Site-Specific Interactions with Copper Promote Amyloid Fibril Formation for λ6aJL2-R24G

Angel E. Pelaez-Aguilar, Gilberto Valdés-Garcia, Leidy French-Pacheco, Nina Pastor, Carlos Amero, and Lina Rivillas-Acevedo*

ABSTRACT: Light-chain amyloidosis (AL) is one of the most common systemic amyloidoses, and it is characterized by the deposition of immunoglobulin light chain (LC) variable domains as insoluble amyloid fibers in vital organs and tissues. The recombinant protein 6aJL2-R24G contains λ6a and JL2 germline genes and also contains the Arg24 by Gly substitution. This mutation is present in 25% of all amyloid-associated λ6 LC cases, reduces protein stability, and increases the propensity to form amyloid fibers. In this study, it was found that the interaction of 6aJL2-R24G with Cu(II) decreases the thermal stability of the protein and accelerates the amyloid fibril formation, as observed by fluorescence spectroscopy. Isothermal calorimetry titration showed that Cu(II) binds to the protein with micromolar affinity. His99 may be one of the main Cu(II) interaction sites, as observed by nuclear magnetic resonance spectroscopy. The binding of Cu(II) to His99 induces larger fluctuations of the CDR1 and loop C′, as shown by molecular dynamics simulations. Thus, Cu(II) binding may be inducing the loss of interactions between CDR3 and CDR1, making the protein less stable and more prone to form amyloid fibers. This study provides insights into the mechanism of metal-induced aggregation of the 6aJL2-R24G protein and sheds light on the bio-inorganic understanding of AL disease.

INTRODUCTION

Light-chain amyloidosis (AL) is a fatal degenerative disease characterized by the extracellular deposition of insoluble aggregates of antibody light chain (LC) proteins. Plasma cells normally produce a small excess of LCs over heavy chains, and these LCs are cleared by the kidneys, but occasionally, free LCs aggregate into pathological forms. LCs misfold and aggregate, depositing in tissues in the form of amyloid fibrils, among other types of aggregates. The LC gene consists of three segments, the variable (V), junction (J), and constant (C) segments. The functional gene is a combination of different VJC genes, which result in a protein with two domains, the variable (VL) and constant (CL) domain. Structurally, the variable domain contains the V and J segments and consists of eight β-sheets of four strands. The sheets are joined by a disulfide bridge. In vitro studies have shown that the VL domain forms amyloid fibers more readily than the CL domain, and there are some LC genes preferentially associated with AL. 6a and JL2, the gene that encodes λ6 proteins. The recombinant protein 6aJL2-R24G contains the λ6a and JL2 germline genes and also contains the Arg24 by Gly substitution. This mutation is present in 25% of all amyloid-associated λ6 LC cases. 6aJL2-R24G protein is less stable and more prone to form amyloid fibers in vitro than the germline protein 6aJL2, while maintaining a similar three-dimensional structure.

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emerged as important players in protein aggregation. Therefore, understanding the properties of the binding and the molecular details of protein−metal complex formation may provide important insights into the pathogenic processes. However, the effect of metal ions in LC and similar proteins is relatively unexplored, as well as the presence of metal ions in the AL patients. The interaction of β2-microglobulin (β2m), a protein from dialysis-related amyloidosis, with metal ions showed that Cu(II) induces amyloid fibril formation, Zn(II) induces oligomerization but not amyloid fibril formation, and Ni(II) does not induce oligomerization or aggregation of the protein.33,34 Also, it has been reported that a recombinant kIV LC (SMA) aggregates when incubated with Cu(II), both in vitro and under high copper concentration conditions within cells.35 Although there are no direct evidences of the role of metal ions in the development of this disease, it has been reported that under the acute phase of an inflammatory response, Cu(II) levels increase in serum36,37 and it becomes acidic,38 leading to a loss of copper binding by ceruloplasmin. These conditions could promote destabilization of plasma proteins, LC among them. Moreover, it is well known that all organs and tissues must have efficient homeostatic mechanisms to prevent the abnormal discompartmentalization of metal ions. The loss of homeostasis has been observed in several neurodegenerative diseases,39−41 and the altered levels of metal ions could lead to an erratic protein behavior, as it is widely studied for those diseases. Also, Cu(II) and Zn(II) levels are altered in type II diabetes patients,42−45 suggesting a link between metal ion homeostasis and diabetes. Hence, if the homeostatic control is lost, there can be abnormal metal−protein interaction in vivo, leading to degeneration into the pathological state.46

