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The Rotating Ring-Disk Electrochemistry of the Copper(II) Complex of Thyrotropin-releasing Hormone

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Abstract

Thyrotropin-releasing Hormone (TRH) forms an electroactive Cu(II) complex in aqueous solution. Rotating ring-disk electrochemistry reveals oxidation at the disk electrode and reduction at the ring electrode. The plot of limiting current vs. square root of rotation frequency deviates from the Levich equation, indicating both preceding and following chemical reactions. The reaction following the oxidation is a multiple-electron ECE-type of process that has been seen before in Cu(II)-peptide electrochemistry. The preceding reaction is unusual. The deviation from diffusion-controlled behavior is more pronounced at higher initial concentration of Cu(II) and peptide. We propose that a non-electroactive dimer, Cu(II)₂-TRH₂, is in a slow equilibrium with the electroactive Cu(II)-TRH. Simulation of the RRDE behavior of the postulated Cu(II)-TRH system has succeeded in matching experimental data. Capillary electrophoresis indicates that there is a negative charge on the dimer. It is suggested that a hydroxo-bridge may link the two Cu(II) centers. Calculations verify that binuclear Cu(II)₂-TRH₂ complexes are possible.

Keywords

thyrotropin releasing hormone; peptide; copper; electrochemistry; biuret; rotating disk electrode

1. Introduction

High performance liquid chromatography with electrochemical detection is an effective method for the determination of peptides. The chromatographic detection of peptides has been established based on the reversible electrochemistry of the Cu(III)/Cu(II) couple in polydentate peptide complexes [1,2], also called biuret complexes. In the dual electrode detector, a Cu(II)-peptide complex is oxidized to a Cu(III) complex at the upstream electrode, which is in turn reduced to Cu(II) at the downstream electrode. The biuret complexes are formed precolumn or postcolumn in both HPLC and CE-based methods[1-8]. In support of this detection method, the RRDE has been used to study the electrochemical kinetics of Cu(II) complexes of oligoglycines as model peptide ligands [9]. The results revealed effects of pH, buffer and rotation frequency on the electrochemistry of Cu(II)-oligoglycine complexes.

While oligoglycine complexes serve to teach us about the essential chemistry of the peptide backbone with Cu(II), their study is not sufficient to teach the complexities of Cu(II) complexes of bioactive peptides. Additional features in peptides including side chains and modified termini add complexity to the coordination reactions and thus the electrochemistry of the

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complexes. The presence of carboxy- and amine-terminal amides impose complications on the biuret reaction and consequently the electrochemical properties of the complex. Generally, N-terminal amides feature slow coordination kinetics and relatively higher redox potentials. The existence of a His residue shifts the redox potential more positive[10]. The prolyl residue is a known break point, being inert in the peptide sequence when coordinating with Cu(II)[11].

Coordination chemistry is not only used in analysis involving HPLC. Copper complexes of peptides are used in mass spectrometry[12].

Aside from their importance in analytical chemistry, peptide-Cu(II) complexes are gaining increased scrutiny in biology. A prolyl-histidyl-containing segment of the prion protein binds copper which influences the protein's properties [13-16]. The interactions of α -synuclein, the protein responsible for the Lewy bodies of Parkinson's disease, with Cu(II) are considered important[17]. The details of the coordination chemistry of copper complexes of peptides are keys to understanding the many roles – both normal and pathological – of copper-peptide systems[16,18,19].

It is thus of considerable importance to study the copper complex of the peptide Thyrotropin Releasing Hormone, TRH or pyroglutamyl-histidyl-prolyl amide (Figure 1). It is a tripeptide blocked at both C- and N- termini (the termini are both amides). It contains a proline and a histidine, both of which can dominate the coordination chemistry and thus the electrochemistry of its Cu(II) complex. The unique structure of TRH makes it interesting from a coordination chemistry and electrochemistry perspective. The probable structure of Cu(II)-TRH has been determined by spectroscopic and electrochemical means [20]. Briefly, Cu(II) binds to TRH through the *p*Glu amide nitrogen, the *p*Glu-His peptide bond nitrogen, the 1-N of the His imidazole and a water. As TRH has lost two protons from two amide functional groups, we denote the complex as Cu(II)-TRH(H₋₂)(H₂O). TRH has been shown to improve functional recovery after neurologic dysfunctions, such as brain trauma and epilepsy, in both humans and animals such as rats, mice and cats [21-25]. We believe that, coupled with online microdialysis and preconcentration, the concentration of TRH can be monitored.

