Electrochemical characterization of biochemically active Cu(II) mixed ligand complex with histidine and cysteine

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Abstract

Copper(II) complexes with cysteine and histidine, amino acids that coordinate copper(II) in human body, were investigated. Cu-His and Cu-Cys complexes were detected in pH range from 5.0 to 9.0 using voltammetric techniques. [CuHis2] complex reduces by two-electron reversible process at   
≈ -0.40 V, while [CuCys] complex by one-electron quasireversible process at -0.6 V, revealing strong adsorption at the electrode surface. When both amino acids are present in the solution, new peak appeared at -0.5 V, which corresponded to the [CuHisCys] complex reduction. Formation and characterization of mixed ligand complex was also supported by UV-Vis spectra recorded at fixed histidine and various cysteine concentrations. Formation of [CuHisCys] complex in the solution was detected and stability constant calculated to amount to log *K*CuHisCys = 16.9 ± 0.3. This study was the first attempt to characterize formation of Cu(II) mixed ligand complexation process with biochemically important amino acids in electron transport and oxygenation reactions in human body.

**Keywords:** copper, histidine, cysteine, mixed ligand complexes, voltammetry

**1. Introduction**

Copper(II) is an essential trace element found in proteins where it is involved in various metabolic mechanisms. Usually, copper ions are ligated by nitrogen ligands or by combination of nitrogen and sulfur donors, which is expected according to HSAB (Hard and Soft Acids and Basis) principle. Copper (I) is a soft acid while copper(II) is borderline, thus, it is expected to bind to sites dominating in amino acids containing side chains with soft or borderline ligands. Amino acids with nitrogen and sulfur donor (soft basis) atoms would therefore, be preferred over amino acids with hydroxyl, carboxyl, or primary amine side chains [1]. These proteins often play role in electron transport (plastocyanin, azurin, laccase), oxygenation reactions (tyrosinase, ascorbate oxidase), and in oxygen transport (hemocyanin). Most biologically active copper centers are found in proteins outside cells or in vesicles. Copper enzymes have been classified into three types, having distinctive geometries and ligand environments surrounding the metal center: Type I, Type II and Type III. Type I centers serve as electron-transfer sites in proteins such as azurin and plastocyanin and they have both cysteine and histidine in their structure. Electrochemical methods could be applied for examination of copper complexes with amino acids to help us understand which conditions are relevant in creation of aforementioned complexes (e.g. pH, ionic strength, concentrations of metal and/or ligand). Learning about copper mixed-ligand complexes with amino acids is also important, due to their key role in active sites of proteins, e.g. active site of the Type I metal center has histidine and cysteine [1,2]

Copper with amino acids complexes were investigated with different electrochemical techniques (cathodic stripping square-wave – SWV and cyclic voltammetry-CV) and UV-Vis spectroscopy. Cathodic stripping voltammetry is a voltammetric method for quantitative and qualitative determination of ionic species. In the accumulation step potential is held at particular potential and the reduced species strip from the electrode by sweeping the potential negatively. The stripping step can be either linear, staircase, square-wave, or pulse [3]. M. Hübneret. al. [4] performed spectroscopic studies of copper complexes with some amino acids, including histidine, in pH range 8.0 – 10.0. Different UV-Vis spectra were observed in case of histidine and copper and histidine complex. In case of Cu (II)-histidine complex, in visible domain (632 nm), a d-d transition was observed and assigned to the 2T2g →2Eg transition, specific for Cu(II) complexes with tetragonal distortion due to Jahn-Teller effect [4]. In case of cysteine, results of UV-Vis measurements showed that the stoichiometry of complexes in the solution gave 1:1 Cu(I)SR complex (*λ*max = 295 nm) [5,6]. According to Davis and Bordelon [7], who proceeded electrochemical measurements, the reduction mechanism for Cu(II)-histidine complex is a simple one-step two-electron reduction process with histidine forming a chelate with copper(II). They used DC polarography and the measurements were performed at pH 8.0. Bilewicz[8] investigated Cu(II)-histidine complexes with various voltammetric techniques, e.g. sampled dc, cyclic, normal pulse and reverse pulse voltammetry, at mercury electrodes at pH 7.2. Multiple voltammetric signals appeared and thus two mechanisms were considered, two-electron reduction of two different but interacting forms of Cu(II) and EE type of mechanism (mechanism involving two successive closely-spaced charge transfer steps) modified by adsorption of the intermediate Cu(I) complex [8]. Pena and Daniele [8] studied Cu-histidine system at platinum conventional and microelectrodes by cyclic voltammetry at pH 6. They reported on two-electron reduction of copper (II)-histidine complexes in SO42─ and ClO4─ solutions and more complex pathway in solutions containing Cl─. Weng and Cheng [10] studied Cu(II)-histidine complexes on glassy carbon electrode using cyclic voltammetry and circular dichroism spectroscopy in pH range 3.0 – 10.0.