In this study, a spectroscopic and computational characterization of the Cu(II)−6aJL2-R24G complexes was carried out. It was found that 6aJL2-R24G can bind Cu(II) with affinities in the sub-micromolar range and that the binding accelerates the protein fibril formation in vitro. In addition, molecular dynamics simulations of the complexes showed binding-site dependent effects that lead to increased flexibility and loss of interactions in key regions of the molecule. Altogether, these observations provide insights into the mechanism of metal-induced amyloid fibril formation of LCs and the bio-inorganic chemistry of AL, which are important to understand the etiology of this disorder.

### RESULTS AND DISCUSSION

**Cu(II) Interaction with 6aJL2-R24G Accelerates the Formation of Amyloid Fibrils.** To characterize the effect of Cu(II) in the amyloid aggregation of 6aJL2-R24G, the kinetics of fibril formation were measured in the absence and presence of Cu(II). Changes were monitored by the thioflavin T (ThT) fluorescence assay. In these assays, an increase in ThT fluorescence intensity at 485 nm indicates the formation of rigid amyloid-like structures capable of binding ThT. Figure

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**Figure 1.** Effect of divalent copper on 6aJL2-R24G amyloid formation followed by ThT fluorescence (A) and thermal stability followed by tryptophan fluorescence (B) in the absence (black) and presence of Cu(II), at molar ratios of 1.0 (blue), 2.0 (red), 3.0 (orange), and 4.0 (green). TEM images of 6aJL2-R24G amyloid fibrils grown in the absence (C) and presence of Cu(II) molar ratios of 1.0 (D), 2.0 (E), 3.0 (F), and 4.0 (G).
The one-way ANOVA analysis shows ±0.23, 30.72 × 7.6 showed that the free protein, and the protein in the presence of Cu(II) decreases the stability of the protein. The refolding curves means. Thus the interaction of Cu(II) with 6aJL2-R24G unfolded with 1.0, 2.0, 3.0, and 4.0 Cu(II) molar ratios, were compared between the unfolding without Cu(II), and the Cu(II) from 1.0 to 4.0, the kinetics without Cu(II) and the kinetics with 1.0, 2.0, 3.0, and 4.0 Cu(II) molar ratios, respectively. This analysis indicates significance differences (loss of intensities larger than 0.4) were noted for residues 3–6, 9, 23, 24, 27–29, 31–33, 35, 48, 71–74, 90, 92, 93, 95, 97, 101, 103, 105, and 107. The intensity changes were mapped onto the protein structure (Figure 2B), and it can be observed that the most affected residues are around His99 (Figure 2A). Placing a Cu(II) atom bound to His99 and calculating the distance between the protein residues and the metal ion shows good correspondence between proximity to Cu(II) and the loss of intensity (Figure 2C). Unfortunately, at higher Cu–protein molar ratios, most of the protein signals are lost (Figure S4), probably because of Cu(II) binding to other sites. All these data together suggest that protein 6aJL2-R24G has at least two Cu(II) binding sites, one of them around His99.

