Inhibition of Proteinase K Activity by Copper(II) Ions†,‡

Lisa A. Stone, Graham S. Jackson, John Collinge, Jonathan D. F. Wadsworth,* and Anthony R. Clarke

MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, U.K.

Received August 14, 2006; Revised Manuscript Received November 3, 2006

ABSTRACT: Disease-related prion protein, PrPSc, can be distinguished from its normal cellular precursor, PrPC, by its detergent insolubility and partial resistance to proteolysis. Several studies have suggested that copper(II) ions can convert PrPC to a proteinase K-resistant conformation; however, interpretation of these studies is complicated by potential inhibition of proteinase K (PK) by copper(II) ions. Here we have examined directly the kinetic and equilibrium effects of copper(II) ions on PK activity using a simple synthetic substrate, p-nitrophenyl acetate. We show that at equilibrium two to three copper(II) ions bind stoichiometrically to PK and destroy its activity (Kd < 1 μM). This inhibition has two components, an initial reversible and weak binding phase and a slower, irreversible abolition of activity with a half-time of 6 min at saturating copper(II) ion concentrations. Copper(II) ions produce a similar biphasic inhibition of PK activity in the presence of brain homogenate but only when the copper(II) ion concentration exceeds that of the chelating components present in brain tissue. Under these conditions, the apparent resistance of PrPC to proteolysis by PK appears to be directly attributable to the inhibition of PK activity by copper(II) ions.

Prion diseases are fatal neurodegenerative disorders that include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia, kuru, and most recently variant Creutzfeldt-Jakob disease in humans (1, 2). Their central feature is the post-translational conversion of host-encoded, cellular prion protein (PrPC)1 to an abnormal pathogenic isoform, designated PrPSc (1, 2). The propagation of transmissible prions can be acquired (by inoculation, or in some cases by dietary exposure) with infected tissue, arise sporadically, or be inherited via germline inoculation, or in some cases by dietary exposure. Several studies have suggested that copper(II) ions can convert PrPC to a PK-resistant conformation; however, interpretation of these studies is complicated by potential inhibition of proteinase K (PK) by copper(II) ions. Here we have examined directly the kinetic and equilibrium effects of copper(II) ions on PK activity using a simple synthetic substrate, p-nitrophenyl acetate. We show that at equilibrium two to three copper(II) ions bind stoichiometrically to PK and destroy its activity (Kd < 1 μM). This inhibition has two components, an initial reversible and weak binding phase and a slower, irreversible abolition of activity with a half-time of 6 min at saturating copper(II) ion concentrations. Copper(II) ions produce a similar biphasic inhibition of PK activity in the presence of brain homogenate but only when the copper(II) ion concentration exceeds that of the chelating components present in brain tissue. Under these conditions, the apparent resistance of PrPC to proteolysis by PK appears to be directly attributable to the inhibition of PK activity by copper(II) ions.

METHODS

Proteinase K. Proteinase K (EC 3.4.21.14) from the fungus Tritirachium album limber was obtained freeze-dried from Merck Biosciences, Ltd. (Nottingham, U.K.). The specific enzymatic activity is approximately 30 Anson units/g, where 1 Anson unit is the amount of enzyme that liberates 1 mmol of Folin positive amino acids/min at pH 7.5 and 35 °C using hemoglobin as a substrate. A stock solution of 70 μM PK was prepared in 25 mM N-ethylmorpholine buffer at pH 7.4 (NEM buffer). Aliquots of this stock solution were used in all experiments and were stored frozen at −20 °C and thawed only once prior to use.

