A combinatorial approach to minimal peptide models of a metalloprotein active site†

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Screening of a "one-bead-one-compound" peptide library containing biomimetic His/Cys ligands has led to the discovery of sequences that hydrolyze ester substrates in combination with Zn^{2+}.

The screening of combinatorial peptide libraries has been used previously to discover both short peptides as well as peptide–metal complexes that function as catalysts for various organic transformations.[1–5] Similar combinatorial strategies have led to the development of well defined peptide–metal complexes as biological probes.[6,7] However, there are still relatively few examples of combinatorial approaches being used to obtain catalysts that have *both* naturally occurring ligands (*e.g.* His, Cys) *and* biologically relevant metal ions (*e.g.* Zn^{2+}).[8] We envision using this approach to discover biomimetic catalysts that can reproduce key structural and functional aspects of metalloproteins.[9] Herein we demonstrate the use of this methodology to select for sequences that contain only proteinogenic metal-binding ligands and catalyze hydrolysis in the presence of Zn^{2+} both on solid support and in solution.

Previous efforts in our lab have been aimed at preparing synthetic analogues of the metal center of peptide deformylase (PDF).[10–12] The bacterial PDF enzyme is an unusual metallohydrolase that contains a mononuclear, four-coordinate Fe^{2+} cofactor bound to the protein by one Cys and two His residues, and a catalytically active water molecule occupying the fourth coordination site.[13,14] Although the Zn^{2+} form of bacterial PDF is nearly structurally identical to the iron(II), cobalt(II), and nickel(II) forms, it is dramatically less active.[15–17] This result is surprising given that PDF carries a conserved sequence motif (HEXXH) found in zinc enzymes, and has an active site structure and hydrolytic function that is normally seen in the mononuclear zinc enzyme family.[18] Interestingly, a recent exception was found in PDF isolated from plants, which is apparently highly active with Zn^{2+}.[19,20] A single bacterial PDF from *Leptospira interrogans* has also been reported to use Zn^{2+}.[21]

We are interested in determining the fundamental role of the metal ion in the mechanism of PDF by constructing and studying small-molecule models of the metal active site. In this paper we have taken a combinatorial approach to this problem by preparing a library of short peptides (11 AAs) that are designed to bind metal ions (*e.g.* Zn^{2+}, Co^{2+}, Fe^{2+}) through a His_2Cys motif. We have successfully screened this library to identify sequences that exhibit Zn^{2+}-induced hydrolysis of phosphate and carboxylic ester substrates.

An earlier report by Berkessel and coworkers[22] described the synthesis of a library of short peptides (11 AAs) with 625 possible unique sequences that was screened on-bead in the presence of Zr^{4+} for the hydrolytic cleavage of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), a well known chromogenic probe.[3] These efforts resulted in the identification of three different peptide–Zr^{4+} constructs capable of hydrolyzing phosphoryl ester substrates. This type of on-bead screening for catalyst discovery has been used previously.[23,24] In light of these results, we chose to make a library of the same size (11 AAs in length, 625 members), with a generalized sequence that is intended to mimic the first coordination sphere of PDF. The architecture of our bead-bound library is shown in Fig. 1. TentaGel-NH_2 resin was used as solid support because it allows for deprotection of the sidechains while leaving the peptide attached to the resin, and because of its good swelling properties in water. As seen in Fig. 1, each member of the library contains one Cys and two His metal-binding ligands fixed in positions (AA_2, AA_6, AA_{10}). The His residues were incorporated as part of the sequence HEXXH, which is a highly conserved motif found in PDF[18] as well as in many zinc enzymes, and invariably forms a chelate ring with the His donors bound to the metal center. A second conserved motif found in PDF is GCLS,[25] which contains the metal-binding Cys ligand, and thus Leu was included next to the Cys. It has been shown previously that specific metal binding properties can be incorporated into synthetic peptides by correctly positioning the ligands in minimally conserved motifs.[26] The diversity of the library was achieved by varying the four X positions over five amino acids, Ser, Arg, Gly, Asp, or Ala, resulting in the desired maximum of 625 (5^4) unique sequences. These amino acids were selected to impart a wide range of properties to the library, including hydrophobic, hydrophilic, ionic and H-bonding characteristics. A phenylalanine at the C-terminus was included for subsequent UV-vis absorption assays in the solution phase.

The peptide library was manually synthesized by standard Fmoc procedures and split/pool methodology. These methods result in a "one-bead-one-compound" library,[3] where each bead contains an undecapeptide of only one sequence, with

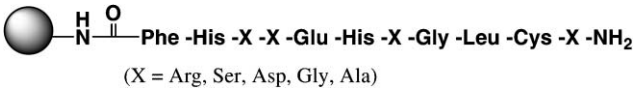

Fig. 1 Combinatorial library of 625 (5^4) peptides synthesized by the split-and-pool method with the X positions varied over the five AAs given in parentheses.

