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Copper inhibits the protease from human immunodeficiency virus 1 by both cysteine-dependent and cysteine-independent mechanisms

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ABSTRACT **The protease of the human immunodeficiency virus is essential for replication of the virus, and the enzyme is therefore an attractive target for antiviral action. We have found that the viral protease is inhibited by approximately stoichiometric concentrations of copper or mercury ions. Inactivation by Cu^{2+} was rapid and not reversed by subsequent exposure to EDTA or dithiothreitol. Direct inhibition by Cu^{2+} required the presence of cysteine residue(s) in the protease. Thus, a synthetic protease lacking cysteine residues was not inhibited by exposure to copper. However, addition of dithiothreitol as an exogenous thiol rendered even the synthetic protease susceptible to inactivation by copper. Oxygen was not required for inactivation of either the wild-type or the synthetic protease. These results provide the basis for the design of novel types of protease inhibitors.**

The aspartic protease encoded by the human immunodeficiency virus (HIV) is essential for the processing of the viral polyproteins encoded by the *gag* and *pol* genes into mature viral proteins (1–3). Mutation or deletion of the protease gene blocks replication of the virus (4, 5), making the protease an attractive target for antiviral therapy of the acquired immunodeficiency syndrome (AIDS). The inhibitors reported thus far are peptides or peptide analogues (6–8), some of which were originally studied as inhibitors of other structurally related aspartic proteases such as pepsin or renin.

Another approach to the inhibition of the protease was suggested by studies of metal-catalyzed oxidation of proteins. Both enzymic and nonenzymic metal-catalyzed oxidation systems are capable of oxidatively inactivating many enzymes (9, 10). Such systems consist of a redox-cycling metal cation such as copper or iron, a reducing agent, and molecular oxygen. Cytochrome P450/NADPH/O_2 is an example of an enzymic system (9), while Fe/ascorbate/O_2 is a well-studied nonenzymic system (11). These systems are capable of reducing the metal cation and of generating hydrogen peroxide. At least for the nonenzymic systems, oxidation of the protein is initiated by the binding of iron to a specific cation binding site on the protein. Oxidation of the reduced form of the metal generates a very reactive oxidizing species, such as the hydroxyl radical. The radical reacts with an amino acid residue very close to its site of generation, generally inactivating the enzyme. In the case of glutamine synthetase, the site specificity has been studied in detail (12, 13). Specificity has been shown to result from the binding of the redox-capable cation to the two binding sites on the enzyme that would normally bind magnesium.

As a general rule, enzymes that possess metal cation binding sites are susceptible to inactivation by metal-catalyzed oxidation systems. However, the aspartic proteases do not require metals for catalytic activity (14, 15). Moreover, three-dimensional structures of the HIV and several other aspartic proteases are available and have not revealed any cryptic cation binding sites (16–19). Nevertheless, the HIV protease could still be targeted for metal-catalyzed oxidation by a bifunctional molecule. One functional region would provide specificity by targeting the protease while the other region would support the oxidative modification. For example, a peptide substrate could be modified to include an iron chelator. While investigating the feasibility of such targeted oxidizing agents, we discovered that the HIV protease is potently inhibited by copper in an oxygen-independent reaction. Inhibition by copper alone requires the presence of cysteine residues in the protease. However, even a synthetic protease lacking cysteine residues could be inactivated by copper when dithiothreitol was added as an exogenous thiol.

MATERIALS AND METHODS

HIV-1 Protease. Production and purification of the wild-type protease in *Escherichia coli* were as described (20). The chemically synthesized protease (21), a generous gift from Stephen Kent, was obtained as a lyophilized powder. Before use, this synthetic protease was dissolved in 6 M guanidine hydrochloride/50 mM Tris, pH 7.8/1 mM EDTA/5 mM dithiothreitol (DTT) and refolded as described below. The specific activity of recombinant preparations varied from 1.3–1.9 μmol/min per mg of protein, while that of the synthetic protease was 1.0 μmol/min per mg of protein, determined with the peptide H_2N-Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-NH_2 (20). One unit of protease activity cleaved 1 μmol of substrate per min at 37°C. Both the recombinant and the synthetic protease were stored at −70°C in 20 mM HCl at protein concentrations of 100–200 μg/ml.

