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The Structure of a Copper Complex of the Growth Factor Glycyl-L-Histidyl-L-Lysine at 1.1 Å Resolution

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Interest in the crystal structure of a copper complex of the growth factor, glycyl-L-histidyl-L-lysine has been stimulated by the tripeptide's ability to facilitate copper uptake in cultured hepatoma cells and by the copper complex's tendency to induce angiogenesis. The coordination polyhedron is a distorted square pyramid, CuN_3O_2, with the four basal ligating atoms bonded to the copper at about 2.00 Å and the apical ligating atom at 2.49 Å. One tripeptide furnishes three of the basal atoms, the glycine amino nitrogen atom, the peptide nitrogen atom of the histidine, and the imine nitrogen atom of the imidazole. A second tripeptide is involved via its terminal carboxyl oxygen atom while the fifth copper ligand is a carboxyl oxygen atom of a third tripeptide. The carboxyl oxygen atoms form bridges between copper centers and thus the system is polymeric in the solid state. The crystal structure can be used to propose a model for the first step in the transport of copper into cells via a copper–tripeptide complex.

Introduction

The role of copper compounds in biological systems has been of interest for many years [1]. Studies involving copper in this context range from nutritional and clinical investigations [2] to physicochemical research on both synthetic and naturally occurring copper complexes [3] to the transport of copper in biological systems [4]. As with any essential element, the ultimate goal is to understand the metabolism of copper at the molecular level that includes the elucidation of details of copper transport *in vivo*.

However, because of the small amount of copper present in living systems and because of the manner of its distribution, there is considerable difficulty in obtaining definitive results concerning transport. For example, plasma contains about 1 ng/ml copper; approximately 90% of this copper is bound to ceruloplasmin in a form that is not readily exchanged while the remainder, which constitutes the metabolically exchangeable fraction, is bound to albumin (~9%) and to low molecular weight components (~1%) [5]. In the fetus, an albumin analog, α-fetoprotein, substitutes as the copper transport protein. The relationship between albumin and the low molecular weight components of plasma on the uptake of copper is not yet understood, although there is evidence that the small amount of copper bound to low-molecular-weight components (~10 ng/ml) does play a role in copper transport [6].

The plasma tripeptide glycyl-L-histidyl-L-lysine (HGHL)* is relatively common ingredient in growth media for cultured cells [7–18] and has been shown to alter the growth rate or the state of differentiation of cultured cells and organisms [19]. For example, GHL has been found to be a contributing factor in the growth of hepatoma cells [20], thyroid follicular cells [21], human kB cells [22], and T-strain mycoplasma [23]. The tripeptide stimulates the growth and differentiation of *Ascaris* larvae [24] and neurons [25], aids in the establishment of 19 cancer cell lines in human tumors [26], inhibits the growth of L929 cells [27], and increases antibody cytotoxicity toward the parasitic worm, *Schistosoma mansoni* [28]. It functions synergistically with copper and iron ions to promote the growth of cultured hepatoma cells, and promotes the incorporation of copper into these cells [29]. *In vivo*, the copper complex

*HGHL denotes the peptide as though it were the classical zwitterion with a protonated amino terminus. In this nomenclature, GHL_- is the deprotonated peptide, $GHLH_{-1}^{--}$ is the species with an additional proton missing from one of the amide nitrogen atoms, and $CuGHLH_{-1}$ is the copper complex.

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induces new capillary formation (angiogenesis) in rabbits [30]. The entire subject has been reviewed in detail recently [31].

A consideration of the large amount of biochemical information covering HGHL and an interest in its copper chelating properties led us to undertake the synthesis and the crystallographic structure determination of a copper complex of the tripeptide. The structure of this complex and its possible relationship to the mechanism of copper uptake into cells are described here.

Synthesis and Structure Determination

The tripeptide (30 mg, 88 μmol) was dissolved in an aqueous solution of Cu(II) acetate (300 μL, 0.30 *M*). Ethanol (1.26 mL) was added and the vessel walls were scratched to initiate the crystallization of dark blue material. The mother liquor was decanted, and H_2O (200 μL) was added to dissolve the crystals. Ethanol (400 μL) was then slowly introduced to reach a cloud point. Upon standing, dark blue octahedral-shaped crystals resulted that were isolated by decanting the mother liquor.

