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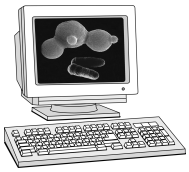

Selective cleavage of an azaGly peptide bond by copper(II). Long-range effect of histidine residue

Reda Mhidia and Oleg Melnyk*

Several reports have highlighted the interest of replacing Gly, a frequent amino acid within bioactive peptides, by azaGly (Agly) to improve their stability, activity or for the design of prodrugs. Because metal catalysis is increasingly used for tailoring peptide molecules, we have studied the stability of Agly peptides in the presence of metal ions. In this study, we show that Cu(II), unlike other metal ions such as Fe(II), Fe(III), Pd(II), or Pt(II), induces the cleavage of Agly peptides at room temperature and pH 7.3. The cleavage occurred in the absence of an anchoring His residue within the peptide but it was accelerated when this amino acid was present in the sequence. The influence of His residue on the cleavage rate was minimal when His and Agly were adjacent, whereas large effects were observed for distant His residues. The reaction between Cu(II) and Agly peptides induced the formation of Cu(I) species, which could be detected using bicinchoninic acid as a probe. The nature of products formed in this reaction allowed suggesting a mechanism for the Cu(II)-induced cleavage of Agly peptides.

Supporting information may be found in the online version of this article

Keywords: azapeptides; azaglycine; Cu(II); cleavage; histidine; long-range effect

Introduction

Azapeptides are peptide analogs in which the α-CH moiety of one or more amino acid residues in the peptide chain is replaced by a nitrogen atom [1,2]. The β-turn geometry induced by the aza residue [3–5] and the increased chemical stability of urea-type bonds compared to amide bonds might explain the better stability of azapeptides in biological medium relative to native peptides [6]. In the same way, Aza-amino acids were used for the design of Ser/Cys protease inhibitors [7]. Several reports have highlighted the interest of replacing Gly, a frequent amino acid within bioactive peptides, by azaGly (Agly) to improve their stability and/or activity [6,8–11]. Another interesting application of Agly residue consists in the design of prodrugs [12].

The synthesis of azapeptides is usually performed using solid-phase methods [13–15]. In particular, Agly residue can easily be introduced into peptides by reacting 1-Fmoc-2-oxoimidazole hydrazine with a peptidyl resin assembled using Fmoc/*tert*-butyl chemistry [8]. Another strategy relies on the site-specific ligation of unprotected peptide fragments. We have recently reported that Agly peptides can be assembled chemoselectively and without racemization using unprotected peptide fragments by silver catalyzed reaction of *C*-terminal peptide hydrazides with *N*-terminal phenylthiocarbonyl peptides [16]. *N*-terminal phenylthiocarbonyl peptides are readily synthesized using standard Fmoc/*tert*-butyl SPPS methods and commercially available phenylthiochloroformate [17]. This ligation method, which opens up the possibility to synthesize large Agly peptides, was also used for the lipopeptides synthesis featuring an Agly residue between the lipid and the peptide chain.

Agly peptides represent an important class of peptidomimetics. Their use in chemical or biological research requires characterization of their reaction or compatibility with well-known chemical reagents. The stability of Agly peptides in the presence of metal ions, and particularly of Cu(II), has never been reported before and is the subject of this communication.

Metal-catalyzed reactions, in particular copper-catalyzed 1,3-dipolar cycloadditions that deliver 1,2,3-triazoles from alkynes and azides, are increasingly used for the assembly of peptide scaffolds, the preparation of peptide-based conjugates or the linkage of peptides to surfaces. These reactions are catalyzed by Cu(I), which is typically generated *in situ* through reduction of Cu(II) ion by ascorbate. Copper(II)/ascorbate-mediated oxidative damage to peptides or proteins has been reported [18,19]. Cu(II)/ascorbate generates oxygen-derived free radical species which mainly cause a modification of histidine residues. Cu(II) as such is known to promote the cleavage of some peptide bonds [20,21]. For example, the peptide sequence $DK_{226}T_{227}HT$ within a recombinant human IgG1 is specifically cleaved by cupric ions between Lys226 and Thr227, i.e. at the second amide bond upstream from His residue (X–Y bond within X–Y-His sequence). Other metal ions such as Pd(II) and Pt(II) also have the property to act as artificial peptidases [22–27]. Overall, the cleavage of peptide bonds by metals such as Cu(II), Pd(II), Pt(II) occurs one or two residues upstream or downstream from the anchor residue (His or Met).

Agly peptides feature a 1,2-dicarbonyl hydrazine motif. The interaction or reaction of Cu(II) salts with hydrazine derivatives such as semicarbazide [28], 1,2-dialkyl semicarbazide [29], 1-phenyl

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Table 1. Agly peptides and control peptides used in this study. His residue is highlighted in bold

Entry	Peptide	Number	Yield (%)[a]
1	H-YKGAAA-Agly-GILKEPVGA-NH_2	**1a**	42
2	H-YKGAAAGILKEPVGA-NH_2	**1b**	35
3	H-YKGAA**H**-Agly-GILKEPVGA-NH_2	**2a**	38
4	H-YKGAA**H**GILKEPVGA-NH_2	**2b**	38
5	H-YKGA**H**A-Agly-GILKEPVGA-NH_2	**3a**	33
6	H-YKGA**H**AGILKEPVGA-NH_2	**3b**	41
7	H-YKG**H**AA-Agly-GILKEPVGA-NH_2	**4a**	40
8	H-YKG**H**AAGILKEPVGA-NH_2	**4b**	32
9	H-YGA-Agly-GILKEPV**H**GA-NH_2	**5a**	31
10	H-YGAGILKEPV**H**GA-NH_2	**5b**	53

[a] After RP-HPLC purification.

semicarbazide [30], or 1,2-diacyl hydrazines [31] has been reported. In this context, the study of Agly peptides stability in the presence of Cu(II) was worth studying. In this communication, we show that Cu(II) induces cleavage of Agly peptides. The influence of a His residue located upstream or downstream from the azaGly bond was examined as this amino acid is known to be an anchor residue for Cu(II). We describe a long-range effect of His residue on the Agly bond rate of cleavage, an effect which has not been reported before. The dependence of the rate and extent of cleavage on the pH or temperature was studied. Evidence is provided for the formation of Cu(I) during the cleavage reaction.

