Characterization of the Binding of Cu(II) and Zn(II) to Transthyretin: Effects on Amyloid Formation†

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ABSTRACT: Although metal ions can promote amyloid formation from many proteins, their effects on the formation of amyloid from transthyretin have not been previously studied. We therefore screened the effects of Cu(II), Zn(II), Al(III), and Fe(III) on amyloid formation from wild-type (WT) transthyretin as well as its V30M, L55P, and T119M mutants. Cu(II) and Zn(II) promoted amyloid formation from the L55P mutant of transthyretin at pH 6.5 but had little effect on amyloid formation from the other forms of the protein. Zn(II) promoted L55P amyloid formation at pH 7.4 but Cu(II) inhibited it. Cu(II) gave dose-dependent quenching of the tryptophan fluorescence of transthyretin and the fluorescence of 1-anilino-8-naphthalene sulfonate bound to it. Zn(II) gave dose-dependent quenching of the tryptophan but not the 1-anilino-8-naphthalene sulfonate fluorescence. Apparent dissociation constants for Cu(II) and Zn(II) binding at pH 7.4 of ∼10 nM and ∼1 μM (∼0.4 μM and ∼5 μM at pH 6.5), respectively, were obtained from the quenching data. Zn(II) enhanced urea-mediated the dissociation of the L55P but not the WT transthyretin tetramer. Cu(II), depending on its concentration, either had no effect or stabilized the WT tetramer but could enhance urea-mediated dissociation of L55P.

Amyloid fibrils form through the self-association of soluble protein molecules. About 20 proteins are known to form disease-associated amyloid; notable examples include Aβ, amylin, α-synuclein, prion protein, and transthyretin (reviewed in (*1*)). Many other proteins can form amyloid under appropriate *in vitro* conditions, and this has led to the proposal that amyloid formation is a generic property of all proteins (*2*). Irrespective of the parent protein, amyloid fibrils share many features. They all bind dyes such as Congo Red and thioflavin T, giving changes in their respective spectroscopic properties. Amyloid fibrils are long, unbranched, and about 10 nm in diameter. They have a characteristic cross-β structure in which individual polypeptide chains form β-strands that are oriented perpendicular to the long axis of the fibril (*3*, *4*).

Transthyretin is an amyloid-forming protein, which has been extensively studied, both from the perspective of amyloid-associated disease and as a model with which to explore the mechanism(s) of amyloid formation. It is a plasma protein, composed of four identical 127-residue subunits, which plays a significant role in the transport of thyroxine. Thyroxine binds in the central cavity of the transthyretin tetramer (*5*, *6*). Each transthyretin monomer is composed of two β-sheets (strands DAGH and CBEF). The formation of amyloid from transthyretin has been linked to three diseases. Senile systemic amyloidosis, which affects about 25% of the over-80 population, is characterized by the formation of amyloid from wild-type (WT[1]) transthyretin (*7*). More than 80 point mutations in transthyretin are associated with the autosomal dominant amyloid diseases, familial amyloid polyneuropathy, and familial amyloid cardiomyopathy, depending on the site of the mutation (*8*). The most common of these is the V30M mutant, in the B strand, and the most aggressive is the L55P mutant, in the D strand (*9*).

The formation of amyloid from transthyretin is initiated by tetramer dissociation; the rates of the dissociation of mutants of transthyretin *in vitro* correlate well with the corresponding disease severity (*10*). Consistent with this, mutations such as T119M, which stabilize the transthyretin tetramer *in vitro* (*11*, *12*), also protect against disease in heterozygotes bearing a disease-linked mutation (*13*). The monomer species generated by dissociation of the transthyretin tetramer must convert into an amyloidogenic intermediate before forming amyloid fibers. The details of this process are incompletely understood, although it has been shown that partial denaturation of the monomer is required (*14*, *15*). In studies of the purified proteins, this partial denaturation is often achieved by allowing amyloid formation to occur at mildly acidic pH (*12*, *16*, *17*).

Metal ions have been shown to interact with a number of amyloid-forming proteins. An extensively studied case is that of the Aβ peptide associated with Alzheimer's disease. Cu(II), Zn(II), and Fe(III) all promote the aggregation of Aβ (*18*), and redox reactions elicited by the binding of Cu(II)

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[1] Abbreviations: WT, wild-type; ThT, thioflavin T; ANS, 1-anilino-8-naphthalene sulfonate; PMSF, phenylmethylsulfonylfluoride; IPTG, isopropylthiolgalactoside; LB, Luria Broth; $K_{d(App)}$, apparent dissociation constant.

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have been proposed to play a role in the pathological effects of Aβ through the production of neurotoxic reactive oxygen species (*19*−*21*). Similarly, Cu(II), Fe(III), Co(III), and Mn(II) promote the formation of amyloid from α-synuclein, which is associated with Parkinson's disease (*22*, *23*), Cu(II) promotes the aggregation of pathogenic immunoglobulin light chains (*24*), Cu(II) and Zn(II) modulate the aggregation and neurotoxicity of prion protein (*25*−*27*), and Cu(II) has been implicated in the formation of amyloid from β2-microglobulin (*28*−*32*). The mechanism(s) by which metal ions can promote the formation of amyloid remain to be fully elucidated. However, structural (*31*) and molecular simulation studies (*32*) of the effects of Cu(II) on β2-microglobulin amyloid formation suggest that Cu(II) catalyzes the formation of a conformationally altered form of the protein, which can then form oligomers that are stabilized by Cu(II).

The effects of metal ions on transthyretin amyloid formation have not been previously studied, though the finding that catalase, which degrades hydrogen peroxide, protects cells against the toxic effects of V30M amyloid (*33*) raises the possibility that metal ion-initiated redox reactions may be important in the transthyretin amyloid diseases and, by inference, that metal ions may modulate transthyretin amyloidogenesis. Below, we report the results of an investigation of the metal ion-binding properties of transthyretin and the effects of metal ions on the formation of amyloid from the protein. We show that Zn(II) and Cu(II) bind to the same binding site(s) on transthyretin with respective apparent dissociation constants in the low micromolar and low nanomolar ranges. These metal ions (depending on the transthyretin mutant and reaction pH) can promote the formation of amyloid; this effect may be a consequence of metal ion-induced destabilization of the transthyretin tetramer.