Anaerobic experiments were performed in the Anaerobic Laboratory of the National Institutes of Health (22). The atmosphere in this laboratory was constantly monitored and never exceeded an oxygen content of 5 ppm. After entry into the anaerobic room, protease and peptide solutions were pump-purged 10 times and buffers were sparged with purified argon for at least 10 min.

Protease was refolded as follows. First, the enzyme was dialyzed against 6 M guanidine hydrochloride/50 mM Tris, pH 7.8/1 mM EDTA/5 mM DTT at ambient temperature for 2 hr. The enzyme solution was dialyzed next at 4°C against 3 M guanidine hydrochloride/50 mM Tris, pH 7.8/1 mM EDTA/1 mM DTT for 2 hr, followed by an additional 2-hr dialysis against 1 M guanidine hydrochloride/50 mM Tris, pH 7.8/1 mM EDTA/1 mM DTT. The final dialysis was into 20 mM HCl, with an additional change of the HCl solution before overnight dialysis. The dialysis tubing (Spectrum Medical Industries) had a nominal molecular weight cutoff of 6000–8000. The ratio of protease volume to dialysate was 1:100 for guanidine solutions and 1:2000 for the HCl.

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Abbreviations: HIV, human immunodeficiency virus; DTT, dithiothreitol.

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Cation Inhibition. Stock solutions of 1 M cations were made by dissolving the salts in water with acidification by HCl to pH 3–5. Typically, protease (2.5 μM) was incubated with 25–100 μM cation for 5 min at 37°C in 10 μl of 150 mM sodium acetate, pH 5.5/10% (vol/vol) glycerol. The assay was started by adding substrate in 2 μl of 150 mM sodium acetate, pH 5.5/6 mM EDTA, yielding 1 mM EDTA in the assay solution. After 20 min at 37°C, products were quantitated by high-pressure liquid chromatography (20).

Sulfhydryl Derivatization. The carboxamidomethylcysteine derivative of the protease was prepared by treatment with iodoacetamide (23). To assure reduction of the cysteine residues, 1 volume of enzyme in 20 mM HCl was mixed with 3 volumes of 8 M guanidine hydrochloride/133 mM Tris/13.3 mM EDTA, giving a final pH of 8.0, and then incubated with 5 mM DTT for 15 min at 37°C. The solution was then made 20 mM in iodoacetamide, incubated at room temperature for 2 hr in the dark, and quenched with excess DTT (10 mM). The sample was then refolded from 6 M guanidine hydrochloride as described above.

Analytical Methods. The protease concentration was calculated from the absorbance at 280 nm, corrected for light scatter (24), using molar absorptivities calculated (25) from the sequence of the protease (ϵ = 12,300). The accuracy of this method was confirmed by amino acid analysis after acid hydrolysis of the protease. Oxidized DTT was prepared by stirring a solution of reduced DTT in room air overnight. Oxidized and reduced DTT were quantitated by high-pressure liquid chromatography with monitoring at 210 nm. These compounds are well-separated from the products and substrate of the protease assay and could therefore be quantified using the same analytical system as for the protease assay (20).

RESULTS

Inhibition by Copper and Mercury. Pepsin and other aspartic proteases are generally not inhibited by divalent cations, including copper (14). However, studies of the susceptibility of the HIV protease to metal-catalyzed oxidation showed that micromolar concentrations of copper or mercury caused marked inhibition of the enzyme (Table 1). Addition of the chelator EDTA 15–30 sec after addition of the metal could not prevent inhibition. When EDTA was added just before the metal, inhibition by copper was blocked but not inhibition by mercury.