Materials and Methods

Synthesis of *N'*-[(9*H*-fluoren-9-ylmethoxy) Carbonyl] 1*H*-imidazole-1-carbohydrazide 6

(9*H*-fluoren-9-ylmethoxy)carbonylhydrazide (Fmoc-$NHNH_2$) was prepared as described elsewhere [32]. About 127.1 mg (0.5 mmol) of Fmoc-$NHNH_2$ and 81.0 mg (0.5 mmol) of CDI were dissolved in 5 ml of anhydrous DMF. The reaction mixture was stirred for 2 h at rt under argon to give reagent **6**. The resulting solution was used directly for the next stage.

Synthesis of Agly Peptides 1–5a

Peptide elongation

Peptide elongation was performed on Rink-PEG–PS resin (NovaSyn® TGR, 0.25 mmol/g, 0.38 g, 0.095 mmol) using standard Fmoc/*tert*-butyl chemistry on a Pioneer synthesizer (Applied Biosystems, Courtaboeuf, France) using TBTU/HOBt/DIEA activation in DMF. A capping step was performed after each coupling with Ac_2O/DIEA. At the end of the synthesis, the Fmoc protecting group of the last amino acid was removed using 20% piperidine in DMF. The resin was washed with DMF (4 × 2 min).

The solution of reagent **6** was then added to the peptidyl resin and the bead suspension was shaken for 20 h. The Fmoc protecting group was removed using 20% piperidine and the peptidyl resin was again subjected to SPPS as described above. Final deprotection and cleavage from the solid support were performed using 20 ml of TFA/anisole/TIS: 95/2.5/2.5 by volume for 1 h. The crude Agly peptide was precipitated in 200 ml of diethyl ether/pentane: 1/1 by volume, solubilized in 10 ml of deionized water and lyophilized. The crude Agly peptide was purified by RP-HPLC on a C18 Nucleosil column, 100 Å 5 µm, 10 × 300 mm, using a linear water/acetonitrile gradient containing 0.05% TFA by volume (6 ml/min, detection at 215 nm). Fractions containing the Agly peptide were collected and lyophilized. The overall yield of the synthesis varied from 33 to 42% depending on the sequence. All peptides displayed the expected molecular ions by MALDI-TOF MS (Table 1 for yields, and Supporting information for all peptide analytical data).

General Procedure for the Cleavage of Agly Peptides by Cu(II)

The Agly peptide (0.21 µmol, 10 mM) was dissolved in 20 µl of a 0.2-M Tris, HCl buffer containing 47 µg or 470 µg (0.21 or 2.10 µmol) of $CuBr_2$. The pH was adjusted with either Tris (for pH >9 sodium carbonate was used instead) or HCl. The reaction mixture was stirred at 22, 37, or 50 °C. Progression of the reaction was monitored by RP-HPLC on a 100 Å 5 µm C18 Nucleosil column using a linear water/acetonitrile gradient containing 0.05% TFA by volume (1 ml/min, detection at 215 nm). Peaks were collected and analyzed by MALDI-TOF MS (see Supporting information).

Other procedures and analytical data are presented in Supporting information.

Results and Discussion

Agly peptides and control peptides used in this study are presented in Table 1. A Tyr residue was inserted at the *N*-terminus to facilitate UV detection of *N*-terminal fragments by RP-HPLC. Agly peptides **1**–**5a** were synthesized as described in Scheme 1. Peptides were assembled using standard Fmoc/*tert*-butyl SPPS protocols on a Rink-poly(ethylene glycol)-polystyrene (PEG-PS) resin [33]. Incorporation of Agly residue was first performed by reacting Fmoc-$NHNH_2$ with CDI to produce 1-Fmoc-2-oxoimidazole hydrazine derivative **6**. Reaction of **6** with protected peptidyl resin **7** gave resin **8**, which was again subjected to Fmoc/*tert*-butyl SPPS. Final deprotection and cleavage in concentrated trifluoroacetic acid in the presence of appropriate scavengers furnished Agly peptides **1**–**5a** (Table 1 for the yields after RP-HPLC purification).

Agly peptides **1**–**5a** and control peptides **1**–**5b** were dissolved in Tris, HCl buffer (pH 10.6) in the presence of 10 equiv of $CuBr_2$ (Figure 1). Cu(II) induced the cleavage of Agly peptides **1**–**5a** but not of control peptides **1**–**5b** lacking the Agly residue. Peptide **1a** without His residue within its sequence was also cleaved in the presence of Cu(II), showing that the presence of an anchoring His residue is not required for the cleavage occurrence. However, data obtained for peptides **2**–**5a** (Figure 1) demonstrate that the presence of a His residue within the peptide sequence had a positive effect on the rate of cleavage. For peptides **2**–**4a** featuring a His residue upstream from Agly residue, the rate of cleavage was in order **2a** > **3a** > **4a**, i.e. increased by the distance between His and Agly residues. The rate of cleavage of Agly peptide **4a** was about twice higher than for Agly peptide **2a**. This positive effect on the rate of cleavage was also observed when His residue was located downstream from Agly residue. Indeed, the rate of cleavage of Agly peptide **5a** (Figure 1) was about ten times the rate of cleavage of peptide **1a**. In this case, His residue is separated from Agly residue by seven amino acids, showing a long-range effect of His residue on the Cu(II)-induced degradation of Agly peptide **5a**.