EXPERIMENTAL PROCEDURES

Expression and Purification of Transthyretin. Plasmid pMMHa, containing cDNA for the L55P mutant of transthyretin, was a gift from Dr. J. W. Kelly (Scripps Institute, La Jolla, CA). The transthyretin insert in this plasmid was back-mutated to WT transthyretin and then to the V30M and T119M mutants using QuikChange site-directed mutagenesis kits (Stratagene, La Jolla, CA), following the manufacturer's instructions. The double mutant, L55PH56G, was constructed in the same way. The integrity of all of these constructs was confirmed by DNA sequencing (Sydney University Prince Alfred Macromolecular Analysis Centre). Plasmids containing these constructs were transfected into competent *E. coli* (BL21 RIG) cells, which were cultured at 37 °C in LB containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The cultures were induced by the addition of IPTG (1.75 mM) and grown for a further 3 h at 37 °C. They were then harvested by centrifugation (8000 rpm, 10 min).

The cell pellets obtained as above were resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, and 1 mM EDTA at pH 8.0) in the presence of lysozyme (1 mg/mL) and PMSF (1 mM). The resuspended cells were lysed by three cycles of freeze−thaw and incubated at 4 °C for 30 min in the presence of DNase I (10 μg/mL), $MgCl_2$ (10 mM), and PMSF (1 mM). Following the removal of cell debris by centrifugation (17,000 rpm, 30 min), the recombinant protein was purified by ammonium sulfate fractionation and anion exchange chromatography as described in ref *17*, except that DEAE-Sephacel was used as the ion exchange matrix. The purity of transthyretin preparations was confirmed by SDS−PAGE, exploiting the fact that unboiled samples migrate as dimer (∼28 kDa), while boiled samples migrate as monomer (∼14 kDa) (*34*). Purified transthyretin was stored at 4 °C as a 90% saturated ammonium sulfate pellet. When needed, samples were dissolved in 25 mM sodium phosphate at pH 7.0 and then desalted by passage through PD-10 columns (Amersham Biosciences) equilibrated by the same buffer. Transthyretin solutions made in this way were stored at 4 °C and were always used within a few days of preparation (*17*).

Effects of Metal Ions on Transthyretin Amyloid Formation. Transthyretin (0.2 mg/mL) in 20 mM MES and 150 mM NaCl at pH 6.5 or 20 mM HEPES and 150 mM NaCl at pH 7.4 in the presence of various metal ions (as chloride salts, except Cu(II) sulfate; all from Sigma Chemical Co.) as indicated in the figure legends were incubated for various time periods at 37 °C. Following the incubation period, samples were removed and assayed for amyloid and insoluble aggregates as described below.

Fluorescence and Light-Scattering Assays. Fluorescence and light-scattering assays were carried out using a Carey Eclipse spectrofluorimeter with the cell-holder maintained at 25 °C. Amyloid formation was monitored using the amyloid-specific dye, thioflavin T (ThT; Sigma Chemical Co.). ThT was added to a final concentration of 10 μM, and fluorescence emission spectra were recorded between 450 and 550 nm (5 nm bandpass), following excitation at 442 nm (5 nm bandpass). The data reported below are fluorescence intensities at the emission maximum (482 nm) and have been corrected for background from fluorescent intensity measurements obtained in the absence of ThT. Total protein aggregation was estimated by the light-scattering of the samples before the addition of ThT, monitored as apparent fluorescence intensity at 450 nm (5 nm bandpass).

Tryptophan fluorescence emission spectra of transthyretin samples (1 μM for Cu(II) titrations and 15 μM for Zn(II) titrations), in the above pH 6.5 or 7.4 buffers, were recorded between 300 and 400 nm (5 nm bandpass) with excitation at 292 nm (5 nm bandpass). The data reported are the relative area under the emission spectra obtained from triplicate scans. The fluorescence of 1-anilino-8-naphthalene sulfonate (ANS; Sigma Chemical Co.) bound to transthyretin was measured at the ANS concentrations indicated in the figure legends, with transthyretin at 15 μM in the above buffer. The emission spectra of ANS were recorded between 400 and 500 nm (5 nm bandpass) with excitation at 370 nm (5 nm bandpass). The data reported are the relative area under the emission spectra obtained from triplicate scans.

Urea-Mediated Unfolding of Transthyretin. Transthyretin samples (0.1 mg/mL in 20 mM HEPES, 150 mM NaCl, and 5 M urea at pH 7.4), either in the absence of added metal ions or with Zn(II), or with 0.2 mM glycine and Cu(II), were incubated in the dark at 25 °C, and at various times up to 96 h, tryptophan fluorescence emission spectra were recorded as above. The extent of unfolding of transthyretin was estimated from the 355:335 nm emission intensity ratio (*35*).

Data Analysis. Fluorescence quenching data obtained at low transthyretin concentrations were analyzed by fitting eq

1, in which F is the measured fluorescence intensity, [M] the metal ion concentration, F_0 the starting fluorescence intensity, F_{amp} the overall change in fluorescence intensity, and $K_{d(App)}$ is the apparent dissociation constant (*36*) by nonlinear regression analysis using SigmaPlot version 8.02 (SPSS, Chicago, IL).

$$F = F_0 - F_{amp}[M]/(K_{d(App)} + [M]) \quad (1)$$

Equation 2, (*36*), in which P_0 is the transthyretin concentration, was similarly used to analyze quenching data obtained at high transthyretin concentrations.

$$F = F_0 - F_{amp}(([M] + P_0 + K_{d(App)}) - \sqrt{([M] + P + K_{d(App)})^2 - 4P_0[M]}/2P_0 \quad (2)$$

The kinetics of urea-mediated unfolding were analyzed by fitting eq 3, where R is the emission ratio, R_0 is the initial ratio, A is the amplitude change, and k is the dissociation rate constant (*10*), to the data by nonlinear regression analysis as described above.

$$R = R_0 + A(1 - \exp(-kt)) \quad (3)$$

RESULTS

Screening Metal Ions for the Enhancement of the Formation of Amyloid from Transthyretin. A number of metal ions, including Zn(II), Cu(II), Fe(III), and Al(III), have been shown to elicit the aggregation of Aβ peptide (*18*). Therefore, we screened these metal ions for their effects on the formation of amyloid from WT transthyretin as well from its V30M, L55P, and T119M mutants. This analysis was carried out at the physiologically acidotic pH of 6.5, as previous studies have indicated a role for physiological acidosis in metal ion-induced amyloidosis (*18*). The results of this investigation (Figure 1) showed that consistent with previous findings the propensity of these transthyretin variants to form amyloid was L55P > V30M > WT > T119M in the absence of added metal ions (*10*, *12*, *37*). Fe(III) and Al(III) did not significantly enhance amyloid formation for any of the transthyretin variants tested, but both Zn(II) and Cu(II) gave significant enhancements of amyloid formation from the L55P mutant.

