**Affinity of copper and zinc ions to proteins and peptides related to neurodegenerative conditions (Aβ, APP, α-synuclein, PrP).**

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**Abstract**

The review describes the state of the art in the field of stability constant determination for Cu(II), Cu(I) and Zn(II) complexes of proteins and peptides involved in neurodegenerative diseases, α-Synuclein (aS), Prion Protein (PrP), Amyloid Precursor Protein (APP) and Amyloid β peptides (Aβ). The methodologies and results are critically analyzed and recommendations are formulated about possible systematic errors in these studies. The possibility of formation of ternary complexes with titration competitors is discussed.

Keywords: Copper, zinc, α-Synuclein, Amyloid β peptide, Prion Protein, Amyloid Precursor Protein.

**1. Introduction.**

The complexity of biological fluids makes it difficult to detect and quantitate interactions between metal ions and biomolecules directly. The traditional methodology of identification of metal binding molecules is based on their isolation from the biological material in the metal-bound state. This can be done only if such a molecule can be isolated intact and in sufficient quantities to perform analysis. In this way, however, only complexes sufficiently abundant, strong and inert to survive isolation procedures can be discovered. Positive examples include metallothioneins, serum albumins and enzymes such as carbonic anhydrase or superoxide dismutase [1-5]. A rule of the thumb is that a subnanomolar or lower dissociation constant is required for such detection. New metal binding proteins can be identified in genomes by homology searches, but this approach will not reveal really novel molecules. While fundamentally useful, these approaches cannot provide a dynamic picture of physiological and pathological interactions of metal ions, which may involve transient binding to a variety of targets, including both macromolecules and low molecular weight ligands. In some cases, like membrane transporters of metal ions, one can design a functional test, and detect and quantitate such interactions indirectly. However, this is rarely possible for pathological targets of metal ions, where there is no function *per se*.

One possible alternative strategy is to determine the affinity of a metal ion to a range of hypothetical biomolecules *in vitro*, and to use such quantitative information to formulate testable hypotheses about the participation of these biomolecules in the physiology or toxicology of this metal (also see a recent review for a detailed description of this approach used to design metal-oriented therapeutic agents [6]). This approach is significantly facilitated if confirmed target biomolecules are available for quantitative studies. Such molecules can be used as benchmarks. E.g. if the metal ion binding to a hypothetical molecule is stronger than that to the confirmed target, then one can hypothesize that such binding may occur *in vivo*.

Affinity can be expressed quantitatively by means of stability constants of various kinds. Therefore, the measurement of stability constants can provide a crucial link between molecular models and the biological reality. In this short review we discuss the use of stability constants in studies of copper and zinc binding to proteins and peptides related to neurodegenerative conditions: Amyloid Precursor Protein (APP), Amyloid β peptides (Aβ), Prion Protein (PrP), and α-Synuclein (aS).

APP and products of its hydrolysis, Aβ peptides, are molecules involved in Alzheimer’s disease, a fatal progressive dementia, predominantly affecting the elderly [7]. PrP is the only recognized protein-only infectious agent, responsible for several neurodegenerative diseases in the mammals, such as scrapie, mad cow disease and the variant of Creutzfeldt-Jacob disease in humans [8]. The relationship between aS and the Parkinson’s disease is not as clearcut, but the contribution of aS deposition to neurodegeneration in this disease seems very likely [9].

**2. Experimental methods of determining stability constants of complexes of biomolecules**

Three techniques can be considered as most productive in quantitating interactions between proteins and metal ions discussed in this review: potentiometry, calorimetry and fluorescence spectroscopy.

2.1. Potentiometry

Potentiometry is the most accurate method of determining binding constants of metal complexes, but is also subject to very strict limitations. Its most common and most versatile version of pH-metric acid-base titration is a direct method for establishing protonation constants. The resolution of glass electrode is up to 0.002 pH units. Therefore, the determination of protonation constants with very low standard deviations of around 0.01 pH units is possible [10]. The potentiometric approach to studying metal ion binding is indirect, because the metal ion coordination is monitored by detecting hydrogen ions displaced from the ligand in this process. Potentiometry is therefore blind to the coordination of metal ions at sites that do not exchange protons. This issue is, however, rarely a problem in the binding of copper and zinc ions to biomolecules, except of rather weak interactions of Cu(II) with Met thioether sulfurs and Cys disulfides [11]. A more relevant disadvantage of this method is its intrinsic inability to handle multiple overlapping deprotonation phenomena with a sufficient accuracy. A practical limit is at about ten exchangeable protons in the studied molecule, with only rare exceptions [12]. In order to obtain a measurable change of pH in the measured sample, one typically needs a millimolar concentration of the studied molecule in a milliliter volume. Together, these limitations reduce the range of applications of potentiometry to lower molecular weight ligands, such as peptides, rather than proteins.

The pH-metric determination of stability constants consists of three series of measurements [13]. First, the electrode calibration experiments are performed by means of strong acid titrations, followed by metal-free ligand titrations to establish its protonation constants. Finally a series of titrations of ligand/metal mixtures is performed, generally at different ligand-to-metal ratios. All samples are required to exhibit the same ionic strength, ensured by inorganic salt, such as KNO3 or NaClO4, typically at 0.1 – 0.2 M.

The data analysis is performed with the use of dedicated software. HYPERQUAD is the current standard of the field [14], although two older programs, PSEQUAD [15] and HYPERQUAD’s predecessor SUPERQUAD [16] are still in use. It has to be noted that HYPERQUAD, as well as PSEQUAD can also use spectroscopic data for the stability constant determination, but they are primarily used to analyze pH-metric titrations. Attempts have been made to overcome the indirectness of the pH-metric method in metal complex studies, by using the metal ion, rather than proton-selective electrodes. This promising approach is currently limited by an insufficient sensitivity of such electrodes. For example, a recent paper demonstrated the usefulness of a Cu(II)-selective electrode to study Cu(II) complexes of peptides, but the lack of linearity of the electrode’s response for Cu2+ concentrations below 10 µM excluded studies of stronger, physiologically relevant complexes [17].

The unique advantage of pH-metry is that the data are obtained over a broad pH range. This leads to the description of the coordination process in terms of sets of pH-independent cumulative stability constants, β, corresponding to all detected species (collectively called the model). These constants express the explicit metal and proton stoichiometries of complexes (see section 3 below). In this way, potentiometry is the only method of binding constant determination which can describe individual components of the chemical equilibrium under given conditions. In particular, potentiometry can reveal the presence of multiple species behind a given single spectroscopic component. Other methods, described above, deal with ensembles of species just being present at the equilibrium (e.g. calorimetry), or with those sharing a physicochemical feature (e.g. spectroscopic methods). In short, potentiometry can provide a very detailed description of a relatively simple system.

