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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

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Abstract: The X-Pro peptide bond (in which X represents any amino acid residue) in peptides and proteins is resistant to cleavage by most proteolytic enzymes. We show that $[Pd(H_2O)_4]^{2+}$ ion can selectively hydrolyze this tertiary peptide bond within the X-Pro-Met and X-Pro-His sequence segments. The hydrolysis requires an equimolar amount of the Pd(II) reagent and occurs under mild conditions—at temperature as low as 20 °C (with half-life of 1.0 h at pH 2.0) and at pH as high as 7.0 (with half-life of 4.2 h at pH 7.0 and 40 °C). The secondary peptide bond, exemplified by X-Gly in the X-Gly-Met and X-Gly-His sequence segments, however, is cleaved only in weakly acidic solution (pH < 4.0) and more slowly (half-life is 4.2 h at pH 2.0 and 60 °C). We explain the sequence-specificity of X-Pro cleavage by NMR spectroscopic analysis of the coordination of the X-Pro-Met segment to the Pd(II) ion. We give indirect evidence for the mechanism of cleavage by analyzing the conformation of the scissile X-Pro peptide bond, and by comparing the rate constants for the cleavage of the tertiary X-Pro peptide bond, the tertiary X-Sar peptide bond (Sar is *N*-methyl glycine), and the typical secondary X-Gly peptide bond in a set of analogous oligopeptides. Methionine and histidine side chains provide the recognition by selectively binding (anchoring) the Pd(II) ion. The proline residue provides the enhanced activity because its tertiary X-Pro peptide bond favors the cleavage-enhancing binding of the Pd(II) ion to the peptide oxygen atom and prevents the cleavage-inhibiting binding of the Pd(II) ion upstream of the anchoring (histidine or methionine) residue. Cleavage can be switched from the residue-selective to the sequence-specific mode by simply adjusting the pH of the aqueous solution. In acidic solutions, any X−Y bond in X-Y-Met and X-Y-His segments is cleaved because the cleavage is directed by anchoring methionine and histidine residues. In mildly acidic and neutral solutions, only the X-Pro bond in X-Pro-Met and X-Pro-His sequences is cleaved because of an interplay between the anchoring residue and the proline residue preceding it. Because Pro-Met and Pro-His sequences are rare in proteins, this sequence-specific cleavage is potentially useful for the removal of the fusion tags from the bioengineered fusion proteins.

Introduction

Protein Cleavage. Hydrolytic cleavage of proteins plays functional and regulatory roles in the control of cell cycle, transcription, signal transduction, antigen processing, and apoptosis.[1] Proteolysis is also a common biochemical procedure in protein sequencing and various new bioanalytical and bioengineering applications. These new methods require protein digestion into small fragments, residue-specific cleavage into large fragments, and even highly selective cleavage at a single site in the sequence. Protein footprinting and studies of folding employ limited, nonselective proteolysis of solvent-exposed segments.[2] Proteomics requires selective digestion of the expressed proteins into long fragments, optimal for mass-spectrometric detection.[3] Semisynthesis involves selective hydrolysis of natural proteins into large fragments, which are then chemically ligated with synthetic peptides.[4] Bioengineering of fusion proteins requires the highest level of proteolytic selectivity—site-specific cleavage that removes the fusion tag from the protein of interest.[5]

Few enzymes and synthetic reagents are commonly used for selective proteolysis. Most of these proteases are residue-specific, and their selectivity can be adjusted by varying the digestion time and the degree of prior unfolding. Proteases are sometimes inadequate because they tend to produce short fragments ill-suited for bioanalytical applications, and because proteases themselves contaminate protein digests. The existing chemical reagents often require harsh conditions and high molar excess and sometimes give partial selectivity and low yields.[6]

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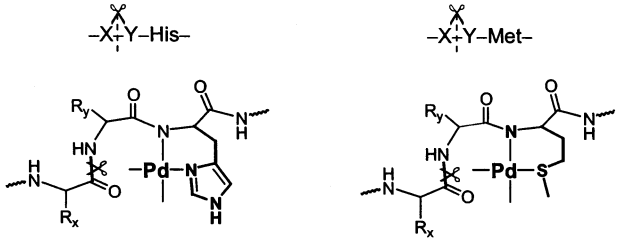
New cleavage reagents having improved efficiency and adjustable selectivity are desired for many emerging applications. Because, however, the peptide bond (i.e., the amide group) is extremely unreactive toward hydrolysis, even nonselective cleavage is hard to achieve. The half-life at room temperature and pH 4−8 is 500−1000 years.[7−9] For controlled and selective cleavage, a formidable task, chemical reagents must selectively recognize or bind one or more amino acid residues in the sequence, and selectively cleave a peptide bond. Relatively mild conditions, an equimolar amount or a small molar excess over the substrate, and easy removal of the reagent after cleavage are desired. Hydrolytic cleavage, which yields pristine fragments, is preferred over oxidative cleavage, which yields irreversibly modified fragments.

Palladium(II) Complexes as Selective Reagents for Protein Cleavage. Complexes of palladium(II) meet the requirements just mentioned. In weakly acidic aqueous solutions, Pd(II) ion in several complexes spontaneously binds to methionine residues and to histidine residues.[10] Upon this residue-specific anchoring, the Pd(II) ion promotes hydrolytic cleavage of a proximal peptide bond. Because histidine and methionine have a combined average abundance in proteins of only 4.5%,[11] cleavage directed by them yields relatively long fragments, suitable for bioanalytical applications. Although free cysteine residues also can bind the Pd(II) ion, they usually exist in proteins as disulfides, which are unknown as ligands for this ion. Our experiments with short peptides show that Pd(II) complexes can also promote cleavage specific to free or alkylated cysteine.[12] If this cleavage is undesirable, free cysteines in proteins can be blocked by covalent modification.[6,13] In nonaqueous solutions, Pd(II) reagents promote tryptophan-directed cleavage of amide bonds,[14,15] a reaction potentially useful for cleavage of lipophillic proteins. The Pd(II) reagent is used in stoichiometric amounts, and it can be removed from the reaction mixture by precipitation with diethyldithiocarbamate,[16,17] or by complexation with common chelators such as glutathione[18,19] or dithiols.[20,21] Upon the removal of Pd(II) ions, the fragments remain pristine.