Effects of Metal Ions on the Time Course of Transthyretin Amyloid Formation at pH 6.5 and pH 7.4. We assessed the effects of Cu(II) and Zn(II) on the time course of transthyretin amyloid formation by monitoring light scattering of the samples (a measure of total protein aggregation) as well as their thioflavin T fluorescence to follow amyloid formation. The light-scattering data (Figure 2A and B) showed that the presence of Cu(II) or Zn(II) had little effect on the aggregation of WT transthyretin at pH 6.5. In contrast, Zn(II) and especially Cu(II) promoted the formation of insoluble aggregates of the mutant L55P. As a step toward assessing which residues in transthyretin might contribute to the metal ion binding site(s) responsible for these effects, we tested the effects of metal ions on the double mutant, L55PH56G. Aggregation of this mutant was indistinguishable from L55P in the absence of added metal ions but both Cu(II) and Zn(II) promoted its aggregation less than they did L55P. This suggests that His56 may contribute to the metal ion binding site responsible for these effects.

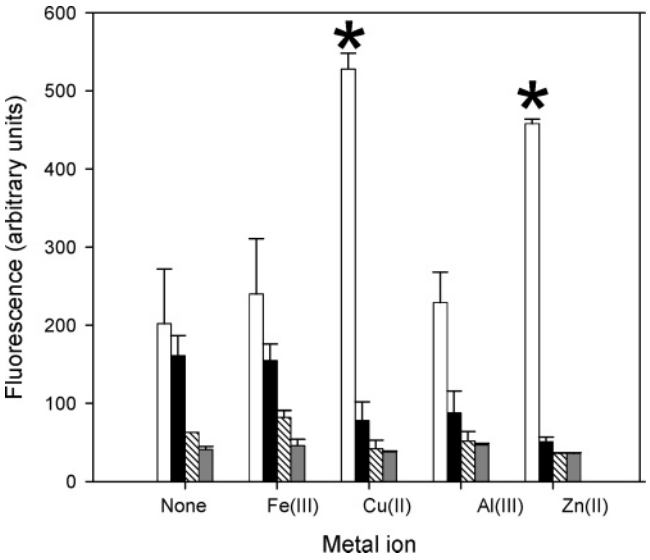

FIGURE 1: Effects of different metal ions on the formation of amyloid from transthyretin. The thioflavin T fluorescence of amyloid formed from the L55P (white bars), V30M (black bars), WT (hatched bars), and T119M forms of transthyretin (gray bars) for 0.2 mg/mL protein samples in 20 mM MES, 150 mM NaCl at pH 6.5 after a 72 h incubation at 37 °C in the presence or absence of 80 μM metal ions was measured as described in Experimental Procedures. The data are the means ± standard deviations of triplicate determinations. Student's *t*-test analyses showed that Cu(II) and Zn(II) significantly enhanced the formation of amyloid from L55P, compared to that with no added metal ions ($p < 0.05$, indicated by *).

A similar pattern of data was observed when the effects of Cu(II) and Zn(II) on amyloid formation at pH 6.5 was measured (Figure 3A and B). Cu(II) and Zn(II) both had no effect on amyloid formation from WT transthyretin but increased the rate of its formation from L55P. The observation that Zn(II) and especially Cu(II) had relatively little effect on the L55PH56G double mutant supports the conclusion drawn from the light-scattering data: His56 may contribute to the metal ion binding site on transthyretin needed for the enhancement of amyloid formation.

In contrast, when similar experiments were carried out at pH 7.4, it was found that although both Zn(II) and Cu(II) promoted the formation of insoluble aggregates (results not shown) only Zn(II) had a similar effect on the formation of amyloid (Figure 4A) with Cu(II) leading to the inhibition of amyloid formation (Figure 4B).

Probing Cu(II) and Zn(II)-Induced Changes in Transthyretin by Fluorescence. The effects of metal ions on amyloid formation presented above prompted us to characterize the Cu(II)- and Zn(II)-binding properties of transthyretin. We assessed whether the binding of Cu(II) and Zn(II) to transthyretin induced changes in its structure by carrying out two series of fluorescence experiments. In the first, we exploited the fact that each transthyretin monomer contains two tryptophan residues, Trp41 and Trp79. The fluorescence of Trp79, which is almost solvent-inaccessible, is largely quenched, whereas Trp41, which is partly solvent-exposed, exhibits appreciable fluorescence (*16*, *37*). Therefore, we examined the effects of titrating Cu(II) and Zn(II) into transthyretin solutions on the tryptophan fluorescence of the protein.

The results of this showed that the addition of both metal ions led to dose-dependent decreases in the tryptophan