2.2 Calorimetry

In many respects, calorimetry is the opposite of potentiometry. There is no limit for the size of the molecule studied, and the metal ion binding to its ligand may be studied successfully with no knowledge on the protonation state of the latter. The binding constants are calculated from the measured reaction heats, *via* enthalpy and entropy determination. The price for this apparent simplicity is a much less comprehensive description of the interaction, and a high susceptibility of this method to systematic experimental errors.

Calorimetry measures the heat released in the course of the interactions studied. In the most common calorimetric method used for the determination of stability constants, Isothermal Titration Calorimetry (ITC), one interaction partner is titrated with another while all other parameters and components of the solution are kept as much constant as possible. In studies of biomolecules this condition requires the use of buffers to stabilize the pH and other additives to stabilize the biomolecule (e.g. a specific salt, reducing agent, or a cofactor preventing protein denaturation). One major disadvantage of ITC is that the heat released in the coordination process must be separated from background effects, resulting from the mixing of the titrated sample with the titrant. These include heats of dilution of interacting and non-interacting components of both solutions, as well as (often significant) heats released by undesired side effects [18]. A highly exo- or endothermic effect may also contribute to the overall process significantly even if it is generated by a small proportion of a reactant or an impurtity. In metal ion studies such phenomena may include precipitation of metal hydroxides and redox reactions. All these heats are additive. Therefore, control titrations must be performed for all individual components. The subtraction of these titration curves from that of the main experiment builds up the experimental errors (cf. ref. [18] for an excellent review of applications of the ITC methodology in studies of metal binding). Unlike in potentiometry, there is no standard software available, except for simple programs provided by ITC manufacturers. As a result, the data analysis is usually limited to simple models, such as a macromolecule with n equivalent or n sequential binding sites, although some progress has been achieved recently with respect to more complicated systems [18-20]. Very importantly, in contrast to potentiometry, the stability constant(s) provided by an individual ITC experiment are conditional constants (c*K*ML for the simplest case of a ML complex, see Eq. 1), determined at a fixed pH value. The quantities of reactants required by ITC depend on the heat effect of the main reaction, but are generally similar to those used in potentiometry (e.g. milliliter volumes of submillimolar concentrations of ligands). One may risk a concluding statement that calorimetry allows for determining affinities of metal binding to a much broader range of macromolecules than potentiometry, but requires a much deeper knowledge of the details of the binding process to avoid systematic errors.

2.3 Fluorescence spectroscopy.

 The application of fluorescence spectroscopy to determine stability constants is by all means analogous to that of any other spectroscopic techniques [21]. In brief, spectroscopic titrations are based on the selection of a unique signal in the spectra that is proportional to the concentration of an individual component of the reaction studied. In the most common application, the titration experiments are performed analogously to calorimetry, by titrating one reactant with another under fixed conditions. The major difference from calorimetry is that the concentration changes of a given species are followed directly, leading to a direct determination of a specific c*K*ML. The capability of this approach to handle complicated coordination equilibria depends on the availability of spectroscopic features that could be assigned to individual species, and is usually limited to very simple forms of interaction. Spectroscopic titrations can also be performed and analyzed in a fashion analogous to pH-metry, as mentioned above, but this very laborious approach has not gained much popularity in metal-biomolecule studies.

 The main advantage of fluorescence spectroscopy is its high sensitivity, which allows one to perform titrations at (sub)micromolar concentrations of reactants. The success of this strategy depends on the presence of an appropriate fluorophore in the biomolecule that reports the binding process. Two most common mechanisms are the change (increase or decrease) of fluorescence due to a change of the biomolecule conformation upon binding [22] and the quenching of fluorescence (total or partial) by increased an relaxation of the excited state, facilitated by a metal ion bonded in the vicinity of the fluorophore [23]. In proteins and peptides one can use Tyr or Trp as native fluorophores. Alternatively, an added synthetic fluorescent probe may serve as a competitor (see below). Regarding the native fluorophores, Tyr is less sensitive, but provides data which are easier to analyze, because the intensity of its fluorescence, but not the emission wavelength is affected. The Trp fluorescence is ca 5-fold stronger than that of Tyr [24], expanding the range of concentrations that can be studied, but usually both the intensity and the wavelength of emission are affected by conformational changes. In summary, fluorescence titrations can provide c*K*ML values only for simple interaction models, but the consumption of biomolecule is minimal, typically less than 1% of that required for potentiometry or calorimetry.

 Table 1 summarizes the advantages and disadvantages of three major methodologies of stability constant determination described above.

2.4 Other experimental methods

In principle, any spectroscopic method can be used for determination of stability constants according to the general rules outlined above. Among these, resonance spectroscopies, NMR and EPR provide a particular advantage of high information content, due to their capability of simultaneous observation of several chemical species. NMR provided invaluable structural data for Cu(II), Cu(I) and Zn(II) complexation by Aβ and PrP related peptides, while EPR contributed very significantly to Cu(II) studies, as reviewed recently [26-28]. NMR is undoubtedly the most informative method for studying protonation equilibria [29], and has been used on many occasions to determine stability constants as well. In the context of this review, the EPR usage is limited to Cu(II) complexes. The applicability of this method for studying solution equilibria of complicated systems has been demonstrated [30]. A low sensitivity, resulting in high material and time consumption, is the major disadvantage of resonance spectroscopies.

Other methods of determination of stability constant that have been used in the context of this review include electronic absorption spectroscopy (UV-vis), circular dichroism (CD), equilibrium dialysis (ED), ESI-MS and surface plasmon resonance (SPR). UV-vis and CD can be applied for the determination of binding constants in multicomponent systems, if the spectra of individual components (complexes) differ from each other sufficiently to allow for a simultaneous determination of their concentrations [31]. However, in studies of complexes of this review, these methods have largely been limited to simple determinations of c*K*ML values, similarly to fluorescence spectroscopy. Optical absorption methods are less sensitive than fluorescence, thus requiring higher concentrations of reactants.

Another frequent and very important application of UV-vis and CD spectroscopies is to follow the pH dependence of complexation, as a tool of validating potentiometric titrations. In some cases only the spectroscopic titration allows one to select the correct potentiometric model [32].

The remaining methods involve significant alteration of the physical environment of the reaction studied: interactions with surfaces in ED and SPR, and transition to gas phase in ESI-MS. For this reason, the applicability of each of these techniques requires validation for every individual reaction studied [33].