After we explained the Pd(II)−peptide coordination modes and the activation of the scissile peptide bond,[22,23] we recently explained the unprecedented selectivity of cleaving synthetic and natural peptides and proteins: *In weakly acidic solutions, the Pd(II) reagent cleaves the second peptide bond upstream from the anchoring methionine and histidine residues, that is, the X-Y peptide bond in the segments X-Y-Met and X-Y-His,* as shown below.[24]



These hydrolytically active complexes are favored by the acidic medium. Because the Pd(II) reagent does not bind to the X and Y side chains, the selectivity of this cleavage is directed by the histidine and methionine anchors. Numerous studies showed that the noncoordinating residues X and Y do not alter the Pd(II) binding modes.[25−34] Consequently, these residues should not alter the cleavage reaction. To test the generality of cleavage, we experimented with natural peptides and proteins containing various X and Y residues: aromatic and aliphatic, polar and nonpolar, charged and neutral.[35] The X-Y bond was cleaved in every X-Y-His and X-Y-Met segment in which X and Y contained noncoordinating side chains, regardless of their nature.

Overview of This Study. We combine the uniqueness of methionine and histidine anchors in directing the cleavage and the uniqueness of proline residues[36,37] in assisting it. The tertiary peptide bond X-Pro in X-Pro-Met and X-Pro-His segments at pH 2.0 is cleaved much faster than the secondary peptide bond X-Gly in similar X-Gly-His and X-Gly-Met segments. Moreover, the proline-assisted cleavage persists at room temperature and at pH 7.0, the first such achievement with Pd(II) reagents. The cleavage can be tuned from residue-selective to sequence-specific simply by adjusting the pH. In acidic aqueous solutions cleavage is residue-selective because it is directed by all anchoring residues, whereas in mildly acidic and neutral solutions the cleavage is specific to X-Pro-His and X-Pro-Met sequences because of the proline assistance. In this study, we achieve and explain the residue-selectivity and, for the first time, the sequence-specificity. Because Pro-His and Pro-Met bonds are rare in proteins, this new sequence-specific cleavage bodes well for site-specific cleavage of fusion proteins.

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Experimental Procedures

Chemicals. Acetonitrile was obtained from Fisher. Palladium sponge, *cis*-[Pd(en)Cl_2] (in which en is ethylenediamine), trifluoroacetic acid (TFA), and α-cyano-4-hydroxy-cinnamic acid were obtained from Aldrich. Methyl phenyl sulfone was obtained from Lancaster. A 0.50 M solution of $[Pd(H_2O)_4]^{2+}$ and a 0.10 M solution of *cis*-[Pd(en)-$(H_2O)_2]^{2+}$ contained perchlorate anions.[38−40]

Peptide Synthesis. The peptides AcLys-Gly-Gly-Ala-Gly-**Pro-Met**-Ala-Ala-Arg-Gly (ProMet-peptide), AcLys-Gly-Gly-Ala-Gly-**Sar-Met**-Ala-Ala-Arg-Gly (SarMet-peptide), AcLys-Gly-Gly-Ala-Gly-**Pro-His**-Ala-Ala-Arg-Gly (ProHis-peptide), AcLys-Gly-Gly-Ala-Gly-**Pro-Ala**-Ala-Ala-Arg-Gly (ProAla-peptide), AcAla-Lys-Tyr-Gly-**Gly-Met**-Ala-Ala-Arg-Ala (GlyMet-peptide), and AcLys-Tyr-Gly-**Gly-Met**-Ala-Ala-Gly-**Pro-Met**-Ala-Ala-Arg-Gly (GlyMet···ProMet-peptide) were synthesized by a standard, manual Fmoc solid-phase procedure[6,41] and purified.[24] Sarcosine (Sar) is *N*-methyl glycine. The found and calculated molecular masses were, respectively, 1019.83 and 1019.53 D for ProHis-peptide, 1013.74 and 1013.51 D for ProMet-peptide, 988.63 and 988.49 D for SarMet-peptide, 954.05 and 954.12 D for ProAla-peptide, 1036.57 and 1036.51 D for GlyMet-peptide, and 1380.98 and 1380.65 D for GlyMet···ProMet-peptide.

Spectroscopic and Analytical Methods. The components of the reaction mixtures were separated by a Hewlett-Packard 1100 HPLC system containing an autosampler and a detector set to 215 nm (peptide bonds) and 350 nm (Pd(II) complexes). Analytical HPLC separations were done with a Supelco Discovery C-18 column (sized 250 mm × 4.6 mm, beads of 5 μm) and the flow rate of 1.0 mL/min. All percentages stated are by volume (v/v). The solvent A was 0.1% TFA in H_2O, and the solvent B was 0.08% TFA in acetonitrile. In a typical run, the percentage of B was kept at 0% for 5 min after the injection of the sample and was raised gradually to 45% over a 35-min period. In the runs for the kinetic measurements, the fraction of B was initially 10% and was raised to 22% over a 13-min period. Preparative HPLC separations were done with a Vydac C-18 column 218TP101522 (sized 250 mm × 22 mm, beads of 10 μm) and the flow rate of 10 mL/min.

The MALDI-TOF experiments were done with a Bruker Proflex instrument. The samples were prepared by a standard dried-droplet procedure: 1.0 μL of the sample was mixed with 9.0 μL of a saturated solution of the matrix (α-cyano-4-hydroxy-cinnamic acid) in a solution containing a 2:1 (v/v) mixture of water and acetonitrile, both containing 0.1% TFA. Angiotensin II and bradykinin were external standards. Molecular masses were calculated by PAWS software, obtained from ProteoMetrics, LLC. The presence of Pd(II) ions was established by the matching molecular mass for peptide plus Pd and by the isotopic distribution diagnostic of palladium.

The ^{1}H NMR spectra were recorded in water at 25.0 °C with a Bruker DRX500 spectrometer and referenced to the methyl signal of DSS in aqueous solutions. For the experiments involving ProMet-peptide, TOCSY (total correlation spectroscopy) spectra in aqueous solution were acquired. The water signal was suppressed with the WATERGATE.[42] Each data set consisted of 256 × 2048 complex points. The mixing time was 70 ms. The ambiguous assignment of the four glycine residues was resolved by ROESY (rotating-frame Overhauser enhancement spectroscopy) experiments. The spin-lock field strength during mixing was 6.4 kHz in TOCSY and 2.5 kHz in ROESY experiments.