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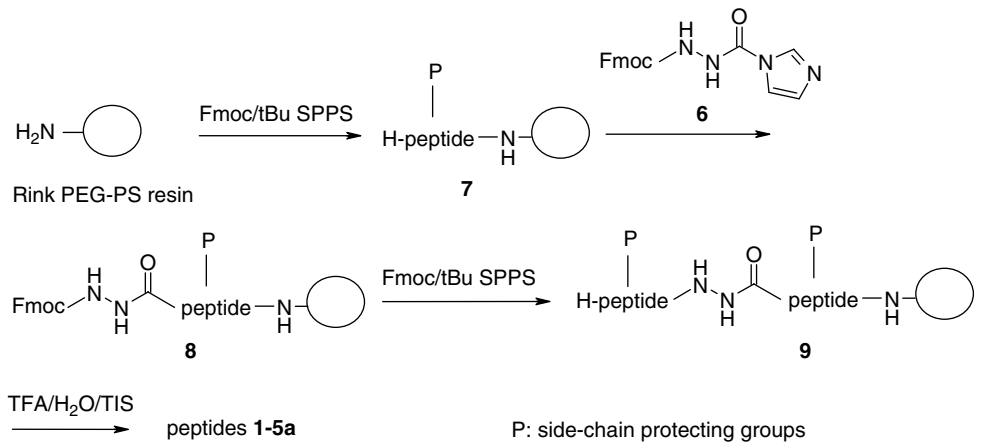


Scheme 1. Solid phase synthesis of Agly peptides **1**–**5a**.

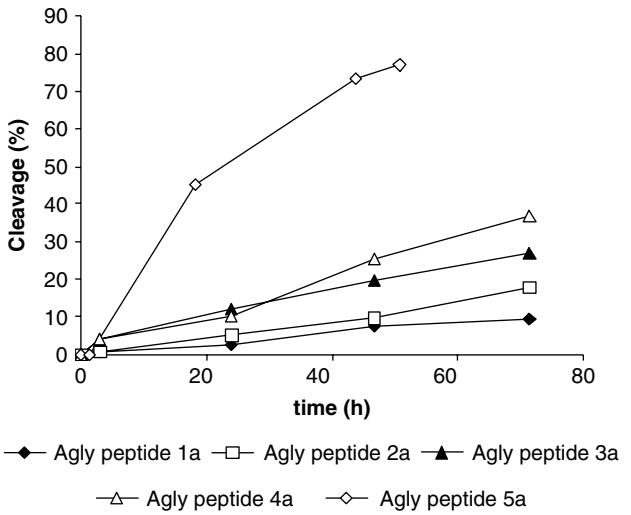
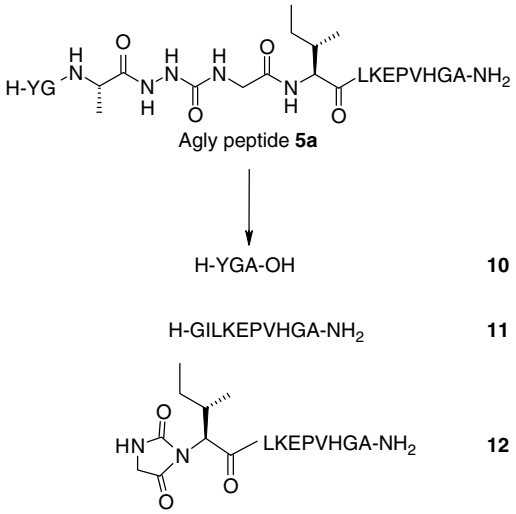


Figure 1. Cleavage of Agly peptides **1**–**5a** (10 mM) in the presence of $CuBr_2$ (100 mM) in Tris, HCl buffer (0.2 M, pH 10.6, 22 °C). Native peptides **1**–**5b** were stable using the same experimental conditions (data not shown). Percentage of cleavage was determined by RP-HPLC.



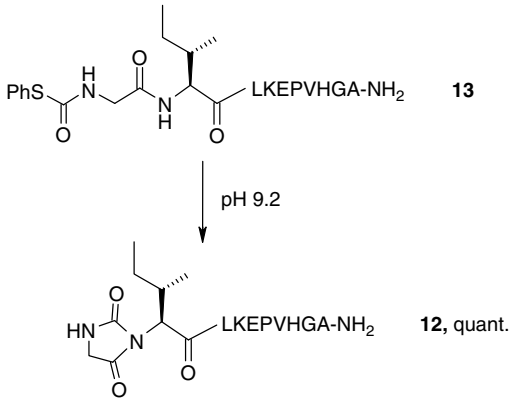
Scheme 2. Products formed in the Cu(II)-induced cleavage of Agly peptides. Peptide **5a** is used as an example.

Scheme 2 shows the products formed in the Cu(II)-induced cleavage of peptides **1**–**5a**, using Agly peptide **5a** as an example. Only three products were observed. The proposed structures are in accordance with MALDI-TOF-post-source decay analyses (PSD). In addition, peptide **12** formed in the Cu(II)-induced cleavage of peptide **5a** by RP-HPLC and MALDI-TOF-PSD was similar to hydantoin peptide prepared by treating phenylthiocarbonyl peptide **13** with base (Scheme 3).

