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Plasmid Relaxation Induced by Copper Metalated Diglycine Conjugates under Heterogeneous Reaction Conditions

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Abstract—This paper reports synthesis and plasmid modification activities of a new class of insoluble copper-metalated diglycine conjugates, containing aliphatic linkers of varying length. Besides providing significant rate enhancement for model phosphate ester cleavage, these constructs also displayed efficient supercoiled plasmid scission, in the absence of co-oxidants, under heterogeneous catalytic conditions.

Gly-Gly-His (GGH) tripeptide is a Cu(II) and Ni(II) binding motif present in serum albumin, neuromedins C and K, human sperm protamine P2a and histatins, which aids transport of small molecules and metal ions.[1] Metal ion coordination ability of GGH motif has been harnessed to engineer semi-synthetic nucleases when attached to larger protein fragments[2] and efforts have been invested to discover GGH variants based on bio-combinatorial approaches.[3] In another approach, conjugation of this tripeptide to an array of small molecules has generated numerous DNA binding and cleavage reagents.[4] Another application of this motif relates to oxidative protein cross-linking in the presence of exogenously added co-oxidants, thus making it a useful biochemical tool.[5]

We have truncated the GGH motif to design a diglycine conjugate (**1**) and its catalytic assistance towards cleavage of non-natural phosphate esters (P-esters) has been reported.[6] This study confirmed that C-terminal histidine could be pruned and consequently, a Cu(II) metalated bis-conjugate (**1**) effectively catalyzed the cleavage of model P-esters: *p*-nitrophenyl phosphate (pNPP), bis-(*p*-nitrophenyl)phosphate (bNPP) and 2-hydroxypropyl-*p*-nitrophenyl phosphate (hNPP), while conforming to the classical Michaelis–Menten kinetic profile. These results provided us an impetus to further investigate structure–nucleolytic activity of analogous, copper-metalated diglycine conjugates, by employing variable length diaminoalkyl linkers, for plasmid modification.

Herein, we present synthesis, characterization and metalation of four new conjugates **2**–**5** and their catalytic potential towards the cleavage of model P-esters and a natural susbtrate, supercoiled plasmid DNA (pBR322). As these metalloconjugates were insoluble in common organic solvents and in aqueous buffers, the present study is a unique example of heterogeneous catalysis of plasmid nicking reaction.

Four diglycine conjugates **2**–**5** were prepared by reacting *N*-benzoylglycylglycine-*p*-nitrophenyl ester with diaminoalkanes such as 1,2-diaminoethane **2**, 1,4-diamino butane **3**, 1,8-diamino-3,6-dioxa octane **4** or 1,4-bis-(3-aminopropoxy)butane **5** (Scheme 1)[7] and metalated with $Cu(OAc)_2 \cdot H_2O$.[8] The extent of copper incorporation was determined by atomic absorption spectroscopy and was reconfirmed by spectrophotometric estimation.[9] Amount of copper incorporated was reasonably close for conjugates **2**–**5** and values were found to be 272, 278, 282 and 332 mg/g, respectively. These values suggest that there are approximately 2.5 copper atoms per bis-dipeptide ligand. It is not possible to determine the crystal structures to verify exact stoichiometry and binding mode due to the insoluble nature of these conjugates.

Pseudo-first-order rate constants (k_{obs}) were determined for model P-ester cleavage assisted by metalloconjugates **2**–**5** (Table 1).[10] Rate accelerations reaching 10^6-fold over the uncatalyzed reaction[11] were observed for bNPP.