The 6aJL2-R24G secondary structure changes after the interaction with Cu(II) were followed by circular dichroism

Figure 2. 1H–15N HSQC spectra of 6aJL2-R24G in the absence (black) and the presence (blue) of Cu(II) at a molar ratio of 0.5 (A). Cu(II)-induced perturbations mapped onto a cartoon of the 6aJL2-R24G 3D structure (PDB entry 2MKW), color-coded according to the chemical shift perturbations via a linear gradient from blue (no change) to red (maximal perturbation) (B). Intensity ratio between 6aJL2-R24G and the Cu(II)–6aJL2-R24G complex (C). The red line indicates the distance between Cu(II), placed near His99, and the protein residues.
(CD) in the UV region. As shown in Figure 3, the CD spectrum of the protein presents a positive signal around 205 nm, a negative band at 220 nm, both typical of the β sheet structure, and a negative band at 230 nm which has been associated with the signal of the aromatic residues of the protein, in particular, tyrosine and tryptophan residues. The CD spectra show some differences after Cu(II) addition, but according to the deconvolution results from BeStSel, the content of β sheet is almost unchanged during titration (Figure 3, Table S1). This means that the secondary structure content of the protein does not change significantly upon Cu(II) binding. Interestingly, at Cu(II) molar ratios above 1.0, the signal at 230 nm shows some changes, suggesting that the side chains of the aromatic residues adopt a different conformation, are exposed to a different environment and/or change their dynamics. CD results suggest that Cu(II) binding does not induce major secondary structure changes but may be inducing conformational changes in the 3D structure of the protein.

Additionally, in order to measure the protein affinity for Cu(II), isothermal titration calorimetry (ITC) experiments were performed. The ITC results were best fit to a two sequential binding site model (Figure 4). The estimated Kds were 0.1 μM for the first binding site and 0.3 μM for the second one. These results show that the protein 6aJL2-R24G binds Cu(II) with moderate affinity. During the titration, there is heat absorption above a Cu(II) molar ratio of 2.5 (Figure S5), however, it is much smaller than the heat absorption for Cu(II) molar ratios below 2.5. Thus, this heat may be due to the first steps in protein aggregation or to Cu(II) binding at other sites with lower affinities. Because the largest effects in the thermal stability and protein aggregation were observed at Cu(II) molar ratios of 1.0 and 2.0, only the heats up to 2.5 were used for curve fitting.

Molecular Dynamics Simulations of the 6aJL2-R24G–Cu(II) Encounter Complex. Following the Cu(II) interaction sites suggested by NMR, 6aJL2-R24G alone and in complex with Cu(II) was simulated to study the molecular effects of the metal ion over the protein dynamics. Three Cu(II)–6aJL2-R24G models were made, Cu(II) bound to His99, Cu(II) bound to His8, and Cu(II) bound to His99 and His8 at the same time. Copper coordination sites comprised ND1H99, OD2D95, and OD1N1 atoms, in the His99 binding site and ND1H8, O H8, GS9 atoms, in the His8 binding site (Figure 5). Distances of these atoms to the Cu(II) ion remain almost unchanged during the simulations (Table 1) and agreed with the results described for the copper model. Moreover, the root-mean-square deviation (RMSD) values of atoms involved in the coordination sites were always below 0.64 Å during the simulations, which means that the mobility of coordinating side chains was decreased upon Cu(II) binding. However, different side chain conformations of residues Asn1, Asp95, and His99 in the Cu(II)–His99 model with respect to the Cu(II)–His99/His8 model were observed (Figure 5A,C). These results suggest that the side chain of residues in the binding site can anchor Cu(II) in different conformations, but once the complex is formed, it is stable.

During the simulation of the protein–Cu(II) complexes, global properties like the total number of hydrogen bonds and radius of gyration are shifted to lower and higher values, respectively (Figure S6). This means that the protein is being destabilized by the presence of Cu(II), which is in agreement with the ITC results.
with that observed experimentally. In order to see if the destabilizing effect after Cu(II) binding was local or distributed over the protein structure, the alpha carbon root-mean-square fluctuations (Cα-RMSF) were calculated for each residue, subtracted from the copper-free fluctuations (Figure S7), and then mapped onto the protein structure (Figure 6). This value can be related to the flexibility of the molecule. The alpha carbon fluctuations of the protein without copper bound showed that the largest backbone fluctuations were at the CDR3 (Figure 6A), while Cu(II) binding to the protein increases the backbone fluctuations mainly in the regions comprising the CDR1 and the C′ loop (Figure 6B). Interestingly, differences depending on the interaction site arose. The Cu(II), when bound to His99, has larger effects on both the CDR1 and the C′ loop regions (Figure 6B,D) than when bound to His8 (Figure 6C), except in the CDR3 where Asp95, a key residue in the coordination site of His99, is located (Figure 6).