Measurement of PK Activity. PK activity was measured using a synthetic substrate p-nitrophenyl acetate (pNA)
(Sigma, Poole, Dorset, U.K.; catalog no. N-8130). Hydrolysis of pNA by PK releases p-nitrophenol that can be monitored by a change in absorbance at 425 nm. For all experiments, the final reaction volume was 1 mL with reactions performed in polystyrene cuvettes (Sarstedt Ltd., Leicester, U.K.; catalog no. 67.742). For experiments performed at equilibrium, PK was diluted from a stock solution into NEM buffer to give final protease concentrations of 0.7, 1.4, and 2.1 μM. Samples were adjusted with 1 mM CuSO₄ (prepared in water), to give final concentrations of copper(II) ions between 0 and 16 μM. Following equilibration at room temperature (~20 °C) for a minimum of 3 h or up to 20 h, samples were transferred to a Jasco V-530 spectrophotometer set to monitor absorbance at 425 nm. At time zero, samples were adjusted with a stock solution of 220 mM pNA (prepared in methanol) to give a final pNA concentration of 2.5 mM in the sample. After rapid mixing had been carried out, the change in absorbance at 425 nm was measured over the subsequent 120 s. The rate of change in absorbance due to PK activity (ΔA per minute) was determined following subtraction of the background rate of pNA hydrolysis in buffer alone or buffer with copper(II) ions.

**Time-Resolved Inhibition of PK Activity.** PK (35 μM) was incubated with a range of CuSO₄ concentrations either in the absence or in the presence of 10% (w/v) normal hamster brain homogenate prepared in NEM buffer. Aliquots (20 μL) of sample were taken at various time points and diluted 50-fold in NEM buffer to give a final volume of 1 mL. Samples were then transferred to a Jasco V-530 spectrophotometer, and PK activity was determined as described above.

**PK Digestion of Brain Homogenate.** Hamster brain homogenate (20%, w/v) prepared in NEM buffer was adjusted with NEM buffer lacking or containing CuSO₄ to a final concentration of 10% (w/v). Samples were digested with PK at a final protease concentration of 20 μg/mL for 30 min at 37 °C. Digestion was terminated by addition of an equal volume of 2X SDS sample buffer [125 mM Tris-HCl (pH 6.8) containing 20% (v/v) glycerol, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 8 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 0.02% (w/v) bromophenol blue] and immediate transfer to a 100 °C heating block for 10 min. Samples were analyzed by electrophoresis on 16% Tris-glycine gels (Invitrogen, Paisley, U.K.). Duplicate gels were electroblotted onto PVDF membrane (Immobilon-P, Millipore) or stained with silver. Immunoblotting with anti-PrP monoclonal antibody 3F4 (15) using high-sensitivity enhanced chemiluminescence and visualization on Kodak BioMax MR film (Anachem, Luton, U.K.) was performed as previously described (16). Silver staining of total protein was achieved using the Plus One silver stain kit (GE Healthcare, Chalfont St Giles, U.K.). Densitometry of silver-stained gels or autoradiography film was performed using a Kodak Image Station 440 CF (Kodak, Hemel Hempstead, U.K.).

**Statistical Analysis.** All experiments were conducted at least three times under optimal conditions. Figures show representative data. Figure legends report dissociation constants or rate constants as the mean ± the standard deviation.

**RESULTS**

**Equilibrium Inhibition of PK Activity by Copper(II) Ions.** In an initial series of experiments, we investigated the residual activity of PK after incubation for 20 h with a range of concentrations of copper(II) ions (Figure 1). The substrate used as a probe for the hydrolytic activity was p-nitrophenyl acetate (pNA), and experiments were performed in a nonchelating buffer [n-ethylmorpholine (NEM)] so that that concentration-dependent effect of copper binding could be examined without undue complications. The reason for using pNA as the substrate, rather than one based on a polypeptide, is its structural simplicity and its relatively poor ability to act as a chelator of transition metal ions. Consequently, the effects of copper(II) ions that we see on the enzymatic activity of the system are due to effects on the enzyme itself rather than on the substrate.

Figure 1 shows that at a stoichiometry of <1:1 there is little inhibition of PK activity by copper(II) ions; however with increasing concentrations of copper(II) ions, a steep descent to zero activity is observed. The inset of Figure 1 shows the extrapolated concentrations of copper(II) ions required to abolish enzymatic activity. The slope of the inset plot indicates that a stoichiometry of two to three copper(II) ions per enzyme molecule is required for complete inhibition of activity. These data are consistent with a system in which copper(II) ions are initially bound through chelation or adsorption events to a high-affinity site on PK that has no effect on enzyme activity. However, upon further titration (to stoichiometries of >1), further binding sites on the enzyme become occupied by copper(II) ions, leading to a complete inhibition of enzyme activity at a copper(II) ion: enzyme ratio of 2–3. As we cannot know the exact inhibitory effect upon binding of each copper(II) ion, the system precludes precise quantitative analysis with respect to affinity, but the relative linearity of the response after initial titration of the noninhibitory high-affinity binding site would suggest that inhibitory copper(II) ion binding is tight with a dissociation constant of <1 μM. This inhibition appears to be specific to copper(II) ions as no measurable decrease in PK.
Inhibition of Proteinase K Activity by Copper(II) Ions