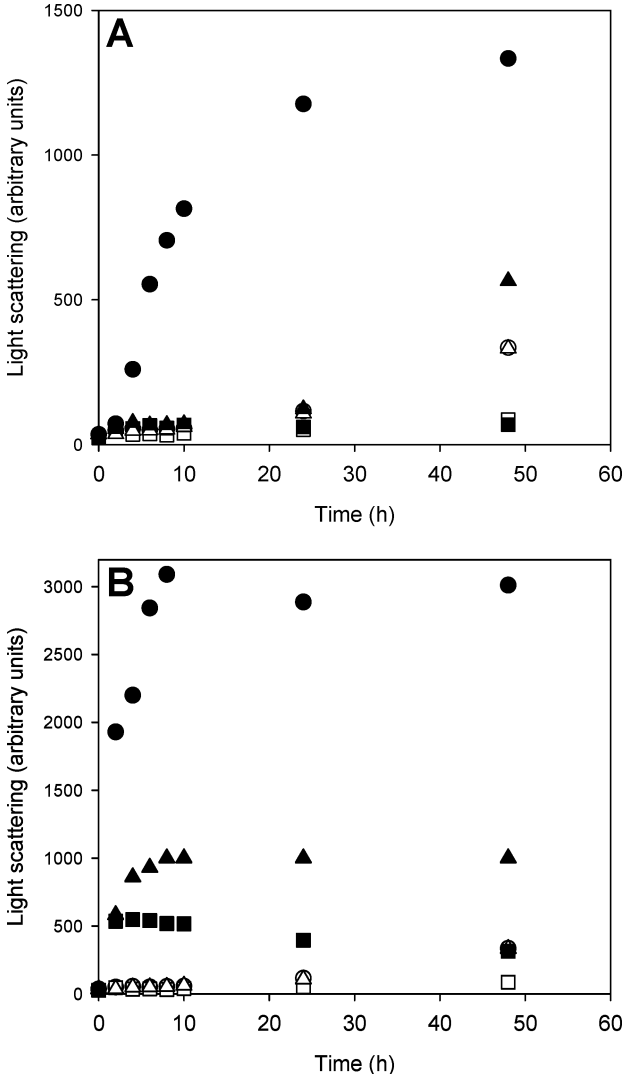

FIGURE 2: Effects of Zn(II) and Cu(II) on the time course of insoluble transthyretin aggregate formation at pH 6.5. The formation of insoluble aggregates from transthyretin (0.2 mg/mL in 20 mM MES and 150 mM NaCl at pH 6.5) at 37 °C was monitored by light scattering in the presence (closed symbols) and absence (open symbols) of 80 μM Zn(II), Panel A, or 80 μM Cu(II), Panel B, as described in Experimental Procedures. The transthyretin samples were L55P (○, ●), WT (□, ■), and L55PH56G (△, ▲). The data shown are representative of three independent experiments.

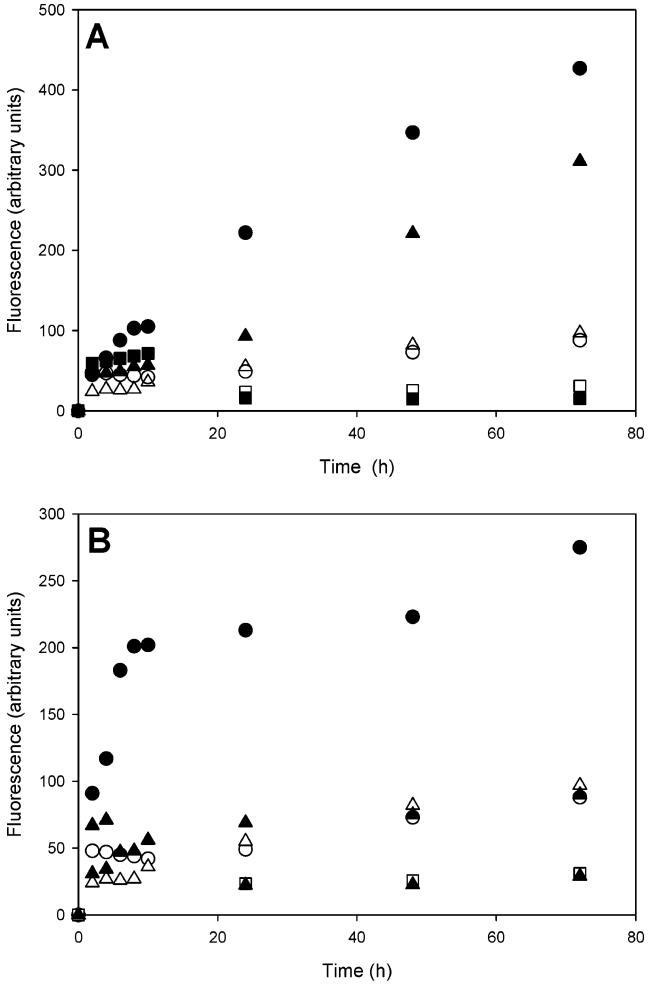

FIGURE 3: Effects of Zn(II) and Cu(II) on the time course of insoluble transthyretin amyloid formation at pH 6.5. The formation of amyloid from transthyretin (0.2 mg/mL in 20 mM MES and 150 mM NaCl at pH 6.5) at 37 °C was monitored by thioflavin T fluorescence in the presence (closed symbols) and absence (open symbols) of 80 μM Zn(II), Panel A, or 80 μM Cu(II), Panel B, as described in Experimental Procedures. The transthyretin samples were L55P (○, ●), WT (□, ■), and L55PH56G (△, ▲). The data shown are representative of three independent experiments.

fluorescence of WT transthyretin, with Cu(II) giving >90% quenching of the initial fluorescence and Zn(II) giving ~30% quenching (Figure 5A and B). Similar extents of quenching were seen for the L55P and V30M mutants, and the extents of quenching were independent of pH over the range 5.5−7.4 (results not shown). These results imply that the binding of both Cu(II) and Zn(II) to transthyretin leads to changes in the local environment of Trp41.

We assessed whether these changes involved an alteration in the solvent exposure of Trp41 by plotting the 355:335 nm fluorescence emission ratio as a function of metal ion concentration. This approach has been used previously to monitor the denaturation of transthyretin; this ratio is proportional to the solvent accessibility of Trp41, being ~0.85 for the native protein, increasing to ~1.3 upon denaturation (*10*, *35*). An analogous strategy was also used by Eakin et al. (*29*) in their investigation of Cu(II) binding sites in non-native states of β2-microglobulin.

We found that the 355:335 nm fluorescence emission ratio of transthyretin was constant at 0.87 ± 0.02, regardless of the presence or absence of Cu(II) or Zn(II), or their concentrations (results not shown). Collectively, these data suggest that the initial binding of both Cu(II) and Zn(II) to transthyretin leads to changes in the microenvironment of Trp41, but these changes do not result in marked changes in the solvent accessibility of this residue.

In the second series of fluorescence experiments, we used the fluorescent probe, 1-anilino-8-naphthalenesulfonate (ANS) to monitor metal ion-induced changes in the surface hydrophobicity of transthyretin. ANS is thought to bind in the central cavity of transthyretin because it is displaced upon the binding of thyroxine (*38*), a property that has been used to quantitate thyroxine binding to transthyretin (*39*, *40*). When Zn(II) was titrated into solutions of transthyretin containing 10 μM ANS, there was no significant change in their ANS fluorescence, which, consistent with the work of