The pH-dependence of the reaction at 22 °C was studied using Agly peptide **5a** (Figure 2, Table 2). The cleavage reaction increased significantly with the reaction mixture pH. At pH 11.0, the cleavage reaction was about seven times faster than at pH 7.3. Allen *et al*. have studied the specific cleavage of histidine-containing peptides by Cu(II) [21]. pH-dependence of the Cu(II)-induced cleavage in Tris, HCl buffer showed a bell-shape curve with a maximum at pH 7. Thus, pH-dependence of the Cu(II)-induced cleavage of Agly peptide **5a** differs markedly from what was observed for native peptides. The influence of the temperature on the rate of cleavage of Agly peptide **5a** was also examined (Figure 3, Table 2). Increasing the temperature from 22 °C to 50 °C at pH 7.3 had a significant effect on the rate of cleavage and led to a decrease of half-life from 180 h to only 7 h.

Finally, we have examined the stability of Agly peptide **5a** in the presence of other metal ions ($FeSO_4$, $FeCl_3$, $PdCl_2$, $Pd(en)Cl_2$, $PtCl_2$, $Pt(en)Cl_2$, $CuSO_4$, $CuBr_2$) in pH 7.3 Tris, HCl buffer at room temperature for up to 48 h of incubation. The reaction mixtures were monitored by RP-HPLC. Neither degradation nor complex formation was observed in the presence of $FeSO_4$ or $FeCl_3$. Complex formation occurred with all Pd(II) or Pt(II) complexes, in particular $PdCl_2$ or $PtCl_2$, but no cleavage of Agly peptide **5a** was observed. Cleavage occurred only in the presence of $CuSO_4$ or $CuBr_2$, the latter being more efficient for inducing the cleavage of Agly peptide **5a**. $PdCl_2$ and $PtCl_2$ were also unable to induce the cleavage of Agly peptide **5a** at 50 °C for up to 24 h of incubation (data not shown).

Several mechanisms might be involved in the Cu(II)-induced cleavage of Agly peptides. First, the metal might catalyze hydrolysis of amide or urea-type bonds. Bivalent metal ions such as Cu(II), Pd(II), or Pt(II) can indeed catalyze hydrolysis of peptide bonds, which are extremely nonreactive toward hydrolysis under standard conditions [21,23,24,26,27]. Usually, the metal ion is captured by an anchoring residue such as His or Met, which promotes hydrolysis



Scheme 3. Synthesis of hydantoin peptide **12** by treating phenylthiocarbonylpeptide **13** with base.

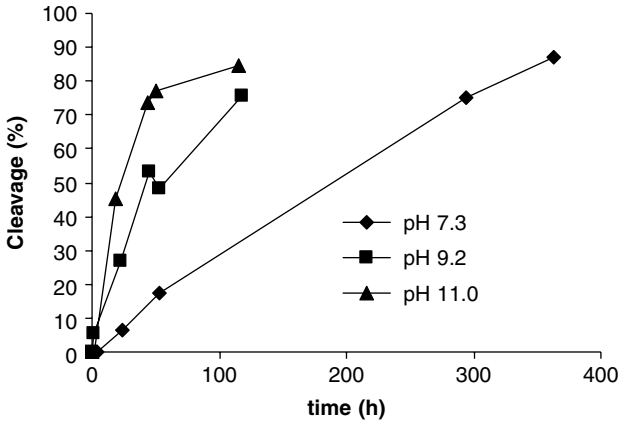


Figure 2. Influence of the Tris, HCl buffer pH on the cleavage rate of peptide **5a**. Control peptide **5b** was stable using the same experimental conditions (data not shown). Percentage of cleavage was determined by RP-HPLC.

of adjacent peptide bonds. The anchored metal complex can bind the oxygen atom of the scissile amide bond, thus activating the carbonyl group toward the external attack by water molecule. Another possible mechanism is the internal attack of the scissile amide bond by an anchored aqua ligand. Both mechanisms are known to promote the cleavage of amide bonds close to the anchoring residue, typically the first or second peptide bond upstream or downstream from the anchoring residue.

The absence of Agly peptide **5a** cleavage in the presence of Pd(II) or Pt(II) complexes at room temperature can be explained by the mild conditions used. Indeed, Pd(II) or Pt(II) complexes were proved to act as synthetic peptidases at acidic pH under thermal or

Table 2. Half-life of Agly peptide **5a** (10 mM) as a function of pH or temperature in the presence of $CuBr_2$ (100 mM) in Tris, HCl 0.2 M buffer

Temperature (°C)	pH	$t_{1/2}$ (h)
22	7.3	180
22	9.2	48
22	11.0	25
37	7.3	32
50	7.3	7

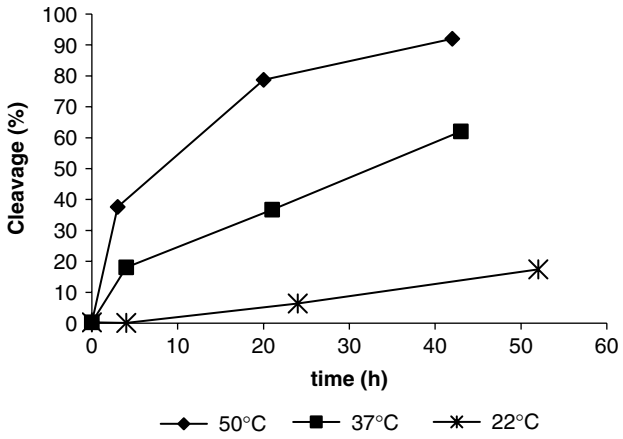


Figure 3. Cleavage of Agly peptides **5a** (10 mM) in the presence of $CuBr_2$ (100 mM) in Tris, HCl buffer (0.2 M, pH 7.3) at 22, 37, or 50 °C. $PdCl_2$ and $PtCl_2$ were unable to induce the cleavage of Agly peptide **5a** at 50 °C for up to 24 h of incubation (data not shown). Percentage of cleavage was determined by RP-HPLC.