Figure 2: Time-resolved inhibition of PK activity by copper(II) ions. PK was incubated with a series of concentrations of copper(II) ions, and aliquots were removed at the times given on the x-axis for immediate assay. The dilution from incubation to assay was 50-fold. Three example progress curves representing residual activity as a function of time are shown at copper(II) ion concentrations of 0 (○), 0.5 (▲), and 1.5 mM (●). The data were fitted to a single-exponential decay function \( [v = v_i \exp(-k_{obs}t)] \) to determine the rate of irreversible loss of enzymatic activity. The observed rate constants \( (k_{obs}) \) are 0.042 ± 0.007 and 0.133 ± 0.025 min⁻¹ at copper(II) ion concentrations of 0.5 and 1.5 mM, respectively.

Our findings demonstrate that the time course of inhibition of PK activity is dependent on the concentration of copper(II) ions and that the phenomenon occurs on two, well-separated time scales. Initially, there is rapid inhibition which occurs on the same time scale as that required to mix the components and withdraw the sample for assay (~15 s). This is then followed by a slower phase of inhibition that leads to a complete loss of proteolytic activity (Figure 2).

The concentration dependence of the initial rapid inhibition of PK activity by copper(II) ions is analyzed in Figure 3A, where the concentration of copper(II) ions is shown on the x-axis. These data appear to fit to a hyperbolic inhibition phenomenon. However, from this experiment alone, it is difficult to tell whether this represents an irreversible inhibition of PK activity by copper(II) ions with a measured \( K_{i,5} \) of ~0.55 mM or a reversible but tighter inhibition that is still operating after the 50-fold dilution of the incubation mix into the assay buffer. To distinguish between these possibilities, we repeated these experiments using the straightforward assay performed under dilute conditions rather than dilution of a concentrated incubation mixture. As shown in Figure 3B, the inhibition is indeed of a relatively high affinity and reversible. The apparent inhibition constant determined under dilute conditions is ~15 μM which compares very closely with the value of 0.55 mM at 50-fold dilution (Figure 3A) that gives an apparent inhibition constant of 11 μM.

Following this rapid and reversible phenomenon, the second phase of inhibition of PK activity by copper(II) ions is slow and irreversible, having a half-time of ~6 min at a copper(II) ion concentration of 2 mM. This phase is apparently first-order; i.e., the activity of the enzyme decays in an exponential fashion. The decay reaches zero activity and is sufficiently slow that it can be properly resolved in time to provide a first-order rate constant. These observed rate constants are plotted as a function of the concentration of copper(II) ions in Figure 4. Although the underlying molecular mechanisms of inhibition may be complicated, the data are consistent with an asymptotic increase in the rate constant with an increasing copper(II) concentration and conform to the following relationship:

\[
k_{obs} = \frac{K}{[Cu(II)] + [Cu(II)]}
\]
The interplay of these factors makes it impossible to produce an exact quantitative analysis, but some simplified conclusions can be drawn.

In initial experiments, we observed that brain homogenate alone has substantial hydrolytic activity when challenged with the assay substrate, but when PK is mixed with homogenate, within 30–60 s the level of hydrolytic activity is reduced to that observed in the presence of PK alone (data not shown). From these data, we conclude that PK is very effective in destroying endogenous proteases present in brain homogenate.