**3. Method-specific issues in determination of stability constants.**

In the above section, we described general features of main methods of determination of stability constants relevant for the title metal ions and biomolecules. In this section we would like to discuss the central issue of the range of values of stability constants which can be determined by these methods. This issue appears to be the source of significant controversies, particularly in Aβ research.

Eq. 1 presents the simplest formulation of the binding process and the corresponding (conditional) binding constant, which describes this process quantitatively.

M + L ⮀ ML; c*K*ML = [ML]/([M][L]) Eq. 1

The range of c*K*ML that can be reliably measured by a given method on the basis of a direct interaction of two reactants, according to Eq. 1, depends on the concentration of the observed species (the complex, the ligand or the metal ion). The reliability criterion is somewhat arbitrary and depends on the character of the collected signal. For example, Hirose suggests the upper limit for c*K*ML in spectroscopic studies as 20 times the reverse concentration of the observed molecule (e.g. 2 × 105 M-1 for 0.1 mM, 2 × 107 M-1 for 1 µM) [18]. Grossoehme et al. are less stringent and suggest the upper limit for the ITC determination of for c*K*ML as 1 × 107 M-1 for 0.1 mM [21]. Our own experience suggests that the limit proposed by Hirose could be pushed by a factor of ten, but only for very good quality (low noise) data [34]. This means that the upper limit for c*K*ML values which can be determined reliably in a direct fashion is ~107 for ITC and ~108 for fluorescence spectroscopy.

To overcome this barrier, a competitive ligand can be introduced. Its function is to lower the equilibrium concentration of the free metal ion so that the apparent stability constant a*K*MLof the observed ligand decreases sufficiently to permit its determination. Appropriate equations were derived and published by many authors (see refs. [35] and [36] for examples). Eq. 2 presents a simple version of such calculations to illustrate the issue. In this example competitor A interacts solely with M, forming only the MA complex.

M + L ⮀ ML, M + A ⮀ MA; [M’] = [M] + [MA]

a*K*ML = [ML]/([M’][L]) → c*K*ML = a*K*ML (1 + c*K*MA [A]) Eq. 2

In principle, using an appropriately strong ligand A one can determine an arbitrarily large c*K*ML, on condition, however, that the principal chemical condition of Eq. 2 is fulfilled. The competing ligand A must not interfere with the target reaction. This issue requires a very careful attention. For every metal ion M, ligand L, and competitor A evidence must be provided that ternary MLA complexes are not formed. Otherwise, the derivation of Eq. 2 is false and the constant(s) determined are wrong. The same condition regards buffers. For example, practically all buffers suitable for the neutral pH range form complexes with Cu(II) ions, which makes them competitors in the sense of Eq. 2 [37, 38]. Interactions of the buffer with both the studied ligand L and the added competitor A must also be considered.

 A suitable example is provided by our study of Cu(II) binding to human serum albumin (HSA) [39]. In that study we used absorption spectroscopy and physiological HSA concentrations of ca. 0.5 mM. According to the criteria outlined above, the direct titrations would allow us to determine the constants not larger than ~4 × 105 M-1. Yet, by using NTA, a competitor with c*K*MA ~1011 M-1, we were able to reliably obtain the c*K*ML = 1 × 1012 M-1 [39]. We could achieve this, however, only thanks to the detection of the formation and quantitation of a ternary complex of Cu(II) with NTA and Hepes buffer.

 Glycine (Gly) has recently gained popularity as competing ligand for ITC and spectroscopic experiments in Cu(II) studies (see, e.g. refs. [35, 36]). It seems therefore necessary to have a closer look at its coordination properties. Gly is the simplest amino acid, equipped with two metal binding sites, and thus capable of forming a favorable 5-membered chelate ring. Structures of typical Gly complexes are presented in Figure 1. It forms two major Cu(II) complexes, CuGly and CuGly2. The IUPAC recommended cumulative stability constants for these complexes are 1.6 × 108 M-1 and 1.2 × 1015 M-2, respectively [40]. The facile formation of the CuGly2 complex makes Gly a very efficient competitor with a strongly non-linear concentration effect. This is shown in Table 2, which presents the values of the competitivity index (CI) [41, 42], calculated for various Gly and Cu(II) concentrations at pH 7.4. Moreover, Gly forms ternary Cu(II) complexes very eagerly. The IUPAC Stability Constants Database [43] contains 73 entries for such complexes, with stability constants for Gly attachement to CuL complexes typically in the range of 103 M-1 – 105 M-1. Two cases seem to be particularly interesting. The binding of Gly to the bidentate Cu(II)-bipirydyl complex is characterized with a cumulative stability constant of c*K*ML+A = 6.3 × 107 M-1 [44, 45], and that to the tridentate Cu(II)-NTA complex with ~2.4 × 105 M-1 [46]. Allowing for the protonation constant of the Gly amine, these constants can be recalculated into c*K*ML+A ~3 × 105 M-1, and ~1 × 103 M-1, respectively. These data illustrate the potential of Gly to form ternary complexes. Therefore, Gly should not be recommended as competitor for c*K*ML determination unless the absence of ternary complexes has been demonstrated [36].

 The pH-metric version of potentiometry is in principle a competition method, in which hydrogen ions are competitors. As such, it can provide reliable binding constants in a very vast range of values. The only significant point of attention is the translation of its cumulative β constants to conditional constants for comparisons. This can be achieved by several methods. The CI approach proposed by us is outlined in Table 2. The alternative, but in principle equivalent approach consists of calculating an “overall” conditional constant for given conditions, without an inclusion of a formal competitor used in CI. This approach was described in detail in recent reviews [28, 47]. One apparently trivial, but still sometimes overlooked issue is that values of stability constants can be compared only when they units are the same, that is, when stoichiometries of the complexes match.

**4. Cu(II) binding to proteins and peptides related to neurodegenerative conditions**

Among the metal ions within the scope of this review, the quantitative data for complexes of paramagnetic, and optically active Cu(II) ions are the most abundant and raise common experimental issues. Therefore, they will be presented in a separate section, ordered by the ligands.

4.1 Cu(II) binding to Amyloid Precursor Protein

Amyloid Precursor Protein (APP) is a transmembrane protein expressed ubiquitously in human tissues. It is processed proteolytically according to alternative pathways. The minor pathway leads to the extracellular formation of Aβ peptides, considered to be the causative factors of Alzheimer’s Disease according to the amyloid cascade concept [48]. APP appears to be an authentic copper-binding protein. It contains a copper binding domain (CBD), structurally analogous to Type 2 non-blue copper centers [49, 50]. As such, it was shown to stabilize Cu(I) vs. Cu(II). The actual function of APP as a copper binding protein remains unknown, but the saturation of CBD with copper reduces the yield of Aβ peptides. A single SPR study indicated a c*K*ML of 108 M-1 for Cu(II) binding to APP [51].