Study of Hydrolysis. All reactions were carried out in aqueous solutions held in 2.0-mL glass vials. A 5.0 mM stock solution of each peptide in water was prepared. In a typical experiment, involving equimolar amounts of the peptide and the Pd(II) reagent, 200.0 μL of the peptide solution was mixed with 2.0 μL of a 500 mM stock solution of $[Pd(H_2O)_4]^{2+}$ or 10.0 μL of a 100 mM stock solution of *cis*-[Pd-$(en)(H_2O)_2]^{2+}$, 20.0 μL of a solution containing 25.0 mg/mL of phenyl methyl sulfone (the internal standard), and 780.0 μL of water. The pH was adjusted with either 1.0 M $HClO_4$ or 1.0 M NaOH. The reaction mixture was kept in a dry bath at 20 ± 1, 40 ± 1, or 60 ± 1 °C. After the reaction was completed, the pH remained within ±0.10 of the initial value. In the control experiments for "background" cleavage, only the Pd(II) reagent was absent. The cleavage fragments from the analytical separations were collected, dried with nitrogen, redissolved in 10.0 μL of water, and analyzed by MALDI-TOF mass spectrometry.

Chart 1. Synthetic Peptides Used as Substrates for Pd(II)-Promoted Cleavage

Symbol	Sequence
GlyMet	AcAla-Lys-Tyr-Gly-**Gly-Met**-Ala-Ala-Arg-Ala
ProMet	AcLys-Gly-Gly-Ala-Gly-**Pro-Met**-Ala-Ala-Arg-Gly
SarMet	AcLys-Gly-Gly-Ala-Gly-**Sar-Met**-Ala-Ala-Arg-Gly
ProHis	AcLys-Gly-Gly-Ala-Gly-**Pro-His**-Ala-Ala-Arg-Gly
ProAla	AcLys-Gly-Gly-Ala-Gly-**Pro-Ala**-Ala-Ala-Arg-Gly
GlyMet···ProMet	AcLys-Tyr-Gly-**Gly-Met**-Ala-Ala-Gly-**Pro-Met**-Ala-Ala-Arg-Gly

For the kinetic measurements at pH ≥ 4.0, successive 40.0-μL samples of the reaction mixture were subjected to reverse-phase HPLC separation. Because the cleavage at pH ≥ 4.0 is slow at room temperature, at which the separation was done, the chromatogram reflected the composition. Because at pH < 4.0 the cleavage is fast, the reaction was quenched by the addition of a solution containing a 10-fold excess of cysteine, which binds Pd(II) ions. The areas under the chromatographic peaks were integrated and normalized to that of methyl phenyl sulfone, with an estimated 5% error. The growth of the peak areas for the cleavage fragments was fitted to the first-order rate law with SigmaPlot v. 5.0, obtained from SPSS Inc. The stated errors in the rate constants correspond to the 95.0% confidence limit. This fitting is correct because Pd(II) reagent rapidly binds to the substrate and because all other processes, including the cleavage, are intramolecular and slower. Representative chromatograms and kinetic plots are shown in the Supporting Information.

Results and Discussion

The Choice of Substrates, Reagents, and Reaction Conditions. Synthetic peptides in Chart 1 contain reactive segments Pro-Met, Pro-His, and Sar-Met to be compared with segments Gly-Met (lacking proline) and Pro-Ala (lacking the anchor). These substrates are realistic examples of protein sequences because the cleavage-directing methionine and histidine residues are remote from the termini. At the same time, these peptides are more convenient than large proteins for exact NMR spectroscopic, HPLC, and kinetic experiments. Cationic residues confer the solubility of peptides and their fragments. The residues flanking the segments of interest are the same in all peptides, to allow comparisons of the selectivity and the rate of cleavage intrinsic to these segments. The cleavage selectivity is the same for all other noncoordinating residues X and Y, because only their backbone, which is common to all residues, interacts with the Pd(II) reagent. Because the residue X preceding the sequence segment of interest is kept the same in all the peptides in Chart 1, we can compare the kinetics of cleavage directed by these segments. For simplicity, we chose glycine as residue X, but as explained above, the selectivity is the same for all other amino acids with noncoordinating side chains.

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Because the first pK_a of $[Pd(H_2O)_4]^{2+}$ is ca. 3.0,[43] the predominant species at pH ≥ 3.0 is $[Pd(H_2O)_3(OH)]^+$. As pH is raised, this complex gradually hydrolyses, forming a mixture of hydroxo-bridged polynuclear products. For the sake of consistency, however, we always use the dicationic formula. The reactions were done at 1.0 ≥ pH ≥ 7.0, to suppress formation of the polynuclear Pd(II) species and nonselective background cleavage by acid.

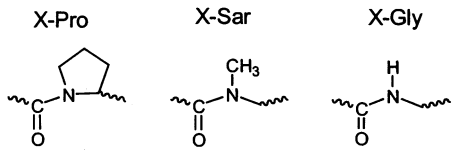
Chromatograms of each peptide (substrate) solution at pH 2.0, 5.0, and 7.0 in the absence of the Pd(II) reagent after 7 days showed only the peak of the intact peptide, evidence against the background cleavage. All the cleavage reactions are attributable to Pd(II) complexes.

Enhancement by Proline of the Rate of Methionine-Directed Cleavage. The chromatogram of an equimolar mixture of ProMet-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.0 and 60 °C after 1.0 h lacked a peak at 18.8 min, due to the intact peptide, and contained peaks at 9.2 and 16.6 min. Both of these peaks were detectable at 215 nm, but only the latter one at 350 nm. Evidently, the first fraction does, while the second does not, contain a bound Pd(II) ion. MALDI spectra showed that these two fractions are, respectively, fragment 1···5 (obsd 431.21 D and calcd 431.22 D) and fragment 6···11 (obsd 601.84 D and calcd 601.30 D). Clearly, the ProMet-peptide is cleaved by the Pd(II) reagent exclusively at the Gly5-Pro6 peptide bond, the second one upstream from the Met7 anchor, as shown below.

AcLys-Gly-Gly-Ala-Gly-**Pro-Met**-Ala-Ala-Arg-Gly

The same selectivity is found with other polypeptides and proteins,[24,35] but the ProMet-peptide is cleaved more quickly. For example, the cleavage of GlyMet-peptide (see Chart 1) at the Gly4-Gly5 peptide bond was completed in 12 h,[24] whereas the cleavage of ProMet-peptide was completed in less than 1.0 h. This rate-enhancing effect of proline is interesting because sequence-specific, fast cleavage is desirable.