Regarding the CD signal at 230 nm and its loss of intensity upon Cu(II) titration, 6aJL2-R24G has one buried and immobile tryptophan and six tyrosines (five of them form an aromatic cluster on one face of the sandwich). Many of these residues are within 1 nm of each other and of the two histidines that bind copper. Given that rotational strength can be affected by changes in the environment and conformation of the aromatic residues, as a first approximation to qualitatively rationalize the changes in the CD spectra, the populated rotamers were calculated and the number of protein–aromatic and aromatic–aromatic contacts within 1 nm for each of the aromatic residues in the four simulations (data available upon request). Differences in the most populated rotamers were found for Tyr33, Tyr37, Tyr90, and Tyr94, which resulted in changes in the distances among themselves and Tyr50 (Figure 7) and also in their interactions with Trp36 and the rest of the protein.

Structural Consequences of 6aJL2-R24G Binding to Cu(II). Divalent metal ions, particularly Cu(II), are considered to be a risk factor for many degenerative diseases. The characterization of Cu(II) binding to the 6aJL2-R24G LC, a model protein for the AL disease, and its effect is reported here for the first time. The binding of Cu(II) to 6aJL2-R24G accelerates amyloid fibril formation kinetics and destabilizes the protein conformation, mainly at Cu(II) molar ratios of 1.0 and 2.0. The fact that Cu(II) accelerates 6aJL2-R24G amyloid fibril formation leads to a new conceptual scheme according to

Table 1. Mean Distance of the Cu(II) to the Coordinating Atoms in the Simulated Protein–Metal Complexes

| interaction       | Cu(II)–His99 (Å) | Cu(II)–His8 (Å) | Cu(II)–His99/His8 (Å) |
|-------------------|------------------|-----------------|------------------------|
| Cu(II)–ND1^{His99} | 2.03 ± 0.03      | 2.03 ± 0.03     | 2.03 ± 0.03             |
| Cu(II)–OD2^{D95}  | 1.83 ± 0.02      | 1.83 ± 0.02     | 1.83 ± 0.02             |
| Cu(II)–OD1^{N1}   | 1.93 ± 0.03      | 1.93 ± 0.03     | 1.93 ± 0.03             |
| Cu(II)–ND1^{His8} | 2.02 ± 0.03      | 2.03 ± 0.03     | 2.03 ± 0.03             |
| Cu(II)–O^{G93}    | 1.92 ± 0.03      | 1.92 ± 0.03     | 1.92 ± 0.03             |
| Cu(II)–OC^{C99}   | 1.93 ± 0.02      | 1.93 ± 0.02     | 1.93 ± 0.02             |

Figure 5. Simulated Cu(II)–6aJL2-R24G complexes. Binding sites of Cu(II)–His99 (A), Cu(II)–His8 (B), and Cu(II)–His99/Cu(II)–His8 (C,D).
which the metal–protein interaction leads to structural effects, as a result of the coordination moieties of the protein. The ITC results suggest a stoichiometry of 2:1 with affinities around 0.1 and 0.3 μM for each site. These affinity values are in the submicromolar range, as well as the affinities of Cu(II) for proteins involved in degenerative diseases such as α-synuclein,30 β-amyloid,52,53 prion protein,54 and Tau.55 The thermogram of copper titration is endothermic, and therefore, the binding process is entropically controlled. The change in the binding entropy could be related to various phenomena: (I) metal ion desolvation with disruption of its hydration shell upon binding, (II) rearrangement of water molecules surrounding the protein, and (III) larger fluctuations or increased flexibility in other regions of the protein. When the Cu(II) molar ratio was above 2.5, the heat absorbed increased above the saturation phase. This behavior could be related to protein unfolding or oligomerization induced by Cu(II) binding.

