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Coordination Chemistry Reviews 249 (2005) 895–909
COORDINATION CHEMISTRY REVIEWS
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Review

The saga of copper(II)–L-histidine

P. Deschamps[a], P.P. Kulkarni[a], M. Gautam-Basak[b,1], B. Sarkar[a,c,*]

[a] *Department of Structural Biology and Biochemistry, The Hospital for Sick Children, 555 University Avenue, Toronto, Ont., Canada M5G 1X8*
[b] *Office of New Drug Chemistry, Center for Drug Evaluation and Research, Food and Drug Administration, Rockville, MD 20857, USA*
[c] *Department of Biochemistry, University of Toronto, Ont., Canada M5S 1A8*

Received 29 June 2004; accepted 16 September 2004
Available online 26 November 2004

Contents

Abstract

Copper is an essential trace element required by all living organisms. Since the discovery in 1966 of copper(II)–L-histidine species in human blood, extensive research has been carried out to determine its role in copper transport. A small fraction of copper(II) bound to L-histidine maintains an exchangeable pool of copper(II) in equilibrium with albumin in human blood. The exchange of copper(II) between L-histidine and albumin modulates the availability of copper to the cell. The role of L-histidine during its interaction with copper(II)–albumin and in the cellular uptake of copper has generated considerable interest to determine the physico-chemical properties and the structure of physiological copper(II)–L-histidine complex. The structure of this complex remained inconclusive for the last four decades despite exhaustive

* Corresponding author. Tel.: +1 416 813 5921; fax: +1 416 813 5022.
E-mail address: bsarkar@sickkids.ca (B. Sarkar).

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0010-8545/$ – see front matter.
doi:10.1016/j.ccr.2004.09.013

characterization studies in aqueous solution. Recently, the physiological copper(II)–*bis*(L-histidinato) complex has been crystallized and the crystal structure has been solved. The structure shows a neutral five coordinate complex with a distorted square planar pyramidal geometry. The unique structural features explain its thermodynamic stability and kinetic reactivity. This review summarizes the overall perspectives encompassing copper(II)–L-histidine coordination chemistry and therapeutic applications of the physiological copper(II)–L-histidine complex. The copper(II)–L-histidine (1:2 complex at physiological pH) has been widely used in the treatment of Menkes disease (a genetic neurodegenerative disorder that leads to early death in the children due to impaired copper metabolism) and more recent use has been reported in the treatment of infantile hypertrophic cardioencephalomyopathy (a condition caused by mutations in *SCO2*, a cytochrome *c* oxidase assembly gene).

Keywords: Copper; Copper–histidine; Menkes disease; X-ray structure; Copper transport; Copper–albumin

1. Introduction

Copper(II)–L-histidine system is of particular interest due to its biochemical and pharmacological properties, as well as its different possible coordination modes. Since the discovery in 1966 of copper(II)–L-histidine species in human blood [1], extensive research has been carried out to determine its role in copper uptake into cells. Copper(II)–L-histidine is shown to be involved in copper transport before its entry into cellular transport systems [2]. The transport properties of copper(II)–L-histidine led to its application in the treatment of Menkes disease, a fatal genetic neurodegenerative disease [2,3]. Recently, the use of copper(II)–L-histidine in the treatment of infantile hypertrophic cardioencephalomyopathy has also been reported [4,5].

The physiological importance of copper(II)–L-histidine and its therapeutic applications are well known [1–5], however the structure of copper(II)–L-histidine complex at physiological pH remained inconclusive for the last four decades despite exhaustive characterization studies in aqueous solution [6–22]. Recently, Deschamps et al. have reported the isolation and the X-ray structure of this complex [23]. This discovery provides the opportunity to present the current understanding of the coordination chemistry of copper(II)–L-histidine system. The knowledge of the X-ray structure of physiological copper(II)–L-histidine complex is expected to provide further advances in its biochemical research. This review will also discuss the insights into structural features in relation to its physiological implications in copper uptake into cells and its action as a therapeutic agent.

2. Detection of copper(II)–L-histidine species in human blood

Copper is an essential trace element required by all living organisms [24,25]. It plays a key role as an integral component of many enzymes (Table 1) [26,27]. While trace amounts of copper are required for normal metabolic processes, it can be extremely toxic in excess [28]. Free copper, even in relatively low concentration, has the ability to generate free radicals through Fenton reaction and oxidize cellular components [28,29]. This places a burden on systems that normally transport copper between organs.

Copper exists only in bound forms in the body both in metalloproteins and in low molecular weight complexes to avoid its inherent toxicity. There are excellent reviews written in recent years, which address various aspects of copper metabolism, particularly the copper transport in human blood [30–33]. Most of the copper in human blood is bound to ceruloplasmin (∼90–95%). However, there is 5–10% of copper in the oxidation state of 2+, which constitutes an exchangeable pool. Bearn and Kunkel [34] first demonstrated that in plasma copper(II) was bound to albumin with a very high affinity. As early as 1954, Earl et al. [35] reported that 0.2–0.4% of unspecified amount of labeled low molecular weight copper(II)-binding substances was ultrafiltrable in serum. But it was not until the reports of Sarkar and Kruck [1] and those of Neumann and Sass-Kortsak [36] that the true nature of these low molecular weight copper(II)-binding substances became known. Neumann and Sass-Kortsak presented the evidence for an amino acid-bound fraction by reconstitution experiments [36]. Sarkar and Kruck detected and

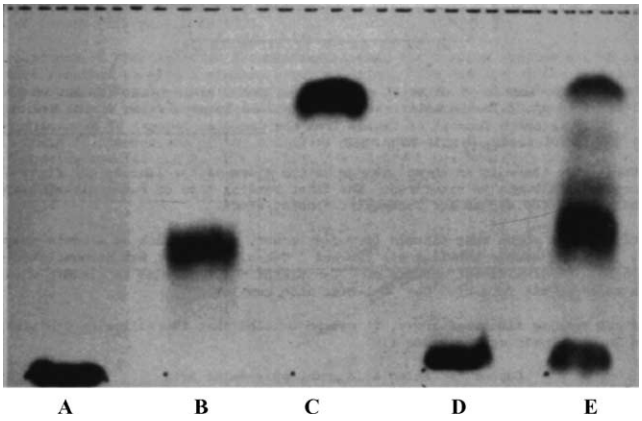


Fig. 1. Identification of copper(II)–L-histidine complex in human blood serum by thin-layer chromatography. Autoradiographic picture of thin-layer chromatograms: (A) $^{64}Cu(OH)_2$; (B) ^{64}Cu-*bis*-L-histidine; (C) ^{64}Cu-*bis*-L-threonine; (D) ^{64}Cu + ultrafiltrate from dialyzed human serum; (E) ^{64}Cu + desalted serum ultrafiltrate. Analysis of the intense band in (E) having a similar R_f value as that of control ^{64}Cu-L-histidine in (B) showed a Cu:L-histidine molar ratio of 1:2 (reprinted with permission from Ref. [1]; Copyright 1966 Academic Press).

Table 1
Copper–containing enzymes in humans

Copper enzymes	Functional role	Known or expected consequences of deficiency
Cytochrome *c* oxidase	Electron transport chain	Muscle weakness, neurological effects, hypothermia
Superoxide dismutase	Free radical detoxification	Uncertain
Tyrosinase	Melanin production	Failure of pigmentation
Dopamine β-hydroxylase	Catecholamine production	Neurological effects, possible hypothermia
Lysyl oxidase	Cross-linking of collagen and elastin	Arterial abnormalities; loose skin and joints
Ceruloplasmin	Ferroxidase	Anemia
Enzyme not known	Cross-linking of keratin	Pili torti

Table 2
Theoretical distribution of copper(II) in the ultrafiltrable fraction of normal blood plasma, as obtained by computer simulation in aqueous solution at pH 7.4 (37 °C, $NaClO_4$ 0.15 mol dm^{-3}), using available stoichiometric stability constants [39,40]

Complex species	log β	Percentage of ultrafiltrable copper[a]
Cu(II)(L-His)(L-Gln)	16.70	19.2
$Cu(II)(L\text{-}His)_2$	17.50	15.5
Cu(II)(L-His)(L-Thr)	17.03	14.7
Cu(II)(L-His)(L-Ser)	16.97	7.5
Cu(II)(L-His)(L-Ala)	17.00	5.4
$Cu(II)(L\text{-}His)(L\text{-}Lys)^{+}$	17.05	4.4
Cu(II)(L-His)(Gly)	16.94	4.3
Cu(II)(L-His)(L-Asn)	16.81	4.2
Cu(II)(L-His)(L-Val)	16.93	3.9

[a] The percentage of these species in the ultrafiltrable fraction of normal blood plasma should be considered with caution because these computational studies did not consider the copper binding to albumin in their calculations.