Forsman investigated Cu (I)-cysteine complexes using cathodic stripping voltammetry (CSV) at pH 4.6 and 9.2 in buffered solutions [11]. In presence of cupric ions, cysteine forms a cuprous complex [Cu(I)RS] at the hanging mercury drop electrode surface. He noticed cysteine possible oxidation to cystine in presence of oxygen and concluded that de-aeration of the solution prior to mixing cysteine and Cu(II) is important in order to minimize oxidation risk [11-13]. Çakir et al. [5] investigated Cu(I)-cysteine complexes by voltammetric techniques at pH 7.4 in the Britton-Robinson buffer. They compared voltammetric behavior of cysteine in absence and presence of Cu(II) in solution.

Electrochemical studies have been dedicated to the investigation of copper(II)-histidine and copper(I)-cysteine complexes, but none of them described mixed ligand complex, particularly interesting as present in active sites of proteins [1,2]. In this work, Cu (II)-histidine-cysteine mixed ligand complex was investigated for the first time using voltammetric methods and UV-Vis spectroscopy to evaluate its qualitative characteristics and stability in aqueous solution.

**2. Experimental**

2.1. Equipment

Experiments were performed using AUTOLAB PGSTAT12 potentiostat (ECO Chemie, Utrecht, Netherlands) equipped with a Metrohm 663 VA stand (Metrohm, Herisau, Switzerland). The instrument was computer-controlled using GPES 4.9 control software. The working electrode was a static mercury drop electrode (SMDE) of 0.25 mm2 drop area, the counter electrode was platinum wire and the reference electrode was Ag/AgCl (sat. NaClO4).

Measurements were carried out in an electroanalytical quartz cell (25 mL) at 25 ± 1°C.

The pH value was checked by means of a combined glass-Ag/AgCl electrode connected to an ATI Orion PerpHecTMeter, model 320 (Cambridge, USA).

Electrochemical techniques applied were square-wave voltammetry (SWV) with the pulse amplitude, *A* = 25 mV; frequency, *f* = 50 s─1; potential step increment, *E*inc = 2 mV and cyclic voltammetry (CV) with scan rate, *v* = 0.05 V s─1. Prior to electrochemical measurements, the solutions were deaerated by extra pure nitrogen.

The UV-Vis absorption spectra were recorded on a Perkin Elmer Lambda 45 spectrophotometer (quartz cuvettes, path length 10 cm) in the wavelength range 300-900 nm. Measurements were performed in water solutions at pH = 7.0 ± 0.05, where pH and constant ionic strength were adjusted by HClO4/NaOH (*I*c = 0.15 mol L-1).

2.2. Chemicals and Solutions

Standard solution of copper(II) nitrate (1,57 × 10─2mol L─1) (FlukaChemie GmbH, Sigma-Aldrich, Buchs, Switzerland) and stock solutions of L-cysteine and L-histidine (Acros Organics, Geel, Belgium) were prepared using Mili-Q water. Solutions pH was adjusted by adding diluted *p.a.*HClO4 or *p.a.*NaOH (Merck, Darmstadt, Germany).

**3. Results and Discussion**

**3.1. SW voltammetric measurements of Cu(II)histidine and Cu(I)cysteine complexes**

SW voltammograms of 1 × 10-5 mol L-1 Cu(II) and various concentrations of cysteine ((0.1 – 2.0) × 10-5 mol L-1) revealed that peak at ─0.15 V, which corresponds to the reduction of free copper (II), decrease by increasing cysteine concentration. At the same time, new reduction peak appeared at ─0.60 V and shifted towards negative potential as the result of complex concentration increment (Fig. 1A). It was ascribed of cuprous cysteinate [CuCys] complex reduction to copper amalgam and free thiol [5]. [CuCys] complex reduction process appeared at same potential in both NaCl and NaClO4, which was expected due to the Cu(I) stabilization in the presence of cysteine. Asymmetric peak shape implied strong adsorption at the electrode surface. At low pH values (from 2 to about 5), two close reduction peaks were observed (≈ -0.2 and -0.3 V) which separated as pH increased. With pH increase, first reduction peak decreased, while second one shifted to more negative potentials (-0.27 – -0.45 V). According to literature data [14] second reduction peak was ascribed to Hg(I)-cysteine complex reduction at the electrode surface. At pH ≈ 5.7 [CuCys] complex reduction peak was registered at -0.6 V (Fig. 2A). In pH range from 5.7 to 9.0, peak potential shifted from -0.6 to -0.7 V with significant reduction current increase.