One of these crystals (of dimension 0.55 × 0.36 × 0.36 mm) was selected for the crystallographic analysis. The crystal system was shown to be tetragonal by oscillation and Weissenberg photographs. The space group is $P4_12_12$ or $P4_32_12$ based on systematic absences in the photographs; *h*00 refections are present only when $h = 2n$, 0*k*0 reflections are present only when $k = 2n$, and 001 reflections are present only when $l = 4n$. (After the structure was solved, the fact that two L-amino acids were used in the synthesis of the tripeptide fixed the space group as $P4_12_12$). The crystal was mounted on a Picker four-circle diffractometer with the c axis parallel to the ϕ axis. Unit cell parameters were determined by a least-squares procedure and found to be $a = b = 14.937(4)$ Å and $c = 25.903(9)$ Å.

Copper K_α radiation was used for the data collection. The $\omega - 2\theta$ scan technique was used with a scan rate of 1°/min in 2θ and a scan width of 1.6° (due to broadened reflections). Seven standard reflections were periodically measured, but no deterioration of the crystal was apparent. Data were collected to a maximum 2θ of 90°. The falloff of the diffracted intensities at higher scattering angle suggested a high overall thermal parameter. No absorption correction was found necessary after measuring a reflection at $\chi = 90°$ at various ϕ values. The X-RAY System of crystallographic programs [32] was used in the structure analysis.

Interpretation of the Patterson map yielded the position of a copper atom that was utilized in the initial phasing model. The remaining atoms in the structure were added to the model on the basis of difference density maps, but attempts to carry out full-matrix least-squares refinement of the model, including anisotropic temperature factors, were not successful. This manifested itself as non-positive definite thermal parameters and unreasonable bond lengths in the peptide. Reinvestigation of the structure determination failed to indicate any alternate model for the structure and lead us to consider recollection of the diffraction intensities. Photographs obtained from additional crystals stored for approximately two years and the one used to collect the original data indicated deterioration of the crystals to the point where they were no longer suitable for data collection. This deterioration could account for the poor refinement behavior of the model.

About 35% of the unit cell is occupied by species other than $CuGHLH_{-1}$, presumably solvent. The density of the crystals was determined by flotation in an ethanol–carbon tetrachloride mixture of known density. From the space group, the volume of the unit cell (5794 $Å^3$), and the density of the crystals (1.40 Mg m^{-3}), it can be estimated that the asymmetric unit contains one $CuGHLH_{-1}$ complex along with about 270 additional daltons of other material. Assuming that the other molecules are water, the formulation of the contents of the asymmetric unit consistent with the density measurement is $CuGHLH_{-1}\cdot 15H_2O$.

The large amount of disordered solvent present in this structure is typical of what has also been observed in crystals of proteins [33] and in a Cu(II)–glycyl–L-histidylglycine crystal [34] and will adversely affect the precision of the structure solution. This situation is not terribly different from that seen in the protein crystal structure refinements of rubredoxin and triclinic lysozyme [35, 36] or in other copper–peptide structure determinations [34] where the presence of solvent molecules has a deleterious effect on the precision of the refined atomic coordinates. The disordered solvent causes R to increase dramatically at low values of $\sin\theta/\lambda$, a behaviour also observed for refined protein structures [37, 38]. Short of applying some sort of continuum model in an attempt to account for the disordered water, the most practical approach for dealing with this type of problem is to omit the most affected low-order reflections. In this case, 20 reflections with $\sin\theta/\lambda < 0.1$ (or out to 5.0 Å resolution) were omitted from further refinement.

Since the goal of this analysis was to determine how the tripeptide binds copper, we decided to use restrained least-squares techniques [39–42] to impose some structural regularity on the tripeptide. The restraints applied were the standard bond lengths, bond angles and torsion angles obtained from more accurate amino acid and peptide structure determinations [42]. No restraints were applied to the copper atom and water oxygen atoms. Individual

isotropic thermal parameters were allowed to refine, but those for connected peptide atoms were restrained as described elsewhere [42]. Population parameters for the 14 water oxygen atoms were not refined, but were assigned values based on the behavior of the thermal parameters after several cycles of refinement with all unit occupancies. The net occupancy of the ordered water is 9.2. Water oxygen atoms were added to the model until the residual difference density being fit reached the noise level. The largest remaining difference density, 0.5 electrons/Å^3, is located near the copper atom and is the size of the lowest occupancy water.