This paper reports on the electrochemistry of the copper complex of TRH. Steady-state voltammetry with the rotating ring-disk electrode (RRDE) comprises most of the electrochemistry in this paper. The RRDE has proven to be powerful in studies of homogeneous kinetics and equilibria [26], and is particularly suited to modeling dual electrode detection [21] (4). While cyclic voltammetry is often the technique of choice in electrochemistry, in this case it is not. The electrochemistry of these complexes is quasi reversible at low sweep rates. The experiments require using low solution concentrations. To access a broad range of time scales requires going to high sweep rates. This creates difficulty for low concentrations (signal/charging current decreases as sweep rate increases) and for quasi reversible reactions (electrochemical, not homogeneous, kinetics become rate limiting).

The disk electrode signal obtained for a simple, mass transport-limited electron-transfer process obeys the Levich equation [26]:

$$i_D = nFADC * / \delta = 0.620nFAC * D^{2/3} \nu^{-1/6} \omega^{1/2} \quad \text{Eq. (1)}$$

where i_D (disk current) is in mA, C^* (concentration of the analyte) is in M, D (diffusion coefficient of the solute) is in cm²/s, ν (kinematic viscosity of the solution) is in cm²/s, ω (rotation frequency) is in rad/s, n is the number of electrons transferred per molecule of solute, and F is the Faraday constant. The ratio of current at the ring to current at the disk is known as the collection efficiency, N_0 .

Homogeneous chemical reactions preceding and following the disk electrode reaction affect the current vs. rotation frequency curve. The model biuret complex Cu(II)-tetraglycine, decomposes following oxidation [27]. The effect of this ECE (electrochemical-chemical-electrochemical) process is seen from extra disk current and reduced ring current at low rotation frequencies. Preceding reactions (CE) include amide deprotonation or ligand exchange [9]. They often cause lower current than expected at high rotation frequencies. For simple peptides such as G₄ (glycylglycylglycylglycine or GlyGlyGlyGly) and G₅, the CE effects can be eliminated by choosing the right pH and buffer systems to obtain a plot of limiting current vs. $\omega^{1/2}$ that obeys the Levich equation at high ω [9].

Preliminary electrochemical investigations in conjunction with determining the structure of the Cu(II)-TRH complex[20] showed that the electrochemistry of this complex is not simple. Because of the implications for electrochemical detection, and for the functioning of the hormone in vivo, we have investigated the electrochemistry of the complex.

2. Experimental

2.1 Chemicals

Thyrotropin-releasing hormone from Bachem (King of Prussia, PA, USA) was used without further purification, but the purity (96%) was taken into account in solution preparation. Copper sulfate pentahydrate (J.T. Baker, Phillipsburg, New Jersey, USA) was recrystallized once from water. Borate buffers were all prepared in 0.25 M KNO₃ (J.T. Baker, USA) with Na₂B₄O₇·10H₂O from Sigma (St. Louis, MO, USA). In pH effect experiments, the borate concentration was 0.10 M. In all other experiments using borate, the borate concentration was 0.05 M. All pH values were adjusted with 0.40 M boric acid (EM science, Gibbstown, NJ, USA) and/or 4 M NaOH (EM science), and measured with an Accumet[®] pH-meter (Fisher Sci, Fairlawn, New Jersey, USA), standardized before each use. Copper-peptide solutions were prepared by diluting a desired amount of stock solution in the order of peptide then copper sulfate in buffer. Molar ratios were 1:1 unless mentioned. All water used was from a Milli-Q A10 Synthesis water purification system (Millipore, Billerica, Massachusetts, USA).

2.2. Instruments and procedures

The rotating ring-disk apparatus consisted of a DT-29 glassy carbon/glassy carbon ring-disk electrode, an ASR-2 analytical rotator, and an RDE-3 potentiostat (all from Pine Instrument Co., Grove City, PA, USA). The ring-disk electrode has a disk area of 0.2475 cm², and geometric collection efficiency of 0.3714. A platinum grid counter electrode and a Ag/AgCl reference electrode (3 M NaCl, Bioanalytical Systems, West Lafayette, Indiana, USA) were used. All potentials here are reported relative to this reference electrode. Before each run the working electrodes were wet polished with 0.05 μ m γ -alumina powder (Buehler Ltd., Lake Bluff, Illinois, USA) and rinsed with Milli-Q deionized water from a wash bottle. The electrode was then placed in the rotator. Background current (buffer used for the sample solution) was measured first and subtracted from sample signal.