The inhibition of the PK activity by copper(II) ions in the presence of brain homogenate occurred in two distinct phases, similar to that observed in the absence of brain homogenate. The rapid and reversible phase of inhibition is analyzed in Figure 6A and shows the initial activity (after a dead time of \( \sim 30 \) s) as a function of the concentration of copper(II) ions. The pattern of inhibition does not conform to a simple inverse hyperbola; rather, there is a threshold below which there is little effect of copper(II) ion concentration. We assume this occurs because there is a relatively high concentration of tight copper(II) ion binding sites in the homogenate that have to be out-titrated before there are sufficient free metal ions to affect PK. The concentration of copper(II) ions required to produce a significant (20%) inhibition of PK activity is \( \sim 1 \) mM. The concentration of protein in a 10% homogenate is approximately 10 mg/mL.

With an average amino acid residue molecular mass of 110, this gives a residue concentration of \( \sim 90 \) mM; this means that, as a crude approximation, there is one tight copper(II) ion site per 90 amino acid residues. From this experiment alone, it is difficult to tell whether this represents an irreversible inhibition with a measured \( K_{0.5} \) of \( \sim 2 \) mM or a reversible but tighter inhibition that is still operating after the 50-fold dilution of the incubation mix into the assay buffer. To distinguish between these possibilities, the experiment was again performed not with a concentrated incubation mixture but via a straightforward assay under dilute conditions. This result is shown in Figure 6B, the analysis of which reveals that the inhibition is, indeed, of a relatively high affinity and reversible. The \( K_{0.5} \) value is now \( \sim 30 \) \( \mu \)M which compares favorably with the value of 2 mM divided by the 50-fold dilution which would provide an apparent inhibition constant of 40 \( \mu \)M.

Finally, the slow phase of inhibition is analyzed in Figure 6C, which shows the first-order rate constant for the loss of enzymatic activity as a function of copper(II) ion concentration. Again, as in the case of the rapid, reversible inhibition, there is no detectable, slow inhibition phase at concentrations of copper(II) ions below 1 mM, presumably because of absorption onto components of the brain homogenate. At 2 mM copper(II) ions, the half-time for this slow, irreversible inhibition is much longer than in the absence of homogenate, being \( \sim 3 \) h as opposed to 6 min.

Inhibition of PK Activity by Copper(II) Ions Is Not Attributable to Changes in pH. PK shows activity over a broad pH range; however, its activity is optimal at basic pH, and a shift from pH 8 to 6 reduces the catalytic activity by approximately 60% \( (17) \). As high concentrations of copper(II) ions in the buffer system will produce a shift to a more acidic pH, we investigated this directly. The pH shift on addition of the highest concentration of copper(II) ions used in experiments lacking brain homogenate (2 mM; see Figures

![Figure 4](link) **Figure 4**: Transient kinetics of slow, irreversible inhibition of PK by copper(II) ions. The observed rate constants for the slow, irreversible loss of enzyme activity on exposure to copper(II) ions (see Figure 2) are plotted as a function of the metal ion concentration in the incubation and fitted to the equation \( k_{obs} = k_u[Cu(II)]/(K + [Cu(II)]) \) (see the Discussion). The value for \( k_u \) is 0.23 ± 0.05 min\(^{-1}\), and the value for \( K \) is \( \sim 1.5 \) ± 0.5 mM.

![Figure 5](link) **Figure 5**: Time course of inhibition of PK by copper(II) ions in the presence of brain homogenate. PK was incubated with a series of concentrations of copper(II) ions in 10% hamster brain homogenate, and aliquots were removed at the times given on the x-axis for immediate assay. The dilution from incubation to assay was 50-fold. Three example progress curves representing residual enzymatic activity as a function of time are shown at copper(II) ion concentrations of 0, 0.5, and 1.5 mM. The fitted values for the constants are 0.0019 min\(^{-1}\) and 0.05 min\(^{-1}\) for \( k_1 \) at copper(II) ion concentrations of 2 and 4 mM, respectively.

The fitted values for the constants are 0.23 min\(^{-1}\) for \( K_u \) and 1.5 mM for \( K \). Both of these values can be considered as empirical constants that allow calculation of residual activity at given times and given concentrations of copper(II) ions. The physical meanings of both \( K_u \) and \( K \) are dependent on the kinetic model; this subject is elucidated further in the Discussion.