The final cycles of least-squares refinement decreased R ($=\Sigma||F_o| - |F_c||/\Sigma|F_o|$) to 0.160 for 1193 reflections with $I > 2\sigma(I)$ out of a total set of 1777 reflections. The refinement of the water molecules had not converged at this point, but it was felt that further expenditure of resources was not justified since the principal purpose of the structure determination was to establish the connectivity of the atoms. The final atomic and thermal parameters are listed in Table I. Atom names for the peptide follow the IUPAC conventions for polypeptides. The standard deviations obtained from the diagonal least-squares and restrained refinement greatly underestimate the errors in the parameters and are not included in Table I. We estimate the errors in the peptide bond lengths to be about 0.05 Å and those in the angles to be 0.5°. Errors in the copper-ligand distances and angles will be less, on the order of 0.02 Å and 0.2°, respectively.

TABLE I. Atomic Coordinates. Isotropic thermal parameters defined by the expression $\exp(-8\pi^2 U)$. Positional parameters $\times 10^4$. Thermal parameter values $\times 10^3$.

	x	y	z	U	Occupancy
Cu	2614	1347	431	77	1.00
N(1)	3537	2264	541	66	1.00
CA(1)	3907	2211	1096	77	1.00
C(1)	3301	1644	1440	71	1.00
O(1)	3431	1625	1917	78	1.00
N(2)	2649	1192	1221	69	1.00
CA(2)	1999	649	1522	75	1.00
CB(2)	1118	611	1236	81	1.00
CG(2)	1148	152	719	102	1.00
ND1(2)	1794	400	342	90	1.00
CE1(2)	1680	−170	−36	112	1.00
NE2(2)	1042	−750	80	125	1.00
CD2(2)	710	−592	562	104	1.00
C(2)	2364	−281	1611	76	1.00
O(2)	1952	−811	1946	78	1.00
N(3)	3073	−569	1337	72	1.00
CA(3)	3362	−1502	1391	72	1.00
CB(3)	4052	−1750	968	102	1.00
CG(3)	3557	−1757	452	140	1.00
CD(3)	4278	−2114	16	197	1.00
CE(3)	3730	−2025	−487	214	1.00
NZ(3)	4271	−1500	−863	289	1.00
C(3)	3737	−1691	1946	65	1.00
$O^1(3)$	4320	−1182	2090	93	1.00
$O^2(3)$	3397	−2346	2187	55	1.00
OW(4)	5761	1897	336	225	1.00
OW(5)	4548	241	646	294	1.00
OW(6)	5721	3770	818	339	0.80
OW(7)	6348	51	952	374	0.60
OW(8)	9202	5026	1242	493	0.60
OW(9)	5183	430	1815	268	1.00
OW(10)	7215	252	−70	350	0.70
OW(11)	4228	4808	−166	315	1.00
OW(12)	6016	5476	308	327	0.75
OW(13)	7512	6736	265	342	0.35
OW(14)	8232	8776	30	139	0.20
OW(15)	7789	1235	813	375	0.60
OW(16)	9404	152	1451	204	0.20
OW(17)	6324	6309	1277	245	0.40

Discussion

Table II contains the bond lengths, bond angles and torsion angles for the final model. As can be seen in Fig. 1, the peptide chain and histidine side chain form a relatively compact, rigid structure connecting several copper atoms. The lysine side chain, however, is extended into the solvent region. The large thermal parameters for the terminal atoms in the lysine side chain may be indicative of static or dynamic disorder. Either interpretation is consistent with the side chain being found in the solvent region where the energetic differences between alternate conformations are small.