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**Figure 1** SW voltammograms of Cu(I)-cysteine (**A**) and Cu(II)-histidine (**B**) complexes in 0.15 NaClO4 with *C*Cu = 1 × 10─5 mol L─1, pH = 7.00 ± 0.05 and *C*Cys  **A**): (1) 0.0, (2) 0.3x10-5, (3) 0.5x10-5, (4) 0.6x10-5, (5) 0.7x10-5, (6) 0.8x10-5, (7) 1.0x10-5 mol L─1. **B**) *C*His: (1) 0.0, (2) 1.0 × 10─4, (3) 2.0 × 10─4, (4) 5.0 × 10─4, (5) 1.0 × 10─3, (6) 3.0 × 10─3 mol L-1;. *f*SW = 50 s─1, *a*SW = 25 mV, *E*inc = 2 mV.

Histidine (1×10─2mol L─1) is an electroinactive ligand and it does not interact with Hg. By adding 1×10─5 Cu(II) to the histidine solution (pH = 7.0) square-wave reduction peak at -0.4 V was recorded, corresponding to the reduction process of Cu(II)-histidine complex [9] (Fig. 1B). Reduction peak shifted towards negative potentials with pH in range from 5.0 to 9.0 (-0.3 ─ -0.6 V) linearly with slope of 50.45 mV/pH. Reduction current increased until pH = 6.0, and in higher pH range remained constant. According to the theoretical distribution at pH higher than 6.0, predominant species was [Cu(His)2] (Fig. 2B).

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**Figure 2** SW voltammograms of Cu(I)-cysteine (**A**) and Cu(II)-histidine (**B**) complexes in 0.15 NaClO4 with *C*Cu = 1 × 10─5mol L─1, *C*Cys = 1 × 10─5 mol L─1 and *C*His = 1 × 10─2 mol L─1 at different pH; *f*SW = 50 s─1, *a*SW = 25 mV, *E*inc = 2 mV.

Reduction mechanisms and electrochemical characteristics of [Cu(I)Cys] and [Cu(II)(His)2]complexes were examined by square-wave voltammetry by inspection of their reduction peak current, potential and half-peak width dependencies on SW frequency and amplitude.

Impact of SW amplitude and frequency on the net peak current (*i*p) and potential (*E*p) was investigated as it could provide a valuable information about redox mechanism. The reduction peak current of the Cu(I)-cysteine complex at pH 7.0 depended linearly on *f*SW in the range 8 – 250 s─1, with slope of 2.14 nA s. This is a characteristic of processes when reactant reduction proceeds from adsorbed state by EC mechanism. The net peak potential remained constant with SW frequency variation. Dependence of the [Cu(His)2] complex reduction peak current on *f*1/2 (range 8 – 250 Hz) was linear up to 25 Hz (slope = 9.32 nA s1/2), which is characteristic of reversible reduction processes with reactant and product adsorption. With the increment of the SW amplitude reduction peak split, indicating reduction process from adsorbed state [15].

Forward-backward SW voltammograms were inspected for both complexes (Fig 3A, 3B). Separation of SW response into forward current, measured before the “down” pulse and backward, reverse current measured at the “down” pulse of SW staircase gave us the information on reversibility of processes in terms of square-wave voltammetry[16]. The [CuCys] complex implied characteristics of the quasireversible process, while [Cu(His)2], with its peaks nearly at the same potentials, of reversible process.

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**Figure 3** SW forward – backward voltammograms in 0.15 mol L─1 NaClO4 for 1 × 10─5 mol L─1 Cu(II) and **A**) 1 × 10─5mol L─1 Cys; **B**) 1 × 10─3 His; pH = 7.00 ± 0.05.

**3.2. SW voltammetric measurements of Cu-histidine-cysteine complex**

Cysteine was gradually added to [CuHis2] complex solution and a new reduction peak appeared at  
 -0.5 V in the pH range from 5.0 to 7.0, which corresponded to the [CuHisCys] complex reduction (Fig 4.). The same reduction process was recorded when histidine was gradually added to the [CuCys] solution.