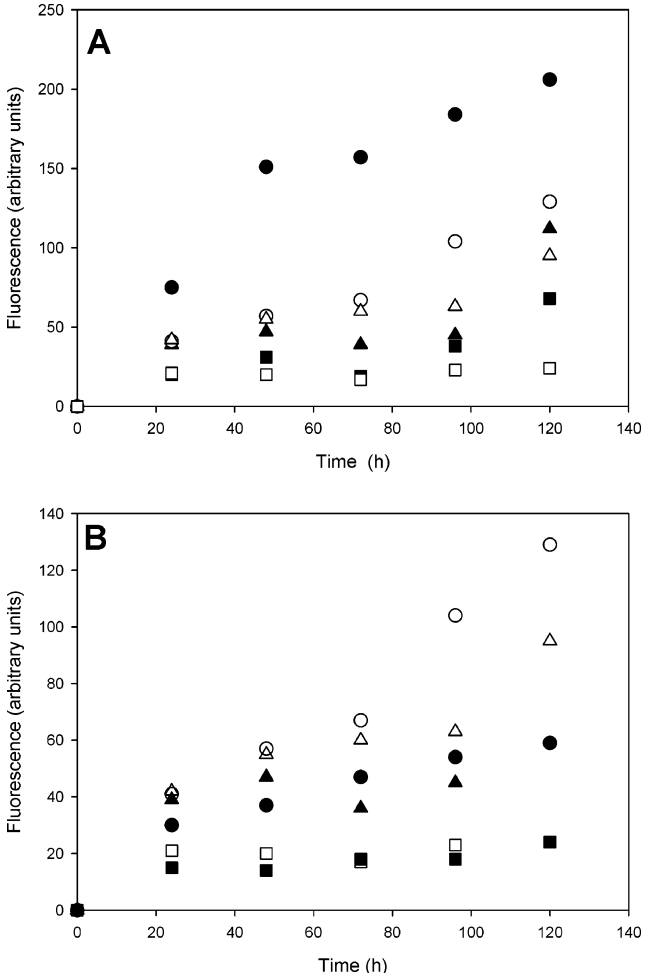

FIGURE 4: Effects of Zn(II) and Cu(II) on the time course of insoluble transthyretin amyloid formation at pH 7.4. The formation of amyloid from transthyretin (0.2 mg/mL in 20 mM HEPES and 150 mM NaCl at pH 7.4) at 37 °C was monitored by thioflavin T fluorescence as described in Experimental Procedures. Panel A: Effects of the presence of Zn(II); amyloid formation from L55P in the presence (●) and absence (○) of 80 μM Zn(II), WT in the presence (■) and absence (□) of 80 μM Zn(II), and L55PH56G in the presence (▲) and absence (△) of 80 μM Zn(II). Panel B: Effects of the presence of Cu(II); amyloid formation from L55P in the presence (●) and absence (○) of 80 μM Cu(II), WT in the presence (■) and absence (□) of 80 μM Cu(II), and L55PH56G in the presence (▲) and absence (△) of 80 μM Cu(II). The data shown are representative of two independent experiments.

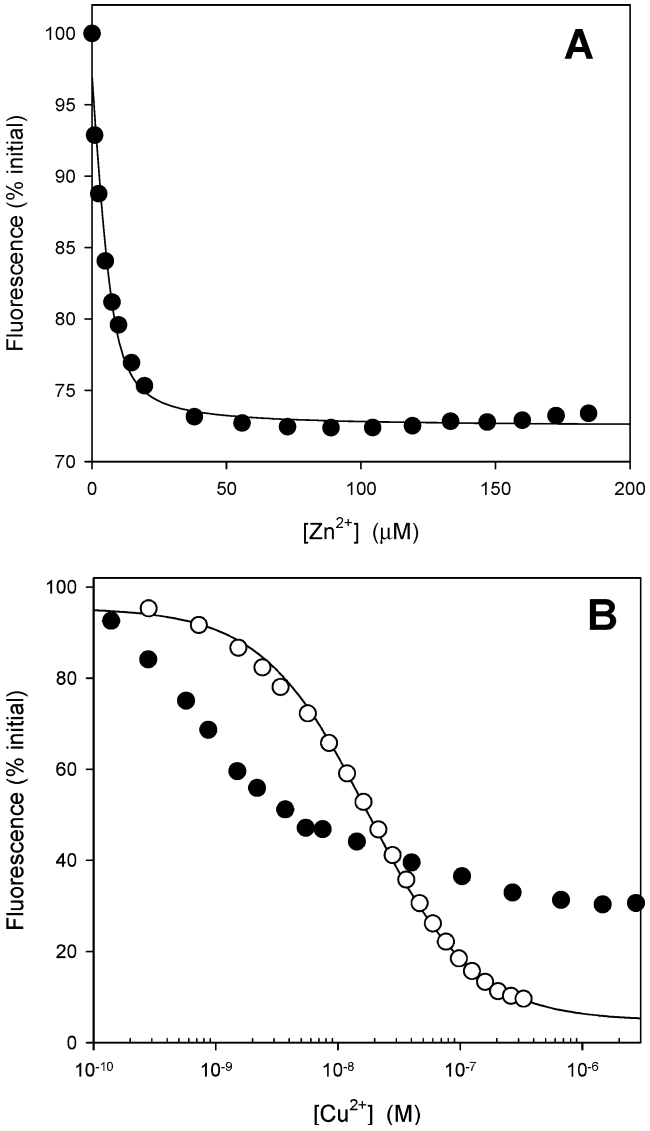

FIGURE 5: Metal ion concentration dependence of quenching of tryptophan and ANS fluorescence. The effects of Zn(II) on the tryptophan fluorescence of WT transthyretin (panel A) as well as the effects of Cu(II) on the tryptophan fluorescence of WT transthyretin (panel B, open symbols) and the effects of Cu(II) on the fluorescence of ANS bound to WT transthyretin (panel B, closed symbols) were measured as described in Experimental Procedures. Equation 1, Zn(II) tryptophan fluorescence intensity data, and eq 2, Cu(II) tryptophan fluorescence intensity data, were fitted to these data by nonlinear regression analysis. The data shown are representative of two independent experiments.

others, had an emission maximum of ~470 nm (*37*). In contrast, the addition of Cu(II) to these solutions gave dose-dependent changes in their ANS fluorescence to ~50% of their initial values, with no significant change in the emission maximum. Zn(II) and Cu(II) had the same effects, regardless of whether WT transthyretin or its L55P and V30M mutants were used. These data collectively suggest that the binding of Cu(II) but not Zn(II) to transthyretin, leads to structural changes in its central thyroxine-binding cavity, with an increase in its hydrophobicity.

