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Palladium(II) Complexes, as Synthetic Peptidases, Regioselectively Cleave the Second Peptide Bond "Upstream" from Methionine and Histidine Side Chains

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Abstract: Palladium(II) complexes promote hydrolysis of natural and synthetic oligopeptides with unprecedented regioselectivity; the only cleavage site is the second peptide bond upstream from a methionine or a histidine side chain, that is, the bond involving the amino group of the residue that precedes this side chain. We investigate this regioselectivity with four N-acetylated peptides as substrates: neurotransmitter methionine enkephalin (Ac-Tyr-Gly-Gly-Phe-Met) and synthetic peptides termed Met-peptide (Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala), His-peptide (Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala), in which a Met is oxidized to sulfone, and HisMet-peptide (Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala). While maintaining protein-like properties, these substrates are suitable for quantitative study since their coordination to Pd(II) ion can be determined (by NMR spectroscopy), and the cleavage fragments can be separated (by HPLC methods) and identified (by MALDI mass spectrometry). The only peptide bonds cleaved were the Gly3-Phe4 bond in methionine enkephalin, Gly4-Gly5 bond in Met-peptide, Gly3-Gly4 in His-peptide, and Gly3-Gly4 and Gly9-Gly10 bonds in HisMet-peptide. We explain this consistent regioselectivity of cleavage by studying the modes of Met-peptide coordination to the Pd(II) ion in $[Pd(H_2O)_4]^{2+}$ complex. In acidic solution, the rapid attachment of the Pd(II) complex to the methionine side chain is followed by the interaction of the Pd(II) ion with the peptide backbone upstream from the anchor. In the hydrolytically active complex, Met-peptide is coordinated to Pd(II) ion as a bidentate ligand − via sulfur atom in the methionine side chain and the first peptide nitrogen upstream from this anchor − so that the Pd(II) complex approaches the scissile peptide bond. Because the increased acidity favors this hydrolytically active complex, the rate of cleavage guided by either histidine or methionine anchor increased as pH was lowered from 4.5 to 0.5. The unwanted additional cleavage of the first peptide bond upstream from the anchor is suppressed if pH is kept above 1.2. Four Pd(II) complexes cleave Met-peptide with the same regioselectivity but at somewhat different rates. Complexes in which Pd(II) ion carries labile ligands, such as $[Pd(H_2O)_4]^{2+}$ and $[Pd(NH_3)_4]^{2+}$, are more reactive than those containing anionic ligands, such as $[PdCl_4]^{2-}$, or a bidentate ligand, such as *cis*-$[Pd(en)(H_2O)_2]^{2+}$. When both methionine and histidine residues are present in the same substrate, as in HisMet-peptide, 1 molar equivalent of the Pd(II) complex distributes itself evenly at both anchors and provides partial cleavage, whereas 2 molar equivalents of the promoter completely cleave the second peptide bond upstream from each of the anchors. The results of this study bode well for growing use of palladium(II) reagents in biochemical and bioanalytical practice.

Introduction

The amide bond in peptides and proteins is remarkably inert toward hydrolysis under usual conditions. For example, the half-life for cleavage of the N-acetylated dipeptide Ac-Gly-Gly at room temperature in neutral solution is ca. 500 years.[1] The half-lives for nonselective hydrolysis of proteins by dilute strong acids and strong bases at room temperature are measured in months and years.

Biochemical procedures involved in protein sequencing, footprinting, folding studies, and protein semisynthesis require partial degradation of proteins via regioselective hydrolysis of peptide bonds.[2] Few proteolytic enzymes and even fewer synthetic reagents are available for this purpose. Proteases cleave proteins selectively and catalytically under mild conditions, but only several of these enzymes are effective enough to be widely used.

Unlike enzymes, the existing synthetic reagents often require harsh conditions. Even when applied in great excess over the substrate, they tend to cleave with partial selectivity and low yields.[3] Cyanogen bromide, the most common chemical reagent for fragmentation of proteins, has several shortcomings. It is volatile and toxic, is applied in 100-fold excess over methionine residues, requires 70% formic acid as the solvent, and gives several side reactions. In the end, cyanogen bromide produces protein fragments that are no longer native because methionine residues in them are irreversibly modified.