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**Figure 4**. SW voltammograms of binary complexes[Cu(His)2] and [CuCys] and ternary complex [CuHisCys] in 0.15 NaClO4 with: *C*Cu = 1 × 10─5, *C*His= 1 × 10─3, *C*Cys = 1×10─5mol L─1; pH = 7.00 ± 0.05.

Impact of the SW amplitude and frequency on the net peak current (*i*p) and potential (*E*p) was investigated as well. [CuHisCys] complex reduction peak current depended linearly on *f* in range 8 – 250 s─1, with slope 1.91 nA, that is characteristic of the reactant reduction from adsorbed state. Additionally, reduction peak splits in two with amplitude increase, what characteristizes electrode process from adsorbed state. The net peak potential did not change with frequency variation, that is, beside appearance of forward – backward voltammograms, an indication of electrode process quasireversibility .

For redox reactions with reactant adsorption peak width at half-height satisfied the relationship Δ*E*p/2 (mV) = (63.5 ± 0.5)/*αn*, where *α* is the average transfer coefficient and n is the number of simultaneously transferred electrons [17,18]. The obtained peak width at half-height of the Cu(I)-cysteine complex was 63.6 mV, which corresponds to α = 0.91, taking into account that Cu(I)-cysteine reduction process is one-electron. In case of Cu(II)-histidine-cysteine complex reduction peak width at half-height was 47.7 mV and *α* = 0.67, respectively, as reduction process is two-electron. Dependence of [Cu(I)Cys] and [Cu(II)HisCys] complexes reduction peak currents on SW amplitude were linear with slopes 6.69 nA/mV and 3.72 nA/mV, respectively.

According to equation:

*i*p = (5 ± 1) × 102*qαn*2*Faf*Δ*EΓ* (1)

amount of reactant adsorbed (*Γ*) was calculated from the slope (Δ*i*p / Δ*a*)a 10) in the range of low SW amplitudes [17]. Used values were *α* = 0.91 and *n* = 1 for the [Cu(I)Cys] and *α* = 0.67 and *n* = 2 for [CuHisCys] complex. Mercury drop area was *q* = 0.0025 cm2, SW frequency *f* = 50 Hz and scan increment Δ*E* = 2 mV. In case of the [CuCys] complex *Γ* was calculated to be 5.88 × 10-11 mol cm-2 while for [CuHisCys] 1.11 × 10-11 mol cm-2.

**3.3. Characterization of Cu(II) complexes by UV-Vis spectrophotometry**

Based on here presented voltammetric results, formation of mixed ligand complex Cu(II)-histidine-cysteine was shown. Mixed ligand complex presence in aqueous solution was also examined using UV-Vis spectrophotometry. Firstly, Cu(II) complexes with each amino acid were characterized. However, Cu(II)-cystein complex solutions were opaque in given experimental conditions, that obstructed its accurate characterization. UV Vis spectrophotometric titration of Cu(II) with histidine was performed in order to determine stability constant and stoichiometry of Cu-His complex. Solutions were of blue colour. Although, voltammetric measurements were conducted at pH 7.0, spectroscopic titration was performed at pH 7.0 to assure histidine imidazolyl moiety deprotonation and formation of CuL2 type complex, predominantly [19, 20]. Set of 14 solutions was prepared: Cu(II) concentration was the same in all solutions (5 x 10-4 mol L-1), while histidine concentration varied   
(7 x 10-4 - 1×10-2 mol L-1). Solutions were equilibrated overnight. Measured spectra showed strong hypsochromic shift (for 200 nm) and increase of absorbance in visible area. Solutions were of green colour. UV-Vis spectra were analyzed through the entire range of measured wavelengths (400-900 nm) by multivariate non-linear least square regression analysis using Specfit program [21 - 23].

The best model that described experimental data involved formation of mostly one Cu(II)-histidine complex: [CuHis2],while proportion of [CuHis]+ was too small to fit the data. Calculated stability constants was log *K*[CuHis2] = 17.5 ± 0.4 that agreed well with literature data [19, 20]. Calculated data were used for mixed Cu(II)-histidine-cysteine complex characterization. Again, set of 14 solutions was prepared: Cu(II) and histidine concentrations remained constant (*c*Cu =5 ×10-4 mol L-1, *c*His = 1×10-3 mol L-1 ), while cysteine concentration varied from 1 x 10-4 to 3 x 10-3 mol L-1. Similar as in the former experiment, spectra were recorded at fixed pH 7 and constant ionic strength 0. 15 mol L-1, adjusted by NaClO4 (Figure 5). Remarkably, displacement of one histidine molecule by cysteine caused strong hypsochromic effect (>60 nm) of mixed complex compared to [CuHis2] combined with absorbance increase. New absorbance maximum in UV Vis area <350 nm upon addition of cysteine appeared. Measured absorbance in UV area was out of instrument range. Spectra were analyzed by multivariate, non-linear, least square regression analysis using Specfit program [21 - 23]. Previously calculated [CuHis2] complex stability constant and amplitude were applied for calculation of [CuHisCys] mixed ligand complex parameters, stability constant and its calculated spectrum. Good agreement between experimental and calculated absorbance was obtained (Figure 5, Inset). Specfit calculations approved formation of [CuHisCys] mixed ligand complex that was registered in voltammetric measurement, as previously described. The best fitting model included coexistence 1:2 Cu:His complex and mixed ligand complex [CuHisCys] in solution, where one histidine was replaced by one cysteine molecule.