4.2 Cu(II) binding to α-Synuclein

α-Synuclein (aS) is a single amino acid chain protein composed of 140 residues, characterized with the lack of strict folding. It contains the N-terminal domain, prone to form amphipathic helical structures upon interaction with membranes (residues 1-67), a central hydrophobic domain (residues 68-95) and the C-terminal acidic region (residues 96-140) [52]. The protein is expressed predominantly in the central nervous system neurons. aS is an intrinsically disordered protein (IDP), Such proteins assume fixed structures only *via* interactions with other macromolecules or structures (e.g. membranes). Aggregated, fibrillar aS is the main constituent of Lewy bodies, pathological structures related to the neuronal death in substantia nigra of Parkinson Disease patients. Aggregation of aS appears to be associated with interactions with metals, including copper and iron [53].

The studies of Cu(II) binding to aS clearly come from two camps. One set of studies indicates that the strong binding site simultaneously involves the N-terminal Met-Asp residues and His-50 [52, 54, 55]. Another proves otherwise, stating that the strong Cu(II) binding is confined to the N-terminal part of the protein, and His-50 either plays no role in this interaction, or provides only a secondary binding site, weaker by ca. 2 orders of magnitude [56-60]. His mutants, such as H50A, H50S and H50N were used to prove the point in both cases. The measurements of stability constants for Cu(II) binding to aS and its mutants are summarized in Table 3. Clearly, the disagreement includes both the character of the site and its affinity. In particular, one ITC study, performed in the presence of Gly competitor, indicated a strong binding, with c*K*ML > 109 M-1 [61], while another, which did not use Gly, proposed a constant c*K*ML ~ 107 M-1 [59] (but the constants were not corrected for the presence of 20 mM Hepes, which is a sufficiently strong competitor to elevate the constant by an order of magnitude [37]). A potentiometric study of N-terminal peptides returned the c*K*ML ~ 4 × 108 M-1 [62].

While it is very difficult to find the reasons for such a clear discrepancy between studies which, each for itself, present a very high skill and sophistication, we think that the problem may lie in the protein. The intrinsic unstructuring of aS, and its tendency to aggregate in the presence of metal ions may lead to its high sensitivity to details of experimental conditions, which could include varied conformations of the N-terminal and His-50 areas of the protein, affecting their availability for Cu(II) binding.

4.3 Cu(II) binding to Amyloid β peptides

 Amyloid β (Aβ) peptides are derived by proteolysis of APP. Aβ40 and Aβ42 are two major physiological peptides, involved in the Alzheimer Disease (AD) pathology. Currently, the less abundant Aβ42 is considered to be the main toxic species in AD [63]. This property is thought to be related to the grater propensity of this peptide to aggregate. Structures as small as dimers may be sufficient in this respect [64]. The binding of Cu(II) to Aβ40 and Aβ42 is considered to augment, or even cause their neurotoxicity [53]. While soluble oligomeric Aβ species appear to be most strongly neurotoxic, significant technical difficulties precluded, so far, the determination of Cu(II) binding modes and affinities in oligomers, despite of the indirect evidence that such complexes do exist [65]. Instead, the elucidation of Cu(II) binding to monomeric Aβ peptides has attracted all attention so far. The structural issues have been reviewed extensively and competently, and there is no need to duplicate this effort [47, 66, 67]. Instead, here we focused our attention on stabilities of complexes. After some initial controversies, a general agreement has been achieved about the location of the strong binding site within the N-terminal 16-peptide sequence, with direct involvement of all three His residues (His-6, His-13, and His-14, and the N-terminal Asp-Ala dipeptide). Two families of dynamic structures, named component I (dominant at pH 6.5, major at pH 7.4) and component II (dominant at pH 9, minor at pH 7.4) were proposed to be present in neutral solutions. The main difference between them is the involvement of the Asp-Ala peptide bond atoms in the coordination of component II. Except of potentiometric studies, all other methods used for measurement of stability constants of Aβ peptides actually measure the average parameter of the mixture of these two families of complexes. Aβ40 aggregates at concentrations above 10 µM, and the Aβ42 monomer is even less stable. On the other hand, all currently available data indicate that the way Cu(II) binds to full length Aβ40 and Aβ42 peptides is preserved in shorter, C-terminally truncated analogs, Aβ28 and Aβ16. Aβ28 aggregates slowly, and Aβ16 does not aggregate in the absence of metal ions, therefore these two peptides have been used frequently in quantitative and structural studies.

 Tables 4 and 5 present the summary of stability constant data for Cu(II)-Aβ complexes. Table 4 provides the binding constants obtained using Tyr-10 fluorescence [68-75], while Table 5 deals with the data derived from ITC [76-79] and potentiometry, which was used to measure stability constants of Aβ28 and Aβ16 peptides [80, 81]. We limited our data review to the strong binding site, because the weaker site, reported predominantly for Aβ16, is not relevant toxicologically [66, 67]. For the sake of simplicity, we also limited the data presentation to unmodified, human Aβ sequences. Recent papers focused on Aβ studies provided in-depth description of results obtained for modified peptides [67, 82]. Among these, the study using PEGylated Aβ16 indicated the binding of multiple Cu(II) ions to a single peptide molecule at the metal excess [81].

The inspection of Tables 4 and 5 shows clearly see that, with few exclusions, the Tyr-10 method delivers the c*K*ML values which are ca. hundred-to thousand-fold lower from those provided by calorimetry and potentiometry. There are two ways to explain these immense discrepancies. One possibility is that there is a systematic error at least on one side, another that the difference in experimental conditions yields different chemical species in Aβ solutions. Calorimetric and potentiometric studies are, by necessity, done at high ligand concentrations around 10-4 - 10-3 M, at which longer Aβ peptides tend to aggregate, while Tyr-10 fluorescence is measured for peptide concentrations of ~10-5 M, at which the aggregation is not observed for Aβ40 and shorter peptides [83]. In our opinion, supported by the data presented in Section 3 and our experimental studies [75, 83], the usage of Gly and other similar competitors leads to the formation of Cu(Aβ)L ternary complexes, with an increase of the apparent stability constant derived from ITC experiments. Notably, a very recent calorimetric study, using ACES buffer, rather than Gly, as a competitor yielded c*K*ML of 109 M-1 [79]. The apparent absence of ternary complexes with ACES thus resulted in a tenfold decrease of c*K*ML, compared to Gly experiments. This point does not, however, explain the very high stability constants produced by potentiometric experiments on Aβ16 and Aβ28 peptides [80, 81]. We interpret the high affinities obtained at higher peptide concentrations in terms of the formation of dimeric/oligomeric complexes rather than the monomeric ones, assumed in the data analysis of these papers. This concept, raised also by others [47], finds support in the report of the concentration-dependent formation of dimeric Cu(II) species [65]. Figure 2 illustrates the relationship between methodologies applied, Aβ peptide concentrations used and Cu(II) affinities reported (expressed as c*K*ML values). Further multi-method research is necessary to clarify this very important issue.