Estimation of the Affinity Constants for Cu(II) and Zn(II) Binding to Transthyretin. The data in Figure 5A show the dose dependence of quenching of WT transthyretin tryptophan fluorescence by Zn(II) at pH 7.4. Fitting of eq 2 to these data yielded an estimate of the apparent $K_{d(App)}$ for Zn(II) binding to transthyretin of 1.2 ± 0.3 μM. An analysis of an equivalent experiment carried out at pH 6.5 gave a $K_{d(App)}$ estimate of 4.8 ± 0.6 μM. The estimated $K_{d(App)}$ values obtained from analyses of experiments carried out using the L55P and L55PH56G transthyretin mutants were similar to these (Table 1). This suggests that the L55P mutation does not perturb the Zn(II) binding site(s) on transthyretin and that His56 does not make a major contribution to these site(s). Our finding that Zn(II) binds to transthyretin with a low micromolar apparent dissociation constant may be compared to $K_{d(App)}$ values of 0.8 and 0.4 μM reported for the binding of Zn(II) to Aβ and PrP52-98, respectively (*36*, *41*).

Before analyzing the tryptophan and ANS fluorescence data to obtain estimates of the affinity constants for Cu(II) binding to transthyretin, we noted that Cu(II) can hydrolyze to form hydroxy or oxy polymers, which may either be unable to bind or may bind to proteins in a nonspecific

Table 1: Estimates of the $K_{d(App)}$ Values for Metal Ion Binding to Transthyretin[a]

	WT		L55P		L55PH56G	
pH	Zn(II)	Cu(II)	Zn(II)	Cu(II)	Zn(II)	Cu(II)
6.5	$4.8 \pm 0.6\ \mu$M	$0.41 \pm 0.02\ \mu$M	$5.7 \pm 1.7\ \mu$M	$0.38 \pm 0.02\ \mu$M	$8 \pm 1\ \mu$M	$0.44 \pm 0.04\ \mu$M
7.4	$1.2 \pm 0.3\ \mu$M	15.7 ± 0.1 nM	$1.5 \pm 0.6\ \mu$M	13 ± 1 nM	$1.0 \pm 0.4\ \mu$M	10 ± 1 nM

[a] The $K_{d(App)}$ values shown were obtained by nonlinear regression analysis of the results of tryptophan fluorescence experiments of the type shown in Figure 5, using the WT, L55P, and L55PH56G forms of transthyretin, as described in Experimental Procedures.

manner, masking high affinity Cu(II) binding sites (*26*). Therefore, following the approach of others in their studies of the binding of Cu(II) to prion protein (*26*, *36*, *42*), all Cu(II) titrations were carried out using buffers containing 200 μM glycine. Glycine serves as a metal ion buffer and also because the affinity constants of glycine for Cu(II) as a function of pH are known (*43*), it is possible to calculate the free Cu(II) concentration from total Cu(II) and glycine concentrations.

The data shown in Figure 5B indicate that quenching of both tryptophan and ANS fluorescence for WT transthyretin at pH 7.4 occurs in a dose-dependent manner when the free Cu(II) concentration varied over the range $10^{-10}-10^{-6}$ M. Fitting of eq 1 to the tryptophan fluorescence intensity data gave an estimate of 15.7 ± 0.1 nM for the $K_{d(App)}$ value. Because the ANS fluorescence intensity curve is biphasic, (see Discussion for an interpretation of this), it was not appropriate to fit eq 2 to these data. However, the half-maximal change in ANS fluorescence intensity occurred at a Cu(II) concentration of $\sim 2-3$ nM. These low nanomolar apparent K_d estimates suggest that transthyretin binds Cu(II) more tightly than α-synuclein and Aβ ($K_{d(App)} \sim 100$ nM in both cases (*23*, *44*) and β2-microglobulin ($K_{d(App)} \sim 3\ \mu$M (*28*)), much more tightly than immunoglobulin L chains ($K_{d(App)} \sim 100\ \mu$M (*24*)), but more weakly than prion protein (K_d values in the femtomolar−nanomolar range; (*36*, *42*)). As was the case for Zn(II) binding to transthyretin, values similar to those shown in Figure 5B that were carried out using the L55P and L55PH56G transthyretin mutants at both pH 6.5 and pH 7.4 (Table 1) for Cu(II) binding were obtained from analyses of equivalent tryptophan fluorescence experiments, implying that the L55P mutation does not perturb the Cu(II) binding site(s) on transthyretin and that His56 does not make a major contribution to these site(s).

Are there Distinct Binding Sites on Transthyretin for Cu(II) and Zn(II)? We sought to establish whether Cu(II) and Zn(II) bind to the same site(s) on transthyretin by exploiting our finding that Cu(II) gave far more pronounced quenching of the fluorescence of Trp41 than Zn(II). From this we reasoned that if Cu(II) and Zn(II) bind to the same site(s) on transthyretin, then titration of Zn(II) into samples of transthyretin that had already received sufficient Cu(II) to quench their tryptophan fluorescence by more than ~30% would lead to partial recovery of fluorescence, with the Zn(II) displacing bound Cu(II). Conversely, if the Zn(II) titration was carried out using transthyretin samples that had received sufficient Cu(II) to quench tryptophan fluorescence by less than 30%, then the addition of Zn(II) would lead to further quenching. In contrast, if the metal ion-induced quenching arose from the binding of Cu(II) and Zn(II) at independent sites, then the titration of Zn(II) into transthyretin samples that had already received Cu(II) would lead to further quenching, regardless of the extent of quenching achieved by the prior addition of Cu(II). The results of an experiment designed to distinguish between these possibilities are shown in Figure 6. From this, it is apparent that when sufficient Cu(II) has been added to quench tryptophan fluorescence by more than ~30%, then the addition of Zn(II) leads to dose-dependent increases in fluorescence (Figure 6, △, ○, and ■). However, when Cu(II) has been added to give less than ~30% quenching, the addition of Zn(II) gives dose-dependent decreases in fluorescence (Figure 6, □). This pattern of data is consistent with there being a single class of binding sites on transthyretin for both Cu(II) and Zn(II).