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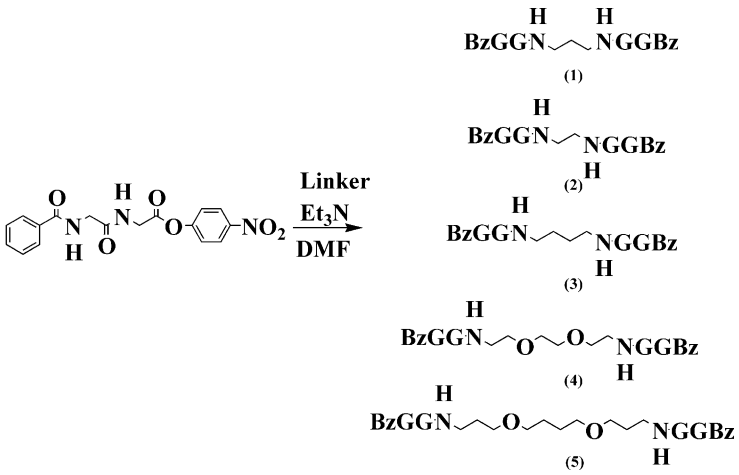

Scheme 1. Synthesis of diglycine conjugates **1**–**5**.

Corresponding k_{obs} for a monophosphate ester substrate, pNPP and an RNA model, hNPP, are mentioned in Table 1. Curiously, all bis-diglycine conjugates exhibited similar rate enhancements thereby indicating a lack of linker length effect. This effect could also be partly ascribed to the fact that all metalated conjugates, except **5**, possess similar copper content. Once again, the insoluble nature of these constructs play a role in imparting similar catalytic features to **5**, despite the fact that it contains more Cu^{2+} than other compounds (**2**–**4**). All unmetalated conjugates were unable to catalyze the cleavage of model P-esters.

Temperature dependence of the cleavage reactions was studied by determining the pseudo-first-order rate constants at four different temperatures (Table 2). As expected, an increase in rate was observed for every 10 °C rise in the temperature. The energy of activation was found to be 19.75 kcal mol^{-1} and the frequency factor was 4.74×10^{10} min^{-1}, as determined from the $-\ln k_{obs}$ versus 1/T plot (Fig. 1). These results confirm high stability of these complexes as the catalysts were able to withstand a temperature of 60 °C, while still maintaining catalytic assistance for pNPP hydrolysis.

In reaction arrest experiment, conjugate **3** was incubated with bNPP (4 mM) for 13 h and the release of *p*-nitrophenolate anion was recorded at regular intervals. After this period, **3** was filtered and change in the absorbance was further recorded over 75 h. As evident from Figure 2, cleavage reaction completely aborted soon after filtration of **3** suggesting a crucial role for coordinated copper ions, and lack of the release of copper ions from the conjugate leading to hydrolysis.

Table 1. Pseudo-first-order rate constants for pNPP, bNPP, hNPP hydrolysis catalyzed by conjugates **2**–**5**[a]

Substrate	Conjugate	k_{obs} (min^{-1})	k_{uncat} (min^{-1})	k_{rel}
pNPP[b]	**2**	6.79×10^{-4}	4.92×10^{-7}	1.38×10^{3}
	3	7.48×10^{-4}		1.52×10^{3}
	4	5.45×10^{-4}		1.11×10^{3}
	5	8.08×10^{-4}		1.64×10^{3}
bNPP[c]	**2**	1.03×10^{-3}	7.8×10^{-10}	1.33×10^{6}
	3	0.86×10^{-3}		1.11×10^{6}
	4	0.80×10^{-3}		1.13×10^{6}
	5	0.54×10^{-3}		0.70×10^{6}
hNPP[c]	**2**	1.62×10^{-3}	1.98×10^{-6}	0.81×10^{3}
	3	1.60×10^{-3}		0.81×10^{3}
	4	1.89×10^{-3}		0.95×10^{3}
	5	1.48×10^{-3}		0.75×10^{3}

[a]Hydrolytic reactions were performed in 0.01 M *N*-ethylmorpholine buffer prepared in 50% aqueous methanol.
[b]Concentration of pNPP was 7 mM, weight of conjugates **2**–**5** were 1 mg/5 mL, corresponding to 0.87, 0.85, 0.88, 1.04 mM of Cu, if all conjugates were to be completely soluble in buffer solution (pH 8.0, 40 °C).
[c]Concentrations of bNPP and hNPP were **10** and 3 mM, respectively and weight of the conjugates **2**–**5** were 1 mg/3 mL, corresponding to 1.46, 1.43, 1.48, 1.7 mM of Cu, if conjugates were to be completely soluble in buffer (pH 7.5, 40 °C for bNPP, 30 °C for hNPP).