Table 1. Effect of cations on wild-type protease activity

Cation (25 μM)	Activity, % control
Al^{3+}	94
Ca^{2+}	100
Co^{2+}	92
Cr^{3+}	96
Cu^{2+}	<2
Fe^{2+}	94
Fe^{3+}	93
Hg^{2+}	<2
K^{+}	92
Mg^{2+}	91
Mn^{2+}	95
Ni^{2+}	94
Pb^{2+}	92
Zn^{2+}	99

Enzyme (2.5 μM) was incubated with 25 μM cation in 150 mM sodium acetate, pH 5.5/10% (vol/vol) glycerol for 5 min at 37°C. The assay for activity was then begun by the addition of substrate in 150 mM sodium acetate/6 mM EDTA, yielding a final concentration of 1 mM EDTA. The incubation was stopped after 20 min of additional incubation.

Table 2. Effect of the order of addition of substrate and protease on inhibition of the wild-type protease

First addition	Second addition	Third addition	Activity, unit(s)
Protease + Cu^{2+}	EDTA	Substrate	0.02
Substrate + Cu^{2+}	EDTA	Protease	1.61
Protease	EDTA	Substrate	1.38

Mixtures were incubated 5 min at 37°C between additions. The final concentration of $CuCl_2$ was 25 μM and that of EDTA was 1 mM.

Both cations have high affinity for amino acids and might inhibit proteolysis by binding either to the protease or to the nonapeptide substrate. Since protease was present at micromolar concentration and peptide at millimolar, the protease was the more likely target. However, a metal–peptide complex could have been the inhibitory species. The protease was shown to be the actual target of copper inhibition by incubating either the peptide or the protease with 25 μM Cu^{2+} for 5 min. Then EDTA was added to 1 mM, followed by protease or peptide to provide a complete assay system. Preincubation of the peptide caused no inhibition of activity, whereas preincubation of the protease led to virtually complete loss of enzymatic activity (Table 2).

Inhibition is rapid, as shown in Fig. 1, and the concentration dependence of inhibition is plotted in Fig. 2. The affinity of binding has not been determined, so one cannot deduce the stoichiometry of binding from the concentration dependence. However, if one assumes that binding is very tight (stoichiometric), then the minimal requirement for inhibition is the binding of about one mercury cation per protease subunit or about two copper cations. Further, the binding of the first copper does not appear to affect activity.

Involvement of Cysteine Residues. Copper and especially mercury tend to bind to the sulfhydryl group of cysteine residues (23). The monomer of the HIV protease has two cysteine residues. Cys^{67} lies on the surface of the enzyme (26), while Cys^{95} participates in forming the dimer interface of the active protease (17). Treatment with DTT restored at least 80% of the activity of the mercury-inhibited enzyme (Table 3). Treatment of the copper-inhibited (25 μM Cu^{2+}) enzyme with DTT gave a variable, but low, recovery of 10–30%. When the copper concentration was increased to

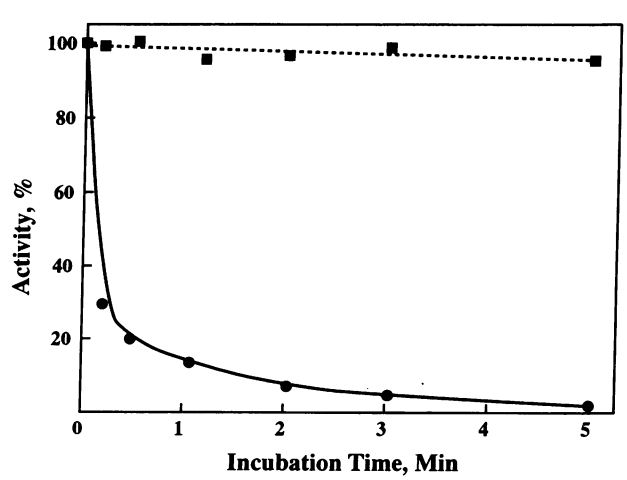

FIG. 1. Time course for inactivation of the wild-type protease by 25 μM $CuCl_2$. Protease (2.5 μM) was incubated with (●) or without (■) copper in 150 mM sodium acetate, pH 5.5/10% (vol/vol) glycerol at 37°C. The activity assay was initiated by transferring 10 μl of the incubation solution to a separate tube containing 2 μl of substrate in 150 mM sodium acetate with 6 mM EDTA.