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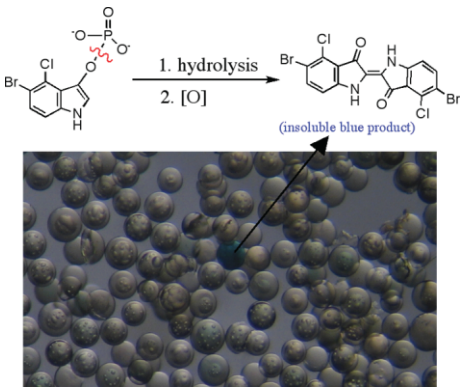

Fig. 2 Microscopic image of a portion of the TentaGel-peptide library incubated with $Zn(OAc)_2$ and BCIP at pH 6, showing a single active bead (dark blue) among inactive beads (colorless).

80–100 pmol of peptide per bead. To screen for hydrolytic activity in the presence of Zn^{2+}, a portion of the library (5 mg, ~2500 beads) was incubated with a 1 mM solution of $Zn(OAc)_2$ in HEPES buffer at pH 6 for 15 min. The substrate BCIP was then added and the library was visually inspected every 6–12 h under a standard stereoscopic microscope. Hydrolysis of the phosphoester group in BCIP leads to rapid oxidative dimerization to give the highly colored, insoluble blue dye shown in Fig. 2. Thus, any beads that actively hydrolyze BCIP are expected to become stained with a dark blue color. Approximately 1–3 dark blue beads had developed after 48 h, and microscopic identification of one of the active beads is shown in Fig. 2. This bead was isolated by microsyringe and its peptide sequence determined by automated Edman degradation. The sequences of two different dark blue beads isolated from separate screening experiments are shown in Fig. 3, along with an inactive sequence obtained from a non-colored bead. Clean single sequences were obtained from each bead, indicating that the coupling steps in the library synthesis were complete. It is interesting to note that the two active sequences are rich in Arg residues in the variable positions. One obvious consequence of Arg selection is that both **A** and **B** are positively charged, which may help to attract the negatively charged BCIP. In contrast, the inactive sequence **C** has an overall negative charge.

The hydrolytically active sequences **A** and **B**, and the inactive sequence **C**, were then resynthesized on a larger scale for solution phase studies. For this purpose, these peptides were synthesized on Wang resin, which allows for cleavage from the solid support. After synthesis and purification by HPLC, these peptides were tested for their ability to hydrolyze BCIP with Zn^{2+} in solution. A solution of the peptide (1 mM) was first incubated with $Zn(OAc)_2$ (1 mM) in HEPES buffer at pH 6 for 1 h. Incubation with Zn^{2+} was performed under anaerobic conditions to avoid disulfide

Fig. 3 Sequences **A** and **B** were determined from microsequencing of two active beads selected from separate BCIP assays. Sequence **C** was determined from an inactive (colorless) bead selected at random.

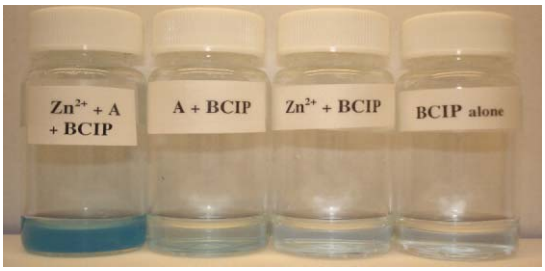

Fig. 4 All solutions contain HEPES (20 mM) at pH 6. From left to right: (1) Sequence **A** + $Zn(OAc)_2$ + BCIP (molar ratio 1 : 1 : 1), (2) **A** + BCIP, (3) $Zn(OAc)_2$ + BCIP and (4) BCIP alone. Photograph taken after 72 h.

formation. BCIP was then added and the solution exposed to air to allow for the colored dye to develop. As seen in Fig. 4, the solution containing the active peptide **A** + Zn^{2+} ions developed a turquoise blue color over 48 h, while only a faint blue color due to background hydrolysis is observed in the control experiments where peptide **A** alone, $Zn(OAc)_2$ alone, or buffer are mixed with BCIP. Similar results were obtained with the active peptide **B**. In contrast, no turquoise color developed over 48 h in the presence of inactive peptide **C** and Zn^{2+} ions. **These results clearly show that it is only the A–Zn^{2+} or B–Zn^{2+} complexes that lead to hydrolysis of the substrate.**

We then tested **A**–Zn^{2+} and **B**–Zn^{2+} to determine if they were capable of hydrolyzing the carboxylic ester *p*-nitrophenylacetate (4-NA), which would further demonstrate their hydrolytic activity in solution, and allow for comparative kinetic measurements. Hydrolysis of 4-NA in the presence of sequences **A** or **B** and Zn^{2+} was examined by methods previously established in our laboratory.[11] The reaction was monitored by observing the appearance of the nitrophenolate product (4-NP) over time in a UV-vis spectrophotometer. The initial rates for **A**–Zn^{2+} and **B**–Zn^{2+} are compared with the background rate of hydrolysis (buffer alone) in Fig. 5. There is a significant rate enhancement for both active sequences, with **A** giving an approximately ten-fold increase in rate, while **B** proceeds approximately 2 times faster than the background reaction. Both **A** and **B** appear to function as catalysts for the cleavage of 4-NA in the presence of Zn^{2+}. Control experiments with $Zn(OAc)_2$ revealed almost no rate enhancement under the same conditions. However, the metal-free peptides **A** and **B** were found to be hydrolytically active toward 4-NA, and exhibit 10 and 100 times faster initial rates than their respective Zn complexes. These results are not surprising because of the well