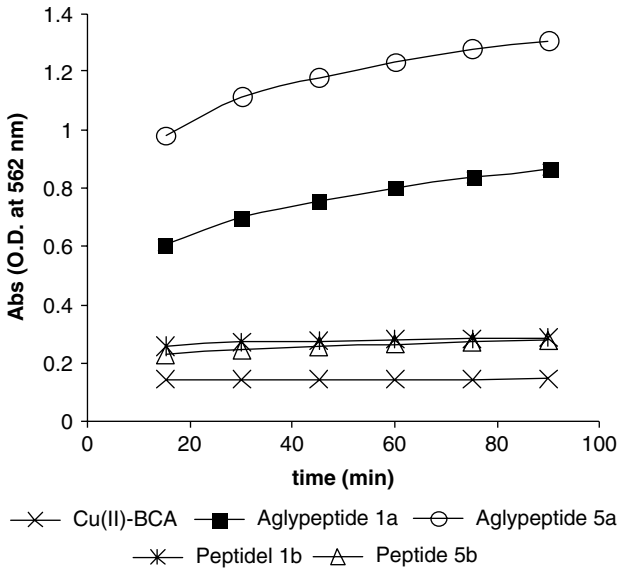


Figure 4. Evidence for formation of Cu(I) species in the Cu(II)-induced cleavage of Agly peptides. Peptides **1**,**5a** and **1**,**5b** (1 mM) were reacted with $CuBr_2$ in pH 9.0 Tris, HCl buffer containing BCA (2.2 mM). BCA forms a stable complex with Cu(I) which absorbs at 562 nm.

microwave heating [22,26]. The stability of control peptides **1**–**5b** in the presence of Cu(II) at room temperature might be due to both the mild experimental conditions used in this study and the peptide sequence chosen. The rate of cleavage of peptide bonds by Cu(II) is indeed known to be highly dependent on peptide sequence, in other words, the presence of a His residue in the sequence is not a sufficient element to induce significant cleavage rates [21]. Moreover, as for Pd(II) or Pt(II) complexes, significant rates of cleavage for Cu(II)-sensitive His-X peptide bonds were observed under thermal heating, typically at 50 °C [21].

In this work, the presence of a histidine residue favored the cleavage reaction of Agly peptides (Agly peptides **2**–**5a**, Figure 1). However, unlike what has been observed previously for metal-based synthetic peptidases, the largest effect was observed for distant His residues separated from Agly residue by more than

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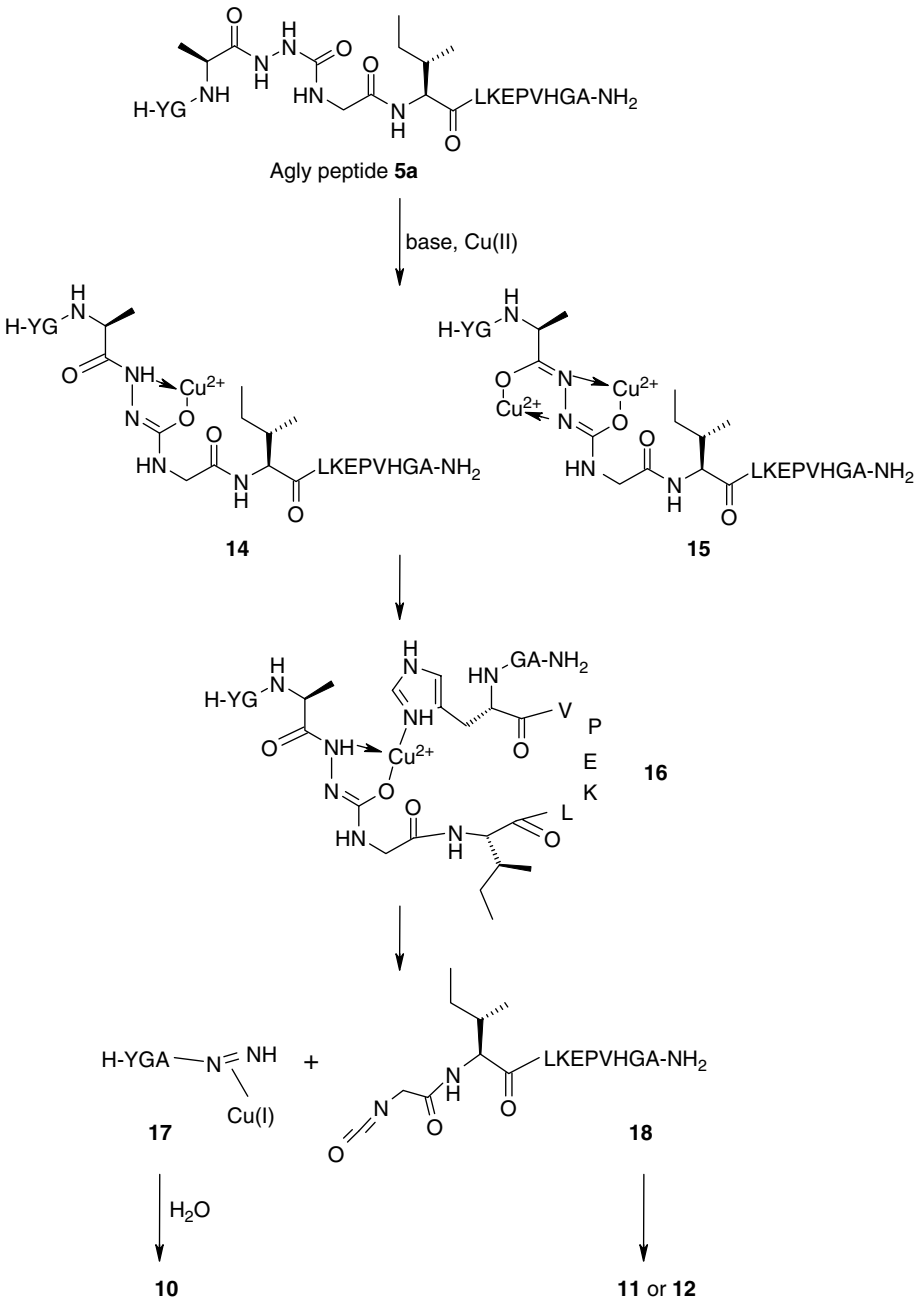