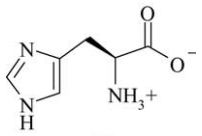
isolated copper(II)–L-amino acid complexes from the normal human blood serum using thin-layer chromatography [1]. They showed that copper(II)–L-amino acid complexes exist predominantly as copper(II)–*bis*-L-histidine complex along with mixed copper(II)–L-amino acid complexes containing L-histidine ligand (Fig. 1).

The formation of mixed copper(II)–L-amino acid complexes has also been demonstrated in in vitro systems [37,38]. Computational studies on copper speciation supported the presence of comparable concentrations of copper(II)–L-histidine complex and mixed copper(II)–L-amino acid complexes involving L-histidine ligand on the basis of stoichiometric stability constants (Table 2) [39,40].

3. Equilibrium between copper(II)–L-histidine species in aqueous solution

3.1. L-Histidine, a potential tridentate ligand

Among the amino acids, L-histidine is one of the strongest metal coordinating ligands and plays an important role in the binding of metal ions by proteins [27]. L-Histidine has three potential metal-binding sites, namely the carboxylate oxygen ($O_{carboxyl}$), the imidazole imido nitrogen (N_{im}) and the amino nitrogen (N_{am}) (Fig. 2). The imidazole nitrogen of L-histidine residues often provides the primary means by which the metal ions are bound to proteins.



(1)

Fig. 2. L-Histidine, a potential tridentate ligand (**HL** form: **1**).

Fig. 3 shows the distribution of four different protonated forms of L-histidine as a function of pH [41]. To be concise, the following abbreviations $\mathbf{H_3L}$, $\mathbf{H_2L}$, **HL**, $\mathbf{L^-}$ corresponding to different protonated forms of L-histidine, will be used here. The pK_a values are 1.8, 6.0, and 9.18, respectively [42]. The dissociation of the second proton of the imidazole group (p$K_a \sim 14$) is difficult to estimate. The major form of L-histidine at physiological pH is **HL**. When the pH value is increased, the amine proton is deprotonated to give a mononegative anionic ligand $\mathbf{L^-}$. On the other hand, when the pH value is lowered, the N_{im} and $O_{carboxyl}$ are respectively protonated. It is most likely that the sequence in which the protons are removed by titration is also the sequence in which the potential donor atoms are used in the metal-binding as the pH increases. However, the use of this criterion alone is risky because the order of pK_a values may not be the same as that of the enthalpy changes accompanying chelate formation, which provides a measure of the relative thermodynamic stabilities of the metal-binding and protonation [43]. Besides proton displacement reaction, several other factors are known to influence the structural arrangement, which include metal–ligand bond strengths (usually expressed in terms of formation constants), stereochemistry of the resultant complex, entropy of chelation and ligand field stabilization energy. The most important negative contributions to the enthalpies of chelation come from the formation of the metal–N_{am} bonds. The enthalpy changes accompanying metal–$O_{carboxyl}$ are not favorable and the chelation of $O_{carboxyl}$ depends on the increase in entropy, which results from the release of aquo-ligands and from the mutual neutralization of the metal and carboxyl group charges [43].

As evident, L-histidine can bind as a mono-, bi-, and tridentate ligand and its mode of coordination implicitly depends on the pH of the solution. All these different coordination modes are known for cobalt(II)–L-histidine system [44,45]. At physiological pH, the tridentate chelation of L-histidine ligand has been established for *bis*-metal complexes of cobalt(II),

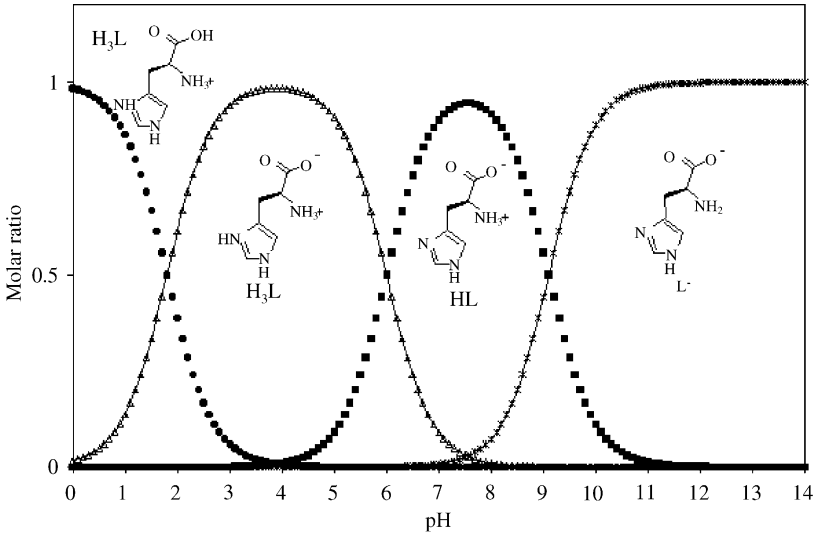


Fig. 3. Distribution of various protonation forms of L-histidine as a function of pH [41].

nickel(II), zinc(II), cadmium(II) [43,46–49]. Table 3 presents the stability constants and enthalpies of formation of complexes involving L-histidine and different divalent metals ions [50]. Copper(II)–L-histidine is the most stable in comparison with the other metal(II)-L-histidine complexes. Further, its highest value of negative enthalpies of formation suggests unique coordination properties.

3.2. Speciation of copper(II)–L-histidine system

The composition of the predominant complexes of copper(II) and L-histidine in aqueous solution depends upon the metal ion to ligand ratio, temperature and pH.

For 1:1 ratio, the $[Cu(His)]^+$ (**ML**) species dominates at pH 6 [51]. Above pH 6 it is converted to **ML**(**OH**) and below pH 5 no species comprises more than 50% of all copper(II) species, the greatest contributor being $[Cu(HisH)]^{2+}$ (**MHL**) which maximizes near pH 3.7 at less than 40%. Most of the remaining ligand is unbound at pH $\leq$ 3.7.

Solutions containing 1:2 or higher molar ratios of copper(II) to L-histidine possess the same species at most pH values [8,20,52]. Fig. 4 shows the equilibrium between these different copper(II)–L-histidine species over a wide range of pH [8]. The copper(II)–L-histidine species detected progressively as pH increases are: $[Cu(HisH)]^{2+}$ (**MHL**), $[Cu(His)]^+$ (**ML**), $[Cu(HisH)_2]^{2+}$ (**MH_2L_2**), $[Cu(His)(HisH)]^+$ (**MHL_2**), $[Cu(His)_2]$ (**ML_2**) and $[Cu(His)_2(OH)]$ (**$MH_{-1}L_2$**). Results of this detailed titration study indicate that among all these species, the major copper(II)–L-histidine species are **MHL**, **MHL_2** and **ML_2**. Relations between these species are summarized in Fig. 5.

The identification of copper(II)–L-histidine species, their stability constants and their quantitative distribution over a wide pH range provide a suitable basis for studying the coordination chemistry of this system in solution. Nevertheless, these results must be used with caution. It is generally accepted that only **ML_2** species is present around physiological pH in aqueous solution (>99% at pH 7.4). But, it is important to note that the experimental conditions clearly influence the copper(II)–L-histidine species distribution as a function of pH values. For example, a change of molar ratio of copper(II):L-histidine from 1:8 to 1:10 at fixed tempera-

Table 3
Logarithms of stability constants and negative enthalpies of formation [50]

		Co(II)	Ni(II)	Cu(II)	Zn(II)	Cd(II)
Stability Constants[a]	$\log K_1$	6.9	8.7	10.1	6.6	5.4
	$\log K_2$	5.5	6.9	8.0	5.5	4.3
Enthalpies of formation[a] (kcal/mol)	$-\Delta H_1$	9.6	16.2	21.0	11.6	–
	$-\Delta H_2$	12.3	16.6	21.3	11.7	–

[a] $M + L \rightleftharpoons ML\,(K_1, \Delta H_1)$, $ML + L \rightleftharpoons ML_2\,(K_2, \Delta H_2)$.