Inhibition of PK Activity in the Presence of Brain Homogenate. Figure 5 exemplifies the inhibition of PK activity observed after its incubation with differing concentrations of copper(II) ions in the presence of a 10% brain homogenate. These results are not as straightforward to analyze or interpret as those collected in the absence of brain homogenate. Indeed, this is further complicated by the possibility of PK activity destroying endogenous proteases in the homogenate (or vice versa) and the possible effect of copper(II) ions on homogenate hydrolases that are capable of cleaving pNA. The interplay of these factors makes it impossible to produce an exact quantitative analysis, but some simplified conclusions can be drawn.

In initial experiments, we observed that brain homogenate alone has substantial hydrolytic activity when challenged with the assay substrate, but when PK is mixed with homogenate, within 30–60 s the level of hydrolytic activity is reduced to that observed in the presence of PK alone (data not shown). From these data, we conclude that PK is very effective in destroying endogenous proteases present in brain homogenate.

The inhibition of the PK activity by copper(II) ions in the presence of brain homogenate occurred in two distinct phases, similar to that observed in the absence of brain homogenate. The rapid and reversible phase of inhibition is analyzed in Figure 6A and shows the initial activity (after a dead time of \( \sim 30 \) s) as a function of the concentration of copper(II) ions. The pattern of inhibition does not conform to a simple inverse hyperbola; rather, there is a threshold below which there is little effect of copper(II) ion concentration. We assume this occurs because there is a relatively high concentration of tight copper(II) ion binding sites in the homogenate that have to be out-titrated before there are sufficient free metal ions to affect PK. The concentration of copper(II) ions required to produce a significant (20%) inhibition of PK activity is \( \sim 1 \) mM. The concentration of protein in a 10% homogenate is approximately 10 mg/mL.

With an average amino acid residue molecular mass of 110, this gives a residue concentration of \( \sim 90 \) mM; this means that, as a crude approximation, there is one tight copper(II) ion site per 90 amino acid residues. From this experiment alone, it is difficult to tell whether this represents an irreversible inhibition with a measured \( K_{0.5} \) of \( \sim 2 \) mM or a reversible but tighter inhibition that is still operating after the 50-fold dilution of the incubation mix into the assay buffer. To distinguish between these possibilities, the experiment was again performed not with a concentrated incubation mixture but via a straightforward assay under dilute conditions. This result is shown in Figure 6B, the analysis of which reveals that the inhibition is, indeed, of a relatively high affinity and reversible. The \( K_{0.5} \) value is now \( \sim 30 \) \( \mu \)M which compares favorably with the value of 2 mM divided by the 50-fold dilution which would provide an apparent inhibition constant of 40 \( \mu \)M.

Finally, the slow phase of inhibition is analyzed in Figure 6C, which shows the first-order rate constant for the loss of enzymatic activity as a function of copper(II) ion concentration. Again, as in the case of the rapid, reversible inhibition, there is no detectable, slow inhibition phase at concentrations of copper(II) ions below 1 mM, presumably because of absorption onto components of the brain homogenate. At 2 mM copper(II) ions, the half-time for this slow, irreversible inhibition is much longer than in the absence of homogenate, being \( \sim 3 \) h as opposed to 6 min.

Inhibition of PK Activity by Copper(II) Ions Is Not Attributable to Changes in pH. PK shows activity over a broad pH range; however, its activity is optimal at basic pH, and a shift from pH 8 to 6 reduces the catalytic activity by approximately 60% \( (17) \). As high concentrations of copper(II) ions in the buffer system will produce a shift to a more acidic pH, we investigated this directly. The pH shift on addition of the highest concentration of copper(II) ions used in experiments lacking brain homogenate (2 mM; see Figures
3 h followed by 50-fold dilution under the standard assay conditions was identical to the activity of PK incubated similarly in NEM buffer at pH 7.4 (data not shown). Thus, transient exposure of PK to pH 6.7 is itself not detrimental to the activity of the protein. Previously, it has been demonstrated that calcium ion depletion of PK reduces its stability as shown by thermal denaturation but that proteolytic activity is unchanged (17). In accordance with these data, we found that the presence of calcium ions, up to 10 mM, had no measurable effect on the copper(II) inhibition or enzymatic activity of the system (data not shown). On the basis of these findings, we conclude that copper(II) ions are directly responsible for inhibition of PK activity and that this is not due to alterations of solvent pH or calcium ion occupancy.

