J|A|C|S
COMMUNICATIONS

Published on Web 12/24/2003

Conjugate of Palladium(II) Complex and β-Cyclodextrin Acts as a Biomimetic Peptidase

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Selective proteolysis can be achieved with enzymes and synthetic reagents, but few of them are practical.[1] Certain transition-metal complexes,[2] especially those of palladium(II),[3−5] are emerging as new chemical proteases. New reagents that recognize or bind side chain(s) and selectively cleave peptide bond(s) are needed. This enzyme-like combination of molecular recognition and reactivity is a familiar concept, but it has seldom succeeded in practice. The reactions are slow, or turnover is absent, or both. Because of the extreme unreactivity of regular amides, hydrolysis studies have been done mostly with esters and activated amides.[6] Cyclodextrins are often used in enzyme mimics because their cavities bind hydrophobic substrates in aqueous solutions.[7] Breslow et al. recently used a Cu(II)−β-cyclodextrin conjugate to hydrolyze a benzyl ester.[8] Hydrolysis of regular (not activated) esters, let alone of amides, remains a challenge.

An internal X-Pro peptide bond is usually so resistant toward enzymatic proteolysis that nature conserves this bond to fortify proteins against degradation.[9] Only one enzyme, HIV protease 1, is known to cleave internal X-Pro bonds, albeit nonselectively.[10] We now report that $[Pd(H_2O)_4]^{2+}$ selectively hydrolyzes X-Pro bonds in neutral solution. Combination of this new reactivity of the Pd(II) aqua complex and the known ability of β-cyclodextrin to recognize aromatic side chains[11] gave the first such conjugate that acts as a sequence-specific peptidase at pH 7.

The solution of AcAla-Ala-Pro-Ala-naphthylamide and 10 equiv of $[Pd(H_2O)_4]^{2+}$ after 24 h at pH 7.0 and 60 °C[12] lacked this tetrapeptide (HPLC[13] peak at 31.9 min) and contained the fragment Pro-Ala-naphthylamide (peak at 29.4 min; calcd mass 312.50 D, obsd mass 312.47 D). In the absence of $[Pd(H_2O)_4]^{2+}$ the control solution contained only the intact tetrapeptide, evidence against "background" cleavage. The Pd(II) reagent selectively cleaved the Ala2-Pro3 bond in the tetrapeptide; the yield was 100%. Both the appearance of Pro-Ala-naphthylamide and the disappearance of the substrate obeyed the first-order rate law,[14] with the rate constant of 0.23 ± 0.03 h^{-1} (Figure S1a).

Chromatographic separations of the fragments and their amino acid compositions and MALDI mass spectra (see Supporting Information) showed that, in similar experiments, the 11-mer AcLys-Gly-Gly-Phe-Ser-Pro-Phe-Ala-Ala-Arg-Ala gave AcLys-Gly-Gly-Phe-Ser (calcd mass 536.26 D, obsd mass 536.39 D) and Pro-Phe-Ala-Ala-Arg-Ala (calcd mass 632.34 D, obsd mass 632.58 D). This time, the Pd(II) reagent selectively cleaved the Ser5-Pro6 bond in the 11-mer; the yield was 80% after 24 h and >95% after 100 h. Similar experiments with bradykinin produced fragments eluting at 4.4, 12.4, 16.7, 18.9, and 19.5 min. This pattern is consistent with the cleavage of all three X-Pro bonds, and none other, as shown below. In all three substrates, the selectivity was the same: Each X-Pro bond in them was cleaved, regardless of the properties of the flanking residues—small and large, polar and nonpolar.

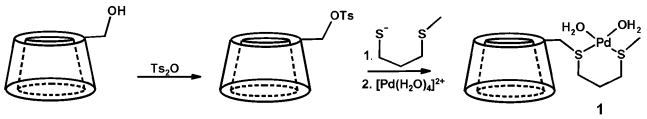
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

We recently showed that in weakly acidic aqueous solutions Pd(II) ion in complexes spontaneously and specifically binds to Met and His residues and then cleaves the second peptide bond "upstream", that is the X-Y bond in the X-Y-Met and X-Y-His segments.[3−5] Now, for the first time, we report the Pd(II)-promoted cleavage not guided by histidine, methionine, or any other anchor for the Pd(II) ion. Moreover, the cleavage occurs at 6 ≤ pH ≤ 9. Conveniently, under these conditions the His-guided and Met-guided cleavage is absent.[3,4]