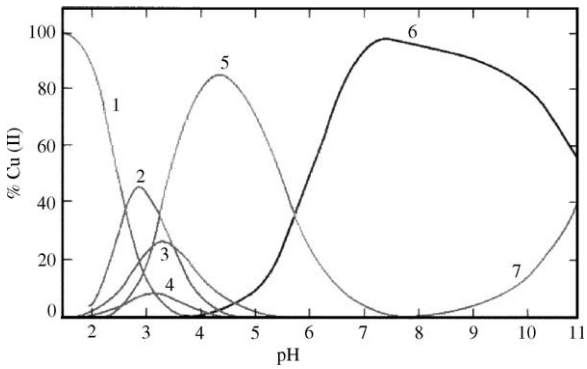


Fig. 4. Species distribution in the copper(II)–L-histidine system as a function of pH in 1:8 copper(II):L-histidine molar ratio. (1) Unbound M; (2) **MHL**; (3) **ML**; (4) **MH_2L_2**; (5) **MHL_2**; (6) **ML_2**; (7) **$MH_{-1}L_2$** (**M**: copper(II), **L**: L-histidine, charges are omitted) (reprinted with permission from Ref. [8]; Copyright 1973). (Please note the detailed structures of these different species are described in Section 4.2.)

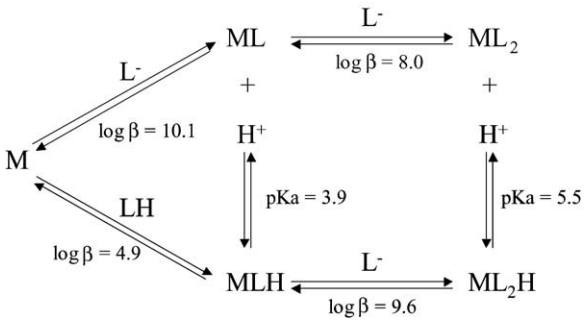


Fig. 5. Relation between the predominant copper(II)–L-histidine species present in aqueous solution. Logarithms of stoichiometric stability constants were determined at 25 °C and 0.1 ionic strength [8].

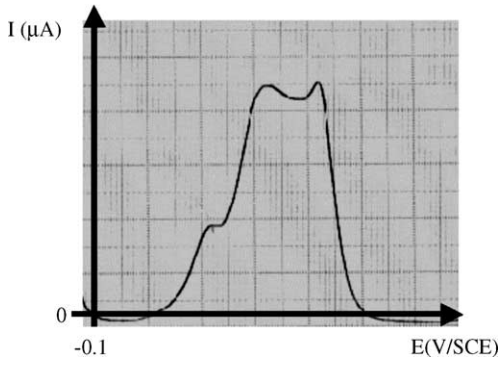


Fig. 6. Polarogram in impulsional mode of a copper(II)–L-histidine solution in 1:20 molar ratio at pH 7.4. Each peak corresponds to the detection of one copper(II)–L-histidine species present in solution ($[Cu^{2+}] = 10^{-3}$ M, $T = 298$ K, phosphate buffer, 1 mL gelatine, 1 cm → 100 mV/SCE and 1 cm → 0.125 μA) [41].

ture and pH values induces a slight shift in the copper(II)–L-histidine species distribution [8]. An EPR study suggested the formation of [Cu(II)(L-His)$_4$] in frozen solution around physiological pH [14]. It is proposed that four equivalent imidazole nitrogen atoms are bound to copper in the presence of excess histidine. In this case, the conditions used: pH of 6.8–7.3, excess of ligand, rapid freezing and immobilized complexes, all have contributed to the formation of this 1:4 copper(II)–L-histidine form. In the same way, a recent polarographic analysis in impulsional mode shows a significant presence of different copper(II)–L-histidine species at physiological pH in a large excess of L-histidine ligand in solution (Fig. 6) [41]. Each copper species has a specific half wave potential value at fixed pH, allowing its detection in solution by polarography in impulsional mode.

4. Coordination chemistry of copper(II)–L-histidine system

4.1. Origin of the saga

A major effort was made to study the stability, speciation and structure of copper(II)–L-amino acid complexes [53,54].

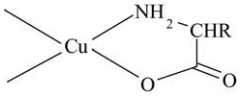


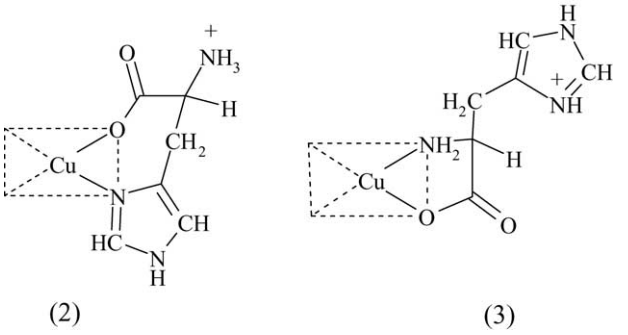
Fig. 7. Common bidentate chelation mode of amino acids with non-coordinating side chains forming thermodynamically stable five-membered chelate ring.

Indeed, the copper(II) interactions with amino acids have been of considerable interest because of their various possible coordination geometries. From the reported crystal structures of copper(II)–L-amino acids complexes [55–60], copper(II) complexed with L-amino acids has been shown to adopt a variety of coordination geometries (from distorted square planar, flattened tetrahedral and distorted square-pyramidal to distorted octahedral).

For the amino acids with non-coordinating side chains, the more common mode of coordination is bidentate chelation giving rise to the more thermodynamically stable five-membered chelate ring (Fig. 7) [54]. For the amino acids with a potential coordinating side chain, the coordination geometry depends on the nature of the metal ions. Many amino acids, being potentially tridentate, binds as bidentate ligands, especially with a metal ion such as copper(II). In addition to the thermodynamic stability of five-membered chelate ring, the bidentate chelation is facilitated by the "softness" of the copper(II) coordination sphere induced by the stereochemical manifestation of the Jahn–Teller effect [61–63]. The coordination compounds of copper(II) generally consist of four nearby donor atoms arranged approximately in a square plane around the metal ion, with the possibility of one or two more distant axial donors [53,64]. At neutral pH, L-glutamine interacts with Co(II) and Ni(II) as a tridentate ligand, but not with copper(II) [54,60].

The stereoselectivity of amino acids is another important factor in the coordination mode of copper(II)–L-amino acid complexes [54]. In the equatorial plane of *bis*-complexes, both *cis*- and *trans*-isomers are possible. If the two L-amino acids are of the same chirality, both side chains will be on the same side of the coordination plane in the *trans*-isomer and on opposite sides in the *cis*-isomer. The formation of the major complex will depend on the relative energy differences between the *cis*- and *trans*-isomers on the one hand and the five-membered chelate ring on the other. The steric control will be governed by whichever is the larger.

The combination of all these factors induces a specific structural arrangement for each copper(II)–L-amino acid complex. Copper(II)–L-histidine system can be considered as a unique case due to the difficulties encountered in the investigation of its coordination modes. L-Histidine may be chelated in bidentate mode. The three donor atoms of L-histidine cannot occupy the same plane and if L-histidine is tridentate, the question arises as to which ones occupies the planar position around copper(II). For the last four decades, the coordination modes of copper(II)–L-histidine has baffled many researchers. Almost all the possible coordination modes have



(2) (3)

Fig. 8. Two different proposed structures of **MHL** [9,11].

been proposed for $\mathbf{ML_2}$ complex. This is the origin of the saga of "copper–histidine".

