled amino acid ligands, as well as complexes containing N- and C-terminal amino acid derivatives. The CID results for the mixed-ligand copper(II)−proline-phenylalanine system and analogous complexes are shown in Table 5. At least one free carboxylate group in the two ligands is necessary for the formation of the $[Cu^{II}(A)_2 - H]^+$ cluster ion, and such a complex can still form when the primary amino group in the amino acids is modified. Decarboxylation is again the major dissociation channel, and further fragmentations are radical-induced, revealing the carboxyl group to be the site of deprotonation. In the case of $[Cu^{II}(Pro)(Phe) - H]^+$, fragments arising from the loss of $C_2H_4$, $C_3H_7\cdot$ and $C_6H_5CH{=}CH_2$ are observed, which suggests that deprotonation can occur at either carboxylic group. This conclusion was further supported by experiments using $^{13}C$-labeled phenylalanine. CID of $[Cu^{II}(Pro)(^{13}C_1\text{-}Phe) - H]^+$ (*m*/*z* 343) results in the loss of both $CO_2$ and $^{13}CO_2$ to form *m*/*z* 299 and *m*/*z* 298, and the triple stage ($MS^3$) spectra of *m*/*z* 299 and *m*/*z* 298 show that CID of the ion *m*/*z* 299 produces *m*/*z* 271 and *m*/*z* 256 via the loss of $C_2H_4$ and $C_3H_7\cdot$, respectively. This corresponds to the CID behavior of the $[Cu^{II}(Pro)_2 - H]^+$ complex, although *m*/*z* 194 (loss of $C_6H_5CH{=}CH_2$) is generated from the dissociation of *m*/*z* 298, which is similar to the dissociation of the $[Cu^{II}(Phe)_2 - H]^+$ complex. Upon the basis of the above results, similar observations by Turecek et al.[36a,37]

and Brodbelt and co-workers,[43] and the known solution chemistry,[44] the copper(II) dimeric cluster ion is proposed to be a tetracoordinate complex (**1**).




**(1)**

According to a longstanding precept of chiral recognition, at least three points of contact should exist between the two chiral ligands within the copper(II)-bound complex in order to bring about the desired chiral recognition. In the structure (**1**), both ligands are bidentate, and multiple interactions between the two ligands mediated by copper(II) provide the basis for efficient chiral distinction. Two of the interactions between the two ligands are copper(II)-mediated interactions that result from the coordination of amino and carboxylate groups to the central copper(II) ion, while the third interaction is between the substituents at the asymmetric α-carbon atoms of two ligands, which is relatively weaker but determines the chiral discrimination. In LEC, in which similar diastereomeric Cu(II) complexes are transiently formed, the third interaction is proposed to be mediated by the solvent, such as water in an aqueous mobile phase, or by the matrix or surface of the stationary phase.[39] The results of the present experiments provide insights into the effects of substituents on the α-carbon of amino acids on the degree of chiral recognition in the absence of any effect introduced by the solvent or the stationary phase.

The superior chiral recognition achieved when the reference ligand has an aromatic side chain (Tables 2 and 3) suggests that $\pi-\pi$ stacking interactions may play a role in the stereospecificity. These interactions are likely to be between the aromatic side chain in the reference ligand and the carboxylate group in the analyte. The conformation in which the aromatic ring is located above the carboxylate group allows electron flow between the aromatic ring and the carboxylate group which coordinates to the Cu(II) cation via a charge transfer (CT) interaction, a model that is similar to that proposed by Yamauchi et al.[45] There may also be a copper(II)−aromatic ring interaction, but prior work shows this contribution is much smaller than the CT interaction.[46] When an L-aromatic amino acid such as L-phenylalanine is used as the reference ligand, such interactions will be disrupted by the side group on the α-asymmetric carbon of the L-analyte, although the side chain group in the D-analyte has little steric effect on the interaction because it is located at the opposite side of the square planar structure. This interpretation is consistent with the observation that the heterochiral dimeric fragment ions are more stable than the homochiral in cases in which an aromatic amino acid is used as either the reference or the analyte. As the size of the side-chain group on the analyte increases, in the series alanine, valine, leucine, and isoleucine, the inhibition of such interactions also increases, and there should be an increasingly large preference for the heterochiral complexes. As predicted, the chiral selectivity ($R_{chiral}$) increases in this series, as shown in Table 3. Proline can be expected to provide a large steric effect, so D,L-proline is efficiently resolved.