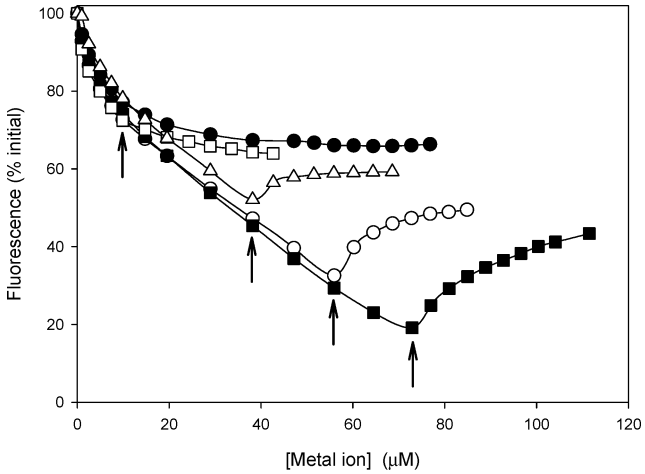

FIGURE 6: Quenching of the tryptophan fluorescence of transthyretin: competition between Cu(II) and Zn(II). The tryptophan fluorescence of WT transthyretin (15 μM in 20 mM HEPES and 150 mm NaCl at pH 7.4) was measured in the presence of either Zn(II) only (●) or Cu(II) at the indicated concentrations as described in Experimental Procedures. At the points in the titrations indicated by the arrows (10 μM added Cu(II), (□); 40 μM added Cu(II), (△); 60 μM added Cu(II), (○); and 80 μM added Cu(II), (■)), Zn(II) was titrated into the samples in 5 μM increments. The lines shown are drawn to guide the eye. The data shown are representative of two independent experiments.

Link between Metal Ion Binding and Enhanced Amyloid Formation. The data presented above show that Cu(II) and Zn(II) can bind to transthyretin and that this can be functionally correlated with enhanced amyloid formation, especially for the L55P transthyretin mutant. The rate-limiting step in the formation of amyloid from transthyretin is the dissociation of the tetramer into native monomer; this is followed by rapid unfolding of the monomer and its subsequent passage down the amyloidogenic pathway. One way in which this has been demonstrated is by measuring the rates of urea-mediated dissociation of the tetramer; these rates correlate well with the rates of amyloid formation from different transthyretin mutants (*10*, *12*, *15*).

Therefore, we examined the effects of the presence of Zn(II) and Cu(II) on the rates of urea-mediated dissociation of WT and L55P transthyretin. The results of this showed that

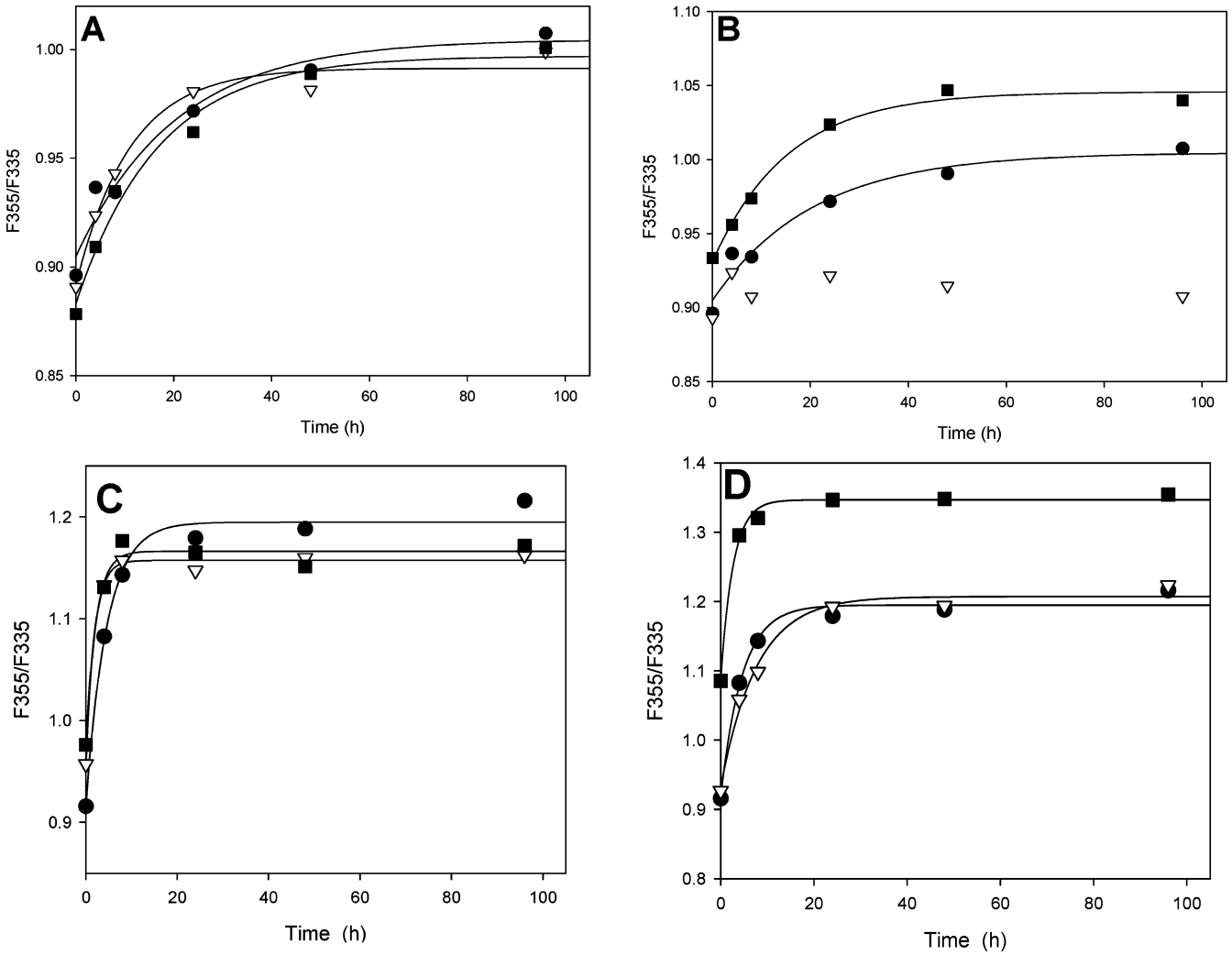

FIGURE 7: Effects of Zn(II) and Cu(II) on urea-mediated dissociation of transthyretin. Urea-mediated dissociation of WT (panels A and B) and L55P transthyretin (panels C and D) was monitored by measuring the ratio of fluorescence emission at 355 to 335 nm as described in Experimental Procedures. Panels A and C show data for transthyretin dissociation in the absence of metals (●), with 10 μM Zn(II) (△), and with 50 μM Zn(II) (■). Panels B and D show data for transthyretin dissociation in the absence of metals (●), with 20 μM Cu(II) (△), and with 100 μM Cu(II) (■). The lines show the results of fitting eq 3 to the data. The data shown are representative of two independent experiments.

the presence of Zn(II) had little effect on the rate of dissociation of WT transthyretin (Figure 7A), whereas Cu(II) at low concentrations (20 μM added) stabilized the tetramer. Higher concentrations of Cu(II) (100 μM added) led to a modest increase in the dissociation rate, which was not statistically significant (fitted rate constants of 0.05 ± 0.01 h^{-1} in the absence of Cu(II) and 0.07 ± 0.01 h^{-1} in the presence of 100 μM added Cu(II)) (Figure 7B).