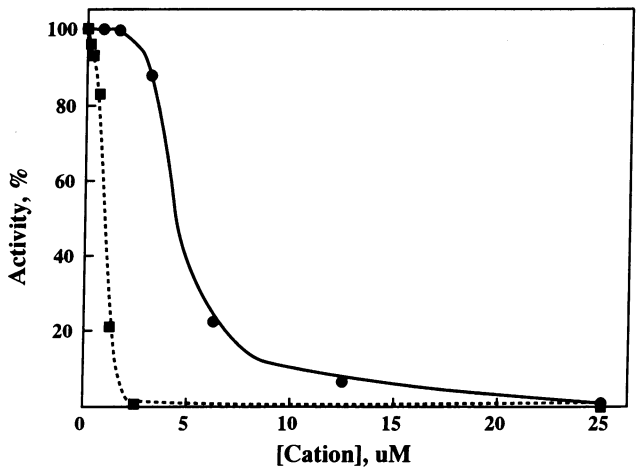

FIG. 2. Concentration dependence of copper-mediated (●) and mercury-mediated (■) inactivation of the wild-type protease. Protease (2.5 μM) was incubated with the cation for 5 min and then assayed as described in Fig. 1.

100 μM, DTT was unable to restore any activity. Regardless, the copper-treated enzyme had not been irreversibly inhibited. Refolding of the enzyme from 6 M guanidine hydrochloride restored activity to the same level as that of a control not treated with copper (data not shown). Recovery of activity from the control varied from 50% to 95%, probably due to variable losses of protein during the dialysis procedure.

We then examined the ability of one metal to displace the other from the enzyme by taking advantage of the observation that the mercury-inhibited enzyme was reactivated by treatment with DTT whereas the copper-inhibited enzyme was not. Neither metal appeared able to displace the other during the 2.5-min incubation (Table 3). This result suggests that the dissociation of bound metal is slow, consistent with the presumed high affinity of binding.

The role of cysteine residues in mediating copper inhibition might be probed with protease in which the cysteines were alkylated. Meek *et al.* (27) reported that the enzyme was catalytically inactive after carboxyamidomethylation, but they did not refold the derivatized enzyme. We found that the carboxyamidomethylated protease was inactive even after refolding, so that the effect of copper could not be evaluated. However, the essential role of cysteine was demonstrated by investigation of a variant protease that lacked cysteine residues. This variant enzyme was produced by solid-phase synthesis, with the two cysteine residues replaced by α-aminobutyric acid (17). It has virtually the same three-dimensional structure and specific activity as the wild-type protein (17, 21). Copper and mercury did not inhibit this variant protease (Table 4).

Table 3. Restoration of protease activity

First addition	Second addition	Third addition	Fourth addition	Activity, % control
None	None	None	DTT	100
None	None	DTT	None	100
Cu^{2+}	None	DTT	None	24
Hg^{2+}	None	DTT	None	82
Hg^{2+}	EDTA	Cu^{2+}	DTT	74
Hg^{2+}	Cu^{2+}	EDTA	DTT	78
Cu^{2+}	Hg^{2+}	EDTA	DTT	23
Cu^{2+}	Hg^{2+}	DTT	None	19

The protease was the wild type. One-microliter additions were made to 19 μl of enzyme solution (2.8 μM), followed by 2.5 min of incubation at 37°C. Stock solutions used for the additions were 500 μM $CuCl_2$, 500 μM $HgCl_2$, 200 mM DTT, and 20 mM EDTA. One microliter of water was substituted when the addition was "none." Activity was assayed for 20 min following the final incubation. The assay was initiated by adding substrate in 4 μl of 150 mM sodium acetate, pH 5.5/6 mM EDTA.