 In summary, we believe that the actual binding constant for the monomeric CuAβ complex at pH 7.4 is close to the *K*dcond value of 57 ± 5 nM, which we reported recently [75], but the actual affinity of Aβ species for Cu(II) in the synaptic cleft will be closer to those determined by calorimetry and potentiometry, due to peptide oligomerization, and/or ternary complex formation. In fact, the analogous mechanism of stability constant increase upon the peptide/complex oligomerization was demonstrated by Tõugu et al. [68] for Aβ interaction with Zn(II) ions (see Section 5).

4.4 Cu(II) binding to prion protein

 Prion protein (Prp) is a small protein of 231 residues, associated with the outer cellular membrane. It is also a unique infectious agent, responsible several fatal central nervous system pathologies in animals and humans. The pathological process involves a conversion of the α-helical domain in the N-terminal part of the protein into the aggregation-prone β-sheet structure (a PrpC to PrpSc conversion) [84]. While PrpSc is the pathogenic form of Prp, the function of non-toxic PrpC remains unclear. Due to the presence of multiple Cu(II) binding sites in its structure, it has been implicated in the regulation of copper physiology in the brain, but crucial evidence for such function is still lacking [28]. Also, Cu(II) interaction does not seem to be related directly to neurodegenerative capabilities of Prp. Nevertheless, the unique Cu(II) binding properties of Prp attracted significant attention.

 Prp contains four repetitive PHGGGWGQ sequences in positions 60-90, known as octarepeats, which were demonstrated to bind Cu(II) ions at the His residue and the adjacent amide nitrogens. This coordination mode is unique, because the presence of a Pro residue preceding the His precludes the formation of the typical “downstream” series of chelate rings, such as observed in albumins and related peptides [85, 86]. Instead, a seven-membered ring is formed by the His imidazole and the amide nitrogen of the next Gly residue. The coordination sphere at neutral pH is completed by the amide nitrogen of the following Gly residue, yielding the 7- and 5- membered couple of fused chelate rings [28, 87, 88]. The four octarepeats can bind Cu(II) independently, but they can also cooperate to bind a single Cu(II) ion *via* their His imidazoles (component 3 [28]). Such binding can occur at a high molar excess of Prp over Cu(II) [89, 90]. Another couple of potential Cu(II) binding sites is present in the C-terminal part of Prp, at His-96 and His-111 residues, and involves the amide bonding. Recent studies indicated that the geometry of the His-111 site is variable, and both upstream and downstream amide nitrogens can be involved [28, 91]. Table 6 gathers the quantitative data about Cu(II) complexes with Prp and its fragments. For simplicity, we only included human sequences. The major difference between the state of the art in Cu(II)-Prp and Cu(II)-Aβ research is that the Cu(II) binding sites in Prp are well characterized structurally. The recent critical analysis of the literature indicates that highest biological importance should be ascribed to the component 3 binding mode to cooperating octarepeats, with *K*dcond of 0.1 nM. At a high Cu(II) availability octarepeats interact with Cu(II) independently with *K*dcond of ~1-10 µM. Other sites do not seem to be relevant, according to spectroscopic studies [28].

Potentiometric studies of C-terminal Prp sequences of various length and their His mutants confirm the above conclusions, and the overall Cu(II) affinities to a certain extent. It has to be noted, however, that the latest of these studies, performed with the hPrP76-114 peptide, indicated a possibility that at physiological pH a mixture of species is present, anchored at various His residues, including His-96 and His-111 outside of the octarepeat sequence, and involving the coordination of adjacent deprotonated amide nitrogens residues [92].

This analysis, and the data presented in Table 6 [92-104] clearly indicate that the usage of Gly as competitor produces impossibly high stability constants. In our opinion, the likely reason for this is, as was in the case of Aβ research, the formation of Gly ternary complexes, facilitated by the partially unsaturated and labile coordination spheres of PrP-bound Cu(II) ions.

**5. Cu(I) and Zn(II) binding to proteins and peptides related to neurodegenerative conditions**

The literature regarding Zn(II) and Cu(I) interactions with proteins and peptides of our interest is much less abundant than that for Cu(II). One reason for the prevalence of Cu(II) data over Cu(I) is that toxicological models developed to account for respective metal-dependent neurotoxicity processes deal predominantly with Cu(II). The majority of considered scenarios occurs in the extracellular space and/or under oxidative stress conditions. Both these factors favor Cu(II) as the basic oxidation state (although an interesting contrary argument was outlined for cerebrospinal fluid recently [105]). The involvement of the much less reactive Zn(II) in these pathologies is also less obvious from the chemical reactivity point of view, and its role is being appreciated only now [106, 107]. Also, Zn(II) and Cu(I) are more cumbersome to study. Zn(II) complexes are generally weaker from those of Cu(II), and are spectroscopically silent. The Zn(II)-Aβ systems also suffer from precipitation problems. In contrast with Cu(II) and Zn(II), Cu(I) aqua ion is not stable under ambient conditions, eagerly disproportionating into Cu(0) and Cu(II). In order to obtain stability constants for Cu(I) complexes of biomolecules, one has to resort to competitive titrations, using strong ligands capable of stabilizing the reduced cuprous state. A recent paper provides an excellent critical account of this methodology [108].

Another reason for pooling Zn(II) and Cu(I) together against Cu(II) is that both are considerably softer ions. The most striking result of this difference is the preference of Cu(II) for coordinating to deprotonated amide nitrogens, observed in Aβ and PrP systems. Neither Zn(II) nor Cu(I) interact in a similar fashion with these peptides.

The Zn(II) binding site in APP is thought to be similar to that of Cu(II). The stability constant of the Zn-APP complex was determined only once, and a relatively high stability constant of 1.3 × 106 M-1 was obtained using a 65Zn2+ radioassay (originally reported as *K*d = 764 nM) [109]. The binding of Cu(I) to this protein has not been studied quantitatively, but the relative stabilization of Cu(I) by APP vs. Cu(II) [50] indicates that this should be expected as higher than 109 M-1.