Proline is the only common amino acid to form tertiary peptide bond, it is the only cyclic amino acid, it can adopt the usual trans and also the cis configuration, it has unusual conformational requirements, and of all natural amino acids it has the most basic amino group. Sarcosine forms a tertiary amide bond, but this residue is acyclic and lacks steric and conformational requirements of proline. Glycine forms a secondary amide bond, common to other natural amino acids. To explain the rate-enhancing effect of proline residue, we compared the reactivity of X-Pro, X-Sar, and X-Gly peptide bonds, shown below.



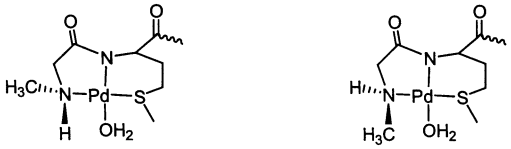
The chromatogram of an equimolar mixture of SarMet-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.0 and 60 °C after 4 h lacked the peak at 16.4 min, due to the intact peptide, but contained peaks at 6.1, 9.5, and 10.0 min. As with ProMet-peptide, all peaks show at 215 nm, but only the latter two show at 350 nm, evidence that the first fraction does, while the second two do not, contain a bound Pd(II) ion. MALDI spectra showed that the fraction eluting at 6.1 min is fragment 1···5 (obsd 431.21 D and calcd 431.22 D), whereas both fractions eluting at 9.5 and 10.0 min are the fragment 6···11 (obsd 576.31 D and calcd 576.28 D). These results prove selective cleavage of the Gly5-Sar6 bond, shown below.

Table 1. Dependence on pH of the First-Order Rate Constants for Cleavage of AcLys-Gly-Gly-Ala-Gly-**Pro-Met**-Ala-Ala-Arg-Gly (ProMet-peptide), AcLys-Gly-Gly-Ala-Gly-**Sar-Met**-Ala-Ala-Arg-Gly (SarMet-peptide), and AcLys-Gly-Gly-Ala-Gly-**Gly-Met**-Ala-Ala-Arg-Gly (GlyMet-peptide) by $[Pd(H_2O)_4]^{2+}$ at 60 °C

	rate constant k /10^{-4} min^{-1}		
pH	ProMet-peptide	SarMet-peptide	GlyMet-peptide
0.9			95(2)
1.2			65(1)
1.5			48(2)
2.0	587(40)	146(8)	28(2)
2.5			17(1)
3.0	150(2)	93(3)	5(2)
4.0	67(3)	57(2)	0
5.0	47(2)	39(4)	0
6.0	43(2)	24(4)	0
7.0	41(3)	21(4)	0

AcLys-Gly-Gly-Ala-Gly-**Sar-Met**-Ala-Ala-Arg-Gly

The two closely spaced HPLC fractions correspond to the two diastereomers expected for the tridentate coordination of the fragment Sar6···Gly11 as shown below.



The selectivity of the methionine-directed cleavage by the Pd(II) reagent is the same regardless of the residue preceding the methionine anchor, be it glycine, sarcosine, or proline, but there are two kinetic differences, shown in Table 1. First, the cleavage of the secondary peptide bond (Gly4-Gly5 in GlyMet-peptide) is virtually absent at pH ≥ 4.0, whereas the cleavage of the analogous tertiary peptide bond (Gly5-Sar6 in SarMet-peptide) occurs at an appreciable rate even at neutral pH. Second, the relative rate constants at pH 2.0 for the cleavage of GlyMet-, SarMet-, and ProMet-peptides are 1:5:20; respective half-lives are 247, 47, and 12 min. Remarkably, SarMet- and ProMet-peptides are cleaved at pH 7.0 at approximately the same rate at which GlyMet-peptide is cleaved at pH 2.5.

An internal X-Pro bond in proteins usually resists enzymatic cleavage because the proline residue has conformational and steric requirements unfavorable for the binding to the active site of common proteolytic enzymes.[44] For this reason, proline residues are often conserved, to fortify the protein against proteolytic degradation.[45] Only one proteolytic enzyme, HIV protease 1, is known to cleave internal X-Pro peptide bonds, but it also cleaves some secondary peptide bonds and is therefore

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somewhat nonselective. *The Pd(II) reagent, however, promotes fast and specific cleavage of this proteolytically inert bond in the X-Pro-Met sequence and does so in weakly acidic and neutral solutions.* We explain this important finding, and also the kinetic trends mentioned above, by comparing structural requirements for the cleavage and analyzing possible reaction mechanisms.

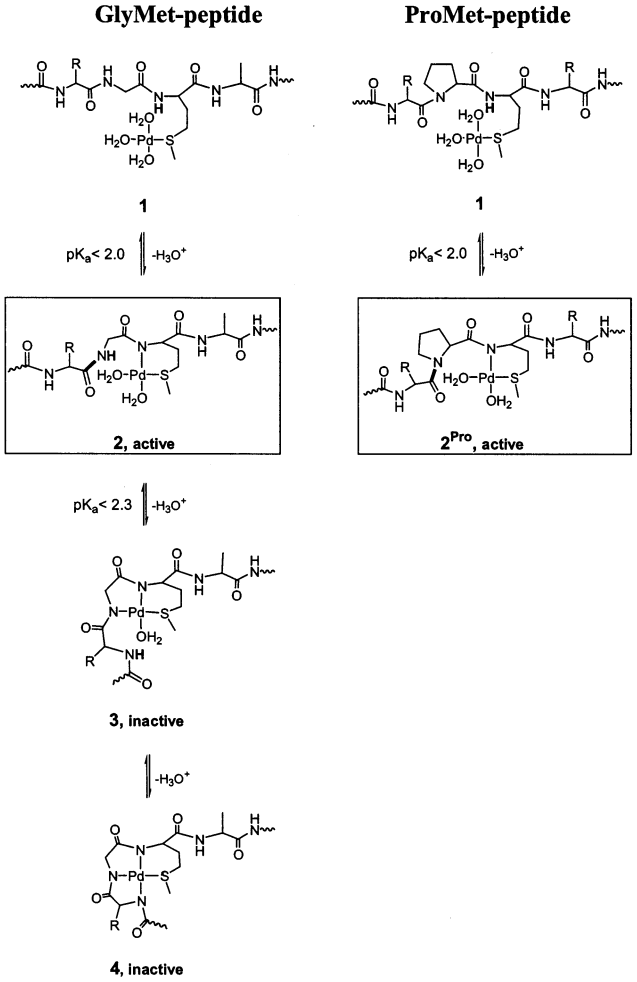
Structural Requirements for Hydrolytic Activity. Selective cleavage requires the formation of the hydrolytically active complex, in which the Pd(II) ion can approach the scissile peptide bond. This approach is guided by the binding of the Pd(II) ion first to the methionine side chain and then to the peptide backbone upstream. Simple binding of the Pd(II) ion to peptides containing a methionine or histidine anchor has been studied in our[22,23,46,47] and other[25,28,48−50] laboratories, but only our studies went beyond binding and revealed subsequent cleavage.