Table 4. Effect of oxygen and DTT on protease activity

Protease	Metal	DTT	Oxygen	Activity, % control
Wild-type	Cu^{2+}	−	−	<2
	Cu^{2+}	−	+	<2
Synthetic	−	+	+	98
	−	+	−	100
	Cu^{2+}	−	−	98
	Cu^{2+}	−	+	98
	Cu^{2+}	+	−	3
	Cu^{2+}	+	+	3
	Hg^{2+}	−	+	93

When added, the concentration of DTT was 10 mM. The final concentration of the wild-type protease was 2.5 μM and that of the synthetic protease (variant without cysteine) was 4.3 μM. The metal ion concentration was 25 μM (for wild-type protease) or 100 μM (for synthetic protease).

Effect of Oxygen. The discrepancy of recovery of activity upon DTT treatment was curious. It seemed likely that both copper and mercury inhibited the protease by direct binding. Addition of DTT to the inhibited enzyme should reverse the inhibition, if the reactions are relatively fast. However, it is known that iron or copper, thiol, and oxygen form a potent metal-catalyzed oxidizing system capable of inactivating many enzymes (28, 29). Thus, addition of DTT to the copper-treated enzyme might also cause oxidative modification of the protease.

This possibility was considered in experiments which examined the effects of oxygen and DTT on the inhibition of the wild-type protease and on the variant protease that lacked cysteine residues. Either anaerobic or aerobic incubation of the wild-type protease with copper caused loss of enzymatic activity (Table 4). Incubation of the synthetic protease with copper alone had no effect on proteolytic activity.

However, the addition of DTT revealed a copper-dependent inactivation that did not require either oxygen or cysteine (Table 4). That is, the synthetic protease was inactivated when incubated with copper and DTT, even in the absence of oxygen. The order of addition of copper and DTT was not important. No significant inactivation occurred when the synthetic protease was exposed to reduced DTT alone or to oxidized DTT with or without copper.

The extent of inactivation by copper and DTT varied with the concentration of copper (Fig. 3). This observation was consistent with several possible mechanisms of inactivation, the simplest of which would be direct inhibition by Cu^{+}, produced through reduction of Cu^{2+} by DTT. However, aerobic exposure of the protease to 100 μM CuCl for 10 min had no effect on proteolytic activity. Thus, neither the cuprous nor the cupric form of copper alone was capable of inhibiting the synthetic protease. However, addition of 10 mM DTT during the last 5 min of incubation with CuCl caused complete loss of activity, paralleling the results with $CuCl_2$.

DISCUSSION

The aspartic proteases, especially pepsin, have been studied in great detail. The active site of this class of proteases always contains two aspartic residues (19), and these could conceivably bind a cation. X-ray crystallographic studies of several aspartic proteases have employed heavy-metal derivatives (17, 18, 26) thus establishing the existence of cation binding sites in these proteins. The effect of metal ions on pepsin has been examined by many groups (14, 30–32). In general,

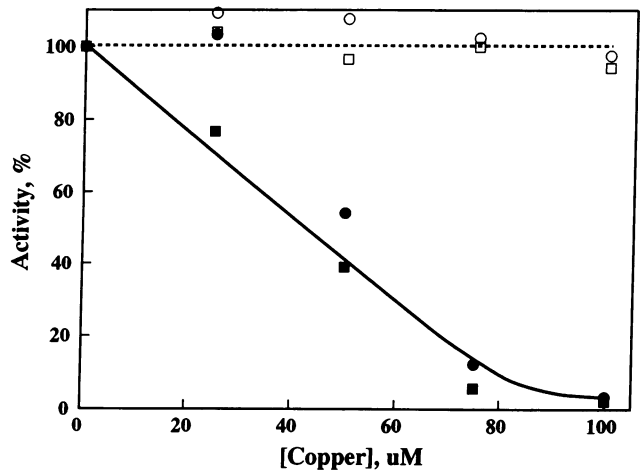

FIG. 3. Concentration dependence of copper-mediated inactivation of the synthetic protease. Enzyme (4.3 μM) was incubated with the cation for 5 min, after which either DTT or water was added. Following an additional 5 min of incubation, protease activity was assayed as described in Fig. 1. Incubations were aerobic (squares) or anaerobic (circles). Open symbols represent incubations without DTT and closed symbols with 10 mM DTT.