The binding of Zn(II) to α-synuclein is considered to be insignificantly weak. This view is based largely on a preliminary fluorescence titration, which indicated that millimolar Zn(II) is required to saturate the protein at a micromolar concentration [110, 111]. It, however, agrees well with the deficit of zinc binding amino acid side chains in α-synuclein, and with the fact that the Cu(II) binding site involves coordination of deprotonated amide nitrogens, and as such is not suitable for a Zn(II) ion [85, 86]. There is no indication that α-synuclein might bind Cu(I), for the same reasons.

In contrast to the abundant literature about binding constants for Cu(II) complexes with Aβ peptides, there are only two very recent papers providing such data for the Cu(I) complexes. First, Feaga et al. published a very high stability constant c*K*ML ~5 × 1014 M-1 for Aβ16 using bathocuproinedisulfonic acid (BC) as a competing ligand and spectrophotometric detection [105]. These experiments were performed in unbuffered solution of unknown (or at least unreported) pH, while a control experiment at pH 7.0 indicated a 10-fold weaker binding. Next, Alies et al. attempted to repeat these experiments, but observed no competition between BC and Aβ16. Instead, they determined the c*K*ML values of 7.5 ± 1 ×106 M-1 and 19 ± 8.2 ×106 M-1 for Aβ16 and Aβ42, respectively, using ferrozine (Fz), as alternative competitor [112]. These experiments were performed at pH 7.4, in a 0.1 M Hepes buffer. Clearly, one of these studies must be wrong. Taking into account that the stability constant for the Cu(II) complex, regarding all controversies described above, is certainly lower than 1012 M-1 and that Aβ peptides stabilize the Cu(II) oxidation state under ambient conditions, our opinion is strongly in favor of the latter results.

The Zn(II)/Aβ literature is more abundant. It is summarized in Table 7 [68, 69, 74, 113-115]. Zn(II) interacts only very weakly with Tris (c*K*ML = 14 at pH 7.4, recalculated from [116]) and does not interact with Hepes at pH 7.4 [19]. Therefore, the *K*dapp values provided by the authors of the cited papers can be recalculated into c*K*ML values by simple reciprocation. In our opinion, these data provide a very consistent picture, explained by the study of Tõugu et al. [68]. The initial weak monomeric complex (c*K*ML values ~1 × 104 M-1) is very prone to aggregate, and this aggregated species, which forms at the time scale of minutes, binds Zn(II) with the unaltered stoichiometry of one Zn(II) ion per peptide molecule, but with an affinity higher by approximately two orders of magnitude (c*K*ML values ~1 × 106 M-1). The intermediate values result from the presence of a mixture of monomeric and soluble aggregated species in the titrated solutions.

The only quantitative study regarding Cu(I) and a prion peptide regards PrP91-124. This study used the titration of the CuI(BC)2 complex with the peptide in a 25 mM phosphate buffer at pH 6.9 [117]. A Met2His2 Cu(I) binding site was identified, with Cu(I) binding through His-96, His-111, Met-109 and Met-112. This site is unlike the Zn(II) or Cu(II) sites. A very broad estimate of the stability constant, 1015 – 1012 M-1 was obtained, which resembles the result obtained for Aβ16 with the same method [105]. Controversial results of these two studies may indicate a problem with using BC for studying peptide complexes.

 The PrP binding site for Zn(II) has not been characterized structurally [111], and only one paper dealing with stability constants was published [98]. The binding constant was determined via Cu(II) competition, using the intrinsic Trp fluorescence, in the presence of high amount of Gly. The *K*dapp value of 100 nM was reported at pH 8.0. This constant is very difficult to interpret, because it was established without considering parallel coordination processes that occurred in the studied sample, such as the Zn(II)-Gly binding.

**6. Concluding remarks**

 The synapse is the main arena of molecular events leading to neurodegenerative disorders which involve the title peptides and proteins. It is therefore worthwhile to confront the data presented above with boundary conditions imposed by this biological structure. APP and PrP are present on the surfaces of neuronal cell extensions that form the synapse and therefore extend into the synaptic cleft. Aβ peptides are then released into this space. Let us consider a glutamatergic synapse, which is thought to be a primary target of Aβ toxicity [118]. The Cu(II) and Zn(II) ions are released into this cleft in the process of Glu-mediated neurotransmission; this fact is at the basis of the concept of AD as a metal ion physiology disorder, and also gives rise to modern approaches in therapy of AD and other neurodegenerative conditions [119, 120]. It is therefore interesting to know that the volume of the glutamatergic cleft is only about 5 aL [121]. This very low volume sets a lower limit on the range of concentrations available, due to a simple fact that by dissolving one molecule in 5 aL of fluid one obtains a “concentration” of 0.33 µM. A release of 300 ions into the cleft results in their average concentration of 0.1 mM, and locally and temporarily, at the site of release this concentration is still higher. Therefore, for example, nanomolar Aβ concentrations detected in the cerebrospinal fluid [122] are not relevant for the synaptic cleft. All chemical processes occur there at concentrations of both peptides/proteins and metal ions that are high, from the point of view of their binding affinities. In this sense it may not matter which side is right in terms of high or low stability constants in disputes presented above). In this context, association and dissociation rates may become more important than equilibrium conditions [123]. The future research should therefore focus on kinetic studies, such as that presented recently for the Aβ-Cu(II) interactions [124]. Another issue is the presence of other metal binding molecules, which can form ternary complexes, or prevent the metal ion binding to title peptides/proteins. As we stated above, we tend to believe that problems of determination of stability constants for Aβ and PrP peptides in experiments with Gly as competitor are due to the formation of ternary complexes. Therefore, amino acids, which are abundant in the synapse as neurotransmitters become natural, while not exclusive partners for such interactions, which need to be studied in the future. Other metal-binding macromolecules, which are known for their metal storage function, may interact not only with metal ions, but also with their complexes. Our concept of HSA as a controller of Cu(II)-Aβ interaction is one example[125], the effects of metallothioneins on Aβ toxicity provide another [126, 127]. Further effort from chemical research to formulate testable hypotheses in neurodegenerative diseases should be expected in the near future.