Table 2. Pseudo-first-order constants[a] for pNPP hydrolysis at variable temperatures assisted by metalloconjugate **2**

Temperature (°C)	k_{obs} (min^{-1})
30	0.31×10^{-3}
40	0.68×10^{-3}
50	2.77×10^{-3}
60	5.30×10^{-3}

[a]Hydrolytic reactions were performed 0.01 M of *N*-ethylmorpholine buffer (5 mL) prepared in 50% aqueous methanol, concentration of pNPP was 7 mM and weight of the conjugate was 1 mg/5 mL, corresponding to 0.87 mM of Cu if the conjugate was to be completely soluble in buffer.

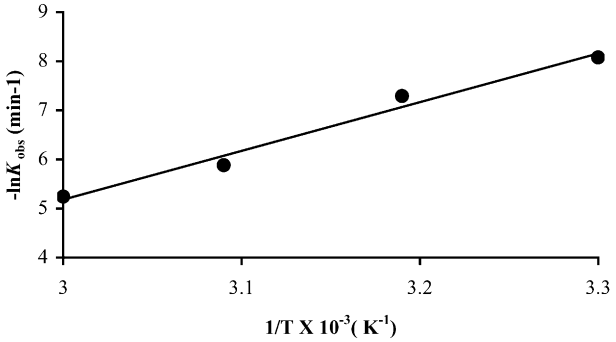

Figure 1. Plot of $-\ln K_{obs}$ versus 1/T, for pNPP hydrolysis catalyzed by **2**.

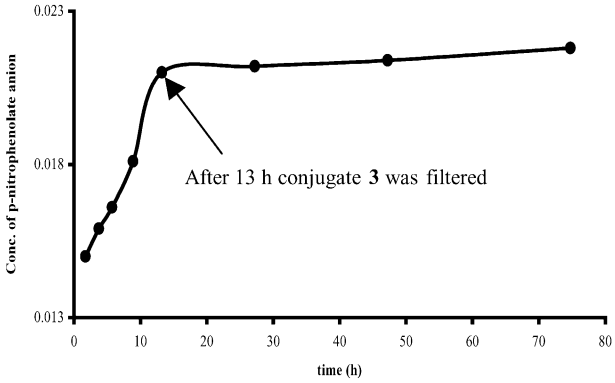

Figure 2. Arrest of bNPP hydrolysis catalyzed by conjugate **3**.

Plasmid modification by copper complexes of tripeptides of type Xaa-Xaa-His-NH_2, have been demonstrated in the presence of exogenously added co-oxidants such as magnesium monoperoxyphthalate, oxone, and H_2O_2/ascorbate system,[2b,d] presumably through the generation of non-diffusible reactive species.

We evaluated the reactivity of insoluble metallobis-dipeptide derivatives using pBR322 supercoiled plasmid relaxation assay.[12] These reactions were performed in the absence of co-oxidants and thus, the observed results are likely to have arisen out of non-oxidative pathway. A time-course analysis of pBR322 cleavage by conjugate **3** displayed complete conversion of supercoiled form **I** to nicked form **II**, in 8 h (Fig. 3). Analogous to the model P-ester cleavage, a similar profile for plasmid modification was observed for conjugates **1**–**5** (Fig. 4), once again suggesting a lack of linker length effect on the catalytic activities of bis conjugates related to plasmid relaxation.

In order to rule out possible involvement of reactive species, this reaction was performed in the presence of radical scavengers and EDTA (Fig. 5). Scavengers, such as *t*-butanol and DMSO, had no visible effect on plasmid relaxation (lanes 6 and 7). These results suggest that the reaction prefers a pathway that remains uninhibited by scavenging reagents. However, the reaction was completely inhibited in the presence of excess EDTA (40 mM) presumably due to copper complexation (lane 8), reinforcing the importance of coordinated copper ions in metalated conjugates.