The GlyMet- and ProMet-peptides differ in the peptide bond immediately preceding the methionine anchor—an ordinary secondary amide in the former but a tertiary amide in the latter. As the left side of Scheme 1 shows,[24] rapid attachment of the Pd(II) complex to the side chain of Met6 gives the unidentate complex **1**. The anchored Pd(II) ion deprotonates the first amide nitrogen atom upstream and binds to it, thus forming the bidentate complex **2**. The pK_a for this deprotonation is estimated to be less than 2.0, far lower than ca. 15, found in the absence of metal ions.[27,29] Palladium(II) ion is the most effective transition-metal ion in deprotonating the secondary amide group and binding to the resulting amidate anion.[25−30,51,52] This extreme ability is important for the exceptional activity of Pd(II) complexes in promoting hydrolytic cleavage of peptide bonds.

In complex **2**, the hydrolytically active species, Pd(II) ion promotes the cleavage of the second peptide bond upstream from the anchor.[24] This complex can, however, convert into hydrolytically inactive species. The Pd(II) ion can successively deprotonate and bind the next two peptide NH groups upstream from the anchor (those of Gly5 and Gly4), in a process driven by successive formation of two five-membered chelate rings. The displacement of these two protons requires progressively higher pH because the Lewis acidity of the Pd(II) ion decreases with each coordination of an additional amidate anion. At pH 2.3, the bidentate complex **2** is a minor and the tridentate complex **3,** a major species.[24] At pH 4.5, the complex **2** disappears, the complex **3** becomes a minor species, and the tetradentate complex **4** becomes the major one.[24] Both **3** and **4** are hydrolytically inactive complexes. Their formation is the reason for which Pd(II) reagents cannot cleave at practical rates the ordinary peptide bonds (i.e., the secondary amide groups) at pH ≥ 3.0.

We studied the modes of ProMet-peptide coordination to Pd(II) ion by one-dimensional and TOCSY ^{1}H NMR spectra of the equimolar mixture of $[Pd(H_2O)_4]^{2+}$ and ProMet-peptide

Scheme 1. Binding of Pd(II) Ion to the Methionine Side Chain (the Anchor) and Stepwise Coordination of the Deprotonated NH Groups (Amidate Anions) in the Peptide Backbone Upstream from the Anchor[a]



[a] In GlyMet-peptide, methionine is preceded by an ordinary amino acid (exemplified by glycine), which forms a secondary peptide bond. In ProMet-peptide, methionine is preceded by proline, which uniquely forms a tertiary peptide bond. The displaceable hydrogen atoms in **1** and the scissile peptide bond in **2 and 2^{Pro}** are highlighted. The complexes **2** and **2^{Pro}** are hydrolytically active and undergo cleavage; the activating role of Pd(II) ion is shown in Scheme 2. The complexes **3** and **4** are favored by higher pH and are hydrolytically inactive; they are present for GlyMet-peptide and absent for ProMet-peptide.

at room temperature and pH 5.0, before and 10 min after the addition of $[Pd(H_2O)_4]^{2+}$. The experiments at pH 7.0 were precluded because the relevant NH resonances were weak. The spectra are shown in Figure 1.

Upon addition of $[Pd(H_2O)_4]^{2+}$, the SCH_3 singlet at 2.00 ppm disappeared, while a broad signal at 2.50 ppm appeared, indicating binding of the Pd(II) ion to the methionine sulfur atom. The methionine NH signal, at 8.47 ppm, and also the signals for its cross-peaks to α-CH, β-CH, and γ-CH, marked in Figure 1a, disappeared, as shown in Figure 1b. Clearly, the methionine NH group becomes deprotonated, evidence for its coordination to Pd(II) ion. The proline δ-CH resonance, at 3.65 ppm, is somewhat broadened but unmoved by the addition of the Pd(II) reagent, evidence that the tertiary peptide nitrogen atom of proline is not bound to the Pd(II) ion. The NH signals of other residues persist in the TOCSY spectrum, evidence that these other amide groups are not bound either.

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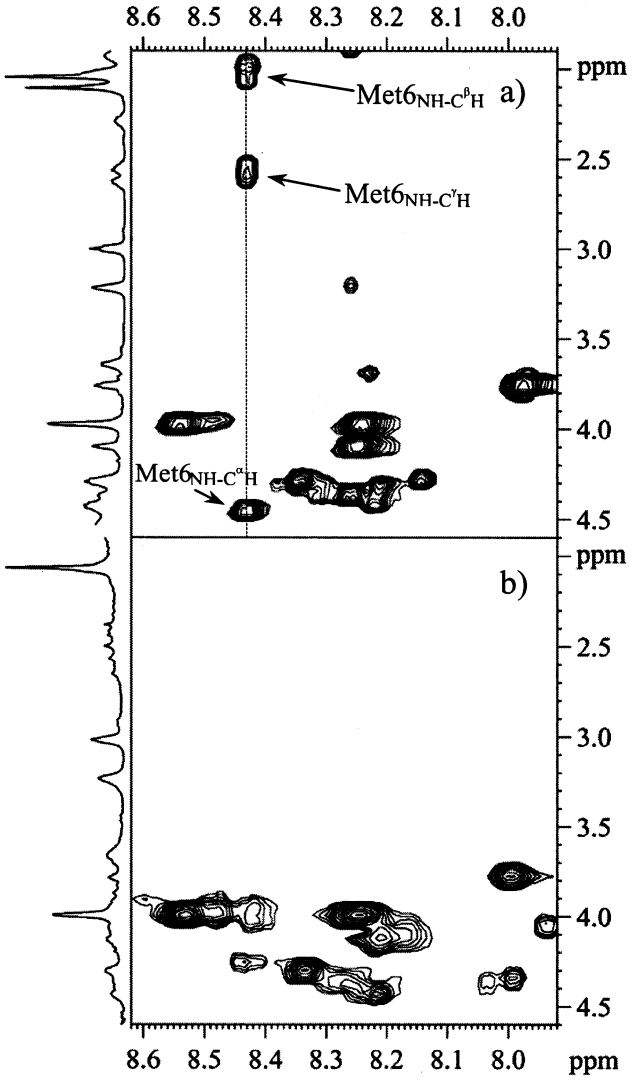
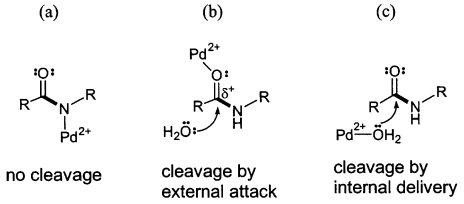