addition of copper to a pepsin/substrate mixture increases the rate of proteolysis, possibly through an effect on the substrate rather than the enzyme. One report suggested that inhibition of pepsin might be observable under certain conditions (32). However, Lundblad and Stein (14) specifically examined the effect of copper on pepsin and reported that there was no change in the catalytic activity. Copper does accelerate the inactivation of pepsin by diazo compounds (14, 30, 31), but this effect was shown to result from the action of copper on the inhibitor (14, 31).

Given these studies on the aspartic proteases, direct and specific inhibition of the HIV protease by copper was unexpected. In addition to direct inhibition, the binding of copper to the protease could render it susceptible to oxidative modification by metal-catalyzed oxidation, presumably by generation of free radicals at the copper binding site (29). Such site-specific oxidative modifications have been reported for many enzymes (10). However, since direct inhibition by copper is not oxygen-dependent, there is currently no evidence for a metal-catalyzed oxidative modification of the wild-type protease.

While inhibition by copper is not oxygen-dependent, it does require the presence of cysteine in the protease. The role of these cysteines in the enzyme is not defined, but they are certainly not essential for catalytic activity (5). Mutant enzymes lacking both Cys^{67} and Cys^{95} have normal catalytic activity (21). Moreover, the protease from HIV-2 strains lacks cysteine (33). Nevertheless, one should note that these cysteine residues are conserved in the protease from almost all HIV-1 strains sequenced thus far.

Cys^{67} is located on the surface of the protein, probably forming a weak hydrogen bond to the single histidine residue, located at position 69 (34). These two residues could well form a metal-binding site, although one cannot predict the effect that binding a cation may have upon enzyme activity. The other cysteine residue is located at position 95 and is intimately involved in the dimer interface of the two subunits (17). Disruption of the dimer interface might well cause loss of activity because dimer formation is essential for proteolytic activity (27, 35). The pK_a for copper inhibition is about 2.5 (data not shown), suggesting the involvement of a very acidic group. Cys^{95} faces the carboxyl-terminal Phe^{99} of the opposite subunit (17), and that free carboxyl group might account for the observed pK_a.

Inactivation of both the wild-type (two cysteines) and the synthetic (no cysteines) protease was dependent on the concentration of copper, consistent with several possible mechanisms of inactivation. Direct inhibition by Cu^+, produced upon reduction of Cu^{2+} by DTT, was a reasonable possibility but was ruled out by the finding that Cu^+ did not inhibit the synthetic enzyme. It remains possible that inhibition was mediated by a copper–DTT chelate. This mechanism deserves experimental investigation as it could lead to the development of inhibitors of the protease. Another potential mechanism of inactivation is the production of the thiyl radical of DTT during the reduction of copper. Direct study of this possibility will require use of electron paramagnetic resonance spectrometry.

Regardless of the mechanisms involved, identification of the copper binding sites may facilitate development of new types of protease inhibitors. These compounds would be designed to target the delivery of cation to a site responsible for inhibition of the protease. However, it is not known whether the DTT-dependent inactivation requires the binding of copper to the protease. This DTT-mediated inactivation might be relevant to the observation by Kalebic *et al.* (36) that glutathione and related thiols suppress HIV replication in a chronically infected cell line.

We conclude that the HIV protease is inhibited by the binding of either copper or mercury, presumably with the direct participation of a cysteine residue(s). However, the protease is also sensitive to inactivation by a copper and DTT, and cysteine residues are not required for this reaction. Oxygen is not required for either mode of inhibition.

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