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**Figure 5.** UV Vis spectra of cCu = 5 × 10−4 mol L─1 and cHis = 1 × 10−3 mol L─1 recorded as a function of cystein concentration at pH = 7.00±0.05; *I*c = 0.15 mol L─1 (NaClO4); **Inset**: comparison of the experimental (●) and calculated (—) absorbances at λ= 450 nm.

Analysis approved [CuHisCys] complex formation with calculated stability constant log *K*[CuHisCys] =16.9±0.3 (Figure 6). Calculated Cu(II) complexes electronic spectra and corresponding distribution diagram is presented in Figure 6 with inset.

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**Figure 6.** Calculated electronic spectra of Cu(II), [CuHis2] and [CuHisCys] complexes. **Inset:** Distribution diagram of [CuHis2] and [CuHisCys] species; *C*Cu = 5 x 10-4 mol L─1, pH = 7.0±0.05.

**4. Conclusions**

Copper(II) as an important cofactor in various metalloenzymes, takes part in electron transport, reactions of oxygenation and oxygen transport. In organisms copper is most often coordinated with cysteine and histidine.

Characterization of Cu(II)-His, Cu(I)-Cys complexes and mixed ligand complex Cu(II)-His-Cys redox processes was performed by SWV, at Ep -0.4 V, -0.6 V and -0.5 V, respectively. By inspection of reduction current and potential dependence on frequency and amplitude it was concluded that [CuHisCys] complex reduction is quasireversible with a reactant adsorption. The amount of reactant adsorbed at the electrode surface was calculated to be *Γ* = 1.11 × 10-11 mol cm-2.

Detailed analysis of UV-Vis spectrophotometric measurements at pH = 7 gave quantitative data on the complexes, offering stability constants: log *K*Cu(His)2 = 17.5 ± 0.4 and log *K*CuHisCys = 16.9±0.3. Speciation calculated from determined stability constants are in good agreement with presumptions arising from voltammetric data.

Therefore, we believe that the described study on Cu (II) complexes with aminoacids and its mixed ligand complex is a precious contribution for understanding of copper bioavalability in the biochemical processes.

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**Figure captions:**

**Fig. 1.** SW voltammograms of Cu(I)-cysteine (**A**) and Cu(II)-histidine (**B**) complexes in 0.15 NaClO4 with *C*Cu = 1 × 10─5 mol L─1, pH = 7.00 ± 0.05 and *C*Cys  **A**): (1) 0.0, (2) 0.3x10-5, (3) 0.5x10-5, (4) 0.6x10-5, (5) 0.7x10-5, (6) 0.8x10-5, (7) 1.0x10-5 mol L─1. **B**) *C*His: (1) 0.0, (2) 1.0 × 10─4, (3) 2.0 × 10─4, (4) 5.0 × 10─4, (5) 1.0 × 10─3, (6) 3.0 × 10─3 mol L-1;. *f*SW = 50 s─1, *a*SW = 25 mV, *E*inc = 2 mV.

**Fig.2.** SW voltammograms of Cu(I)-cysteine (**A**) and Cu(II)-histidine (**B**) complexes in 0.15 NaClO4 with *C*Cu = 1 × 10─5mol L─1, *C*Cys = 1 × 10─5 mol L─1 and *C*His = 1 × 10─2 mol L─1 at different pH; *f*SW = 50 s─1, *a*SW = 25 mV, *E*inc = 2 mV.

**Fig**. **3**. SW forward – backward voltammograms in 0.15 mol L─1 NaClO4 for 1 × 10─5 mol L─1 Cu(II) and **A**) 1 × 10─5mol L─1 Cys; **B**) 1 × 10─3 His; pH = 7.00 ± 0.05.

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