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**Tables**

**Table 1**

Advantages and disadvantages of main experimental methods used for determination of stability constants of metal ion complexes with biomolecules

|  |  |  |  |
| --- | --- | --- | --- |
| Method | Advantages | Disadvantages | Concentration of biomolecule |
| potentiometry | * solves complicated stoichiometric models
* delivers universally applicable absolute stability constants
 | * limited to relatively small molecules
* consumes much material
 | 10-3 M |
| ITC | * applicable to molecules of all sizes
* determines entropy and entalpy
 | * delivers apparent stability constants
* sensitive to systematic errors
* limited to simple interaction models
 | 10-4 M |
| fluorescence spectroscopy | * very sensitive, consumes little material
* can follow native biomolecule or added competitor
 | * suitable only for very simple complex stoichiometries
* delivers apparent stability constants
 | 10-5 - 10-7 M |

**Table 2**

Competitivity index (CI)a values for Gly binding of Cu(II) at pH 7.4b.

|  |  |  |
| --- | --- | --- |
| Gly:Cu(II) | cCu(II) = 10−4 M | cCu(II) = 10−3 M |
| 1 | 6.03 | 6.00 |
| 2 | 7.11 | 7.98 |
| 4 | 7.96 | 8.92 |

a computational entity, defined as the logarithm of the conditional stability constant of MZ (the metal complex of a theoretical molecule Z), such that Σ*ijkl*([M*i*H*j*L*k*A*l*] = [MZ] [41, 42].

b The binding constants from ref. [40] were used for calculations.

**Table 3.**

Literature values for the stability constants of Cu2+ complexes with α-synuclein (aS) and its mutants. WT denotes wild-type aS. Only the strong binding site is described.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| protein | *K*dapp (µM) | c*K*ML (M-1) | pH | T (°C) | aS conc. (μM) | Method | Buffer | ref. |
| WT |  | 2.3 × 109 | 7.2 | 37 | 70-90 | ITC | 20 mM PIPES, 103 mM NaCl, 4 mol. eq. of Gly per Cu(II) | [61] |
| A53T | 2.4 × 109 |
| A30P | 1.6 × 109 |
| WT | 4.7 × 109 | 7.4 |
| A53T | 5.0 × 109  |
| A30P | 3.6 × 109 |
| MDVFMK | 0.11 |  | 7.5 | 15 | 70 | ITC | 20 mM Hepes, 100 mM NaCl | [59] |
| WT | 0.11 |
| H50A | 0.12 |
| WT | 0.12 |  | 6.5 | 15 | 300 | CD | 20 mM Mes, 100 mM NaCl | [57] |
| F4WF4W/H50S | 0.100.11 |  | 7.0 | 25 |  | fluorescence decay | 20 mM MOPS, 100 mM NaCl | [58] |
| WTH50A |  | 9.96 × 1092.5 × 109 |  |  |  | EPR, pentaGly competition |  | [55] |
| aS1-39 |  | 4 × 108 | 7.4 | 25 |  | potentiometry | 100 mM KNO3 | [62] |

**Table 4.**

Summary of stability constants for Cu(II)-Aβ complexes, obtained from Tyr-10 fluorescence data. The c*K*ML values were calculated from the literature *K*dapp data by Tõugu et al. [68], except for ref. [75].

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Aβ peptide | Kdapp (μM) | c*K*ML (M-1)  | Buffer / pH | Competitor | ref. |
| 40 | 1.6 | 1.4 × 108 | 10 mM Tris, 100 mM NaCl / 7.4 | - | [69] |
| 42 | 2.0 | 1.1 × 108  | 10 mM Tris, 100 mM NaCl / 7.4 |
| 28 | 0.01-0.1 | 107 - 108 | H2O / 7.8 | Gly/His | [70] |
| 28 | 28 | 3.1 × 107 | 100 mM Tris, 150 mM NaCl / 7.4 |  | [71] |
| 40 | 11 | 8.3 × 107 |
| 40 | 8 | 3.1 × 106 | 50 mM PBS, 100 mM NaCl / 7.4 |  | [72] |
| 16 | ~ 0.1 | 107 | H2O / 7.8 | Gly/His | [73] |
| 28 | 2.5 | 2.7 × 107 | 10 mM Hepes / 7.2 |  | [74] |
| 28 | 0.4 | 1.3 × 107  | 10 mM phosphate / 7.2 |  |
| 40 | 0.5 | 2.7 × 107 | 10 mM Tris, 100 mM NaCl / 7.3 |  | [68] |
| 1.2 | 2.9 × 107 | 20 mM Tris, 100 mM NaCl / 7.3 |  |
| 3.8 | 4.2 × 107 | 50 mM Tris, 100 mM NaCl / 7.3 |  |
| 30 | 1.9 × 107 | 100 mM Tris, 100 mM NaCl / 7.3 |  |
| 0.6 | 2.8 × 107 | 20 mM Hepes, 100 mM NaCl, pH 7.4 |  |
| 0.9 | 4.2 × 107 | 50 mM Hepes, 100 mM NaCl, / 7.4 |  |
| 2.5 | 2.9 × 107 | 100 mM Hepes,100 mM NaCl / 7.4 |  |
| 42 | 0.8 | 2.1 × 107 | 20 mM Hepes, 100 mM NaCl / 7.4 |  |
| 40 |  | 1.8 × 107 | 20-100 mM Hepes / 7.4 |  | [75] |

**Table 5.**

Summary of stability constants for Cu(II)-Aβ complexes, obtained from ITC and potentiometric data (ref. [80]). The *K*dapp  and c*K*ML values are given as provided in the original papers, except for ref. [80], where  c*K*ML values were calculated from the source log β constants by the CI approach [41, 42].

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Aβ peptide | *K*dapp (μM) | c*K*ML (M-1) | Buffer / pH | Competitor | ref. |
| 40 |  | 1.1 × 1010 | 20 mM Hepes / 7.2 | Gly | [76] |
|  | 2.4 × 1010 | 20 mM Hepes /7.4 | Gly |
|  | 9 × 1010 | 20 mM Pipes / 7.2 | Gly |
| 16 |  | 1.5 × 1010 | 20 mM Hepes / 7.2 | Gly |
|  | 2.9 × 1010  | 20 mM Hepes /7.4 | Gly |
|  | 3.0 × 1010 | 20 mM Pipes / 7.4 | Gly |
| 16 |  | 1.5 × 1010 | 20 mM Hepes / 7.2 | Gly | [77] |
| 16 | 0.1 |  | 50 mM Hepes, 100 mM NaCl / 7.4 | - | [78] |
| 28 | 0.1 |  | 50 mM Hepes, 100 mM NaCl / 7.4 | - |
| 16 | 0.1 |  | 50 mM Tris / 7.4 | - |
| 16 |  | 1.1 × 109 | 20 mM ACES, 100 mM NaCl / 7.4 |  | [79] |
| 16 |  | 1.04 × 109 | 50 mM ACES, 100 mM NaCl / 7.4 |  |
| 16 |  | 1.06 × 109 | 100 mM ACES, 100 mM NaCl / 7.4 |  |
| 28 |  | 0.58 × 109 | 20 mM ACES, 100 mM NaCl / 7.4 |  |
| 28 |  | 4.1 × 1010 | 100 mM KNO3 | - | [80] |
| 16 |  | 4.8 × 109 |
| 16 |  | 8.3 × 109 | 200 mM KCl |  | [81] |