Scheme 4. Hypothetical mechanism for the Cu(II)-induced cleavage of Agly peptides. Peptide **5a** is used as an example.

two amino acids (Agly peptides **4a** and **5a**). These data, combined with the fact that cleavage of Agly peptides occurred as well in the absence of an anchoring His residue (Agly peptide **1a**, Figure 1), suggest that the catalysis of amide bond hydrolysis by Cu(II) is not the main pathway involved in Cu(II)-induced cleavage of Agly peptides.

An alternative mechanism might involve the potential oxidation of Agly moiety by Cu(II). Indeed, interaction and/or reaction of hydrazino compounds with Cu(II) have been thoroughly studied [34]. For example, oxidation of 1-aryl-2-acylhydrazines by Cu(II) into corresponding aryl diazene is on the basis of the safety-catch linker developed by Millington *et al*. [35]. In this reaction, Cu(II) is reduced to Cu(I) which is then oxidized to Cu(II) by molecular oxygen, making the reaction catalytic in Cu(II). *N*,*N*′-diacylhydrazines are less susceptible to oxidation into corresponding diazenes and require stronger oxidants such as fuming nitric acid, mercuric chloride, or silver nitrate combined with iodine or bromine or *N*-bromosuccinimide [34]. Reaction of semicarbazide (SC) with Cu(II) affords stable complexes of types $Cu(SC)_2X_2$ and $Cu(SC)_2X_2$, (X = Cl, Br, NO_3, ClO_4, or $1/2SO_4$), whereas reaction of 1-phenylsemicarbazide with copper leads to oxidation of the hydrazino group and formation of azo derivatives [30]. Little attention has been devoted to reaction of 1-oxosemicarbazide derivatives with Cu(II) or other metal ions.

Oxidation of Agly residue by Cu(II) might result in the formation of Cu(I). To test the potential formation of Cu(I) species during the Cu(II)-induced cleavage of Agly peptides, Agly peptides **1a** and **5a** and the corresponding control peptides **1b** and **5b** were reacted

with Cu(II) as usual in the presence of bicinchoninic acid (BCA). BCA is a sensitive, stable, and highly specific reagent for Cu(I) [36]. It forms a BCA/Cu(I): 2/1 complex that absorbs strongly at 562 nm. Thus, absorbance of the reaction mixture at 562 nm reveals the *in situ* formation of Cu(I) species. Figure 4 shows that absorbance of the reaction mixture at 562 nm increased significantly for peptides **1a** and **5a** but not for control peptides **1b** and **5b** or $CuBr_2$/BCA mixture without peptide. Interestingly, absorbance at 562 nm was significantly higher for peptide **5a** than for peptide **1a**, in accordance with the higher rate of cleavage observed for the former due to the long-range effect of His residue. This experiment showed the formation of Cu(I) species during the Cu(II)-induced cleavage of Agly peptides (Scheme 4). Thus, the reaction could first involve formation of type **14** or **15** complexes. A type **14** complex was proposed as an intermediate in the reaction between 1-phenylsemicarbazide and Cu(II) [30]. Formation of the type **15** complex can be proposed as well because similar complexes are formed when 1,2-diacylhydrazines are reacted with Cu(II) in the presence of a base [31]. Formation of these complexes is probably favored by the ease of 1,2-diacylhydrazines deprotonation (p*K*a 10.9 for 1,2-diacetylhydrazine) [37]. By comparison, the p*K*a for deprotonation of the amide group in *N*-methylacetamide, a model for the peptide bond, has been estimated to 18 [38]. This p*K*a explains why the biuret reaction between polypeptides and Cu(II) is performed at high pH, typically in 0.1 M aqueous NaOH [39]. Moreover, deprotonation of the peptide bond is facilitated by coordination of Cu(II) to the amide oxygen [40]. Precoordination of Cu(II) to the Agly moiety might lower the p*K*a of hydrazine nitrogens similarly allowing the reaction to proceed at pH 7.3 (Figure 2).