The potentiostat signals were filtered with a WaveTek 852 filter (WaveTek, San Diego, CA, USA) set for low pass with 1 Hz cutoff frequency. RRDE data were collected using EzChrom chromatography software with a DT2802 chromatography board (Data Translation, Inc., USA), or PeakSimple software with a SRI202 board (SRI Instruments, Torrance, CA, USA). Data collection was triggered manually while the voltage was simultaneously applied to the electrode. All files then were converted to ASCII format and processed with Excel.

For hydrodynamic voltammetry, the disk potential was swept linearly from 0 mV to 1000 mV. The sweep rate was always 0.5 V/min unless otherwise specified. The ring electrode was maintained at a constant potential of 0 mV. RRDE rotation frequency studies were conducted

with the disk electrode potential held at 900 mV and the ring electrode at 0 mV. These potentials were identified by voltammetry as being in the limiting current region. In each run, the electrode was rotated initially at a moderate rotation frequency before potentials were applied. The non-Faradic current was permitted to decay no less than 2.5 min. The rotation frequency then was varied in a random sequence with some of them repeated 1 or 2 times. Data collected within the initial period of current decay were ignored. The rotation frequency was maintained at each value long enough to ensure steady state.

The mass transport properties of the system were tested from 10 to 5000 rpm using 0.20 mM ruthenium(III) hexamine trichloride (Aldrich, Milwaukee, WI, USA) as a reversible one-electron standard. The solution was prepared in 0.4 M carbonate buffer, pH 9.8. The complex was reduced at the disk ($E_{\text{app}} = -280$ mV) and then oxidized at the ring ($E_{\text{app}} = 0$ mV). Data yielded a linear Levich plot ($R^2 = 0.9945$, $n = 39$) over the entire rotation frequency range tested at both disk and ring electrodes. The diffusion coefficient obtained, $4.05 \times 10^{-6} \text{ cm}^2/\text{s}$, agreed with the value $3.91 \times 10^{-6} \text{ cm}^2/\text{s}$ obtained by the Taylor-Aris flow injection method. Room temperature was 22 ± 2 °C during all RRDE experiments.

Spectroscopic measurements employed an HP 8453 spectrophotometer with HP 8453E operating software (Agilent, USA). Spectra of copper – peptide complexes in 1 cm quartz cuvettes were measured over 400 – 1100 nm in general scanning mode at 0.5 s integration time, using Milli-Q water as a solvent blank in all cases.

Determination of diffusion coefficients by laminar flow axial dispersion was according to the procedure of Beisler [3]. Briefly, a rectangular plug of solute is allowed to disperse in a 25 μm inside diameter capillary under pressure-induced flow. Analysis of the shape of the formerly rectangular zone as it passes through a downstream detector yields the diffusion coefficient. The aqueous flow solution containing 0.1% trifluoroacetic acid, 3% 1-propanol and 25% acetonitrile was pumped with a Waters 600 E quaternary pump. A splitter tee was used to decrease the flow rate to 2–5 $\mu\text{L}/\text{min}$. The TRH stock solution was diluted in mobile phase to make a concentration of 10 μM and injected with an Upchurch microinjector. Injections were combined with the biuret solution containing 5.00 mM CuSO_4 , 30.0 mM sodium tartrate in 1.2 M carbonate. The analyte was detected at 0.80 V versus Ag/AgCl with a 10 μm carbon fiber electrode 1 mm in length (CV-27 Potentiostat, BAS, W. Lafayette, IN, USA) and current was measured with a Keithly (Cleveland, OH) 427 Picoammeter. Signals from the ammeter were collected at 50 Hz by EzChrom (Scientific Software, San Ramon, CA). Data obtained were exported and smoothed using PeakFit software. The first derivative was determined in PeakFit using Savitzky-Golay filtering. The statistical moments were determined by fitting the 5-parameter GEMG function to the data based on the least squares criterion. The Scheibel correlation allowed D to be corrected for solvent composition[28]

Capillary electrophoresis experiments were carried out on an ISCO 3850 Capillary Electropherograph (ISCO Inc., Lincoln, NE), with PeakSimple Chromatography Data System with a SRI202 board (SRI Instruments Inc., Torrance, CA, USA) for data collection. The absorbance detector was set at either 210 nm or 590 nm. The fused silica capillary (Polymicro Tech, LLC, Phoenix, Arizona, USA) had a 50 μm inner diameter and 70 cm total length with 50 cm from the injection end to the detector. 20 kV were applied across the capillary containing 10.0 mM pH 9.84 borate buffer. Sample solutions of 1.0 mM each Cu(II) and peptide prepared in the running buffer were vacuum-injected for 5 s. 5% acetonitrile (v/v) served as a neutral marker.