We also performed the cleavage reaction under rigorous anaerobic conditions[13] (argon-filled glove bag) to investigate the role of ambient oxygen.[14] It was found that the supercoiled plasmid (form **I**) converted to its closed circular form (form **II**) under such conditions (Fig. 6, lane 2), confirming oxygen-independent cleavage.

The results presented here indicate that the plasmid relaxation is occurring either via non-diffusible radicals or through hydrolytic pathway. Interestingly, these constructs oxidatively cleaved plasmid in the presence of co-oxidants, such as magnesium monoperoxy pthalate (data not shown). Hydrolytic and oxidative cleavage duality of copper based artificial nucleases has been previously discussed.[14a,b,15] However, hydrolytic cleavage by artificial nucleases is more desirable for nucleic acid modification as the products so formed are amenable to further biochemical manipulations.[16]

Detailed investigations regarding mechanism and cleavage product identification are currently under progress. In short, we have synthesized a series of bio-inspired nucleolytic reagents that demonstrate efficient plasmid modification under heterogeneous reaction conditions. The latter property, imparted to the conjugates by molecular design, enables their convenient removal at the end of reaction thus obviating the use of additional purification steps, prior to subsequent biochemical transformations. Finally, incorporation of a recognition element for selective DNA cleavage will also be carried out and reported in due course of time. It is expected that such reagents will broaden the scope of chemical nucleases in many chemico-biological applications.

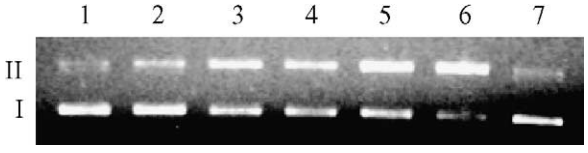

Figure 3. Cleavage of supercoiled pBR322 plasmid DNA by conjugate **3**. lane 1: pBR322 (0 h); lanes 2–6: pBR322 + **3** (1, 3, 5, 7, 8 h), respectively; lane 7: pBR322 (8 h).

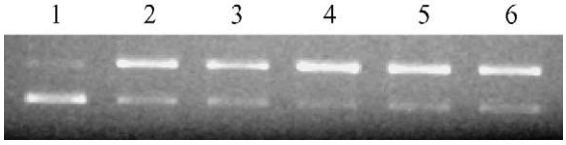

Figure 4. Cleavage of supercoiled plasmid pBR322 by metalated conjugates. Lane 1: pBR322 (blank); lanes 2–6: pBR322 + conjugates **1**–**5** (8 h), respectively.

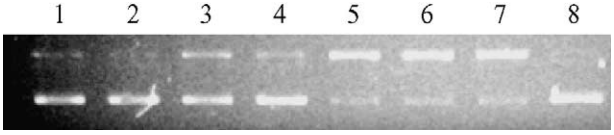

Figure 5. Radical scavenger experiment for pBR322 plasmid cleavage assisted by **3**. Lanes 1–4: pBR322; pBR322 + unmetalated **3**; pBR322 + Cu^{+2} (50 μM); pBR322 + Cu^{+2} (50 μM) + EDTA (2 mM), respectively; lane 5: pBR322 + **3**; lanes 6 and 7: pBR322 + **3**, in presence of *t*-butanol (100 mM) or DMSO (100 mM); lane 8: pBR322 + **3**, in presence of EDTA (40 mM).

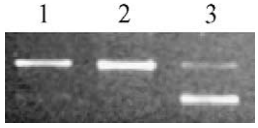

Figure. 6. pBR322cleavage with conjugate **3** under anaerobic conditions. Lane 1: pBR322 + **3** (8 h); lane 2: pBR322 + **3**, under anaerobic conditions (8 h); lane 3: pBR322 (blank, 8 h).