The X-Pro group is the only tertiary amide group in the polypeptide backbone. Because this group cannot be deprotonated, and thus cannot form the hydrolytically inactive Pd(II)−amidate complex,[5,15] suppression of this binding by acid is not needed, and the reaction can occur at 6 ≤ pH ≤ 9. Because the X-Pro group contains the most nucleophilic amide oxygen atom in proteins,[16] this atom can displace a H_2O ligand and bind to the Pd(II) ion without the benefit of prior anchoring. This binding of the amide oxygen atom to the Lewis acid enhances the electrophilicity of the amide carbon atom, stabilizes the tetrahedral intermediate formed in the nucleophilic attack by an external water molecule, and thus promotes the hydrolytic cleavage of the X-Pro bond.[15]

The cleavage by the simple Pd(II)−aqua complex at pH 7 is residue-selective, i.e., it occurs only at X-Pro bonds and at all of them. Biochemical applications, however, often require sequence-specific cleavage. To achieve this formidable goal, we combined the newly discovered ability of Pd(II) ion to promote hydrolysis of X-Pro bond and the known ability of cyclodextrins to bind, albeit weakly, the aromatic side chains (Ar) of Phe, Tyr, and Trp.[11] In search of an artificial peptidase specific to X-Pro-Ar sequences, we synthesized conjugate **1** (Scheme 1 and the Supporting Information) and characterized it (Figure S2 and more, in Supporting Information).

Scheme 1. Synthesis of the Conjugate **1**, Containing β-Cyclodextrin and a Pd(II) Aqua Complex[a]



[a] For details, see Supporting Information.

The mixture of AcLys-Gly-Gly-Phe-Ser-Pro-Phe-Ala-Ala-Arg-Ala and 10 equiv (to enhance host−guest binding) of **1** was kept at pH 7.0 and 60 °C for 24 h. The chromatograms showed that 81% of the 11-mer was consumed, while its fragments, eluting at 17.1 and 19.0 min, appeared. The cleavage yield eventually became >95%. These elution times and molecular masses (Figure S3)

10.1021/ja038404p CCC: $27.50

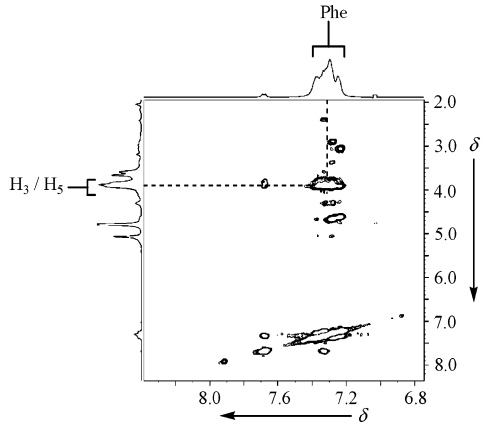


Figure 1. A region of the ROESY ^{1}H NMR spectrum of the equimolar mixture of AcLys-Gly-Gly-Phe-Ser-Pro-Phe-Ala-Ala-Arg-Ala and conjugate **1** dissolved in a 0.100 M phosphate buffer at room temperature and pH 7.0 containing 90% H_2O and 10% D_2O.

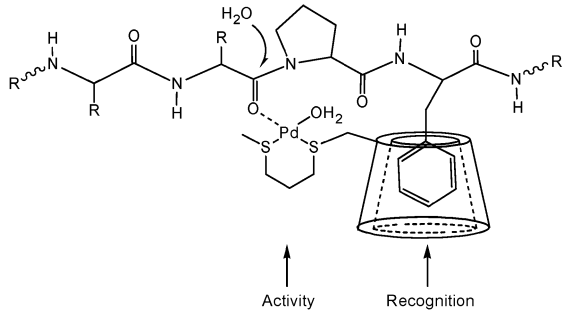


Figure 2. Sequence-specific, hydrolytic cleavage of the X-Pro bond promoted by the conjugate **1**. After the β-cyclodextrin (weakly) binds the aromatic side chain and thus brings (a fraction of) the Pd(II) aqua complex near the scissile bond, the Lewis acid Pd(II) ion binds the carbonyl oxygen atom and thus activates the amide group toward nucleophilic attack by the solvent. One of the various possible substrate−reagent orientations is shown.

confirmed the fragments to be AcLys-Gly-Gly-Phe-Ser and Pro-Phe-Ala-Ala-Arg-Ala, the same ones obtained with nonconjugated reagent, $[Pd(H_2O)_4]^{2+}$. Clearly, the Pd(II) ion in conjugate **1** remains hydrolytically active.