2.3 Simulation

Molecular simulation was carried out with the software CAChe (Fujitsu, Ltd., Japan) version 6.1.1. Structures were first checked for correct valences and hybridizations, then optimized

using Allinger's standard MM3 force field model [29,30]. The following factors were taken into account when optimizing: bond stretch, bond angle, dihedral angle, improper torsion, van der Waals interaction, electrostatics interaction, hydrogen bond, torsion stretch, and bend-bend interactions.

Electrochemical simulation was carried out with software DigiSim (BAS, USA), version 3.03b, under hydrodynamic conditions.

3. Results and discussion

The diffusion coefficient of the Cu(II)-TRH(H₂O)(H₂O) complex (D_{TRH}) determined by axial dispersion [3,31,32] is $5.86 \times 10^{-6} \text{ cm}^2/\text{s}$. This value is used in the Levich equation to determine the expected current response for a diffusion controlled one-electron transfer reaction of the Cu-TRH(H₂O)(H₂O) complex. In pH 9.84 borate buffers, equimolar solutions of Cu(II) and TRH show a characteristic bluish-purple color, with a maximum absorbance (λ_{max}) at 588 nm. The voltammetric wave generated by RRDE gives a half-wave potential, $E_{1/2}$, of 785 mV at both disk and ring electrodes. Shown in Figure 2, the absorption band does not shift with varying pH in the range of pH 7.4 to pH 11 [20], while the $E_{1/2}$ displays a linear decrease with a slope of -70 mV/pH . When either of the two reagents is in excess, the numerical value of slope gets closer to -59 mV .

The data in Figure 2 are consistent with proton transfer accompanying electron transfer, reaction 2.

For now, we do not specify details about the lost proton in the formula for the Cu(III) complex. A proton can be lost from Cu(II)-TRH(H₂O)(H₂O) to give an electroactive complex as a preceding reaction before the oxidation, or from Cu(III)-TRH as a following reaction. Peptide bonds in Cu(II) complexes of peptides can deprotonate. However, this process, for a simple peptide like tetraglycine, is slow enough to show two waves in RRDE voltammetry, representing two electroactive complexes Cu(II)-NNNO (Cu-GGGG(H₂O)) and Cu(II)-NNNN (Cu-GGGG(H₃)) [9]. The complex with four nitrogen donors has a lower oxidation potential. This is clearly not the case here with TRH. We determined earlier that equatorially bound water in Cu(II)-TRH(H₂O)(H₂O) can lose one proton [20]. This deprotonation is likely responsible for the shift $E_{1/2}$ with pH. The Cu(III) form is thus represented as Cu(III)-TRH(H₂O)(OH).

A Levich plot, in the case of a mass transport-limited oxidation, is a straight line with zero intercept. Figure 3a shows an experimental Levich plot of Cu(II)-TRH(H₂O)(H₂O). It clearly deviates from the theoretical lines at both disk and ring electrodes. Figure 3b and 3c are replots of the same data. Figure 3b is the rotation frequency-dependent “experimental collection efficiency N_K ” obtained by dividing i_R (limiting current at ring) by i_D (limiting current at disk) at each rotation frequency. Figure 3c is the “apparent number of electrons transferred (ANE)” obtained by dividing each experimental current by the theoretical value.

Based on rotation frequency, the data set can be divided into two parts. When the rotation frequency is low ($\omega^{1/2} < 10$), N_K is significantly less than N_0 . Woltman et al. have shown similar RRDE behavior with Cu(II)-oligoglycines [9]. It is consistent with an ECE process recognized and studied by Margerum et al. [27]. Briefly, the Cu(III)-peptide as the product of the first electron transfer reaction decomposes and produces more oxidizable species, P_{red} , as shown in reactions 3 and 4.

The irreversible oxidation of these species gives extra current at the disk and at the same time reduces the ring current because it removes Cu(III)-peptide. Correspondingly, in Figure 3b N_K is close to zero at low frequencies.