**PK Inhibition by Copper(II) Ions Monitored by SDS-PAGE and PrP Immunoblotting.** In a final series of experiments, we investigated PK digestion of PrPC in hamster brain homogenate in the presence of increasing concentrations of copper(II) ions. PrPC was visualized by immunoblotting and total protein by silver staining. As shown in Figure 7A, PK digestion of PrPC is inhibited in a concentration-dependent manner by copper(II) ions at concentrations of ≥1 mM. This effect is not, however, unique to PrPC as PK digestion of other proteins in the brain homogenate (visualized by silver stain) was similarly prevented at the same concentrations of copper(II) ions (Figure 7B). Densitometry of PrPC signal intensity and the intensity of three other selected silver-stained proteins showed a closely similar pattern of apparent resistance to PK digestion (Figure 7C). Importantly, the concentration of 1–2 mM copper(II) ions required to see significant inhibition of PK activity in the presence of brain homogenate using the pNA assay (Figure 6A) correlates similarly in NEM buffer at pH 7.4 (data not shown). Thus, transient exposure of PK to pH 6.7 is itself not detrimental to the activity of the protein. Previously, it has been demonstrated that calcium ion depletion of PK reduces its stability as shown by thermal denaturation but that proteolytic activity is unchanged (17). In accordance with these data, we found that the presence of calcium ions, up to 10 mM, had no measurable effect on the copper(II) inhibition or enzymatic activity of the system (data not shown). On the basis of these findings, we conclude that copper(II) ions are directly responsible for inhibition of PK activity and that this is not due to alterations of solvent pH or calcium ion occupancy.

**DISCUSSION**

In the experiments described here, we aimed to make a quantitative assessment of the concentration dependence of PK inhibition by copper(II) ions. The analysis has resolved this process into two distinct time domains, one phase of inhibition being reversible and occurring within seconds and the other being irreversible and occurring over longer time scales that we could easily resolve kinetically. One practical relevance of these findings is that inhibition occurs over the same metal ion concentrations and incubation periods used to distinguish PrPC from PrP Sc on the basis of their differential susceptibility to digestion by PK. As a consequence, if copper(II) ions are investigated for the ability to convert the PrPC conformation to a PrP Sc-like form, then these effects must be considered.

Our initial experiments showed that saturation of PK with copper(II) ions was a complex phenomenon with multiple binding sites and a plateau phase at low copper(II) ion concentrations. Such complexity rules out orthodox binding analysis because there are too many variables in the system; i.e., the curve would have to be fitted to an equation that described the degree of inhibition upon occupation of a given site and the dissociation constant defining the affinity. Given
that there are multiple sites and that we cannot assume weak binding conditions, this puts the system beyond simple analytical solution. This means that the analysis of inhibition has to be somewhat empirical because we cannot precisely define a molecular mechanism. However, what we can conclude from these low-concentration equilibrium data is that it requires between two and three copper(II) ions to bind to the enzyme to abolish its activity and that binding is micromolar or submicromolar with respect to the equilibrium dissociation constant.

The next question we wished to address was the time dependence of inhibition. In most classical inhibition studies using small ligands, the time required for the inhibitory effect is very short. For instance, with freely diffusing ligands, the time taken for interaction with a surface site is determined by the second-order rate constant describing the diffusion-collision process (typically $10^7$–$10^8$ M$^{-1}$ s$^{-1}$) and the ligand concentration. Hence, even at a low ligand concentration such as 1 mM, the binding event happens over a period of 10–100 ms. However, inhibition wrought by the binding of transition metals may occur more slowly if the metal has to penetrate the body of the protein rather than associate rapidly with a surface-exposed site. This type of “burrowing” event may require the transient and infrequent opening of regions of the protein structure or, possibly, the global unfolding of the molecule. Hence, such processes may occur over slow time scales. Indeed, the data show that part of the inhibition by copper is not instantaneous; there is a fast phase in which 80–85% of the enzymatic activity is lost and a slow phase in which the remainder disappears. The fast phase represents reversible binding with an effective $K_I$ of $10^{-20}$ M. The slower phase, in which the rate constants are easily measurable, has a different mechanistic origin. The observed rate of this phase rises with the concentration of copper(II) ions but then reaches an asymptotic maximum. Although we cannot be certain of the mechanism, a reasonable physical model that can explain this kinetic pattern is described by the following scheme:

$$F = U \rightarrow U \cdot Cu(II)$$

where the folded state (F) can undergo slow and rate-limiting opening events to a more unfolded state (U) that can then form tight, irreversible interactions with the transition metal. If we define the rate constant for unfolding (F $\rightarrow$ U) as $k_u$, the rate constant for refolding or closing as $k_c$, and the bimolecular rate of copper(II) ion association as $k_a$, then the observed rate constant ($k_{obs}$) for the formation of the dead-

**Figure 7:** Analysis of the effects of copper(II) ions on PK activity in the presence of brain homogenate by PrP immunoblot or SDS-PAGE silver stain. Normal hamster brain was prepared as a 20% (w/v) homogenate in NEM buffer and diluted to 10% in NEM buffer alone or containing 0.1, 0.5, 1, 2, 3, and 6 mM CuSO$_4$. PK digestion was performed at a final protease concentration of 20 µg/mL for 30 min at 37 °C, and equivalent aliquots were analyzed by SDS–PAGE. (A) Immunoblot probed with anti-PrP monoclonal antibody 3F4 showing that the signal intensity of PrP$^\text{C}$ increases with increasing CuSO$_4$ concentrations. The lane denoted with an asterisk shows an identical volume of brain homogenate analyzed in the absence of PK digestion. Densitometry of the PrP$^\text{C}$ signal is shown in part i of panel C. (B) Silver stain showing the total homogenate protein. Again, the signal intensity increases with increasing CuSO$_4$ concentrations, and three prominent bands, ii, iii, and iv, were selected for densitometry as shown in parts ii–iv of panel C.
Inhibition of Proteinase K Activity by Copper(II) Ions

end inhibited state [U-Cu(II)] is defined by the following equation:

\[ k_{\text{obs}} = k_u[Cu(II)]/\{K + [Cu(II)]\} \]  

(1)

where the value of \( K \) is given by the \( k_d/k_u \) ratio and is measured in terms of the molarity of copper(II) ions in the incubation; it can also be defined as the concentration required to give half the maximal rate of irreversible copper(II) ion inhibition. The data fit this empirical model tolerably well (see Figure 4) so that eq 1 serves as a useful tool in quantifying the effects of copper(II) ions at a range of concentrations and incubation times.

The rapid reversible loss of activity shown in Figure 3 can be quantified by the relationship

\[ A = K_I/[\{Cu(II)] + K_I} \]  

(2)

where \( A \) is the fractional residual activity and \( K_I \) is the apparent inhibition constant. Equation 1 gives the time dependence of the slow, irreversible loss of activity so that the first-order decay can be described by

\[ A = \exp(-k_{\text{obs}} t) \]  

(3)

where, again, \( A \) is the fractional residual activity and \( k_{\text{obs}} \) the observed first-order rate constant defined by eq 1. Integrating eq 3 yields an expression for the total percentage activity (\( A\% \)) over an incubation time (\( t \)) for the slow phase of inhibition:

\[ A\% = 100[1 - \exp(-k_{\text{obs}} t)]/k_{\text{obs}} \]  

(4)

Equations 2 and 4 can then be combined to describe the percentage of the uninhibited activity (\( A\% \)) at any incubation time (\( t \)) at any concentration of copper(II) ions:

\[ A\% = 100(K_I/[\{Cu(II)] + K_I}) \]  

\[ [1 - \exp(-k_{\text{obs}} t)]/k_{\text{obs}} \]  

(5)

\( k_{\text{obs}} \) is defined by eq 1.