According to the NMR results, one of the Cu(II) binding sites could involved His99 and Asn1 side chains, as well as Asp95 backbone. This binding site is located between CDR1 and CDR3 (Figure 3B), and it is similar to the Cu(II) binding site in the β2m, a protein with a high structural similarity with 6aJL2-R24G. The β2m binding to Cu(II) has been widely studied, and mass spectroscopy suggests that the protein binds Cu(II) at His31 in the native state, whereas the non-native states of β2m bind Cu(II) at His13, His51, and His84.56,57 The binding site at the native state of β2m involves the N-terminal amine, the amide of Gln2, His31, and Asp59.56 The Cu(II) binding to 6aJL2-R24G at His99 may be accelerating the aggregation of the protein by inducing a conformational disruption along the CDR3 and CDR1 segments. His99 is localized in the CDR3, which in the 6aJL2 protein interacts with the CDR1 and is stabilized by the Arg24 residue. The substitution of Arg24 for Gly decreases the protein stability, which makes it more amyloidogenic.19 The binding of Cu(II) to His99 induces larger fluctuations of the CDR1 and loop C″ (Figure 6), as shown by the increase in the RMSF values in the molecular dynamics simulations. This may be due to an even bigger loss of interactions between CDR3 and CDR1, making the protein less stable and more prone to form amyloid fibers. These results are in agreement with previous reports, pointing out the importance of loop C″ in the antibody stabilities, and specifically in 6aJL2-R24G.56,59 Moreover, although the CD spectra indicate that the secondary structure of the protein does not change during the Cu(II) titration, the 230 nm signal does change. This signal has been proposed to arise from the side chain of aromatic residues, in particular, tyrosine and

Figure 6. RMSF values mapped onto the 6aJL2-R24G structures. Values were calculated for 6aJL2-R24G alone (A), Cu(II)–His99 (B), Cu(II)–His8 (C), and Cu(II)–His99/Cu(II)–His8 (D). The loops were solid colored as they showed the largest changes upon Cu(II) binding. Color scale, blue-gray-red, shows the RMSF values ranging from low (blue) to high (red).
tryptophan. There is a cluster of tyrosines close to His99 and His8, thus Cu(II) binding may be inducing a reorganization of these tyrosines (Figure 7). Also, Tyr33 is located at the end of the CDR1 and is connected with the CDR2 loop, which contains Tyr50. Thus, according to the molecular dynamics simulations and the CD spectra, Cu(II) binding to 6αJL2-R24G could be disturbing the side chain of several Tyr residues, leading to changes in the 230 nm signal. On the other hand, NMR spectra also showed residues around His8 to be affected at substoichiometric concentrations of Cu(II). This may be indicating that Cu(II) binds to His99 and His8 at the same time because the affinities estimated by ITC for two binding sites were very similar.

Histidine is the most common residue involved in Cu(II) binding. Thus, in this work, it is proposed that His99 and His8 are the anchor residues for copper binding. According to the ALbase database (http://albase.bumc.bu.edu/aldb), His8 is highly conserved (94%) among the 6α LCs of patients, while His99 is present in 27% of them because it lies at the junction between the V and J gene segments (Figure S8). Moreover, it is clear by the $T_m$ decrease that the addition of the second molar ratio of Cu(II) makes the protein less stable. The binding of Cu(II) to both histidines, His8 and His99, showed the largest swelling of the protein and the greatest loss of hydrogen bonds in the molecular dynamics simulations (Figure S7), indicating a greater destabilization of the protein with respect to Cu(II) binding to only one of the histidines. The pattern of flexibility changes upon copper binding is not additive either qualitatively or quantitatively and shows that binding at each site has repercussions across the whole domain (Figure S6). An interesting finding is that binding of both coppers increases the fluctuations in loop EF and the loops connecting strands A−B and C−C′, in addition to the already high fluctuation in loop C″. This balance in the dynamics of loop C″ and loop EF is very interesting and has been shown to happen also to the germline protein 6αJL2 under acidic pHs.