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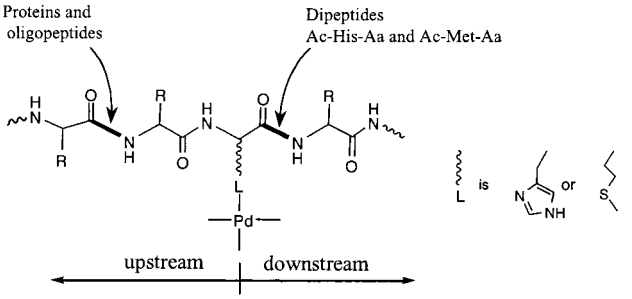
New chemical reagents for efficient and selective cleavage of proteins are needed. Some transition-metal complexes are beginning to fill this need.[4−15] Their properties can, in principle, be adjusted for particular applications. They can cleave proteins into large, unmodified fragments, which can be conveniently sequenced or chemically recombined into semisynthetic proteins.[16] Because transition-metal complexes are small and have few or no sterical requirements, they can probe the conformation or accessibility of protein regions.[7,8,17−22] Besides serving practical needs, study of the proteolytic activity of metal complexes can advance our understanding of natural metalloproteases.

After initial anchoring of the metal ion to the terminal amino group or a side chain, binding of this metal ion to the amide group in the peptide backbone can activate or deactivate this group toward hydrolysis.[23,24] Binding to amide oxygen atom enhances the electrophilicity of the amide carbon atom, stabilizes the tetrahedral intermediate formed in the nucleophilic attack by water, and promotes hydrolysis. Binding to (deprotonated) amide nitrogen strengthens the C−N bond, makes the amide carbon less susceptible to nucleophilic attack, and inhibits hydrolysis.

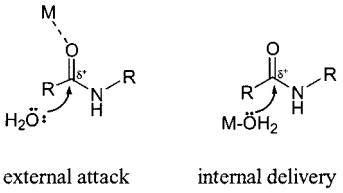
Metal complex can be anchored to a side chain in two ways: via a synthetic tether attached to a ligand coordinated to the metal ion,[17] or simply by direct binding of the metal ion to nitrogen and sulfur atoms in the side chains. Either anchoring mode leads to selective hydrolysis of a proximate amide bond, but the latter method is simpler than the former. The regioselective anchoring is a prerequisite for the regioselective cleavage because the anchor assists the interaction between the pendent metal complex and the amide bond. Complexes of palladium(II) can bind to the thioether group in methionine (Met) and to the imidazole group in histidine (His) residues. In the dipeptides of general formula Ac-His-Aa and Ac-Met-Aa (in which Ac is N-acetyl group and Aa is a leaving amino acid), the anchored Pd(II) ion hydrolyzes the peptide bond involving the carboxylic group of the anchoring amino acid, that is, the first peptide bond downstream from the anchor (see Scheme 1).[26−33] The cleavage

Scheme 1. Palladium(II) Complexes Act with Different Regioselectivity in Cleaving Dipeptides and Proteins[a]



[a] The sites of cleavage are highlighted. The anchoring side chain is coordinated to the Pd(II) ion via the group L; the remaining three ligands are not specified. The direction of the cleavage is defined with the anchor as the reference point.

Chart 1. Possible Limiting Mechanisms for Pd(II)-Promoted Hydrolysis of Peptide Bond



of dipeptides Ac-His-Aa is moderately catalytic;[31,32] a turnover as high as 14 was achieved with a binuclear Pd(II) complex as a catalyst.[34]

The peptide bond can be hydrolyzed by two kinetically indistinguishable limiting mechanisms, shown in Chart 1. The anchored Pd(II) ion either binds the oxygen atom of the scissile amide group, thus activating the carbonyl group toward the external attack by a water molecule, or delivers an aqua ligand to the scissile amide group. Regardless of the hydrolytic mechanism, that is, for either case in Chart 1, the likely rate-determining step in the hydrolysis of peptide bond is the breakdown of the tetrahedral intermediate.[5,6]

The regioselectivity of cleavage depends on the stereochemistry of coordination. The anchored Pd(II) complex must approach the peptide bond if the complex is to promote cleavage of this bond. Because this is a study of regioselectivity, it concerns the stereochemical factors. The results of this study are valid whether the cleavage occurs by external attack or internal delivery.