4.2. Structures of the species in the copper(II)–L-histidine system

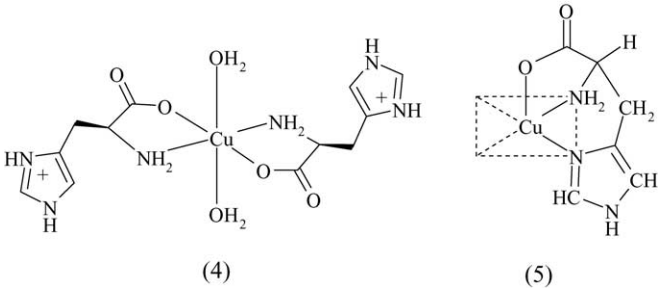
In aqueous solution, copper(II)–L-histidine system assumes various coordination modes depending on the pH value. Both *mono*- and *bis*-L-histidine complexes have been identified. Here, we describe the predictive structures, based on the studies carried out in solution and those determined by single-crystal X-ray diffraction method.

4.2.1. *MHL species*

At pH 2, the predominant species is **MHL**. The location of the ionizable proton in **MHL** is uncertain. The imidazole nitrogen N_{im} and the carboxyl oxygen $O_{carboxyl}$ bindings seem to be evident from the IR results [9]. The corresponding structure **2** is shown in Fig. 8. This proposed coordination mode is the most accepted [51,65], but another chelation mode has also been suggested [6,11]. Casella and Gullotti [11], using nearly identical conditions, proposed the coordination mode **3** for **MHL** because their CD studies suggested that below pH 3, the imidazole group of L-histidine is not involved in binding to copper(II) (Fig. 8). The coexistence of several species present in solution may explain the uncertainty about coordination mode of L-histidine in **MHL**.

4.2.2. *MH_2L_2 and ML species*

$\mathbf{MH_2L_2}$ and **ML** species show maximum concentration at almost the same pH. For a long time, the only X-ray structure available was that of $\mathbf{MH_2L_2}$. The X-ray structure of this species which crystallized from a solution at pH 3.7 has been solved by Evertsson [66]. The structure of cationic *trans*-isomer $\mathbf{MH_2L_2}$ (**4**) species showed that two N_{am} and two $O_{carboxyl}$ atoms are coordinated to the copper(II) ion (Fig. 9). The N_{im} atoms, being protonated, are not bound to copper(II). The mode of coordination around copper(II) center is square planar with the four donor atoms situated at 1.93–2.00 Å. There are two water molecules, one above and one below this plane, at distances of 2.46 and 2.78 Å, respectively. This binding mode, involving N_{am} atoms in the first coordination shell, shows that the sequence in which the protons of L-histidine are removed is not the sequence in which potential donor atoms are used in the metal-binding. It was suggested by Makinen et al. [67] that copper(II) reacts with the neutral zwitterionic L-histidine followed by a rapid proton transfer from the amino group to the imidazole ring.



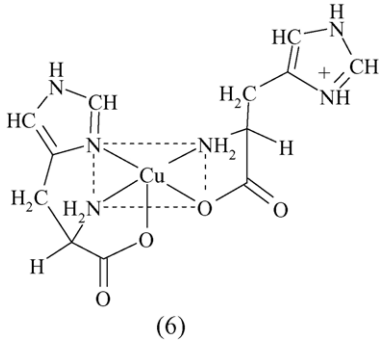
(4) (5)

Fig. 9. X-ray structure of the complex ion $\mathbf{MH_2L_2}$ $[Cu(C_6H_9O_2N_3)(H_2O)_2]^{2+}$ (**4**) and predictive structure of **ML** (**5**).

It is difficult to elucidate the structure of **ML** since the amount of this species is always exceeded by either **MHL** or $\mathbf{MH_2L_2}$. Nevertheless, ESR, CD and electronic data established the presence of two nitrogen donor atoms in the copper(II) coordination environment in a square planar arrangement [9–11]. The IR data also showed carboxylate group involvement in the first coordination shell [9]. Nevertheless, some studies suggested that the carboxylate group is only weakly bound [6]. The predictive structure **5** of **ML** is shown in Fig. 9.

4.2.3. *MHL_2 species*

The structure of $\mathbf{MHL_2}$ is considered to be a combination of one tridentate L-histidine ligand with a weakly coordinated axial carboxylate group and one bidentate L-histidine ligand in solution [9–11,19]. The visible absorption and CD spectra near pH 5 are similar to that of the mixed complex with L-histidine and L-1-methylhistidine, a bidentate ligand with a non-chelatable imidazole group [50]. A simple acid–base relation between $\mathbf{MH_2L_2}$ and $\mathbf{MHL_2}$ is observed. The predictive structure **6** of $\mathbf{MHL_2}$, deduced from IR, ESR, CD and UV–vis spectroscopies, is presented in Fig. 10 [9–11,19].



(6)

Fig. 10. Proposed structure of $\mathbf{MHL_2}$ (**6**).

*4.2.4. Physiological **ML$_2$** species*

4.2.4.1. Structural studies in solution. Extensive research has been carried out to determine the structure of physiological copper(II)–L-histidine complex. Despite efforts by many laboratories to crystallize the **ML$_2$** complex, such attempts often led to the formation of dark solution due to the sensitivity of this complex to light and oxygen. These unsuccessful results to crystallize **ML$_2$** complex led many researchers to focus on studies in aqueous solution by various spectroscopic techniques such as IR, ^{13}C- and ^{1}H NMR, ESR, ENDOR, Raman, UV–vis, CD and EXAFS [6,7,9–22]. Even with all these studies, the greatest uncertainty remained for the structure of **ML$_2$** in aqueous solution. This complex has been variously described as coordination mode involving (a) the amino and imidazole groups in the square plane, (b) the amino and carboxylate groups in the square plane. Yet other studies have suggested mixed binding modes for L-histidine ligands. This saga of coordination chemistry of copper(II)–L-histidine at physiological pH was further compounded by many studies reporting that an equilibrium between two different coordination configurations may also be possible.

The involvement of $O_{carboxyl}$ in axial position was beyond speculation for some studies. The formation constant [19], enthalpy of formation [68] and degradability by H_2O_2 [65] of copper(II)–L-histidine complex at pH 7.4 are very similar to those of copper(II)–histamine complex formed under the same conditions. Histamine has no carboxyl group, so it was suggested that the metal must be bonded only to the N_{am} and N_{im} atoms. Nevertheless, some studies suggested that the $O_{carboxyl}$ of first L-histidine ligand may be coordinated in an apical position, though it is strained [2].

*4.2.4.2. Model compounds of the structural arrangement of **ML$_2$** complex.* Several X-ray crystal structures of copper(II) complexes provided insights into a possible arrangement between copper(II) and L-histidine. In earlier studies from our laboratory, we have crystallized the complex [Cu(II)(L-His)(D-His)$(H_2O)_2$]·4H_2O (**7**) at pH 8 (Fig. 11) [69]. The copper atom is octahedrally coordinated by N_{am} and N_{im} atoms of L-histidine and D-histidine in a square planar arrangement and by oxygen atoms of water molecule at the axial positions. The Cu(II)–N distances are 2.03 Å (N_{am}) and 2.00 Å