The 11-mer contains only one X-Pro bond, followed by a Phe residue. Can the cyclodextrin moiety in conjugate **1** enhance the selectivity of Pd(II) ion and make it sequence specific? In bradykinin (see above) one of the three X-Pro bonds is flanked by an aromatic residue, Phe. In a mixture containing bradykinin and 10 equiv of **1** after 48 h at 60 °C the amount of bradykinin, eluting at 24.7 min, decreased, while only two fragments, eluting at 19.4 and 20.9 min, appeared (Figure S1b). Their amino acid analyses and MALDI mass spectra identified Pro-Phe-Arg (calcd mass 418.23 D, obsd mass 418.76 D) and Arg-Pro-Pro-Gly-Phe-Ser (calcd mass 660.34 D, obsd mass 660.12 D). Clearly, **1** cleaved only the Ser-Pro peptide bond in bradykinin, as shown below.

Arg-Pro-Pro-Gly-Phe-Ser┆Pro-Phe-Arg

The cleavage at Pro2 and at Pro3 was absent. The unconjugated Pd(II) aqua complex cleaved all X-Pro bonds, whereas conjugate **1** cleaved this bond in the X-Pro-Phe segment exclusively.

Figure 1 shows cross-peaks between the aromatic protons in Phe (at 7.22−7.41 ppm) and H-3 and H-5 protons in β-cyclodextrin (at 3.8−4.0 ppm). Evidently, the β-cyclodextrin moiety in **1** forms an inclusion complex with this aromatic side chain. Although the exact host−guest orientation is unknown, a complex clearly is present. The SCH_3 singlet stayed at 2.04 ppm, evidence that the sulfur atom remained coordinated to the Pd(II) ion.

Figure 2 qualitatively explains the sequence specificity of the conjugate **1**. After the β-cyclodextrin moiety (weakly) binds the aromatic side chain and thus brings (a fraction of) the Pd(II) aqua moiety near the scissile bond, the Lewis acid Pd(II) ion binds the carbonyl oxygen atom and thus activates the amide group toward nucleophilic attack by the solvent.[15] The conjugate **1** is the first reagent capable of sequence-specifically cleaving the regular, unactivated amide bond. Our future studies will test the selectivity of cleavage and explain its origins.

Acknowledgment. This work was supported by the NSF, CHE316868. We thank D. Bruce Fulton for help with NMR spectroscopy.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) In a typical experiment, 20.0 μL of a 500 mM solution of $[Pd(H_2O)_4](ClO_4)_2$ were added to 1.00 mL of a 1.00 mM solution of the peptide in 0.10 M phosphate buffer at pH 7.0, and the pH was adjusted to 7.0 with NaOH. After the reaction was completed, the pH remained within ±0.1 of the initial value. In this and other control experiments for "background" cleavage, only the Pd(II) reagent was absent.

(13) The reaction mixtures were separated by a Hewlett-Packard 1100 HPLC system, with detection at 215 nm. In the reverse-phase separations, an analytical Supelco Discovery C-18 column (sized 250 mm × 4.6 mm, beads of 5 μm), and a preparative Vydac C-18 column 218TP101522 (sized 250 mm × 22 mm, beads of 10 μm) were used. The solvent A was 0.10% (v/v) CF_3COOH in H_2O, and solvent B was 0.08% (v/v) CF_3COOH in acetonitrile. In a typical run, the percentage of solvent B was kept at 0.0% for 5 min after the injection, and then raised to 45.0% over a 35-min period.

(14) In the kinetic measurements, the areas under the chromatographic peaks were normalized to that of the internal standard, methyl phenyl sulfone, to compensate for evaporation and possible errors in the injection volume. The integration error was ca. 5%. The plots of the peak areas for the cleavage products versus time were fitted to the first-order rate law with *SigmaPlot* v. 5.0. Because the cleavage reaction is very slow at room temperature, the species distribution in the chromatographic separations corresponded to those in the digests.

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JA038404P