Each copper atom has pseudo-square pyramidal coordination (Figs. 1 and 2). The tripeptide functions as a planar, tridentate ligand bonded to a given copper atom through its glycine amino nitrogen atom (N(1)), the first peptide nitrogen atom (N(2)), and through the imine nitrogen atom (ND1(2)) on the imidazole ring of the histidine. The Cu–N bond distances are 1.96 Å, 2.06 Å, and 1.88 Å respectively, which are in the range expected for Cu–peptide complexes [43]. One of the carboxyl terminal oxygen atoms from another complex ($O^2(3)$) fills the remaining copper coordination site in the basal plane ($Cu–O^2(3) = 1.96$ Å). The carboxyl terminal oxygen ($O^2(3)'$) from yet another complex (where ′ denotes a symmetry related atom) fills the apical copper coordination site ($Cu–O^2(3)' = 2.49$ Å).

The Cu–GHL portion of the crystal structure is easily visualized as helices of –Cu–tripetide–Cu–

TABLE II. Bond Lengths and Angles.

Bond Lengths, (Å)

Cu–N(1)	1.96	NE2(2)–CD2(2)	1.36
Cu–N(2)	2.06	CD2(2)–CG(2)	1.35
Cu–ND1(2)	1.88	CA(2)–C(2)	1.51
Cu–$O^2(3)$	1.96	C(2)–O(2)	1.33
Cu–$O^2(3)'$	2.49	C(2)–N(3)	1.34
N(1)–CA(1)	1.54	N(3)–CA(3)	1.47
CA(1)–C(1)	1.53	CA(3)–CB(3)	1.55
C(1)–O(1)	1.25	CB(3)–CG(3)	1.53
C(1)–N(2)	1.31	CG(3)–CD(3)	1.65
N(2)–CA(2)	1.49	CD(3)–CE(3)	1.55
CA(2)–CB(2)	1.51	CE(3)–NZ(3)	1.49
CB(2)–CG(2)	1.51	CA(3)–C(3)	1.57
CG(2)–ND1(2)	1.42	C(3)–$O^1(3)$	1.22
ND1(2)–CE1(2)	1.31	C(3)–$O^2(3)$	1.27
CE1(2)–NE2(2)	1.32		

Bond Angles (°)

N(1)–Cu–N(2)	89.1	CB(2)–CG(2)–CD2(2)	129
N(1)–Cu–ND1(2)	175.5	ND1(2)–CG(2)–CD2(2)	110
N(1)–Cu–$O^2(3)$	84.1	Cu–ND1(2)–CG(2)	124
N(1)–Cu–$O^2(3)'$	89.3	Cu–ND1(2)–CE1(2)	132
N(2)–Cu–ND1(2)	93.0	CG(2)–ND1(2)–CE1(2)	105
N(2)–Cu–$O^2(3)$	103.1	ND1(2)–CE1(2)–NE2(2)	110
N(2)–Cu–$O^2(3)'$	174.3	CE1(2)–NE2(2)–CD2(2)	111
ND1(2)–Cu–$O^2(3)'$	100.3	NE2(2)–CD2(2)–CG(2)	104
ND1(2)–Cu–$O^2(3)'$	92.6	CA(2)–C(2)–O(2)	119
Cu–N(1)–CA(1)	111	CA(2)–C(2)–N(3)	120
N(1)–CA(1)–C(1)	111	O(2)–C(2)–N(3)	121
CA(1)–C(1)–O(1)	120	C(2)–N(3)–CA(3)	119
CA(1)–C(1)–N(2)	118	N(3)–CA(3)–CB(3)	111
O(1)–C(1)–N(2)	122	N(3)–CA(3)–C(3)	111
Cu–N(2)–C(1)	113	C(3)–CA(3)–CB(3)	112
Cu–N(2)–CA(2)	124	CA(3)–CB(3)–CG(3)	107
C(1)–N(2)–CA(2)	123	CB(3)–CG(3)–CD(3)	107
N(2)–CA(2)–C(2)	110	CG(3)–CD(3)–CE(3)	102
N(2)–CA(2)–CB(2)	109	CD(3)–CE(3)–NZ(3)	108
C(2)–CA(2)–CB(2)	111	CA(3)–C(3)–$O^1(3)$	117
CA(2)–CB(2)–CG(2)	115	CA(3)–C(3)–$O^2(3)$	115
CB(2)–CG(2)–ND1(2)	121	$O^1(3)$–C(3)–$O^2(3)$	128