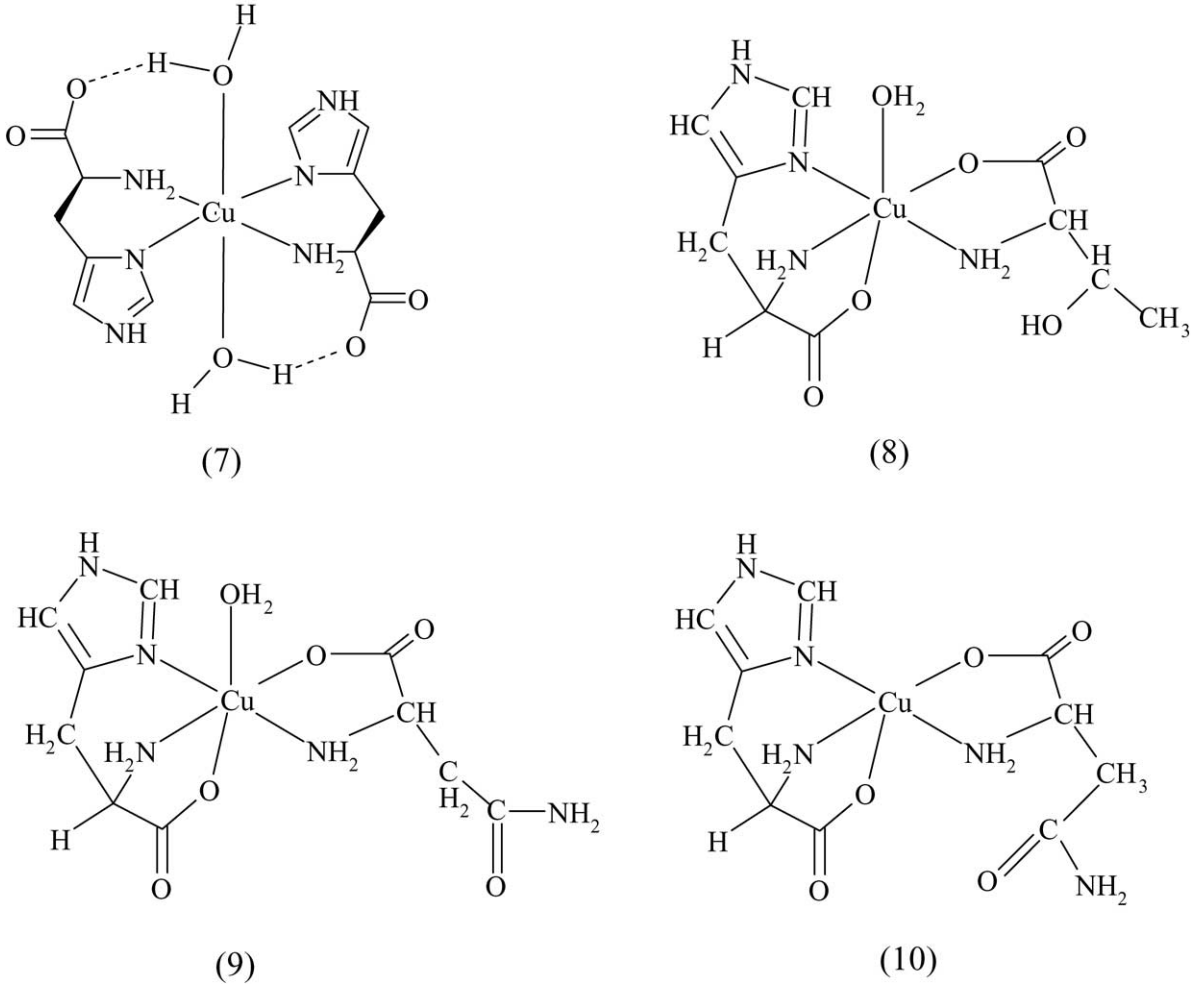


Fig. 11. X-ray crystal structures of [Cu(II)(L-His)(D-His)$(H_2O)_2$]·4H_2O (**7**), [Cu(II)(L-His)(L-Thr)(H_2O)]·H_2O (**8**), [Cu(II)(L-His)(L-Asn)(H_2O)]·3H_2O (**9**), and [Cu(II)(L-His)(L-Asn)] (**10**) [69,71–73].

(N_{im}) and the Cu–OH_2 separation is 2.57 Å. The carboxylate groups of histidine molecules secondarily coordinate to the copper(II) through hydrogen bonding to the axial water molecules. This structure shows the involvement of both imidazole groups in *trans*-geometry. It is interesting to note that the crystal structure of $[Cu(II)(histamine)_2](ClO_4)_2$ has also been determined and the structural features are very similar to **7** [70]. Four nitrogen atoms occupy the coordination plane with two axial perchlorates. The nitrogen donor atoms from two planar imidazole rings occupy *trans*-coordination positions at 1.98 Å from the copper(II) center.

In mixed copper(II)–L-amino acid complexes, such as [Cu(II)(L-His)(L-Thr)(H_2O)]·H_2O (**8**), [Cu(II)(L-His)(L-Asn)(H_2O)]·3H_2O (**9**), and [Cu(II)(L-His)(L-Asn)] (**10**) complexes, L-histidine acts as a tridentate ligand (Fig. 11) [71–73]. The X-ray analyses of these complexes revealed that they have the same structural features. Interestingly, the structures **9** and **10** show different conformations of the polar side chain of L-asparagine. For the tetrahydrated complex, the side chain is stretched while the anhydrous complex presents a bent side chain. Both amide groups of L-asparagine ligand are H-bonded to adjacent complex molecules and/or water molecules. It has been suggested that the amide nitrogen of this polar side group may form a H-bond with the axially coordinated $O_{carboxyl}$ of L-histidine ligand by rotation around the C_α–C_β bond and a slight deformation of the chelate ring for the anhydrous complex [74].

During our recent efforts to elucidate the structure of copper(II)–L-histidine species at physiological pH, first we crystallized a novel copper(II) complex with modified L-histidine ligand (Fig. 12) [75].

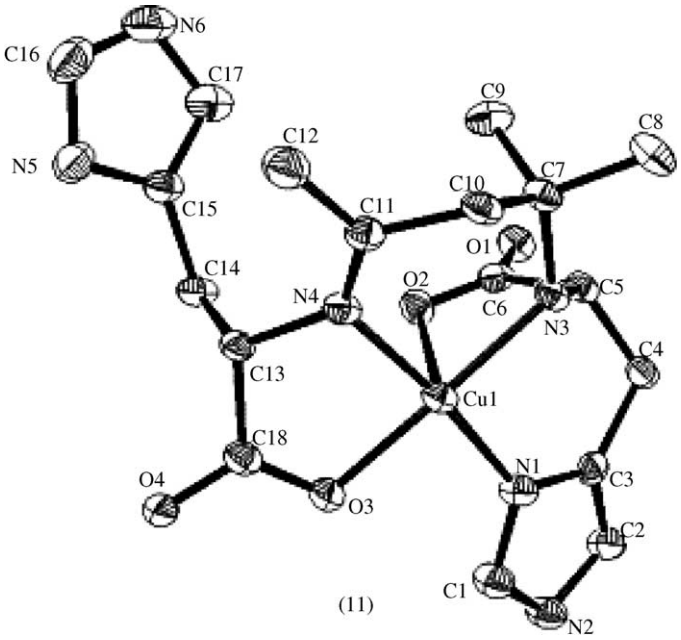


Fig. 12. X-ray crystal structure of **Cu(II)A** with **A** = 2-[(1,3-dimethylbutylidene)-3-*N*(1-(1H-imidazol-4-yl)ethanoic acid)]-3-(1H-imidazol-4-yl) propanoic acid [75].

The copper(II) ion is coordinated by a tetradentate ligand with the amino and imidazole imido nitrogen atoms on one side versus imino nitrogen and carboxylate oxygen atoms on the other side in a distorted square-planar geometry. The novel ligand is obtained by the reaction between the L-histidine molecules coordinated to copper(II) and 4-hydroxy-4-methylpentan-2-one formed by aldol condensation of acetone. Two oxygen atoms complete the distorted octahedral coordination sphere. The first oxygen atom is originated from carboxylate group of the first L-histidine ligand at 2.50 Å and the other oxygen atom is provided by symmetry. This compound presents similar structural features that those of mixed copper(II)–amino acid complexes [75].

4.2.4.3. X-ray crystal structure of $\mathbf{ML_2}$. Recently, Deschamps et al. reported the isolation and the X-ray structure determination of the physiological $\mathbf{ML_2}$ species [23]. Fig. 13 shows the X-ray crystal structure of $\mathbf{ML_2}$ (**12**), a neutral five coordinate distorted square-pyramidal complex. One of the L-histidine ligands acts as a monoanionic bidentate form through N_{am} and $O_{carboxyl}$ atoms. While the other binds in monoanionic tridentate ligand towards copper(II) center through its N_{am}, N_{im}, and $O_{carboxyl}$ atoms. The $O_{carboxyl}$ atom lies in an axial position.