**Table 6.**

Published *K*dapp and c*K*ML constants of Cu2+ complexes with Prp and its peptide fragments. The  c*K*ML values for refs. [92] and [103] were calculated from the source log β constants by the CI approach [41, 42], with the inclusion of mononuclear complexes only.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| PrP fragment | *K*dapp (μM) | c*K*ML (M-1) | Method | Buffer / pH | Competitor | ref. |
| hPrP60-91(Octa4) | 6.7  |  | fluorescence | PBS / 7.4 |  | [93] |
| hPrP23-98(Octa4) | 5.9 |  | equilibrium dialysis | NEM 25 mM, KCl 0.15 M / 7.4 | Gly  | [94] |
| hPrP51-75(Octa2) | ̴6 |  | CD | phosphate or N‑Ac‑morpholine / 7.4 |  | [95] |
| hPrP60-67(Octa1)  | 1‑5 |  | CD/EPR | NEM 25 mM, KCl 0.15 M, glycerol 20% / 7.4 |  | [96] |
| hPrP60-75(Octa2) |  |
| hPrP60-90(Octa4) |  |
| hPrP23-98 | ~2 C‑term~6 Octa4 |  | fluorescence, ESI‑MS | MOPS 20 mM, NaCl 100 mM, DTAC 1mM / 7.2 |  | [97] |
| hPrP23-112 |  |
| hPrP60-91(Octa4) |  | 1 mM NEMO/formic acid / 7.4 |
| hPrP60-109 |  |
| hPrP(23-28,57‑91)(contains Octa4) | 1 × 10-4low occup. |  | EPR/ fluorescence quenching | NEM 25 mM, NaCl 100 mM, DTAC 1mM / 7.4 | various amino acids | [90] |
| 7.0-12.0 high occup. |  |
| hPrP52-98(Octa4)  | 8 × 10-9 |  | fluorescence | MOPS 5mM / 8.0 | Gly | [98] |
| hPrP91-231 | 40 × 10-9 |  |
| hPrP23-231 | 10-6-10-9 |  | ITC/CMCA | no buffer / 7.0 | Gly | [99] |
| hPrP57-91(Octa4) | 3 × 10-3 |  | equilibrium dialysis | Tris/HCl / 7.4 | Gly  | [102] |
| hPrP91-115 | K1 ~0.1K2 ~0.14 |  |
| hPrP23-231 | ̴~10-3 |  | fluorescence, NMR | MES 5mM / 5.5 | Gly | [101] |
| hPrP91-231 |  |
| hPrP91-126 | 90 |  | fluorescence | NEM 50 mM / 7.4 | Gly | [102] |
| hPrP106-114 |  | 7×106 | potentiometry |  |  | [103] |
| hPrP84-114 |  | 3×106 | potentiometry |  |  | [104] |
| hPrP91-115 |  | 7×104 | potentiometry |  |  |
| hPrP76-114 |  | 1.2×1010 | potentiometry |  |  | [92] |

**Table 7.**

Summary of stability constants for Zn(II)-Aβ complexes. (\*) denotes the initial complex, according to Tõugu et al. [68]. *K*dapp values were taken from the literature, and recalculated into c*K*ML values.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Aβ peptide | *K*dapp (µM)  | c*K*ML (M-1) | Buffer / pH | method | ref. |
| 40 | 300 | 3.3 × 103 | 10 mM Tris, 100 mM NaCl / 7.4 | Tyr-10 fluorescence | [69] |
| 42 | 57 | 1.8 × 104 |
| 16 | 22 | 4.5 × 104 | 20 mM Tris, 100 mM NaCl / 7.4 | ITC | [113] |
| 28 | 10 | 1.0 × 105 |
| 40 | 7 | 1.4 × 105 |
| 16 | 14 | 7.1 × 104 | 20 mM Hepes, 100 mM NaCl / 7.4 | Zincon competition |
| 28 | 12 | 8.3 × 104 |
| 40 | 7 | 1.4 × 105 |
| 42 | 7 | 1.4 × 105 |
| 28 | 6.6 | 1.5 × 105 | 10 mM Hepes / 7.2 | Tyr-10 fluorescence, Cu(II) competition | [74] |
| 28 | 3.2 | 3.1 × 105 | 10 mM Na phosphate / 6.5 |
| 28 | 1.1 | 9.1 × 105 | 10 mM Na phosphate / 7.2 |
| 40 | 1.2 | 8.3 × 105 | 10 mM Na phosphate / 7.2 | NMR, Cu(II) competition |
| 40 | 60\* | 1.7 × 104 | 10 mM Tris, 100 M NaCl / 7.4 | Tyr-10 fluorescence | [68] |
| 40 | 184\* | 5.4 × 103 | 100 mM Tris, 100 mM NaCl / 7.4 |
| 40 | 65\* | 1.5 × 104 | 20 mM Hepes, 100 mM NaCl / 7.4 |
| 42 | 91\* | 1.1 × 104 | 20 mM Hepes, 100 mM NaCl / 7.4 |
| 40 | 3.2 | 3.1 × 105 | 10 mM Tris / 7.4 | 65Zn2+ displacement | [114] |
| 3.2 | 3.1 × 105 | 10 mM Hepes / 7.4 |
| 40 | 5.2 | 1.9 × 105 | 20 mM Tris / 7.4 | 65Zn2+ displacement | [115] |

**Figure 1.**

Typical structures of Cu(II) glycine complexes; clockwise from the top left: CuGly and CuGly2 binary complexes, a ternary complex with a bidentate ligand L-L and a ternary complex with a tridentate ligand L-L-L. Water molecules are omitted for the sake of simplicity.



**Figure 2.**

The log-log relationship between Aβ peptide concentrations and reported c*K*ML values for Cu(II) binding, according to various experimental methodologies. The data are taken from Tables 4 and 5. Boxes represent the ranges of concentrations and stabilities for all peptides. The dashed line indicates a putative relationship between the concentration and the apparent stability constant.