The biuret complex between polypeptides and Cu(II) involves deprotonation of four peptide bonds, resulting in the binding of four imidic nitrogens to Cu(II) [41]. The mild experimental conditions used in this work do not favor such complex formation because peptides **1**–**5b** were unable to react with Cu(II) (Figures 2 and 3). Assuming that Cu(II) binds to a deprotonated form of the 1,2-dioxohydrazine moiety of Agly peptides **1**–**5a** only and that no deprotonated peptide bonds participate in the ligand shell of Cu(II), there is place for the imidazole ring of His residue to bind Cu(II) and stabilize the Cu(II)–Agly peptide complex (complex **16**, Scheme 4). The conformational constrains imposed at the Agly moiety in complex **14** or **15** are expected to disfavor the participation of neighboring His residues as observed in this work and might explain the long-range effect of His residue on the rate of cleavage. The next step is the oxidation of the hydrazo moiety by Cu(II), which has been demonstrated to generate Cu(I) species (Figure 4). Formation of isocyanate **18** is proposed based on the study of Heyman *et al*. [29]. In this report, the oxidation of alkyl semicarbazides with $CuCl_2$ was shown to produce isocyanates by the elimination of the diazene group. Hydrolysis or cyclization of isocyanate **18** is expected to yield peptides **11** and **12**, respectively, whereas hydrolysis of diazene **17** is expected to produce peptide **10**.

In conclusion, we show that Cu(II) but not other metal ions such as Fe(II), Fe(III), Pd(II), or Pt(II) induces the cleavage of Agly peptides at room temperature and pH 7.3. The cleavage occurs in the absence of an anchoring His residue within the peptide but it is accelerated when this amino acid is present in the sequence. The distance between the His and Agly residues has an important impact on the rate of cleavage. The highest rates were observed for His residues distant from Agly moiety. The reaction between Cu(II) and Agly peptides leads to the formation of Cu(I) species, which could be detected using BCA as a probe. A cleavage mechanism is proposed based on previous reports and on data presented. Besides the usefulness of this report for those involved in azapeptide synthesis, Agly peptides might be used as cleavage linkers whose cleavage is triggered by the presence of Cu(II).

Acknowledgements

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Supporting information

Supporting information may be found in the online version of this article.

References

1 Gante J. Peptide and azapeptide synthesis by means of a new *N*-activated amino acid derivative. *Chem. Ber.* 1966; **99**: 1576–1579.
2 Gante J. Azapeptides. *Synthesis-Stuttgart* 1989; **17**: 405–413.
3 Lee HJ, Park HM, Lee KB. The beta-turn scaffold of tripeptide containing an azaphenylalanine residue. *Biophys. Chem.* 2007; **125**: 117–126.
4 Didierjean C, Del Duca V, Benedetti E, Aubry A, Zouikri M, Marraud M, Boussard G. X-ray structures of aza-proline-containing peptides. *J. Pept. Res.* 1997; **50**: 451–457.
5 Zouikri M, Vicherat A, Aubry A, Marraud M, Boussard G. Azaproline as a beta-turn-inducer residue opposed to proline. *J. Pept. Res.* 1998; **52**: 19–26.
6 Dutta AS, Furr BJ, Giles MB, Morley JS. Synthesis and biological activity of alpha-azapeptides: alpha-aza-analogues of luteinizing hormone releasing hormone. *Clin. Endocrinol. (Oxf)* 1976; **5**: (Suppl): 291S–298S.
7 Din H, Gray CJ, Ireson JC, McDonald R. Inhibition of trypsin by *t*-butyloxycarbonyl-alpha-aza-(4-aminophenyl)alanine phenyl ester. *J. Chem. Technol. Biotechnol.* 1991; **50**: 181–189.
8 Bondebjerg J, Fuglsang H, Valeur KR, Kaznelson DW, Hansen JA, Pedersen RO, Krogh BO, Jensen BS, Lauritzen C, Petersen G, Pedersen J, Naerum L. Novel semicarbazide-derived inhibitors of human dipeptidyl peptidase I (hDPPI). *Bioorg. Med. Chem.* 2005; **13**: 4408–4424.
9 Verhelst SH, Witte MD, Arastu-Kapur S, Fonovic M, Bogyo M. Novel aza peptide inhibitors and active-site probes of papain-family cysteine proteases. *ChemBioChem* 2006; **7**: 943–950.
10 Weber D, Berger C, Eickelmann P, Antel J, Kessler H. Design of selective peptidomimetic agonists for the human orphan receptor BRS-3. *J. Med. Chem.* 2003; **46**: 1918–1930.
11 Steinmetzer T, Schweinitz A, Kunzel S, Wikstrom P, Hauptmann J, Sturzebecher J. Structure–activity relationships of new NAPAP-analogs. *J. Enzyme Inhib. Med. Chem.* 2002; **17**: 241–249.
12 Wipf P, Li W, Adeyeye CM, Rusnak JM, Lazo JS. Synthesis of chemoreversible prodrugs of ara-C with variable time-release profiles. Biological evaluation of their apoptotic activity. *Bioorg. Med. Chem.* 1996; **4**: 1585–1596.
13 Liley M, Johnson T. Solid phase synthesis of azapeptides utilising reversible amide bond protection to prevent hydantoin formation. *Tetrahedron Lett.* 2000; **41**: 3983–3985.
14 Melendez RE, Lubell WD. Aza-amino acid scan for rapid identification of secondary structure based on the application of *N*-Boc-aza(1)-dipeptides in peptide synthesis. *J. Am. Chem. Soc.* 2004; **126**: 6759–6764.
15 Boeglin D, Lubell WD. Aza-amino acid scanning of secondary structure suited for solid-phase peptide synthesis with fmoc chemistry and aza-amino acids with heteroatomic side chains. *J. Comb. Chem.* 2005; **7**: 864–878.