When the rotation frequency is greater than 1000 rpm ($\omega^{1/2} > 10$), ANE values of the disk and ring electrodes agree with each other, and N_K reaches a plateau. Equal disk and ring ANE values and a constant N_K represent a situation where little or no reaction occurs in the gap. Under this condition, all of the Cu(III) complex generated at the disk is reduced at the ring. The lifetime of the Cu(III) species depends on many factors, one of which is substitution at the α -carbon in the amino acids making up the peptide. Glycines contribute to instability, while peptides from 2-methylalanine (i.e., no α -hydrogens) have more stability [33-35]. For triglycine, the rotation frequency at which the plateau is reached is near 5000 rpm [9], greater than the value of 1000 rpm seen with Cu-TRH consistent with the greater α -carbon substitution in TRH.

As can be seen in Figure 3 (a and c), the number of electrons transferred at disk and ring electrodes agree with each other at high frequencies, but both are significantly smaller than 1 (Figure 3c). This behavior at high rotation frequencies is consistent with a CE (chemical-electrochemical) process. In such a process, the electroactive species is generated by a chemical reaction prior to the oxidation. As rotation becomes faster, less time is given for this reaction, so less reactant is provided to the disk electrode causing a lower disk current. The limiting current is related to both the preceding reaction's equilibrium constant, and its relaxation time (related to its rate constants). The value of K determines the fraction of electroactive species present in the absence of the perturbation caused by the electrochemical reaction. The reaction's relaxation time determines how rapidly the equilibrium responds to the electrochemical perturbation.

The preceding reaction could in principle be the Cu(II) plus peptide binding equilibrium. The conditional Cu(II)-TRH(H_2O)(H_2O) binding constant at pH 9.8 has been estimated to be at least 10^5 M^{-1} [20]. This corresponds to 90% or more bound at concentrations of 1.00 mM each Cu (II) and TRH. This value is substantially bigger than the ANE values at high ω shown in Figure 3c. This means that if the Cu-peptide binding equilibrium is the only preceding reaction, even at an infinitely small rate constant, the current should still be higher than the experimentally obtained value. If the Cu-TRH binding equilibrium was responsible for the observations, increasing the Cu and peptide concentrations should lead to ANE closer to 1. We recorded limiting current data at various concentrations. As shown in Figure 4, the result is the opposite – there is less deviation at lower concentrations. At high rotation frequencies, the normalized limiting current decrease when concentration increases. Thus, we rule out the simple Cu-TRH binding as the preceding reaction.

Levich pointed out that with a first order preceding reaction, under conditions that $(D/\nu)^{1/6} \ll k/\omega$, the value $i/\omega^{1/2}$ is proportional to the value i and the Levich equation can be rewritten as [26]:

$$i/\omega^{1/2} = \frac{i_L}{\omega^{1/2}} - i \left(\frac{D^{1/6}}{1.61\nu^{1/6} K k^{1/2}} \right) \quad \text{Eq. 5}$$

where i_L is the limiting current without a preceding reaction, K is the equilibrium constant and k is the rate constant of the preceding reaction. Because of Cu(III)-TRH decomposition, only data representing a pure CE process ($\omega^{1/2} > 10$, in the case of Cu(II)-TRH complex) can be used.

Because Eq. 5 describes current in the presence of a first order preceding chemical reaction process, the parameter $Kk^{1/2}$ is a constant regardless of concentration. This parameter can be determined from the slope of $i_{\text{disk}}/\omega^{1/2}$ vs i_{disk} . Our “ $Kk^{1/2}$ ” values calculated, however, vary with concentration. Figure 5 shows decreasing “ $Kk^{1/2}$ ” with increasing concentration. Apparently, the C-reaction in the case of Cu(II)-TRH is not a first order reaction. Eq. 5 does not work in this particular case.

The data are consistent with the presence of a higher order, electroinactive complex that must dissociate to form the electroactive Cu(II)TRH(H₂O)(H₂O) (Eq. 6).

This hypothesis of higher order reaction is plausible and consistent with the electrochemical and spectroscopic data. However, neither visible spectroscopy nor electrochemistry is capable of verifying it. Spectroscopy cannot distinguish the two forms while electrochemistry only recognizes one of them. Cu(II)TRH(H₂O)(H₂O) is a neutral species [20]. If the electroinactive complex differs in the charge, capillary electrophoresis should tell more.