Figure 1. Aliphatic region of the one-dimensional ^{1}H NMR spectrum and a part of the NH-to-aliphatic region of the TOCSY ^{1}H NMR spectrum of the aqueous solution of AcLys-Gly-Gly-Ala-Gly-**Pro**-**Met**-Ala Ala-Arg-Gly (ProMet-peptide) at room temperature and pH 5.0: (a) before addition of $[Pd(H_2O)_4]^{2+}$ and (b) 10 min after addition of an equimolar amount of $[Pd(H_2O)_4]^{2+}$.

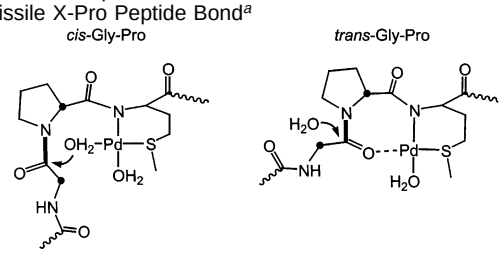
The right side of Scheme 1 concerns ProMet-peptide. As with GlyMet-peptide, the initial anchoring of Pd(II) ion to the methionine sulfur atom gives the unidentate complex **1**, and subsequent binding to deprotonated methionine nitrogen atom in the backbone produces the bidentate complex designated $\mathbf{2^{Pro}}$. The Pd(II) ion in $\mathbf{2^{Pro}}$, however, cannot continue to bind to the amide nitrogen atoms further upstream, because the amide group of Pro6 lacks a hydrogen atom and thus cannot be converted into the amidate anion required for the coordination. The next peptide nitrogen upstream also cannot bind to the Pd(II) ion because this binding requires formation of a five-membered chelate ring. Because the proline residue disrupts further "wrapping" of the peptide backbone around the bidentately bound Pd(II) ion,[53,54] the complex $\mathbf{2^{Pro}}$ persists at pH 5.0 (and likely also at pH 7.0), unlike the analogous complex **2**, which

Chart 2. Possible Interactions between Pd(II) Ion Anchored to a Side Chain of Methionine or Histidine and a Proximate Amide Group[a]



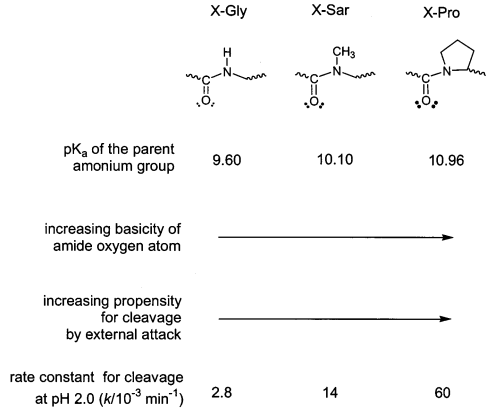
[a] For clarity, the anchor is not shown. (a) Binding to the amidate nitrogen atom requires the deprotonation of the NH group, makes the carbon atom less electrophilic, and inhibits the hydrolytic cleavage; (b) binding to the oxygen atom makes the carbon atom more nucleophilic toward solvent water and thus activates the amide group toward hydrolytic cleavage; and (c) close approach by the Pd(II) ion aids delivery of its aqua ligand to the carbon atom and hydrolytic cleavage of the peptide bond.

Scheme 2. Orientations of the C=O Group with Respect to the Pd(II) Ion in Complex $\mathbf{2^{Pro}}$ in the cis and trans Conformations of the Scissile X-Pro Peptide Bond[a]



[a] For simplicity, the Gly-Pro segment of the Gly-Pro-Met sequence is shown. The cis conformation, which is not observed, would activate the X-Pro bond for hydrolysis by the internal delivery of the water ligand. The trans conformation, which is the only one observed for this tertiary peptide bond and for the secondary (ordinary) peptide bonds, activates the scissile bond for hydrolysis by the attack of the external water molecule. The scissile C−N bond and the α-C atoms in glycine and proline are highlighted.

Scheme 3. Correlation between the Basicity of the Amino Group That Is the Precursor of the Scissile Amide Bond; the Intrinsic Metal-binding Ability of the Amide Oxygen Atom (Indicated by the Progression in the Size of the Dots Representing the Lone Electron Pairs); and the Rate Constant for the Pd(II)-Promoted Hydrolytic Cleavage of Gly-Gly, Gly-Sar, and Gly-Pro Peptide Bonds (These Correlations Give Evidence for the Mechanism b in Chart 2)



is only a minor species even at pH as low as 2.3 and disappears well below pH 5.0.

