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Selective hydrolysis of peptides promoted by metal ions: a positional scanning approach†

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Rapid, microplate-based fluorogenic screening of a positional scanning combinatorial library accurately predicts the sequence specificity of metal-assisted peptide hydrolysis.

Reagents that mediate the selective cleavage of peptides and proteins have become increasingly important in amino acid sequencing[1] and in the study of protein function and solution structure.[2] While the extreme stability of the peptide amide bond has placed limits on the number of available reagents, there is emerging evidence that metal ions and/or complexes of Ce^{IV}, Co^{III}, Cu^{II}, Ni^{II}, Pd^{II}, Pt^{II} and Zn^{II} effectively hydrolyze unactivated amide bonds in small peptides.[3] Cleavage is frequently accomplished at relatively mild temperature and pH,[3a,e,g] rendering some metals very promising for structural studies of folded proteins in solution. For example, work with small peptides has lead to the development of specific Cu^{II}, Co^{III} and Pd^{II} complexes that hydrolyze intact proteins.[3e,4] Although significant progress has been made, hydrolytically active metals are limited in number, and there is a growing interest in the discovery of new metal-based reagents endowed with novel sequence specificities and enhanced cleavage efficiencies.

A screening procedure affording rapid detection and characterization of metal-assisted peptide cleavage would greatly facilitate the discovery of useful metal ions and complexes. (Conventional methods based on HPLC and NMR have serious limitations that prevent the simultaneous analysis of multiple hydrolysis reactions.) To this end, we introduce a fluorescamine-based positional scanning strategy that permits rapid identification of hydrolyzed amino acid sequences and rapid screening of metal ions and complexes. Positional scanning represents a powerful combinatorial tool for the identification of enzyme substrates and protein ligands in complex peptide libraries.[5] This technique involves the solid phase synthesis of sub-libraries containing two or more samples. In each sample, one peptide position (O) is defined and each remaining position (X) contains equimolar amounts of various amino acids. Screening of the library samples automatically identifies the preferred amino acid at each defined position.

In our procedure, multiple samples within a positional scanning peptide library are treated with metal. The samples are then reacted with fluorescamine in a single 96-well plate, which is scanned in a microplate reader in 58 s. Detection is based on the fluorescence produced by derivatization of amino acids released upon hydrolysis of the peptide amide bond. (Fluorescamine is intrinsically nonfluorescent, but reacts in seconds with primary aliphatic amines to yield highly fluorescent derivatives.[6]) Because the library is spatially addressed, the fluorescence signal automatically identifies the sequence of hydrolyzed peptides. Furthermore, the microtiter plate format employed affords rapid analysis of multiple peptides, metal ions, and metal complexes in a near simultaneous fashion.

A positional scanning library comprised of nine acetylated dipeptides was prepared from 2-chlorotrityl chloride resin by 'split-and-mix' synthesis using Gly, Leu and Met Fmoc protected amino acid monomers. (This choice of amino acids was inspired by chemistry of Kostić and coworkers in which Pd^{II} promotes sequence specific hydrolysis of acetylated dipeptides containing methionine.[3b,c]) Standard DIPEA/HOBt/PyBOP peptide synthesis protocols† [7] were employed and all coupling, Fmoc deprotection, and acetylation reactions proceeded to greater than 99.5% completion as accessed by ninhydrin and/or chloranil tests.[7b] Coupling and Fmoc deprotection were further confirmed by derivatization of peptides with dabsyl chloride and subsequent reversed-phase HPLC analysis.† After acetylation of α-NH_2 groups (acetic anhydride–pyridine–DMF = 1:2:3),[5] the peptides were cleaved from the resin (TFA–TIS–H_2O–EDT = 94:1:2.5:2.5).†[7b] As shown in Fig. 1, the positional scanning library is composed of two sub-libraries that each contain three samples. All nine acetylated dipeptides are represented in each of the two sub-libraries and are divided into the three samples based on the identity of the

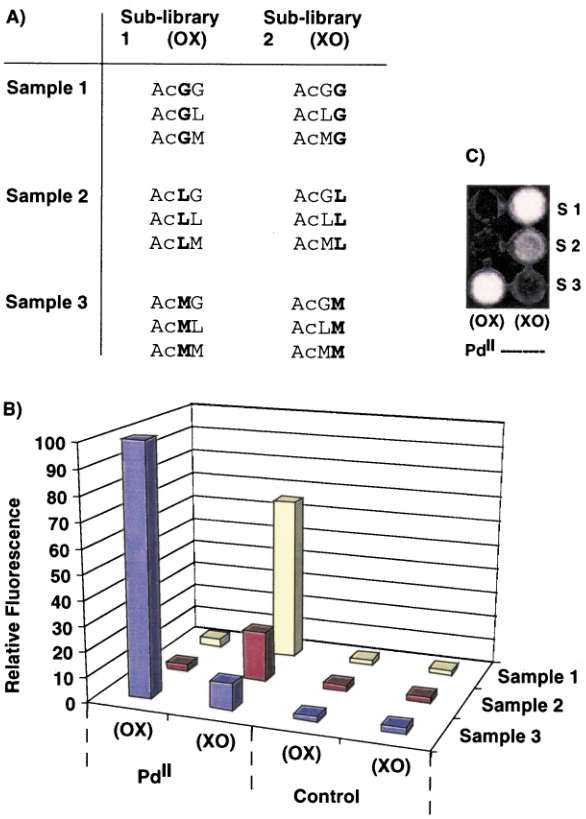

Fig. 1 (a) In this positional scanning library, nine acetylated dipeptides are divided into three samples within each of two sub-libraries. (b) Relative fluorescence intensities produced by individual library samples in the presence and absence of Pd^{II}. (Data is averaged over three trials.) (c) A photograph of a corresponding microplate in which the Pd^{II} reactions are visualized on a transilluminator set at 365 nm.