The X-ray structure obtained is different from all the structures suspected for copper(II)–L-histidine species at physiological pH in solution to date. The important variables such as pH and ligand-to-metal ratios were not considered sometimes in previous studies, but this aspect alone is not sufficient to explain the inconclusive structural results in solution for the last four decades. It is likely that the exact nature of **12** was difficult to determine in solution due to the fact that each technique allowed the identification of some structural features but not all. The strong fingerprint of the binding Cu(II)–N_{im} could be misleading in many spectroscopic studies, such as CD and UV–vis analyses. The involvement of carboxylate groups was indicated by IR studies, but the num-

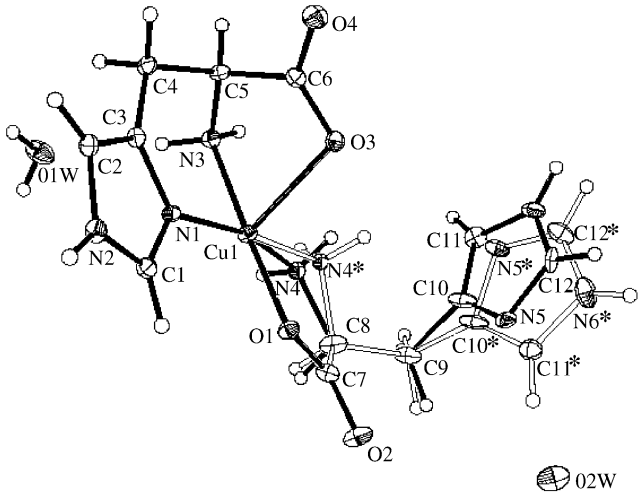


Fig. 13. X-ray crystal structure of physiological $\mathbf{ML_2}$ complex (**12**) [23].

ber of $O_{carboxyl}$ group(s) involved in the first coordination shell of copper(II) was not conclusive.

The structural arrangement in the complex **12** is the result of a combination of steric effect induced by the tridentate L-histidine ligand and the preference of copper(II) ion for a square planar geometry. Only one imidazole group is bound to copper(II) center and the other imidazole is trapped in an unfavorable position closed to the axial $O_{carboxyl}$ group of the tridentate L-histidine ligand. This pendant imidazole group further fails to rotate in proximity to the remaining axial site in the coordination sphere due to the *S*-stereochemistry of L-histidine ligand. The stereoselectivity is a crucial factor in the coordination mode of L-histidine ligands. It induces electrostatic interactions, such as H-bonding involving the pendant imidazole and the carboxylate group of tridentate L-histidine and could explain the excellent stability of complex **12**. The presence of racemic ligands in [Cu(II)(L-His)(D-His)$(H_2O)_2$]·$4H_2O$ decreases the steric effects, explaining in this case the involvement of both imidazole groups in *trans*-geometry. This factor was not considered with the use of histamine as model and it explains why the coordination mode is different with L-histidine in solution.

The intense blue crystals of **12** were obtained from an aqueous solution of copper(II)–L-histidine in 1:2 molar ratio at pH 7.4. A volume of *N*,*N*-dimethylformamide (DMF) was added to make 50/50 v/v water/DMF mixture. The crystallization induced by slow diffusion of ethanol yielded small clusters. Isolation of **12** was facilitated by its solubility and presence of pendant basic imidazole group that assist the crystal growth through non-covalent interactions. The crystal structure of aquocopper(II)–L-amino acid complexes is greatly dominated by intermolecular hydrogen bonds formed between water hydrogen atoms and carbonyl oxygen atoms from the surrounding molecules [76]. When two or more water molecules are present in crystal lattice, the copper complexes are additionally linked through a network of hydrogen bridges between water molecules and the amino acid's oxygen and nitrogen atoms. In our crystallization process, the addition of DMF reduces H-bonding interactions involving the pendant imidazole group and anions in aqueous solution facilitating the crystallization process preceded by the salt precipitation.

The bidentate L-histidine ligand shows a positional disorder. The observed disorder is due to the presence of two conformations of the side chain of the pendant L-histidine ligand. The water molecule (O2WB) was only half occupied in the crystal structure of **12** and it appears that it caused the reorientation of the unbound imidazole when it was present due to H-bonding to N(5). The use of other solvents could help to resolve the observed disorder in **12** facilitating either the stretched or the bent configuration by reducing or growing H-bonding interactions involving the pendant imidazole group.

4.2.5. $MH_{-1}L_2$ species

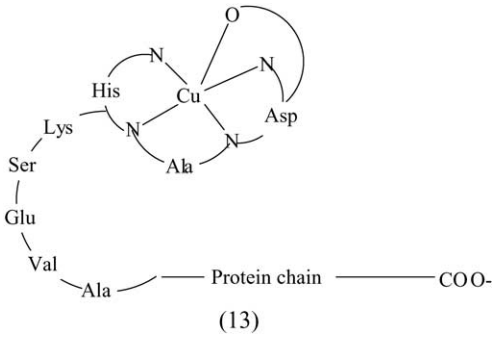
The species $\mathbf{MH_{-1}L_2}$ is formed at a pH greater than 8. No structural assignment of this species is proposed. This is because of the instability of the copper(II)–L-histidine system in the pH range above 9. At high pH, the loss of proton could result either from the addition of OH^- or the pyrrole hydrogen ionization.

5. Copper(II)–L-histidine complex as a copper transporter in humans

5.1. Equilibrium in the exchangeable pool of copper(II) in human blood

In human blood, albumin binds copper with very high affinity and thus acts as a buffer against copper toxicity [24,77]. Equilibrium dialysis, visible spectroscopy and proton displacement studies demonstrated the presence of a single high affinity copper(II)-binding site on albumin [78,79]. The N-terminal region of albumin, with the involvement of the amino acid sequence Asp–Ala–His, provide a specific binding site for copper(II) ion (Fig. 14) [80–84]. The copper(II)-transport site on albumin was suggested to involve the N_{amino}, two $N_{peptide}$, and the $N_{imidazole}$ of the histidine residue in position 3 in a square planar geometry. The carboxyl side chain of the amino-terminal aspartyl residue has also been reported to be involved in copper(II) chelation for albumin [80]. A more recent study on the binding of copper(II) to human albumin suggested also the involvement of the lysine residue [85]. This ^{1}H NMR analysis shows that the paramagnetic effects due to copper(II) caused specific perturbation of the resonances for the three N-terminal residues Asp–Ala–His as well as those for Lys.

A small fraction of copper bound to L-amino acids maintains an exchangeable pool of copper in equilibrium with albumin in human blood [1]. This fraction is constituted almost exclusively of binary copper(II)–L-histidine complex and mixed copper(II)–L-amino acid complexes involving L-histidine ligand [1,37,38,86]. Copper(II)–L-histidine complex and mixed copper(II)–L-amino acid complexes involving L-histidine ligand have similar stoichiometric stability constant values (log $\beta \sim 17$–18). The similarity of structural features between copper(II)–L-histidine complex (**12**) and the mixed copper(II)–L-amino acid complexes involving L-



(13)

Fig. 14. Proposed structure of the Cu(II)-binding site of human serum albumin (**13**).

Table 4
Structural features of the first coordination shell of copper for some copper(II)–amino acid complexes involving L-histidine ligand

Copper complexes	Coordination binding of L-histidine ligands	Atoms involved	Distances Cu–X (Å)	Ref.
Cu(II)(L-His)$_2$	Tridentate	N_{am}	2.003	[23]
		N_{im}	1.984	
		$O_{carboxyl}$	2.277	
	Bidentate	N_{am}	2.034	
		$O_{carboxyl}$	1.957	
Cu(II)(L-His)(L-Thr)	Tridentate	N_{am}	2.01	[71]
		N_{im}	1.95	
		$O_{carboxyl}$	2.58	
	Bidentate	N^{*}_{am}	2.00	
		$O^{*}_{carboxyl}$	1.97	
Cu(II)(L-His)(L-Asn)	Tridentate	N_{am}	2.03	[73]
		N_{im}	1.94	
		$O_{carboxyl}$	2.39	
	Bidentate	$N^{\#}_{carboxyl}$	1.95	
		$O^{\#}_{carboxyl}$	1.99	

* and # are associating to atoms originating from L-threonine and L-asparagine, respectively.

histidine ligand is consistent with similar stability constant values of these complexes (Table 4). This could explain why it is suggested that these different complexes are in comparable concentrations in human blood [39,40].