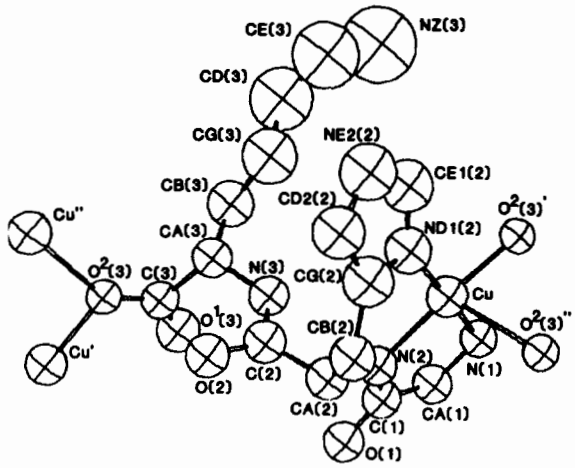

Fig. 1. ORTEP plot showing the thermal ellipsoids and atomic numbering scheme.

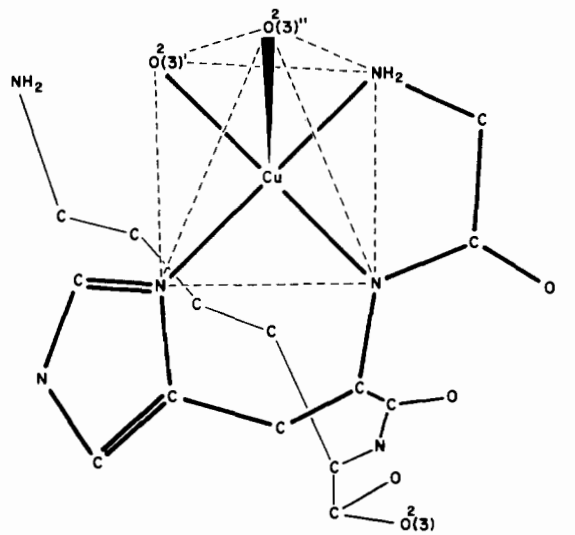

Fig. 2. Schematic summarizing the coordination of the copper atom.

tripeptide– centered on each four-fold screw axis. Copper atoms related by the screw axis are connected by tripeptides bound by N(1), N(2) and ND1(2) to one copper, and with $O^2(3)$ bound to the next symmetry related copper along the screw axis. The only interaction between adjacent four-fold helices is through the long $O^2(3)$–Cu bond of 2.49 Å. Fig. 3 is a stereoscopic drawing of the molecular arrangement of $CuGHLH_{-1}\cdot 14H_2O$ in the $P4_12_12$ unit cell.

The explicit planar, triaza, tridentate type of coordination of the glycyl–L-histidyl (GH) moiety in $CuGHLH_{-1}\cdot 14H_2O$ is also observed in crystals of four other Cu(II) complexes containing the N terminal GH unit (Table III). In each of the other complexes, as well as that reported here, a relatively short Cu–O (carboxylate) bond completes the 'square' of coordination also involving the triaza group. In one of them, namely $CuGHGH_{-1}\cdot 12H_2O$, the coordination polyhedron is essentially the same as that of $CuGHLH_{-1}\cdot 14H_2O$ [44]. In the other three, there are either two longer axial bonds to copper or one, as in the case of $CuGHH_{-1}\cdot 1.5H_2O$, but in the latter, the fifth donor atom is an oxygen atom of water. In none of these example do the potential ligating atoms of the rest of the peptide coordinate to the same copper atom as does the tridentate GH moiety. Given that the same tridentate ligating function has been observed in five crystals containing Cu(II) and the N-terminal GH unit, this linkage would be expected to be seen in solution (but not necessarily as the only form of GHL–Cu binding.) Furthermore, it is likely that the polymeric nature of the