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16 Ollivier N, Besret S, Blanpain A, Melnyk O. Silver-catalyzed azaGly ligation. Application to the synthesis of azapeptides and of lipid-peptide conjugates. *Bioconjug. Chem.* 2009; **20**: 1397–1403.
17 Besret S, Ollivier N, Blanpain A, Melnyk O. Thiocarbamate-linked peptides by chemoselective peptide ligation. *J. Pept. Sci.* 2008; **14**: 1244–1250.
18 Uchida K, Kawakishi S. Site-specific oxidation of angiotensin I by copper(II) and L-ascorbate: conversion of histidine residues to 2-imidazolones. *Arch. Biochem. Biophys.* 1990; **283**: 20–26.
19 Uchida K, Kawakishi S. 2-Oxo-histidine as a novel biological marker for oxidatively modified proteins. *FEBS Lett.* 1993; **332**: 208–210.
20 Smith MA, Easton M, Everett P, Lewis G, Payne M, Riveros-Moreno V, Allen G. Specific cleavage of immunoglobulin G by copper ions. *Int. J. Pept. Protein Res.* 1996; **48**: 48–55.
21 Allen G, Campbell RO. Specific cleavage of histidine-containing peptides by copper(II). *Int. J. Pept. Protein Res.* 1996; **48**: 265–273.
22 Dutca LM, Ko KS, Pohl NL, Kostic NM. Platinum(II) complex as an artificial peptidase: selective cleavage of peptides and a protein by cis-$[Pt(en)(H_2O)_2]^{2+}$ ion under ultraviolet and microwave irradiation. *Inorg. Chem.* 2005; **44**: 5141–5146.
23 Milovic NM, Dutca LM, Kostic NM. Transition-metal complexes as enzyme-like reagents for protein cleavage: complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ as a new methionine-specific protease. *Chemistry* 2003; **9**: 5097–5106.
24 Milovic NM, Dutca LM, Kostic NM. Combined use of platinum(II) complexes and palladium(II) complexes for selective cleavage of peptides and proteins. *Inorg. Chem.* 2003; **42**: 4036–4045.
25 Milovic NM, Kostic NM. Interplay of terminal amino group and coordinating side chains in directing regioselective cleavage of natural peptides and proteins with palladium(II) complexes. *Inorg. Chem.* 2002; **41**: 7053–7063.
26 Milovic NM, Kostic NM. Palladium(II) complexes, as synthetic peptidases, regioselectively cleave the second peptide bond "upstream" from methionine and histidine side chains. *J. Am. Chem. Soc.* 2002; **124**: 4759–4769.
27 Milovic NM, Kostic NM. Palladium(II) complex as a sequence-specific peptidase: hydrolytic cleavage under mild conditions of X-Pro peptide bonds in X-Pro-Met and X-Pro-His segments. *J. Am. Chem. Soc.* 2003; **125**: 781–788.
28 Campbell MJ, Grzeskowiak R. Some copper(II) complexes of semicarbazide. *J. Inorg. Nucl. Chem.* 1968; **30**: 1865–1871.
29 Heyman M, Bandurco VT, Snyder JP. Azoalkane synthesis by direct oxidation of semicarbazides with copper halide. *Chem. Commun.* 1971; 297–298.
30 El-Dessouky MA, Amira MF, Abu El Amayem MS, Iskander MF. Coordination compounds of hydrazine derivatives with transition metals-IX. The reaction of 1-semicarbazide with copper(II) salts. *J. Inorg. Nucl. Chem.* 1976; **38**: 463–469.
31 Qing-bao S, Zhong-lin L, Xiao-li W, Yong-xiang M. *N*-Ferrocenylcarbonyl-*N*′-benzoyhydrazine and its transition metal(II) complexes. *Transition Met. Chem.* 1994; **19**: 503–505.
32 Zhang RE, Cao YL, Hearn MW. Synthesis and application of Fmoc-hydrazine for the quantitative determination of saccharides by reversed-phase high-performance liquid chromatography in the low and subpicomole range. *Anal. Biochem.* 1991; **195**: 160–167.
33 Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res.* 1990; **35**: 161–214.
34 Newbold BT. Oxidation and synthetic uses of hydrazo, azo and azoxy compounds. In *The Chemistry of the Hydrazo, Azo and Azoxy Groups*, Patai S (ed.). John Wiley: London, 1975; 541–597.
35 Millington CR, Quarrell R, Lowe G. Aryl hydrazides as linkers for solid phase synthesis which are cleavable under mild oxidative conditions. *Tetrahedron Lett.* 1998; **39**: 7201–7204.
36 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985; **150**: 76–85.
37 Radushev AV, Chekanova LG, Gusev VY, Sazonova EA. Determination of hydrazides and 1,2-diacylhydrazines of aliphatic carboxylic acids by conductometric titration. *J. Anal. Chem.* 2000; **55**: 445–448.
38 Eriksson MA, Hard T, Nilsson L. On the pH dependence of amide proton exchange rates in proteins. *Biophys. J.* 1995; **69**: 329–339.
39 Legler G, Muller-Platz CM, Mentges-Hettkamp M, Pflieger G, Julich E. On the chemical basis of the Lowry protein determination. *Anal. Biochem.* 1985; **150**: 278–287.
40 Pushie MJ, Rauk A. Computational studies of Cu(II)[peptide] binding motifs: Cu[HGGG] and Cu[HG] as models for Cu(II) binding to the prion protein octarepeat region. *J. Biol. Inorg. Chem.* 2003; **8**: 53–65.
41 Saurwein J. The biuret reactions of the hexapeptide, pentaglycylglycine, and of the heptapeptide, hexaglycylglycine. *J. Am. Chem. Soc.* 1937; **59**: 2177–2178.