The tridentate chelation of one of the L-histidine ligands is an important factor in the thermodynamic stability of copper(II)–L-amino acid complexes involving L-histidine ligand. This provides an additional stability over the other binary copper(II)–L-amino acid complexes. The lower stoichiometric stability constant values of other copper(II)–*bis*-L-amino acid complexes ($\log \beta \sim 12$–13) can explain the negligible concentrations of these complexes detected in human blood [55–60]. Nevertheless, the enhanced stability of copper(II)–*bis*-L-histidine ($\log \beta \sim 18$) cannot be attributed only to the five coordinate geometry. The asymmetry around the central copper(II) and the involvement of carboxylate group in axial position may reflect themselves thermodynamically in terms of entropy change in the system. The non-covalent bonds may also have a considerable influence on the stability of these complexes. Enhanced stability of several mixed copper(II)–L-amino acid complexes has been attributed to electrostatic ligand–ligand interactions [87].

5.2. Tissue and cellular uptake of copper facilitated by L-histidine

Experiments with hepatic tissues demonstrated that L-histidine enhances the uptake of copper in hepatic cells [88–90]. Similar results have been obtained in placental cells [91]. In vitro studies have demonstrated that L-histidine can facilitate copper uptake in mammalian brain [92]. The recent knowledge of the structure of the physiological copper(II)–L-histidine complex is a major breakthrough in our understanding of the biochemical aspects of its role in copper transport [23]. The concurrence of the high stability associated with tridentate ligation and the kinetic lability of the bidentate binding of L-histidine ligand highlight the contribution of complex **12** in the copper transport across cell membranes. The structural elucidation of **12** gives insights to understand the possible role of hydrophobicity, surface charge distribution in recognition as well as its binding on the cell membrane receptor. Further research in this area should explore the role of pendant protonated imidazole in the transport of copper(II)–L-histidine complex across cellular membrane. The formation of copper(II)–L-histidine complex with the involvement of weak intramolecular H-bonding interactions should be particularly favored in the transport of copper in the hydrophobic environment of biological membranes. The basic character of imidazole group and H-bonding interactions of the bidentate L-histidine ligand can also be used for the effective delivery of radio imaging agents across cell membranes.

5.3. Kinetics of copper exchange with albumin

Copper transport in the tissues is facilitated by L-histidine [93] while albumin inhibits the copper uptake into the cells [94,95]. The exchange of copper(II) between L-histidine and albumin modulates the availability of copper to the cell. Evidence has been presented for the existence of a ternary coordination complex between albumin, copper(II) and L-histidine under physiological conditions [79]. The equilibrium and spectroscopic studies of the ternary system: copper(II), the native sequence tripeptide Asp–Ala–His-*N*-methyl amide and L-histidine show the formation of mixed ligand complexes **CuH$_{-1}$AL** (**15**) and **CuAL** (**14**) (where **A** and **L** denote the anionic forms of Asp–Ala–His-*N*-methyl amide and L-histidine, respectively) at physiological pH [82,83]. Tabata and Sarkar [82,83] studied the kinetics for copper-transfer reaction of the native sequence tripeptide, Asp–Ala–His-*N*-methyl amide and L-histidine over a pH range of 6.5–10.0. The reaction mechanism for the copper-transfer reaction involving copper(II), Asp–Ala–His-*N*-methyl amide and L-histidine can be described in the light of recent X-ray crystal structure of physiological **ML$_2$** species (Fig. 15). For the copper-transfer from copper(II)–L-histidine complex to na-

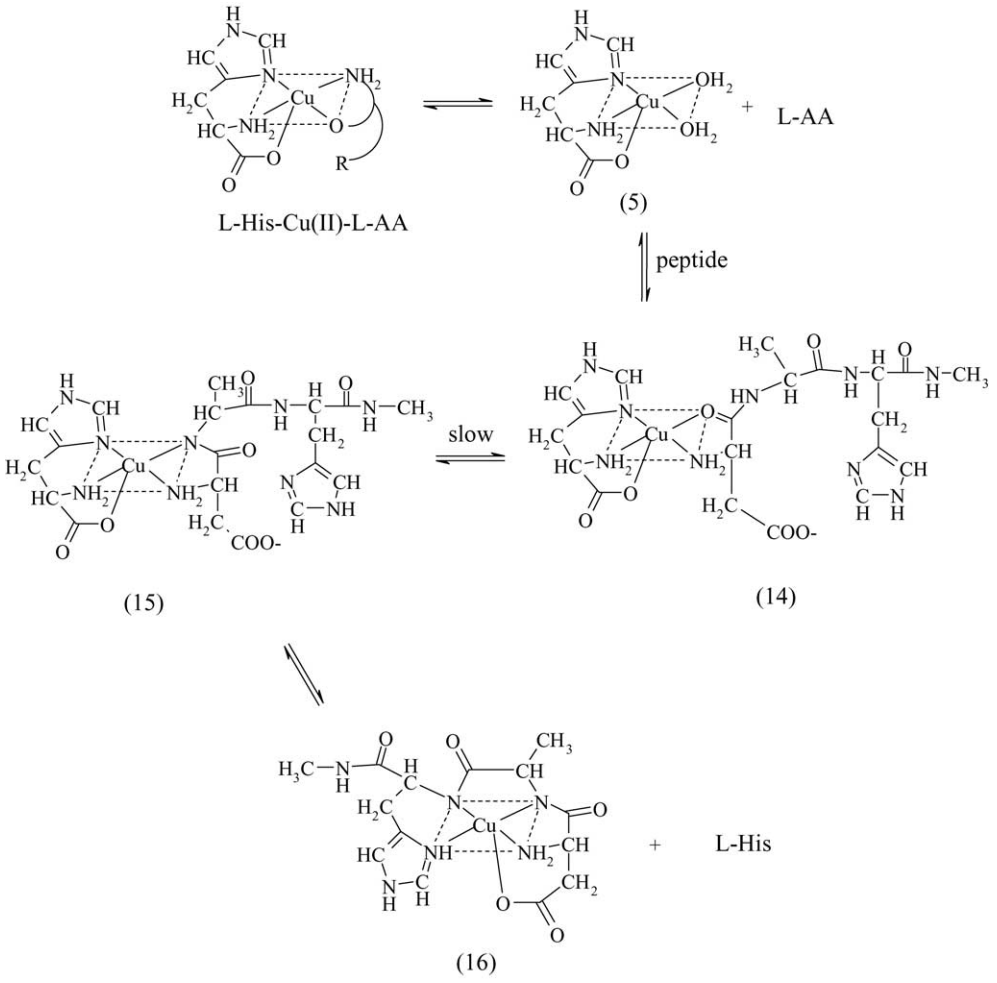


Fig. 15. Proposed reaction mechanism for the copper-transfer reaction involving copper(II), Asp–Ala–His-*N*-methyl amide and L-histidine. L-AA corresponds to both L-histidine and another L-amino acid (adapted from Ref. [82]).

tive sequence peptide, the rate-determining step is a bond formation between copper(II) and peptide nitrogen to form **CuH$_{-1}$AL** (**15**) from **CuAL** (**14**) by deprotonation of peptide nitrogen atom. For the copper-transfer reaction from copper(II)–peptide complex to L-histidine, the rate determining step involves a bond breaking between copper(II) and peptide nitrogen to form **CuAL** (**14**) from **CuH$_{-1}$AL** (**15**) by protonation to a peptide nitrogen. According to the structural features of copper(II)–L-histidine complex, a similar mechanism may be considered in the case of mixed copper(II)–L-amino acid complexes involving L-histidine ligand.