A recent Cryo-EM study shows the structure of AL55 amyloid fibrils, acquired during autopsy from a patient affected by systemic AL λ AL. AL55 is a LC protein that belongs to the 6α subgroup and includes the R24G mutation. The reported

Figure 7. Tyrosine cluster conformation in the absence (A) or presence of copper bound to His99 (B), His8 (C), or both (D), shown for representative structures of each condition. The protein backbone is shown in gray ribbons and the tyrosine and histidine residues in ball and stick representation with CPK colors. Copper ions are shown in brown.
fibril structure shows that both His8 and His99 are located in the outer part of the \( \beta \)-sheet structure.\(^{52} \) Thus, assuming that \( \lambda \)-derived fibers will acquire similar structures, both histidines could stabilize the fiber by binding Cu(II).

A correlation between fibril formation and decreased thermodynamic stability has been well documented,\(^{53,64} \) and ligand binding is one of the many mechanisms by which a LC can respond to its physiological environment and aggregate.\(^{65} \) Thus, the conformational changes induced by Cu(II) binding become relevant in the LC aggregation pathway. The evidence presented in this work gives new insights into the bioinorganic chemistry of AL disease because as mentioned before, Cu(II) has been implicated in the pathogenesis of degenerative diseases, with affinities in the same range as those found for 6aJL2-R24G in this study. Thus, Cu(II) binding to 6aJL2-R24G can promote aggregation at physiological conditions, establishing a link with other amyloid disorders such as Alzheimer, Parkinson, and prion diseases.

### ACCESSION CODES

UniProtKB—Q5NV88.

### EXPERIMENTAL SECTION

**Reagents.** All chemicals were of reagent grade and were used without further purification. MQ-Grade water with a resistivity of 18 M\( \Omega \) cm was used.

**Protein Expression and Purification.** Recombinant VL domain 6aJL2-R24G was expressed in BL21 Escherichia coli cells (DE3), transformed with plasmid pET27b+-6aJL2-R24G, and incubated in 2XYT medium with 60 \( \mu \)g/mL kanamycin, agitating at 200 rpm and 37 °C, until an OD\(_{600} \) of 0.9 was reached. Then, over-expression was induced with 1 mM isopropyl\( \alpha \)-thiogalactoside (IPTG) and incubation overnight at 20 °C. The cells were harvested by centrifugation at 4000 rpm for 30 min and 4 °C. The pellet was lysed by osmotic shock, placing the cells in a cold solution of 20% (w/v) sucrose, 100 mM Tris, and 1 mM EDTA (pH 8.0) for 20 min. The cells were recovered by centrifugation at 4000 rpm for 20 min and 4 °C and resuspended in 30 mL of distilled cold water, incubated on ice for 20 min, and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.22 \( \mu \)m pore size and loaded onto a Superdex S-200 GE size-exclusion column, using an AKTApurifier (GE Healthcare). Purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein concentration was determined by UV absorption using an extinction coefficient of 14,565 M\(^{-1}\) cm\(^{-1}\) determined using ProTparam.\(^{66} \) The protein was obtained in a 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES) buffer and 75 mM NaCl, pH 7.4. All the spectroscopic and calorimetric studies were made under these buffer and pH conditions.

**\( ^{15} \text{N} \)-Labeled Samples.** Recombinant protein was expressed in 1 L of 2XYT. When cells reached an OD\(_{600} \) of 0.9, they were transferred to 250 mL of minimal M9 medium containing 1 g/L \( ^{15} \text{NH}_{4} \text{Cl} \) (CIL) as the sole nitrogen source. After incubating 1 h at 37 °C, the cells were induced by adding 1 mM IPTG and then incubated at 20 °C and 100 rpm for 12 h. The lysis and purification was done as described above.