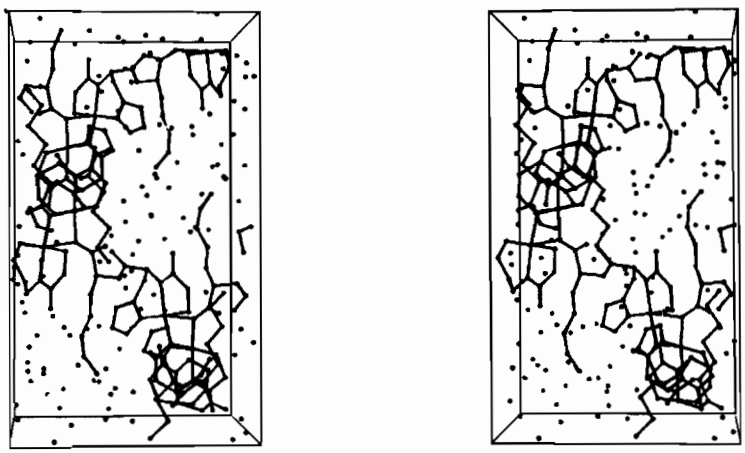

Fig. 3. Stereo diagram indicating the conformation of the copper–tripeptide complex as well as the packing of the solvent molecules in the unit cell.

TABLE III. Copper Complexes Containing the Glycylhistidyl Moiety.

Compound	Coordination number	Donor atom in 'square' with triaza group[a]	Axial donor atom[b]	References
$CuGHH_{-1}\cdot 1.5H_2O$	5	O(carboxylate)	O(water)	43
$CuGHGH_{-1}\cdot H_2O\ NaClO_4$	6	O(carboxylate)	O(water) O(carboxylate)	44
$CuGHGH_{-1}\cdot 2(1/2H_2O)$	6	O(carboxylate)	O(water) O(carboxylate)	47
$CuGHGH_{-1}\cdot 12H_2O$	5	O(carboxylate)	O(carboxylate)	34

[a]This donor atom makes a relatively short bond to Cu (<2.1 Å). [b]These donor atoms make a relatively long bond to Cu (>2.46 Å).

complexes (observed in the crystalline state) will not persist in dilute solution. This follows from basic considerations of chemical equilibria (R. Österberg, private communication) and was not explicitly stated in an earlier note concerning this work [29]. It may be noted that the nature of the Cu(II) complex of the tripeptide has been studied in solution by optical, electron paramagnetic [45], and electron spin-echo spectroscopies [46]. A 1:1 complex exists in which the Cu(II) ion is probably bound to three nitrogen atoms as described here, but no Cu(II) oxygen-bridged pair exists over a broad pH range.

There remains the critical question of whether the mode of coordination between GHL and copper as observed in the crystal will be the dominant one in solution at physiological pH. The single set of equilibrium measurements reported to date indicate that the tripeptide is 'starting to bind Cu(II) at about pH 3, and liberating a maximum of 3 protons in a range of less than 2 pH units' [48]. This observation is consistent with the binding observed in the crystal or via other combinations of ligating atoms including the ϵ amino group of the lysine [48]. The observed formation constants of GHL–Cu, GH–Cu and GHG–Cu have been used to implicate the involvement of the lysine amine int he binding of GHL to Cu(II) [48]. In the latter two cases, GH–Cu and GHG–Cu, it is highly likely that the triazatridentate form of bonding is the dominant one at physiological pH. Clearly, similar equilibrium constants for these three peptides would suggest or be consistent with a common mode of binding, and grossly different equilibrium constants would suggest other modes of binding. The actual values were determined to be $10^{16.44}$, 10^{6}, and $10^{4.54}$, respectively, and it was concluded that the 'closed' form of the complex must be dominant one [48].

However, because of the larger number of proton acceptors on GHL^- (as compared to GH^- and GHG^-), great care must be taken in performing such a comparison. A case in point is shown in Fig. 4. The equilibrium constant for Experiment I is $10^{16.44}$ (for $GHL^- = A^-$), a number about 10^8 times greater than those measured for GH^- and GHG^-. However, one model or interpretation (Postulate I) of the actual process taking place in this experiment (see Fig. 4)