The above quantitative arguments apply to systems in which there is no strong copper chelator in the system and no tissue homogenate, but essentially the same phenomena occur in the presence of a 10% brain homogenate; i.e., there is a rapid reversible binding of copper that inhibits the enzyme activity and a slow irreversible phase. Under these circumstances, PK is protected from copper(II) ion inhibition by the presence of the proteolytic breakdown products of the tissue components and probably by other species present in this complex mixture. Thus, in the presence of 10% brain homogenate, it is not until a threshold of \( \sim 1 \) mM copper(II) ions has been exceeded that we see significant inhibition of the proteolytic activity, either in the reversible or in the irreversible modes.

Comparisons of the effects of copper(II) ions on PK activity using SDS-PAGE and immunoblot or silver staining show that the effects using pNA were relevant to the prion field. The analysis of the PK resistance of PrP\(^{C} \) is normally visualized via an immunoblot probed with a PrP specific antibody. Examining total protein effects by using a simple method such as silver stain shows whether copper(II) ions specifically effect PrP or act directly on the enzyme itself.

Under circumstances involving a purified sample of PrP that cannot be compared with other proteins, we provide the following mathematical equations to subtract the levels of inhibition of PK produced by copper(II) ions.

**Practical Quantification of the Inhibition of Proteinase K.** By integrating eq 5 between limits of zero time and the incubation time (\( t \)), an expression can be derived from our data that approximates the percentage activity \( [\text{Act(\%)}] \) over time when a sample is incubated over a given period (\( t \)) with a given concentration of copper(II) ions (\( C \)) in millimolar. This value of \( \text{Act(\%)} \) refers to the exposure of the system to PK activity as a percentage of that which would occur in the absence of copper(II) ions. In a nonchelating buffer, the following equation applies:

\[ \text{Act(\%)} = 100[1 - C/(C + 0.015 \text{ mM})] \]  

\[ [1 - \exp(-k_{\text{obs}} t)]/(k_{\text{obs}} t) \]

where \( k_{\text{obs}} = 0.23 \text{ min}^{-1} \times C/(C + 1.5 \text{ mM}) \). The first part of the above expression represents the instantaneous and reversible inhibition, and the second part represents the slow irreversible phase that required the integral solution. Hence, if one were to look at a proteinase K digestion in the presence of 1 mM copper(II) for 60 min, these variables can be substituted into the above to reveal the degree of inhibition. If the experimental system contains a copper(II) chelator (e.g., by virtue of the buffer), then the concentration available to inhibit the enzyme \( C \) (in the expression given above) is given by

\[ C = [Cu^{2+}]_{\text{total}} \times K_d/([K_d + [\text{chelator}]_{\text{total}}] \]

where \( K_d \) is the equilibrium dissociation constant for the chelator-copper complex. This relationship holds as long as \([\text{chelator}]_{\text{total}} \) is greater than \([Cu^{2+}]_{\text{total}} \). In the presence of 10% brain homogenate, we can also provide an estimate of the residual percentage activity. From our data, it appears that the homogenate can neutralize 1 mM copper(II) ions, and above 4 mM, we see a dramatic precipitation of components within the homogenate. However, when the copper(II) ion concentration is between 1 and 4 mM, the following relationship can be applied:

\[ \text{Act(\%)} = 100[1 - C/(C' + 0.03)] \]  

\[ [1 - \exp(-k_{\text{obs}} t)]/(k_{\text{obs}} t) \]

where \( C' = [Cu^{2+}] - 1 \text{ mM} \) and \( k_{\text{obs}} = C' \times 0.0027 \text{ min}^{-1} \text{ mM}^{-1} \). In this expression, the value of 0.0027 \text{ min}^{-1} \text{ mM}^{-1} is defined by the slope of the rate plot shown in Figure 6C.

Alternatively, those working on the acquisition of protease resistance of PrP and prion infectivity should in the future consider using a protease not effected by copper(II) ions or simply address prion infectivity itself, as it has not been shown that there is a direct correlation between copper(II) ion-coordinated PrP conformation and prion infectivity.

**ACKNOWLEDGMENT**

We are grateful to R. Young for preparation of the figures.

**REFERENCES**


BI061646S