**In Vitro ThT Fluorescence Assay.** The kinetics of 6aJL2-R24G amyloid fiber formation were monitored by the ThT fluorescence assay, following the increase in fluorescence emission at 485 nm, which occurs when the amyloid-specific dye ThT binds to an amyloid fiber. The assay was performed using a Cary Eclipse fluorimeter, at an excitation wavelength of 440 nm. The protein was diluted in 10M MES and 75mM NaCl buffer, pH 7.4, and 25 \( \mu \)M ThT, to a final concentration of 100 \( \mu \)M, and the appropriate amount of CuCl\(_2\) to have a molar ratio of 0.0, 1.0, 2.0, 3.0, and 4.0 \( \mu \)M of Cu(II). Fluorescence emission at 485 nm was monitored overnight at 37 °C and constant agitation. Three replicates were made. The normalized intensities were plotted as a function of time. The lag times were obtained by adjusting a linear regression in the nucleation and elongation phases of the fibril formation kinetics. The intersection between the two lines corresponds to the lag time. The lag time from the three replicates was averaged, and the standard deviation was calculated. Also, a one-way ANOVA against the no-copper condition was made, and \( p < 0.05 \) were considered significantly different.

**Transmission Electron Microscopy.** After the ThT assay, the solutions of fibrils were decanted overnight at 4 °C. Then, the pellet was washed several times with deionized water to remove the ThT as much as possible, and it was decanted overnight at 4 °C. The pellet was gently resuspended in 100 \( \mu \)L of water, and 10 \( \mu \)L of this suspension was loaded onto Formvar-coated copper grids for 1 min, washed twice with 5 \( \mu \)L of MQ water, and then negatively stained with 2.0% uranyl acetate for 1 min. Samples were imaged with a Zeiss transmission electron microscope.

**Thermal Stability.** Because the fluorescence emission of the only tryptophan present in 6aJL2-R24G is quenched by the close presence of the disulfide bridge when the protein is folded, the unfolding of the protein can be followed by the fluorescence intensity increase at 350 nm.\(^{67} \) The unfolding of the protein was induced by increasing the temperature from 25 to 60 °C, at a rate of 1 °C/min, using a Cary Eclipse fluorimeter, at an excitation wavelength of 290 nm. The protein was diluted to a final concentration of 10 \( \mu \)M in the absence and presence of Cu(II) with molar ratios from 1 to 4. Thermal unfolding curves were fit to a single monophasic transition. Three replicates were made and the \( T_m \) of each replicate was averaged, and the standard deviation was calculated. Also, a one-way ANOVA against the no-copper condition was made, and \( p < 0.05 \) were considered significantly different.

**NMR Spectroscopy.** All NMR spectra were recorded at 25 °C for \(^{15} \text{N} \)-labeled samples on a Bruker 500 MHz spectrometer equipped with a cryogenically cooled triple-resonance pulsed field gradient probe at the Laboratorio Nacional de Estructuras de Macromoléculas (LANEM). Backbone resonance assignments for 6aJL2-R24G were obtained from BioMagResBank entry 19798.\(^{68} \) All NMR spectra were recorded at 25 °C in 10 mM MES and 75 mM NaCl, pH 7.4, and 15% D\(_2\)O, in the absence and presence of Cu(II), at molar ratios of 0.2, 0.5, and 0.8. Spectra were processed with NMRPipe\(^{69} \) and analyzed using CARA software.