† Electronic supplementary information (ESI) available: Fig. S1, details of peptide synthesis, fluorescamine reactions, and of HPLC analyses. See http://www.rsc.org/suppdata/cc/b2/b202285k/

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spatially addressed amino acid 'O', which alternates between Gly, Leu and Met.

The six positional scanning library samples were treated with $CdCl_2$, $CeCl_3$, $CrCl_3$, $K_2[PdCl_4]$, $FeCl_3$ and $MnCl_2$, in a total of 36 separate reactions. In a typical experiment, 6 mM of each sample (500 nmol total peptide) in 10 mM metal salt and 68 mM TFA pH 1.7 was treated at 45 °C for 5 h, after which metal was chelated by the addition of equivalent volumes of 100 mM EDTA (for Cd^{II}, Ce^{III}, Cr^{III}, Fe^{III} and Mn^{II}) and 100 mM ethanedithiol (for Pd^{II}). In control reactions, metal solutions were replaced by equivalent volumes of water. Hydrolyzed peptides were then detected by simultaneous treatment of each of the 36 reactions in 0.03% (wt/v) fluorescamine (5 min, 22 °C, 5 mM sodium borate buffer pH 8) followed by rapid quantitation in a fluorescence microplate reader.†[6c] While five of the six metals generated negligible levels of fluorescence, significant peptide hydrolysis was detected in several of the palladium(II) reactions (Fig. S1 in ESI).† The relative fluorescence intensities produced by these samples revealed that three of the nine peptides in the library were hydrolyzed in the order AcMet-Gly > AcMet-Leu > AcMet-Met (Fig. 1). Virtually no fluorescence was detected in negative controls in which Pd^{II} was substituted by water. The hydrolysis products free glycine, leucine and methionine were then identified in the library samples by subsequent amino acid analyses with dabsyl chloride.†[6b] The HPLC retention times of the derivatized hydrolysis products were in exact agreement with dabsyl derivatized Gly, Leu and Met amino acid standards. In addition, HPLC peak heights were found to be in excellent agreement with the corresponding positional scanning relative fluorescence intensity values. Finally, the dabsylated standards were added to the Pd^{II} reaction mixtures and were shown to enhance HPLC peaks corresponding to free Gly, Leu and Met.

In order to verify the accuracy of positional scanning, the nine acetylated dipeptides were synthesized in-parallel, after which a total of 2 mM of each dipeptide was reacted with 10 mM $K_2[PdCl_4]$ and fluorescamine as described above. A comparison of relative fluorescence intensities confirmed the order of reactivity indicated by the positional scanning data. The dipeptide AcMet-Gly was shown to be the most reactive followed by AcMet-Leu and then AcMet-Met (Fig. 2). This ordering was further verified by dabsylation and HPLC analyses of the nine hydrolysis reactions.†[6b]

Overall, our findings are in strong support of data obtained by conventional means. Of the six metal salts tested, only

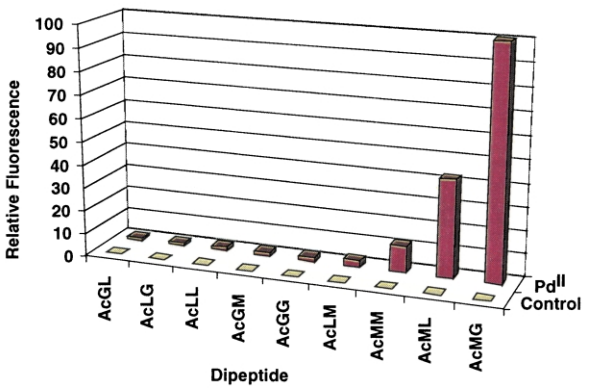

Fig. 2 Average relative fluorescence intensities produced by hydrolysis of individual acetylated dipeptides over three trials.

$K_2[PdCl_4]$ is known to be hydrolytically active.[3,4] In NMR studies of AcMet-Gly, AcMet-Leu and other dipeptides, Kostić and coworkers clearly established that (i) Pd^{II} promotes hydrolysis of amide bonds at the C-terminal end of anchoring methionine residues and that (ii) the rate of this hydrolysis is inversely proportional to the bulk of the leaving group.[3b,c]

Fluorescamine is a well known reagent for the quantitative fluorometric determination of peptides, proteins and amino acids (with the exception of proline).[6] Under the derivatization conditions employed here, the detection limit of fluorescamine for individual amino acids is in the 17–100 pmol range.[6c] In a large positional scanning library containing all possible acetylated dipeptide sequences (400 dipeptides), each of the 40 library samples would contain 20 dipeptides. Therefore, if only one of the 20 peptides were hydrolyzed, approximately 25 nmol of amino acid would be released, which is well within the detection capabilities of fluorescamine, as we have shown in previous experiments.[6c] It is reasonable to anticipate that the positional scanning strategy presented here will be extended to the design, synthesis and screening of larger peptide libraries.

In conclusion, fluorogenic screening of positional scanning libraries serves as a rapid, alternative means of surveying metals and of characterizing metal-assisted peptide hydrolysis reactions. In comparison to HPLC and NMR, the microtiter plate format employed represents a major advantage, allowing multiple peptides and metals to be scanned in only 58 s. We envisage that positional scanning methodology will lead to the discovery of new and efficient metal complexes for use in protein structural studies.

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