The Pd(II) ion in the complex of type **2** promotes the cleavage of the second peptide bond upstream from the anchor.[24] The differences in the coordination modes for GlyMet- and ProMet-peptides clearly explain the selective cleavage of Gly-Pro bond in ProMet-peptide at mildly acidic to neutral conditions. *The cleavage can occur only if the hydrolytically active complex, **2***

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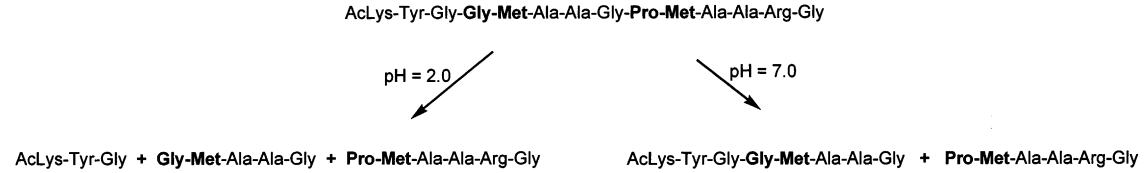
Scheme 4. Two Levels of Regioselectivity in Pd(II)-Promoted Cleavage of GlyMet···ProMet-peptide[a]



[a] At pH 2.0, the cleavage is directed by both Met5 and Met10 anchors. At pH 7.0, cleavage is directed only by Met10, owing to the presence of a proline residue immediately preceding it.

or $\mathbf{2^{Pro}}$*, is present: at pH < 3.0 for the X-Y-Met sequences, in which the scissile X-Y linkage is a secondary peptide bond, and throughout the range 1.0 < pH < 7.0 for the X-Pro-Met sequence, in which the scissile X-Pro linkage is a tertiary peptide bond.* Because analogues to the hydrolytically inactive complexes **3** and **4** cannot form with the X-Pro-Met segment, the X-Pro peptide bond is cleaved even in mildly acidic and neutral solutions.

Three other findings strengthen these conclusions. First, the cleavage of SarMet-peptide persists as the solution pH is raised from acidic to neutral. Because the scissile Gly5-Sar6 linkage is a tertiary peptide bond, a sarcosine analogue of the hydrolytically active complex $\mathbf{2^{Pro}}$ probably persists at pH ca. 7.0 since its conversion to the inactive complexes of types **3** and **4** is thwarted. Second, the ProAla-peptide (see Chart 1) is not cleaved by $[Pd(H_2O)_4]^{2+}$at pH 2.0, because it lacks the anchor. Third, ProMet-peptide is not cleaved by an equimolar amount of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ complex at pH 5.0. Because the Pd(II) ion already carries a bidentate ligand, this ion becomes "locked" in an inactive bis(bidentate) complex that resembles $\mathbf{2^{Pro}}$ but lacks the aqua ligands, necessary for cleavage.[35]

Possible Mechanisms of Cleavage. Because the peptide bond, i.e., the amide group, is a poor ligand, the Pd(II) ion interacts with this group in aqueous solution only if this metal ion (as a part of a complex) is already anchored to a side chain or to the terminal amino group. The mechanism by which the anchored Pd(II) ion in the hydrolytically active complexes **2** or $\mathbf{2^{Pro}}$ activates the scissile peptide bond is unknown. The anchored Pd(II) ion containing at least one accessible coordination site (weakly occupied by an aqua ligand) can variously interact with the amide bond, as shown simplistically (without the anchor) in Chart 2. This interaction can either inhibit or promote hydrolytic cleavage. The anchored Pd(II) ion can deprotonate the amide NH group and bind the nitrogen atom of the resulting amidate anion (Chart 2a).[29,55] The negative charge of the (coordinated) amidate anion strengthens the C−N bond, makes the amide carbon atom less susceptible to nucleophilic attack, and inhibits hydrolysis of this peptide bond. This Pd(II) binding to the amidate group explains the inactivity of complexes **3** and **4** in Scheme 1.

Binding of the anchored Pd(II) ion to the oxygen atom (Chart 2b) 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 cleavage of this peptide bond.[29,55] The anchored Pd(II) ion can promote cleavage of the proximal amide group even without binding to it (Chart 2c), by delivering an aqua ligand to the scissile group. Both mechanisms (b) and (c) in Chart 2 can explain the hydrolytic activity of complexes **2** or $\mathbf{2^{Pro}}$, but these two mechanisms cannot be distinguished by purely kinetic methods.[56] In the next subsection, we give two lines of indirect evidence for the external attack (Chart 2b) as the mechanism of the hydrolytic step.

Indirect Evidence for External Attack as the Cleavage Mechanism. The TOCSY ^{1}H NMR spectrum of ProMet-peptide shows only one set of signals for the Pro6 residue, indicating that only one of the two conformers of the Gly5-Pro6 peptide bond exists. The ROESY ^{1}H NMR spectrum of ProMet-peptide shows the cross peak between the α-CH signal of Gly5 (at 4.10 ppm) and δ-CH signal of Pro6 (at 3.65 ppm), evidence for the trans conformer, and lacks the cross peak between the α-CH signal of Gly5 and α-CH signal of Pro6, evidence against the cis conformer.[45] Upon binding of Pd(II) ion to the peptide, the δ-CH signal of Pro6 remains unshifted, evidence that the conformation of the Gly5-Pro6 peptide bond remains trans.

Scheme 2 shows that the orientation of the Pd(II) ion in complex $\mathbf{2^{Pro}}$ with respect to the crucial C═O group of the scissile Gly5-Pro6 peptide bond depends on the conformation of this bond. The cis conformer brings the Pd(II) ion toward the carbonyl carbon atom, the orientation favorable for the cleavage as in Chart 2c. The trans conformer brings the Pd(II) ion toward the carbonyl oxygen atom, the orientation favorable for the cleavage as in Chart 2b. The observed cleavage of the *trans*-Gly5-Pro6 peptide bond is therefore an indirect evidence for the external attack by the solvent water. Moreover, secondary peptide bonds are almost always trans. The fact that the Pd(II) reagent promotes their cleavage supports our conclusion.

Further indirect evidence for external attack as the cleavage mechanism comes from comparing cleavage of Gly-Pro bond in ProMet-peptide, Gly-Sar bond in SarMet-peptide, and Gly-Gly bond in GlyMet-peptide. The rate constants at pH 2.0 are 6.0×10^{-2}, 1.4×10^{-2}, and 2.8×10^{-3} min^{-1}, respectively. At this pH value, the dominant Pd(II)−peptide species for all three peptides is the hydrolytically active complex of type **2**. Clearly, at pH 2.0, the decreasing trend in the rate constant is not due to the existence of the inactive complexes of types **3** and **4**; they form at higher pH values.