In contrast, the presence of 10 or 50 μM Zn(II) enhanced the rate of dissociation of the L55P tetramer (respective fitted rate constants of 0.53 ± 0.08 h^{-1} and 0.46 ± 0.11 h^{-1} compared to 0.22 ± 0.03 h^{-1} in the absence of metal ions; Figure 7C). Low concentrations of Cu(II) (20 μM added) had little effect on the dissociation rate, but higher concentrations (100 μM added) led to enhanced dissociation (fitted rate constant of 0.38 ± 0.05 h^{-1} compared to 0.22 ± 0.03 h^{-1} in the absence of metal ions; Figure 7D).

DISCUSSION

Our data show that transthyretin is a metal-binding protein, capable of binding both Zn(II) and Cu(II) at a single class of binding site(s), with affinities comparable to those reported for other amyloid-forming proteins. The low micromolar apparent dissociation constant for Zn(II) binding to transthyretin, coupled with the finding that plasma Zn(II) concentrations are typically about 15 μM (*45*), suggests that the binding of Zn(II) to transthyretin may be physiologically relevant. The potential physiological relevance of the binding of Cu(II) to transthyretin is more difficult to assess. Plasma Cu(II) levels typically fall in the range 7−40 μM, with a median of 17 μM. Most plasma Cu(II) is tightly bound to proteins, including caeruloplasmin, albumin, and transcuprein (*46*, *47*). However, removal of the protein fraction of plasma by ultra-filtration or polymer adsorption has allowed estimates of the free plasma Cu(II) levels to be made; these lie in the range 0.3−0.8 μM (*48*). A comparison of this range with the $K_{d(App)}$ of transthyretin for Cu(II) of ∼10 nM (Table 1) suggests that the transthyretin−Cu(II) interaction may be physiologically relevant.

Binding of these metal ions to transthyretin has both structural and functional consequences. The data in Figure 5A and B show that binding of both Zn(II) and Cu(II) to

transthyretin leads to dose-dependent decreases in its tryptophan fluorescence intensity. Consistent with the fact that only one of the two tryptophan residues in each transthyretin monomer (Trp41) exhibits significant fluorescence (*16*, *37*), the tryptophan fluorescence curves in Figure 5A and B show a monophasic dependence on metal ion concentration. This implies that metal ion binding to transthyretin leads to perturbation of the local environment of Trp41. In addition, binding of Cu(II) but not Zn(II) to transthyretin leads to dose-dependent decreases in the fluorescence intensity of the hydrophobic probe, ANS (Figure 5B). Given that ANS binds in the central thyroxine-binding pocket of transthyretin (*38*), this implies that the region of the protein is perturbed upon binding of Cu(II). However, inspection of the ANS fluorescence intensity curve in Figure 5B shows that in contrast to the tryptophan fluorescence intensity curve the ANS fluorescence intensity shows a biphasic response to increasing concentrations of Cu(II). Equilibrium dialysis, difference absorption spectroscopy, and fluorescence enhancement studies have shown that the transthyretin tetramer can bind two molecules of ANS, with dissociation constants in the low micromolar range (*38*, *49*). It is therefore possible that the biphasic ANS fluorescence intensity curve in Figure 5B reflects differential effects of the binding of Cu(II) on the local micro-environments of the two ANS binding sites.

Moreover, both metal ions can enhance amyloid formation from transthyretin, in a manner that varies with the pH and mutations in transthyretin. A comparison of the patterns of data shown in Figures 3, 4, and 7 suggests a possible mechanism for this enhancement of amyloid formation by metal ions. In the case of WT transthyretin, neither Cu(II) nor Zn(II) had any effect on the rate of amyloid formation at pH 6.5 or 7.4 (Figures 3 and 4, respectively). Zn(II) had no effect on the rate of urea-induced dissociation of the WT transthyretin tetramer (Figure 7A), and Cu(II), depending on its concentration, either had no effect on the dissociation or actually stabilized the tetramer (Figure 7B). In contrast, Zn(II) promoted amyloid formation from L55P at pH 6.5 and 7.4 (Figures 3A and 4A); this can be correlated with the finding that the L55P tetramer dissociated more rapidly in the presence of Zn(II) than in its absence. Given that the rate-limiting step in transthyretin amyloidogenesis is tetramer dissociation (*10*−*12*, *50*), the enhancement of tetramer dissociation by Zn(II) would be expected to lead to an increased rate of amyloid formation, as was found experimentally. Similar reasoning may be applied to the enhancement of amyloid formation from L55P by Cu(II). This ion, at least at relatively high concentrations, promoted urea-induced tetramer dissociation and thus might be expected to similarly promote amyloid formation.

There are two seemingly paradoxical aspects of our findings. One is that although the L55PH56G mutant shows impaired metal ion enhancement of amyloid formation, compared to that of L55P (Figures 3 and 4), its ability to bind Cu(II) and Zn(II) is indistinguishable from that of L55P (Table 1). This implies that if the region of transthyretin centered on this residue forms part of its metal ion binding site(s), His56 may not be a major part of it. Also, although both Zn(II) and Cu(II) promoted amyloid formation from the L55P mutant at pH 6.5 (Figure 3A and B), only Zn(II) promoted amyloid formation at pH 7.4, with Cu(II) being inhibitory (Figure 4A and B). These data may be interpreted as suggesting that at this pH Cu(II) may direct transthyretin down a pathway leading to the formation of amorphous aggregates rather than amyloid. Similar pH-dependent variations in the formation of amyloid from theL55P mutant have been noted previously; the incubation of L55P at pH 5−5.5 leads to amyloid formation, but amorphous species are generated when it is incubated under more acidic conditions (*17*).

A deeper understanding of the role of metal ions in transthyretin amyloidogenesis will require knowledge of the structure(s) of the metal ion binding site(s) on the protein. Preliminary crystallographic data reported by Sato et al. (*51*) suggest that Cr(III) binds close to Glu54; our studies of the L55PH56G transthyretin mutant also suggest that this region of the protein may provide a metal ion binding site. As a first step toward testing this conjecture, crystallization screening trials of WT and transthyretin mutants in the presence of both Zn(II) and Cu(II) are underway in this laboratory.

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