We used two TRH analogs (Figure 6) as reference compounds. MeHisTRH and TRH-OH have the same amino acid sequence as TRH but differ in one substituent. Their spectroscopic and electrochemical properties suggest identical equatorial binding under the current conditions [20]. TRH-OH and its complex should carry one more negative charge than the corresponding analogs due to the dissociation of the C-terminal carboxylic acid group.

Both TRH and the 1:1 mixture of Cu(II) and TRH absorb maximally at 210 nm. The complex absorbs in the visible region while the peptide does not. In CE with ‘normal’ polarity, negatively charged species migrate at a lower velocity than the electroosmotic flow. The top electropherograms in Figure 7 show a peak for TRH (Cu(II) absent) at pH 9.84 migrating with the neutral marker (arrow). The Cu(II)-TRH mixture is shown as a broad band with a plateau. This is because we have injected a solution containing several components in an equilibrium, and the separation disturbs the equilibrium [36,37] Krylov calls this ‘non equilibrium capillary electrophoresis of equilibrium mixtures’ or NECEEM. To interpret the data, it is helpful to consider the lower traces ($\lambda = 590$ nm). These traces show only Cu(II) complexes (note the lowest trace shows no response from TRH, and Cu(II) itself is not soluble at this pH). There is a small peak about 3.9 min corresponding to a neutral complex and a sharp peak at 4.25 min as well as an exponentially decaying zone with a maximum at about 4.1 min. We interpret these curves within the framework of NECEEM[36,37]. The neutral Cu(II)-TRH complex gives a peak at 3.9 min. An anionic complex also exists with its peak at 4.25 min. The exponentially-tailed peak in between represents the dissociation of the anionic complex to form the neutral complex.

Complexes formed from MeHisTRH and TRH-OH give different CE traces (Figure 8). The TRH-OH complex should have a charge of -1, as does the peptide itself. The CE traces are completely consistent with this. The MeHisTRH complex acts like the TRH complex.

Hence, there is a slow equilibrium involving two different forms of Cu(II)-TRH (and Cu(II)-MeHisTRH) complexes. One component of the equilibrium has some negative charge. TRH-OH, on the other hand, binds Cu(II) with overall charge unchanged.

What proton is lost? Deprotonation of the N-terminal amide is the first to be considered. Evidence for such deprotonation in a peptide is rare. The pK_a of a secondary amide is usually much higher than 10. Coordination to Cu(II) may promote the deprotonation, as for the peptide

backbone amide deprotonation in a typical biuret reaction. However, the deprotonated N-terminal amide cannot reach the Cu(II). This ring formation is essential in stabilizing a biuret complex. We conclude that deprotonation of the N-terminal amide in TRH and MeHisTRH to introduce one negative charge to their Cu(II) complexes is not likely. The best possibility lies in the equatorial water ligand, which can deprotonate to become a hydroxide. Another Cu(II) center may lose a water ligand and take the place of the missing proton in this hydroxide.

Intermolecular aggregation of Cu(II)-TRH complexes serves as an explanation. Though the exact number in the aggregate is unknown, the simplest case of intermolecular aggregation – dimerization, is thermodynamically and also sterically more favored than larger clusters. We propose that a Cu(II)-TRH dimer exists, linked by a hydroxo-bridge. The presence of the dimer and the following reactions serve to explain the Levich plot curve shape and the electrophorograms. Equations 8 - 10 describe the hypothesis: We have dropped the details of protonation state in the formulas for clarity.

No simple solution for rotation frequency-dependent current is available for this particular condition. We sought help from the simulation tool DigiSim to verify that a system following equations 8 - 10 describes the observations. The following parameters have been used in the simulation:

1. Concentrations, electrode geometry, and voltammetry parameters are the same as in actual measurements.
2. Diffusion coefficients (D): The diffusion coefficient of the electroactive form of the Cu(II)-TRH complex (monomer) is $5.86 \times 10^{-6} \text{ cm}^2/\text{s}$; those of Cu(III)-TRH and TRH are assumed the same; the diffusion coefficient, D, of Cu(II)_{aq} has been calculated to be $6.5 \times 10^{-6} \text{ cm}^2/\text{s}$ based on the constant $D\eta/T = 2.23 \pm 0.37 \times 10^{-10} \text{ cm}^2\text{poise/Ks}$ [38]; the value of $3.8 \times 10^{-6} \text{ cm}^2/\text{s}$ has been used for Cu(II)₂-TRH₂, an estimation based on the monomer value $5.86 \times 10^{-6} \text{ cm}^2/\text{s}$ and the expression $D \propto (\text{molar volume})^{-0.6}$ [28], assuming that molar volume of the dimer is twice as that of the monomer.
3. K_1 (Eq. 8) is about 10^5 M^{-1} [20]. Simulation trials were made within range $1 \times 10^5 - 3 \times 10^5 \text{ M}^{-1}$, and K_2 (Eq. 9) within the range $2 - 3.3 \times 10^3 \text{ M}^{-1}$. The two parameters were then narrowed down so that the unbound Cu(II) and TRH fractions were consistent with the spectroscopic data, and the simulated current matched experimental data. The values $K_1 = 1.0 \times 10^5 \text{ M}^{-1}$ and $K_2 = 2.8 \times 10^3 \text{ M}^{-1}$ are optimum.
4. We assign subscript 1 for the parameters in Eq. 8 and subscript 2 for those in Eq. 9. Thus, the reaction rate for Eq. 8 is $v_1 = k_{f1}[\text{Cu(II)}][\text{TRH}] - k_{b1}[\text{Cu(II)-TRH}]$, and the reaction rate for Eq. 9 is $v_2 = k_{f2}[\text{Cu(II)-TRH}]^2 - k_{b2}[\text{Cu(II)}_2\text{-TRH}_2]$. Our simulation of a simple binding equilibrium as the preceding reaction confirmed that k_{f1} values in the range of 10^{-3} to $10^5 \text{ M}^{-1} \text{ s}^{-1}$ only caused about a 1% difference in the current at 0.06 mM Cu(II) and TRH each at 3000 rpm. So a value of $500 \text{ M}^{-1} \text{ s}^{-1}$ has been chosen for k_{f1} . The value of k_{b1} is 0.005 s^{-1} , based on k_{f1} and K_1 .

5. With all of the above parameters settled, the optimal value of k_{f2} has been found to be $3.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The value of k_{b2} is 12 s^{-1} .

Figures 9 and 10 compare simulated and experimental currents vs. concentration and rotation frequency. Final deviations ($\text{absolute}(i_{\text{sim}} - i_{\text{exp}})$) shown are no more than 5%. Since the simulated currents match the experimental values rather well in the applicable concentration or rotation frequency range, we have demonstrated that a dimer complex of $\text{Cu(II)}_2\text{-TRH}_2$ is possible.

Hydroxo-bridges in Cu(II) complexes are not rare. There are many μ -hydroxo- Cu(II)_2 examples [41-45] with one or two hydroxo-links. The geometry around the two Cu(II) centers is not always the same in each complex, but typically the Cu-Cu distance is $2.8 - 3.1 \text{ \AA}$ and the bond angle at the OH^- is $95^\circ - 105^\circ$.

CAChe software helped to demonstrate possible structures of $\text{Cu(II)}_2\text{-TRH}_2$ dimers. The linkage Cu-OH-Cu is bent due to the sp^3 hybridization of the oxygen. Figure 11 shows one plausible structure in which the lactam oxygen of the pGlu residue in the peptide bound to one Cu(II) occupies an axial position at the other Cu(II) center. The bond geometries are in rather good agreement with the literature: Cu-Cu distance: $3.06 \text{ \AA}$, and $\text{Cu}_1\text{-OH}^-\text{-Cu}_2$ angle: 95.1° . A water molecule may also act as a bridge [46,47]. Calculations also suggested a four membered ring formed by Cu-OH-Cu- H_2O , in which the water is an apical ligand brought relatively closer to the metal center.

In all calculated structures, the previously coplanar, equatorially arrayed donor atoms are no longer coplanar. The square planar structure of the equatorial donor atoms is distorted. This distortion is not favored by the $d^8 \text{ Cu(III)}$, meaning that the $\text{Cu(II)}_2 \text{ TRH}_2$ molecule will be harder to oxidize than the Cu(II) TRH . The binuclear complex is likely to be electroinactive in the applied potential range, which fits our hypothesis.

The hypothesis that dimer formation is responsible for the behavior of Cu(II)-TRH and the analogous MeHis complex, but that there is no analogous behavior of TRH-OH is consistent with the observations as well. We interpret the difference in the behavior of the latter complex (compared to the former two) based on electrostatics. In the case of the anionic TRH-OH , there is an added electrostatic barrier to dimer formation, inhibiting it.