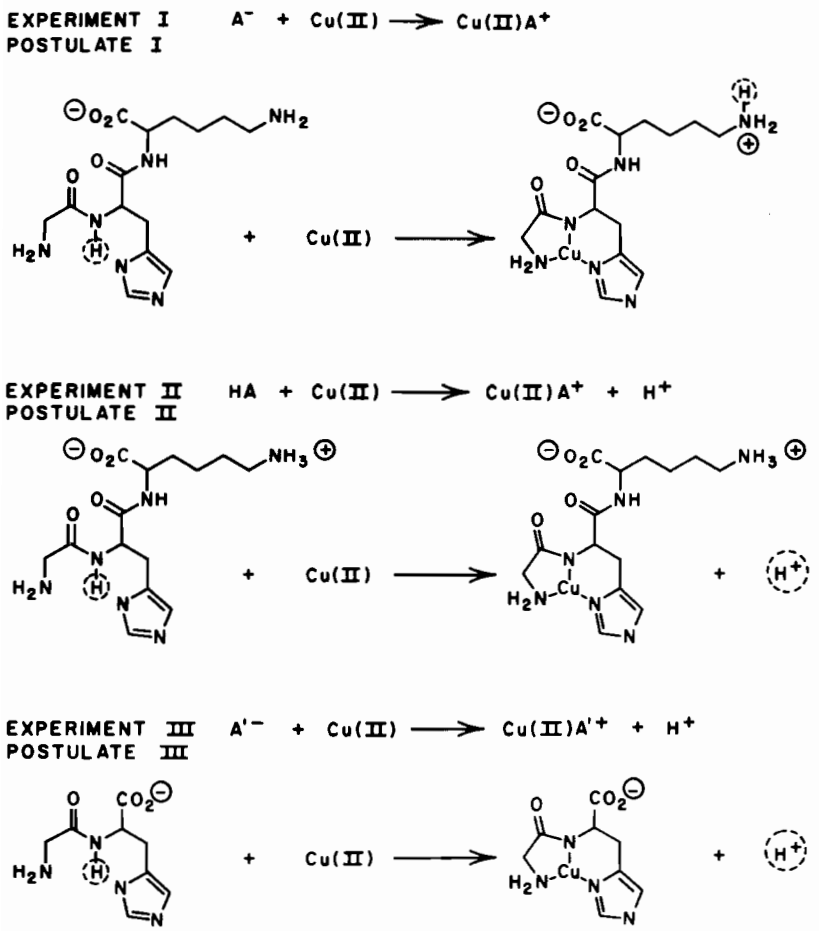

Fig. 4. Expt. I, Postulates, etc.

involves the generation of the triazatridentate mode of binding for GHL and the transfer of a peptide proton to the ϵ amino group. Since GH^- and GHG^- do not have a free amino group to accept a proton from a peptide nitrogen atom there can be no definitive, appropriate comparison between GHL^- and either GH^- or GHG^- using equilibrium data for Experiment I. Alternatively, for Experiment II and the model (Postulate II) proposed for it, the ϵ amino group is protonated throughout and this requires that the peptide proton be displaced to the solution upon the binding of Cu(II). This situation reflects what must happen for GH^- in Experiment III, Postulate III. In fact, the three equilibrium constants for the actual Experiments II and III with GH^- and GHG^-, respectively are 10^6, $10^{4.54}$ and $10^{5.32}$ [48]. These numbers are similar enough to entertain the possibility that all three peptides bind copper in the same manner in solution at physiological pH. The data, of course, do not prove this point, but only allow it to be retained as a possibility.

The X-ray structure of the $CHLH_{-1}$–copper complex indicates that the lysyl side-chain is clearly not essential for binding the copper. This result is similar to the conclusions obtained in binding studies of albumin and α-fetoprotein in which the histidyl residue at position three of the polypeptide chain seems to be essential for the binding of copper. However, the lysyl residue at position four in albumin or the arginyl residue at position four in α-fetoprotein are not required for such binding [49], while in GHL, the lysyl residue is essential for the retention of bioactivity [50]. Thus, it is probable that the basic amino acid residue adjacent to the copper-binding histidine in GHL, as well as in the larger proteins [29], may provide an NH_3^+ group at physiological pH for specific binding to a receptor site. Comparison of copper-tetrapeptide complexes with albumin and α-fetoprotein indicate that these proteins serve at least as planar tetra-dentate ligands for copper [4]. However, $GHLH_{-1}$ serves as a tri-dentate ligand leaving free one of the four major coordination positions on the copper for potential binding to another receptor site. Thus complexes of the tridentate ligand can clearly provide two different types of chemical moieties for attachment, the metal and the ligand.

This model makes use of the coordinately unsaturated metal complex (with respect to the tripeptide) and is different in this sense from some of those involving hexadentate siderophores in iron transport [51].

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