6. Applications of copper(II)–L-histidine complex as a therapeutic agent

6.1. Menkes disease

Menkes disease is a fatal X-linked genetic disorder which causes rapidly progressive cerebral degeneration [96]. The disease manifests itself with severe mental retardation, convulsions, growth retardation, hypothermia, skeletal changes, pili torti, and connective tissues abnormalities [3,97–108]. Children usually die before the age of three. The biochemical basis of this disease is related to a widespread defect in intracellular copper transport [98,109,110]. Menkes disease fibroblasts in culture show a significant accumulation of copper, but the efflux of copper from the cell is reduced [111,112]. Serum copper and ceruloplasmin levels are reduced due to the impairment of the intestinal absorption of copper. The lack of available copper leads to decreased levels of developmentally important copper enzymes (Table 1) [24]. Deficiencies of copper enzymes are thought to account for the clinical manifestations of this disease [3]. There is clinical heterogeneity in Menkes disease. Although there are mild forms such as occipital horn syndrome, most patients (90–95%) present the severe form [113]. The responsible gene (*ATP7A* or *MNK*) was isolated in 1993 [114–116]. It is expressed in all tissues although only in trace amounts in liver [116]. It encodes a copper-transporting P-type ATPase responsible for the transportation of copper across cell membranes. However, this role alone would not fully explain the

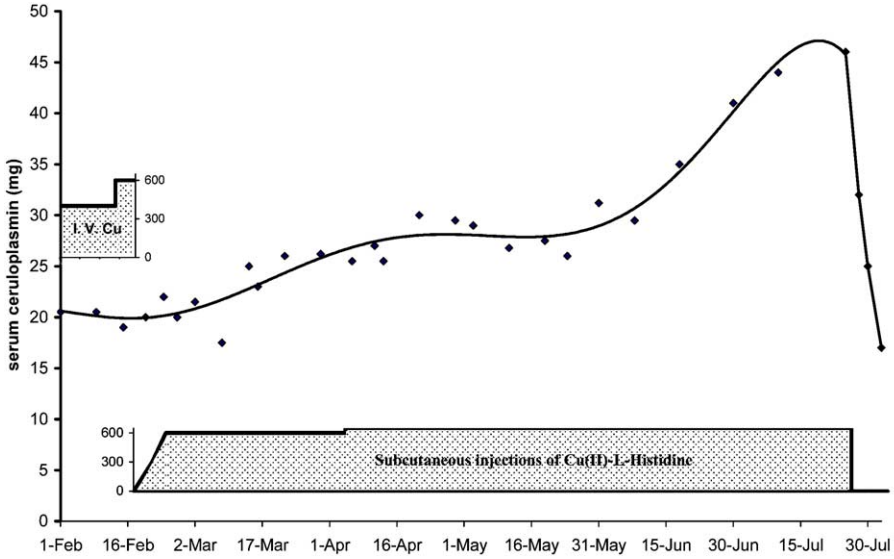


Fig. 16. Effect of subcutaneous administration of copper(II)–L-histidine on serum ceruloplasmin level in a Menkes patient (adapted from Ref. [119]). The patient was unsuccessfully treated with daily intravenous injections of copper chloride during the three 3 months. From age 3 months (Feb 16th) treatment was changed to daily subcutaneous injections of copper histidine. A normalization of ceruloplasmin level was observed. Copper–histidine treatment was temporarily suspended for 1 month at age 11 months because the plasma ceruloplasmin level became supranormal.

clinical manifestations of Menkes disease. It is likely that the protein is also involved in the transport of copper into some intracellular organelles [30,117].

Treatments for Menkes patients have concentrated on restoring normal copper levels in the body by administering copper. Since intestinal copper absorption is extremely low in Menkes patients, copper must be administrated parenterally. There is another obstacle in delivering copper into the cells. As described earlier, albumin binds copper with a very high affinity in human blood and inhibits the copper uptake into the cells [24,77,94,95]. The administration of copper salts induces the formation of copper–albumin complex in blood and therefore copper in this form is not bio-available to the cells [118]. The patients treated with intravenous injections of copper salts did not show a normalization of ceruloplasmin level (Fig. 16) [118,119]. This explains why the parenteral therapy with various copper forms (copper chloride, copper sulfate, copper EDTA and copper albumin) commencing either before or after neurological impairment, has not been successful in resisting the progressive neurodegeneration caused by the disease [118]. To achieve an efficient treatment of Menkes disease, the equilibrium of copper-transfer reaction between albumin and the administrated copper form must be in favor of the formation of the bio-available form of copper. As reported earlier, kinetic studies demonstrated the exchange of copper(II) between albumin and L-histidine in the presence of an excess of L-histidine. The transport properties of the copper(II)–L-histidine complex led us to its application as a therapeutic agent to treat Menkes disease [113,120–123]. We have previously reported a long-term follow-up of four patients treated with copper(II)–L-histidine [122]. These patients have shown significant clinical and biochemical improvement. They all show relatively good neurological outcome and the serum copper, ceruloplasmin levels have been normalized (Fig. 16) [119]. They represent the world's longest surviving Menkes patients. Despite these significant improvements in the copper(II)–L-histidine treatment of Menkes disease patients, connective tissue disorders continue to persist, indicating that lysyl oxidase levels are not restored by copper(II)–L-histidine treatment. Subcutaneous injections of copper(II)–L-histidine when initiated very early in life, have been tolerated best and seem to be the most rational and effective treatment in preventing some of the serious complications of Menkes disease [122]. The efficacy of early treatment may be the result of making copper available during a critical period of development of the central nervous system. The copper(II)–L-histidine complex is a relatively unstable inorganic complex in aqueous solution. To our knowledge, many formulations of copper(II)–L-histidine are currently used [2,41,124]. The isolation of copper(II)–L-histidine in the solid state allows us to consider a novel and better formulation for the treatment of Menkes disease.

6.2. *Infantile hypertrophic cardioencephalomyopathy*

Human mitochondrial disorders are clinical entities associated with abnormalities of oxidative phosphorylation [125]. Mutations in *SCO2*, a cytochrome *c* oxidase assembly gene, result in a fatal infantile hypertrophic cardioencephalomyopathy (HCM) with severe cytochrome *c* oxidase deficiency [4,126]. All patients reported so far have died from heart failure due to HCM. Human *SCO2* seems to be involved in the insertion of copper atoms into the mitochondrial cytochrome *c* oxidase holoenzyme. Several studies in yeast, bacteria and fibroblasts, myoblasts from patients with *SCO2* mutations have shown that copper supplementation can restore cytochrome *c*

oxidase activity to almost normal levels [125,127,128]. These results suggested a possible therapy for the early treatment of this fatal infantile disease by increasing the intracellular content of copper in critical tissues. On the basis of the successful treatment of Menkes disease, Jaksch and co-workers [5] recently suggested subcutaneous copper(II)–L-histidine supplementation for a patient with *SCO2* mutations resulting in severe hypertrophic cardioencephalomyopathy. This study described the first patient who showed a reversal of HCM and who survived significantly longer (42 months) compared to all the previously reported patients (longest 13 months). Copper(II)–L-histidine supplementation might be the most probable explanation for the improvement of cardiac function in this patient [5]. However, further studies are required to generalize the use of this treatment.

7. Conclusion

Copper(II)–L-histidine was discovered in human blood. Subsequently, it was demonstrated that L-histidine enhances the cellular uptake of copper and the exchange of copper between L-histidine and albumin modulates the availability of copper to the cell. A major effort was made to study the copper(II)–L-histidine system to establish the distribution, stabilities and structures of different species. All these studies led to the use of copper(II)–L-histidine in the treatment of Menkes disease and HCM. Nevertheless, the mechanism by which the copper(II)–L-histidine complex provides copper to crucial enzymes remained unclear. Recently the isolation and the X-ray crystal structure of physiological copper(II)–L-histidine complex have been reported. The concurrence of the high stability associated with tridentate ligation and the kinetic reactivity of the bidentate ligation of L-histidine highlight the contribution of this complex in copper transport in the presence of albumin in human blood. Further, the pendant imidazole observed in the copper(II)–L-histidine complex may be the key to its interaction on the cell membrane receptor ultimately modulating the availability of copper to the cell. The knowledge gained from these studies will provide further advances in our understanding of the role of copper(II)–L-histidine in normal physiology and in its applications as a therapeutic agent in diseases.

Acknowledgements

Research in the laboratory of B. Sarkar was supported by the Medical Research Council of Canada and the Canadian Institutes of Health Research. Authors thank many colleagues and co-workers whose names appear in the references.

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