**Table 6.** Chiral Selectivity at Different Collision Energies

| | | $R_{chiral}$ | | |
|---|---|---|---|---|
| ref* | A[a] | energy 9% | energy 10% | energy 11% |
| L-Phe | Ala | 2.1 | 2.0 | |
| | Val | 4.9 | 4.5 | 4.1 |
| | Leu | 2.4 | 2.3 | 2.3 |
| | Ile | 5.2 | 4.8 | 4.5 |
| | Pro | | 5.3 | 4.5 |
| | Asp[b] | 2.7 | 2.8 | 2.6 |
| | Glu[b] | 3.1 | 2.9 | 2.8 |
| | Ser | | 1.5 | 1.5 |
| | Thr | | 1.8 | 1.7 |
| L-Trp | Met | | 7.6 | 7.4 |
| | Phe | 10 | 8.3 | 8.2 |
| | Tyr | 10 | 11 | 9.7 |
| | Asn | | 1.8 | 1.7 |
| | Gln | | 6.8 | 5.7 |
| L-Asn | Trp | | 1.8 | 1.7 |
| L-Arg | His | | 0.47 | 0.35 |
| L-His | Lys[c] | 0.53 | 0.56 | 0.55 |

[a] A = analyte amino acid [b] For Asp and Glu, CID energies were 10%, 10.5%, and 11% [c] For Lys, CID energies were 8%, 8.5%, and 9.5%.

In the case of tyrosine, the OH group on the ring tends to increase the electron density, which makes the CT interactions stronger and, thus, increased chiral recognition is observed. Tryptophan, whose indole residue should provide good overlap with the $\pi$-orbital of the carboxylate group, shows a similar effect.

In LEC, entropy effects are extremely important because of the number of species involved in forming the complexes and the fact that complex formation typically occurs on the stationary phase surface. These effects, as noted by Davankov, are reflected in a slight increase in the resolution of enantiomers of N-benzylproline as the temperature increases.[47] Such entropic effects appear not to be as significant in the present gas-phase study, as shown in Table 6, which presents selectivity as a function of collision energy. With increasing collision energy, selectivity decreases gradually. This is an expected result and a common phenomenon in cases in which thermochemical distinctions are made using the kinetic method. The higher the excitation energy supplied, the smaller the relative rate ratio for dissociation to form the two competitive products.[48] Because the energy range is not defined accurately under the current experimental conditions (multiple collisions in most cases in the ion trap), such a trend is only of qualitative value and does not allow separation of the entropic and the energy terms. Further experiments under different collision conditions on other instruments are needed to define the entropic contributions.[49]

## Conclusions

Insights into the structures of the copper(II)-bound cluster ions generated by ESI mass spectrometry are obtained through

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examination of their dissociation behavior by tandem mass spectrometry. The kinetic method provides a basis for rationalizing the thermochemical factors which control dissociation of the parent trimeric cluster ions, and it predicts a linear relationship between the logarithm of fragment ion abundance ratio and the enantiomeric composition of the sample. On the basis of these structural and thermochemical considerations, the kinetics of dissociation of copper(II)-bound cluster ions offers a powerful new method for observing large chiral effects that readily allow distinction of almost all of the natural chiral α-amino acids, something not achieved in any of the previous mass spectrometric studies on the chirality of amino acids. Most significantly, it allows quantitative measurements of optical purity in the absence of solvation effects or stationary-phase interactions. The present results show interesting differences and parallels with condensed-phase chiral selectivity data obtained via LEC. The measurements are simple and rapid, use standard ESI mass spectrometry and tandem mass spectrometry, and require very small amounts of material for analysis. The results are largely independent of relative concentrations of the analyte and the chiral reagent. The enantiomeric excess of mixtures of enantiomers can be assayed using this method, resulting in high accuracy and sensitivity to small differences in ee.