**CD Spectroscopy.** Room-temperature CD spectra in the UV–visible region were recorded using a Jasco J-815 CD spectropolarimeter. Spectra were recorded in quartz cells with 0.1 cm path lengths. The protein was diluted to a final concentration of 10 \( \mu \)M and it was titrated from 0.0 to 3.0 molar ratio of Cu(II). The secondary structure analysis was made with the BeStSel software.\(^{49,50} \)

**Isothermal Titration Calorimetry.** All the samples were exchanged into identical buffer to ensure minimal buffer mismatch. The ITC experiment was performed on a Malvern...
ITC200 instrument at 25 °C, with the cell containing 100 μM 6aJL2-R24G and the syringe containing 2.1 mM CuCl2. Each experiment consisted of 1.5 μL injections, with an injection spacing of 180 s. Three replicates were made. To account for the heat of dilution, the background titration, consisting of the identical titrant solution into only the buffer solution, was subtracted. The thermogram was integrated with the NPTIC software package. Data sets were fitted using a nonlinear least-squares algorithm for a two noninteracting binding site model by SEDPHAT. Graphics were done using the GUSSI program.

**Molecular Dynamics Simulations.** The initial coordinates for 6aJL2-R24G were taken from its atomic structure (PDB entry 2MKW). Encounter complexes of 6aJL2-R24G with Cu(II) were constructed manually by placing the metal ion close to the His99 and/or His8 side chain. The protein–metal complex was solvated using TIP4P water in a cubic box with 12 Å to the edge and 0.1 M NaCl. Molecular dynamics simulations were performed using the OPLS-AAL71,72 force field with the previously described special parameters for Cu(II)51 within GROMACS v5.1.3.73 Systems were equilibrated, prior production of molecular dynamics, using 50,000 steepest descent steps, followed by 100 ps of NVT and NPT dynamics. Electrostatic interactions were treated using the particle mesh Ewald method74 with 12 Å cutoff for short-range electrostatic interactions. The van der Waals interactions were switched to zero between 10 and 12 Å. The temperature was kept at 298 K with a modified Berendsen thermostat, and the pressure was kept at 1 bar via a Parrinello–Berendsen barostat. Bonds were constrained using the LINCS thermostat, and the pressure was kept at 1 bar via a Parrinello–Berendsen barostat. The temperature was kept at 298 K with a modified Berendsen thermostat, and the pressure was kept at 1 bar via a Parrinello–Berendsen barostat. The simulations scheme included 500 ns of 6aJL2-R24G alone, with Cu(II) bound to His99, with Cu(II) bound to His8 and with Cu(II) bound to His99 and His8 simultaneously. Trajectories were analyzed using GROMACS v5.1.373 tools. Molecular graphics were done with VMD.77

**ASSOCIATED CONTENT**

1. Supporting Information
   The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03220.

   6aJL2-R24G fibril kinetic replicates; thermal refolding of 6aJL2-R24G in the absence of Cu(II); 6aJL2 fibrillation kinetics and thermal unfolding; 6aJL2-R24G titration with Cu(II) followed by NMR; ITC of 6aJL2-R24G titration with Cu(II); global property distribution among the simulated systems; differences in backbone dynamics per residue; 6aJL2-R24G alignment; and BeSTSel deconvolution results (PDF)

2. **AUTHOR INFORMATION**

   **Corresponding Author**
   Lina Rivillas-Acevedo — Centro de Investigación en Dinámica Celular-IICBA, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos 62209, Mexico; orcid.org/0000-0002-1633-2064; Phone: (+52)7773297000, ext. 3615; Email: lrvilllas@uaem.mx

   **Authors**
   Angel E. Pelaye-Aguilar — Centro de Investigación en Dinámica Celular-IICBA, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos 62209, Mexico

   **Gilberto Valdés-García** — Department of Molecular Medicine and Bioprocesses, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, Mexico
   **Leidy's French-Pacheco** — Centro de Investigaciones Químicas-IICBA, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos 62209, Mexico
   **Nina Pastor** — Centro de Investigación en Dinámica Celular-IICBA, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos 62209, Mexico;
   **Carlos Amero** — Centro de Investigaciones Químicas-IICBA, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos 62209, Mexico;

   Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b03220

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   **ABBREVIATIONS**
   NMR, nuclear magnetic resonance; CD, circular dichroism; ITC, isothermal titration calorimetry; ThT, thioflavin T; TEM, transmission electron microscopy

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