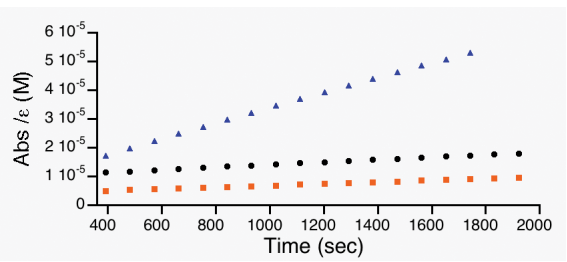

Fig. 5 Initial rates for the hydrolysis of 4-NA. All reactions done in HEPES at pH 7.5 with 1 mM 4-NA added to initiate the reaction. **A** + $Zn(OAc)_2$ (▼); **B** + $Zn(OAc)_2$ (●); background (■).

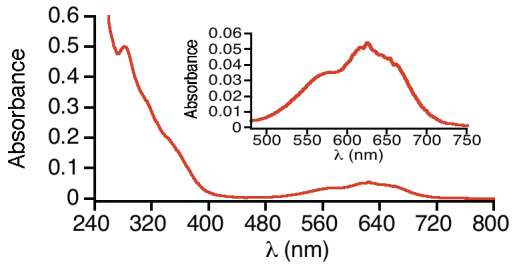

Fig. 6 UV-vis spectrum of **B** + $CoCl_2$ in HEPES (20 mM) at pH 7.5. Inset: expanded region showing the d–d transitions.

known catalytic activity of His residues toward the hydrolysis of carboxylic esters, and similar activity has been seen for apo zinc finger peptides *vs* the zinc-loaded peptide.[27,28] The lower activity in the presence of Zn^{2+} suggests that the Zn^{2+} ion is in fact coordinated to the His residues of **A** and **B**, blocking them from direct participation in the hydrolysis of 4-NA.

The library design and screening techniques presented here have clearly resulted in the discovery of short peptides with proteinogenic metal-binding ligands that, in combination with the most common biological metal ion used for hydrolysis, Zn^{2+}, function as hydrolytic catalysts both on solid support and in solution. To elucidate the structure of the catalytic species, Co^{2+} was added in place of Zn^{2+} as a spectroscopic probe. This type of substitution has been used extensively to obtain structural information on zinc metalloproteins, and relies on the fact that Zn^{2+} and Co^{2+} typically exhibit similar coordination geometries for a given ligand set. Addition of $CoCl_2$ to peptide **A** or **B** resulted in the appearance of an intense blue color, indicative of the formation of a four- or five-coordinate Co^{2+} complex. The UV-vis spectrum of **B**–Co^{2+} is shown in Fig. 6, and reveals peaks characteristic of Co^{2+} d–d transitions at 570, 625, and 660(sh) nm. This spectrum matches quite well with that obtained for tetrahedral zinc proteins, including zinc finger proteins with four-coordinate, CCHH ligation at the metal center.[29] In addition, there are strong absorbances at 283, 317(sh) and 360(sh) nm which do not appear in the spectrum of the free peptide or that of $CoCl_2$ alone. These latter peaks can be assigned to Cys-to-Co^{2+} LMCT bands. Similar peaks are observed for Cys-to-Co^{2+} LMCT bands in cobalt-substituted zinc fingers.[29]

The Co^{2+} data provide good spectroscopic evidence that both peptides **A** and **B** coordinate to Co^{2+} through the designed His_2Cys motif. However, we cannot rule out other coordination modes based on these data alone. Titration experiments with Co^{2+} were attempted in order to shed further light on the stoichiometry and strength of metal binding, but interpretation of these data were hampered by the fact that the Co^{2+} complex slowly decays over time to unidentified cobalt species. Although these studies were done anaerobically, the instability of the peptide–Co^{2+} complexes is likely due to oxidative degradation from trace amounts of dioxygen. We have previously observed extreme air-sensitivity in $(N_2S(thiolate)Co^{II}$ model complexes.[10]

In summary, on-bead screening of a combinatorial peptide library has led to the discovery of short peptides that utilize Zn^{2+} to hydrolyze ester substrates both on solid support and in solution. Although the explicit structure of the catalytically active peptide–metal complex has not been determined, these peptides bind Co^{2+}, and by analogy Zn^{2+}, through natural metal-binding ligands specifically incorporated to mimic the first coordination sphere of the metallohydrolase PDF. Combinatorial peptide libraries may be useful for obtaining models of other types of metalloenzymes, provided a proper reactivity screen can be developed.

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