4. Conclusion

The oxidation of $\text{Cu(II) TRH(H}_2\text{O)}_2$ is complex. At long time scales, low rotation frequencies, intramolecular redox processes lead to destruction of the one-electron, one proton oxidation product $\text{Cu(III)TRH(H}_2\text{O)}_2(\text{OH})$ in the interelectrode space. Electroactive fragments are formed. Increased current at the disk anode and decreased current at the ring cathode result. At frequencies $\geq 1000 \text{ rpm}$, a CE process becomes evident. The simplest explanation in agreement with the spectroscopy, electrophoresis and RRDE results is that an electroinactive hydroxyl-bridged dimer exists in a slow equilibrium with $\text{Cu-TRH(H}_2\text{O)}_2$.

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TRH

Figure 1.
TRH structure.

Figure 2.

pH-dependent spectroscopy (♦, right y-axis) and electrochemistry (□, left y-axis) of 1:1 Cu-TRH. All solutions contain 1.00 mM (in spectroscopic measurements) or 0.10 mM (in electrochemical measurements) Cu(II) and TRH in borate buffers. Dashed horizontal lines in the left figure define the upper and lower limits of λ_{max} for NNNO- and NNNN-bound Cu(II) calculated from Billo's equation [48]. The left plot is adapted from [20] with permission.

Figure 3.

Experimental and predicted diffusion controlled currents plotted against $\omega^{1/2}$. Solutions contain 1.00 mM each Cu(II) and TRH in pH 9.84 borate buffer. (a). Normalized currents. The upper trace is of disk signals, the bottom is of ring. Straight lines are predicted ($D = 5.86 \times 10^{-6} \text{ cm}^2/\text{s}$; n = number of electrons transferred) from the Levich equation, taking the total concentration of Cu(II) ion as electroactive C^* . (b). Experimental collection efficiency, calculated as i_R/i_D . (c). Apparent number of electrons involved in the redox reactions on disk (upper data set) and ring electrode (lower data set).

Figure 4.

Concentration effect on RRDE behavior of 1:1 Cu(II)-TRH. All solutions are in pH 9.84 borate buffer. Dashed arrows indicate concentration increase. (a). Normalized currents. (b).

Experimental collection efficiency, calculated by i_R/i_D . (c). Apparent number of electrons involved in the redox reactions on disk and ring electrode.

Figure 5. “ $Kk^{1/2}$ ” values decrease with increasing initial concentration of TRH (which is equal to Cu (II)). These values are derived from data in Figure 4 and Eq. 5.

Figure 6.
Structures of TRH, TRHOH and Me-His2-TRH.

Figure 7.

Capillary electrophoresis signals of TRH and Cu(II)-TRH 1:1 mixture in 50.0 mM pH 9.84 borate buffer. 1.00 mM each. Upper trace: detected at 210 nm. Bottom trace: detected at 590 nm.

Figure 8.

Capillary electrophoresis signals of TRH, TRHOH and MeHisTRH and their copper complexes in 50.0 mM pH 9.84 borate buffer. All solutions contain 1.00 mM each Cu(II) and peptide. Detection by absorbance at 210 nm. Arrows point to the neutral marker.

Figure 9.

Simulation of CE process following Eq.8 – Eq.10: current vs. concentration at 3000 rpm. $K_1 = 1 \times 10^5 \text{ M}$, $k_{f1} = 500 \text{ M}^{-1}\text{s}^{-1}$, $k_{b1} = 0.005 \text{ s}^{-1}$; $K_2 = 2.8 \times 10^3 \text{ M}^{-1}$, $k_{f2} = 3.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_{b2} = 12.1 \text{ M}^{-1}\text{s}^{-1}$.

Figure 10.

Simulation of CE process following Eq.8 – Eq.10: current vs. square root of rotation frequency.

$K_1 = 1 \times 10^5 \text{ M}$, $k_{f1} = 500 \text{ M}^{-1}\text{s}^{-1}$, $k_{b1} = 0.005 \text{ s}^{-1}$; $K_2 = 2.8 \times 10^3 \text{ M}^{-1}$, $k_{f2} = 3.4 \times 10^4 \text{ s}^{-1}$, $k_{b2} = 12.1 \text{ M}^{-1}\text{s}^{-1}$.

Figure 11.

CAChe simulation of a dimerized $\text{Cu(II)}_2\text{-TRH}_2$ complex. An OH^- acts as a bridge. The lactam oxygen of the pGlu residue from the right monomer coordinates with Cu(II) of the left monomer in the apical position.