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7. *Synthesis of conjugates* **2**–**5**: *N*,*N*′-bis-(*N*-Benzoylglycylglycine)-1,2-ethanediamine (**2**). *N*-benzoylglycylglycine *p*-nitrophenyl ester (2.24 g, 6.3 mmol)[6] was dissolved in dry DMF (35 mL). A mixture of 1,2-diaminoethane (0.21 mL, 3.5 mmol) and triethylamine (0.87 mL, 6.3 mmol) in dry DMF (4 mL) was drop-wise added at room temperature with vigorous stirring, which was continued for 2 h. A precipitate was formed, which was filtered and washed with MeOH (4×10 mL) followed by acetone (3×10 mL). The product was recrystallized from hot DMF (1.35 g, 87%). Similar reaction procedure was followed to prepare *N*,*N*′-bis-(*N*-benzoylglycylglycine)-1,4-diamino butane (**3**), *N*,*N*′-bis-(*N*-benzoylglycylglycine)-1,8-diamino-3,6-dioxaocatane (**4**), *N*,*N*′-bis-(*N*-benzoylglycylglycine)-1,4-bis(3-aminopropoxy)butane (**5**), by using corresponding diaminoalkane linkers.
8. Conjugates **2**–**5**, were metalated with excess of $Cu(OAc)_2 \cdot H_2O$ in aqueous methanol (25 °C). After 48 h, blue precipitates so formed were washed with hot DMSO (4×10 mL), methanol (4×10 mL), followed by acetone (4×10 mL). Conjugates were air dried and subjected to atomic absorption and spectrophotometric analyses to determine copper contents.
9. *Copper estimation*: ***AAS method***: 0.1 g of the sample was weighed accurately and digested in aqua regia (1 mL) and heated to 50 °C. Solutions were filtered through Whatman filter paper (No. 42) and solution was made up to 100 mL using ultra-pure water. The extract was analyzed using commercially available standards and a blank solution.
Spectrophotometeric method: The amount of copper incorporated in conjugates **2**–**5** was ascertained by digesting a known amount of sample in 8 M HNO_3. A standard curve was prepared by using known concentrations of copper acetate in 8 M HNO_3.
10. *Kinetics*: All hydrolytic reactions were performed in duplicate in centrifuge tubes thermostatted at 40 °C, unless otherwise mentioned. Total assay mixture contained 3 or 5 mL of substrate solution of appropriate concentration prepared in 0.01 M *N*-ethylmorpholine buffer (pH 8.0 or pH 7.5) in 50% aqueous solution without metaloconjugate, to correct for background hydrolysis. Amount of conjugates used for hydrolysis were 1 mg/5 mL or 1 mg/3 mL of buffer contains substrate and due to insoluble of these conjugates, copper concentrations present in **2**–**5** were taken as catalyst concentration. Initial velocities were determined from time-dependant release of *p*-nitrophenolate anion ($\varepsilon = 1.65\times10^4$ M^{-1} cm^{-1}) and pseudo-first-order rate constants were determined from $(A_\infty/A_\infty - A_t)$ versus time plots. All reactions were performed over eight half-lives.
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12. *pBR322 plasmid relaxation assay*: All cleavage reactions were performed in sodium cacodylate buffer (10 mM, pH 7.5, 25 °C). Supercoiled plasmid pBR322 (0.2 μg) and metaloconjugates (50 μg) were suspended in a total reaction volume of 20 μL. The reactions were terminated by adding the gel loading buffer (100 mM EDTA, 50% glycerol in Tris–HCl, pH 8.0, 5 μL) and reactions were loaded onto 0.7% agarose gel containing ethidium bromide (1 μg/mL) and electrophoresed for 1 h at constant current (80 mA). Gels were imaged on PC-interfaced Bio-Rad Gel Documentation System 2000.
13. *Plasmid cleavage under anaerobic conditions*: Nitrogen gas was purged through cacodylate buffer and it was subjected to four freeze–thaw cycles. Transfer of reagents and initiation of cleavage reactions were carefully performed in an argon-filled glove bag. The reactions were terminated as in ref 12.
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