The decrease in the rate constants can be explained in terms of the cleavage mechanism. In a series of dipeptides, the basicity and the metal-binding ability of the amide oxygen atom closely parallel the basicity of the amino group that is the precursor of the peptide bond of interest.[55] As the pK_a values in Scheme 3 show, proline has the most basic amino group. Consequently, the peptide bond involving the proline nitrogen atom has the most basic amide oxygen atom. Basicity, and the concomitant electron density, of the amide oxygen atom would disfavor the unassisted attack by a nucleophile, as in Chart 2c. Relatively high electron density can lead to an enhanced reactivity only if the amide oxygen atom binds to a Lewis acid, thus activating the amide carbon atom toward nucleophilic attack by a water

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Table 2. Kinetic Parameters for Cleavage of AcLys-Gly-Gly-Ala-Gly-**Pro-His**-Ala-Ala-Arg-Gly (ProHis-peptide) by $[Pd(H_2O)_4]^{2+}$ at Different Temperatures and pH Values

	rate constant $k/10^{-4}$ min^{-1}		
pH	at 20 °C	at 40 °C	at 60 °C
1.0	36(2)	164(8)	431(20)
2.0	115(8)	311(11)	940(80)
3.0	68(3)	181(8)	396(20)
4.0		34(4)	92(7)
5.0		11(2)	32(3)
6.0		19(1)	27(5)
7.0		28(4)	36(5)

molecule, as in Chart 2b. Parallel correlations in Scheme 3 give additional evidence that the Pd(II) ion acts as a Lewis acid and indirectly support the notion of the nucleophilic attack by the external water molecule.

Enhancement by Proline of the Rate of Histidine-Directed Cleavage. Histidine and methionine behave similarly as anchors and provide the same selectivity of cleavage.[24] Now we ask whether cleavage guided by both of these anchors will be similarly accelerated by the presence of a proline residue preceding the anchor.

The chromatograms of the equimolar mixture of ProHis-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.0 and 60 °C after 1.0 h and also after 24.0 h lacked the peak at 14.8 min, due to the intact peptide, and contained peaks at 6.1 and 13.1 min. MALDI mass spectra identified these two fractions, respectively, as fragment 1···5 (obsd 431.21 D and calcd 431.22 D) and fragment 6···11 (obsd 608.19 D and calcd 608.32 D). The cleavage is shown below.

Again, the Pd(II) reagent promoted selective cleavage of the second peptide bond upstream from the anchor, this time histidine. A long fragment of the protein myohemerythrin, containing segment Val-Pro-His, was cleaved at the Val-Pro bond, confirming that the cleavage is independent of the residue X in the X-Y-His reactive segment.[16] Cleavage of the tertiary X-Pro peptide bond is much faster than cleavage of similar peptides containing a secondary X-Y bond within an ordinary X-Y-His segment. Clearly, proline exerts its accelerating effect on hydrolytic cleavage regardless of the anchor (methionine or histidine) that follows it.

This effect is quantitated in Table 2. As Table 1 showed for ProMet-peptide, this table shows that at 60 °C the cleavage of ProHis-peptide is relatively fast at low pH (the half-life at pH 2.0 is 7.3 min) and persists even in neutral solution (the half-life is 191 min). The cleavage occurs at practical rates at 40 and even 20 °C (the half-life is 60 min at pH 2.0). These results confirm that the hydrolytically active complex is the histidine analogue of $\mathbf{2^{Pro}}$. The anchoring and the binding to the peptide backbone upstream from the anchor are unaffected by the neighboring side chains, provided these side chains do not coordinate to Pd(II) ion. Therefore, *cleavage at mildly acidic to neutral pH is expected whenever the anchoring residue (methionine or histidine) is preceded by a proline residue or another N-alkylated residue*, which forms a tertiary peptide bond.

Sequence-Specific Cleavage of X-Pro Peptide Bond. The chromatogram of the mixture containing Gly-Met···Pro-Met-peptide and 2 equiv of $[Pd(H_2O)_4]^{2+}$ after 12 h at pH 2.0 differed from the chromatograms of the same mixture at pH 5.0 and 7.0, which were alike. At pH 2.0 there were peaks at 12.9, 4.1, and 16.2 min. MALDI mass spectra showed the following fragments: 1···3 (obsd 409.11 D and calcd 409.20 D), 4···8 (obsd 406.28 D and calcd 406.17 D), and 9···14 (obsd 601.76 D and calcd 601.72 D). At pH 5.0 and 7.0, there were only peaks at 16.2 and 17.7 min, corresponding respectively to fragments 1···8 (obsd 795.20 D and calcd 795.36 D) and 9···14 (obsd 601.76 D and calcd 601.72 D).

As Scheme 4 shows, cleavage is directed by both Met5 and Met10 at pH 2.0, but only by Met10 at pH 5.0 and 7.0. This adjustable selectivity is easily explained. At pH 2.0, the Pd(II) reagent forms complex **2** with Met5 and complex $\mathbf{2^{Pro}}$ with Met10. Both complexes are hydrolytically active, and cleavage directed by both methionine anchors occurs. At pH 7.0, the Pd(II) reagent forms the hydrolytically inactive complex **4** with Met5, whereas the hydrolytically active complex $\mathbf{2^{Pro}}$ with Met10 persists. Consequently, the cleavage directed by Met5 is suppressed, while that directed by Met10 remains. Evidently, *selectivity of Pd(II)-promoted cleavage can be switched from anchor-selective to sequence-specific simply by changing the pH of the solution.*

Conclusions and Prospects

Although the X-Pro bond is highly resistant to cleavage by most proteolytic enzymes, $[Pd(H_2O)_4]^{2+}$ selectively cleaved this bond in the X-Pro-Met and X-Pro-His sequences. This sequence-specific cleavage occurs owing to the ability of the side chains of methionine and histidine to anchor the Pd(II) ion and the ability of proline residue to form a tertiary peptide linkage. The cleavage is possible under mild conditions—at temperature as low as 20 °C and at pH values from mildly acidic to neutral. This selectivity is a consequence of coordination of the Pro-Met segment to the Pd(II) ion. Cleavage can be switched from residue-specific to sequence-specific simply by adjusting the pH.

Because ordered pairing of a proline residue (average occurrence, 5.2%) and a methionine (2.2%) or histidine (2.3%) residue is improbable,[11] the segments Pro-Met and Pro-His are rare in proteins. Either can be introduced into bioengineered fusion proteins between the desired protein and the C-terminal tag. The Pd(II) reagent can cleave exclusively at the N-terminal end of this segments, releasing the intact protein of interest. Because most proteolytic enzymes are merely residue-selective, few of them are fit for this application. Our simple, inexpensive Pd(II) reagent has the sequence-selectivity unlike that of other proteases, requires relatively mild conditions, and works in equimolar mixture with the substrate. We will pursue biotechnological applications of this site-selective reaction.

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Supporting Information Available: Representative chromatograms and kinetic plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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