The success of these experiments is related to the facts that (i) copper(II)-bound complexes provide multiple interactions for chiral recognition, (ii) direct interactions between the chiral reference and the analyte in the environment of the mass spectrometer allow the optimization of chiral recognition by choosing appropriate chiral references, and (iii) dissociation rates are sensitive to small changes in critical energy. The observed ion abundance ratios are transformed, using the kinetic method, into a thermochemical term that is related to copper(II) affinities, namely, the energy quantity, $\Delta(\Delta Cu^{II}BDE)$, which reflects stereochemical interactions in the cluster ions. This quantity is argued to be linearly proportional to the optical purity, and the predicted linear relationship between ln(*R*) and the optical purity has been verified and utilized for the quantification of amino acids. Independent justification for the assumption of the linear relationship is included in the Supporting Information.

The present method for the practical chiral analysis is based on certain assumptions. Although it is proposed that the chiral distinction is due to the different stabilities of dimeric diastereomers, chiral discrimination in the formation of the trimeric cluster ions (e.g., $[Cu^{II}(ref^*)_2A - H]^+$) is still possible,[50] and this would change the outcome of the experiments. Formation of isomers of trimeric cluster ions as a result of different deprotonation sites might also influence the measurement of relative intensity ratios. So too, would entropic effects on the rates of competitive trimer ion dissociations. However, the use of two competitive measurements will cancel most errors. For quantitative analysis, the quotient ratio method using the $[Cu^{II}(A)_2(ref^*) - H]^+$ system is preferred, because every calibration point is obtained by two measurements, as shown in Figure 6b. Note, however, that no less accuracy has been recorded for the single ratio method in the cases so far examined.

A significant outcome of the present investigation on the mechanism of chiral recognition of copper(II)−amino acid complexes is that the interaction between the side groups on the α-asymmetric carbon need not be mediated (by solvent or stationary phase) to observe chiral discrimination, even though aromatic side chains are desirable to observe large enantioselectivity for bidentate ligands. Indeed, the intrinsic chiral selectivity of the present experiments is much greater than that which has been achieved in corresponding solution studies.

The present methodology is general and practically useful for chiral analysis of amino acids, and its extension to other important biological molecules is anticipated. Peptides are ongoing candidates, and their chiral analysis will be described in a future publication.[50] The method should also apply to other metal ions such as nickel(II),[51] iron(II), zinc(II), cobalt(II), and palladium(II). More fundamental features need to be further clarified, including the thermodynamic contributions to chiral recognition. In addition, it may be possible to use this method to examine drug−receptor interactions mediated by metal ions. Furthermore, progress reported here on amino acid chirality determination and parallel progress on miniaturization of mass spectrometers suggest a potential use of this instrumentation for chiral recognition in astrobiological experiments.[52] Overall, the present results suggest that the kinetic method has the potential to become a powerful technique for quantitative chiral analysis with broad applications.

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**Supporting Information Available:** Figures S1 and S2, the relationship of $[Cu^{II}(A)(ref^*) - H]^+/\{[Cu^{II}(A)(ref^*) - H]^+ + [Cu^{II}(ref^*)_2 - H]^+\}$ vs molar fraction of the D-isomer (α) using the same experimental data as that utilized in Figure 6a, plotted using a linear and polynomial plot, respectively. The data conform best to a nonlinear relationship, in agreement with the linearity of the plot of ln(*R*) and α (Figure 6a). Supporting Information S1, derivation of the linear relationship between ln(*R*) and α; Supporting Information S2, derivation of the linear relationship between ln(*R*′) and α; Supporting Information S3, derivation of the relationship between the composition of a mixture of enantiomers and the critical energy for dissociation. All materials are available free of charge via the Internet at http://pubs.acs.org.

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