After our several studies with dipeptides and tripeptides, the pattern of their cleavage remained puzzling and unpredictable; these short substrates were reproducibly cleaved downstream, upstream, or on both sides of the anchoring residue. For example, in the dipeptide Ac-His-Gly, both the first upstream (Ac-His) and the first downstream (His-Gly) peptide bond were cleaved in the presence of $[PdCl_4]^{2-}$ complex.[35] However, in the tripeptide Ac-Gly-Gly-His, the second bond upstream from the anchor (the Gly-Gly bond) was cleaved by *cis*-[Pd(en)-

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$(H_2O)_2]^{2+}$.[36,37] A study involving a histidine-containing 19-residue fragment of the protein myohemerythrin, which can exist as an α-helix or a random coil, showed that the regioselectivity and the rate of cleavage are independent of the overall conformation.[36] In this long substrate, the only cleaved site was the second peptide bond from the N-terminal side of one of the two histidine residues, that is, the second bond upstream from the anchor (see Scheme 1).

Palladium(II) complexes proved remarkably effective in promoting fairly regioselective cleavage of cytochrome *c*,[13] myoglobin,[14] three albumins,[38] and several other proteins.[25] Because histidine and methionine residues have a combined average abundance in proteins of only 5.5%, the cleavage usually will give large fragments, convenient for sequencing and other biochemical applications. Upon removal of Pd(II) ions by precipitation or chelation, the peptides (protein fragments) remain pristine and can be used further. Despite these successes, the regioselectivity of the Pd(II)-promoted cleavage of proteins is not yet understood enough to be controlled. The cleavage sometimes occurred at the first bond downstream from the anchor, but in most cases at the second peptide bond upstream from it.

The present study is a step toward the understanding of Pd-(II)-promoted cleavage of proteins. We explain the difference in regioselectivity between dipeptides on the one side and oligopeptides and proteins on the other. This issue had to be resolved before our Pd(II) complexes could become accepted as practical proteolytic reagents.

To explain protein cleavage, we now report cleavage of longer peptides that in relevant aspects resemble proteins. While maintaining the protein-like properties, peptides are more suitable for detailed study since the structural information can be obtained (by NMR spectroscopy), and the fragments can be separated (by HPLC methods) and identified (by MALDI mass spectrometry). Placement of the anchoring residue(s) at some distance from the termini makes these peptides realistic models for protein segments. Indeed, the cleavage regioselectivity reported here matches that observed recently in proteins; in all cases, if pH is kept at 1.5 or higher, the exclusive site of cleavage is the second peptide bond upstream from the methionine or histidine residue.

We explain this consistent result by studying the coordination modes of the anchored Pd(II) ion before, during, and after the cleavage reaction. We also investigate the effect of the solution pH on the kinetics of cleavage. Palladium(II) complexes offer regioselectivity not achievable by other synthetic reagents – *the cleavage occurs not at the peptide bond involving the anchoring residue, but at a specific proximal peptide bond*. Now that we finally understand the regioselectivity of protein cleavage, we will be able to control it.

Experimental Procedures

Chemicals. Distilled water was demineralized and purified to a resistivity higher than 16 MΩ·cm. Palladium sponge, *cis*-[Pd(en)Cl_2] (in which en is ethylenediamine), $K_2[PdCl_4]$, piperidine, triisopropylsilane, trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid, and *N*,*N*-diisopropylethylamine were obtained from Aldrich Chemical Co. Methionine enkephalin (Tyr-Gly-Gly-Phe-Met) was obtained from Sigma Chemical Co. Methyl phenyl sulfone was obtained from Lancaster Synthesis Inc. Acetonitrile of HPLC grade, dichloromethane, *N*,*N*-dimethylformamide (DMF), diethyl ether, and 1,2-ethanedithiol were obtained from Fisher Scientific Co.

Each amino acid had its amino group protected by Fmoc group; those containing reactive side chains had them protected as well. The α-amino acids are Fmoc-Ala, Fmoc-Arg(Pmc), Fmoc-Gly, Fmoc-His-(Trt), Fmoc-Lys(Boc), Fmoc-Met, Fmoc-Phe, Fmoc-Tyr(tBu), and Fmoc-Val. They, the *N*-α-Fmoc-Ala-Wang resin, HBTU, and HOBt were obtained from Calbiochem-Novabiochem Corp. Above, Fmoc is 9-fluorenylmethoxycarbonyl, Pmc is 2,2,5,7,8-pentamethylchroman-6-sulfonyl, Trt is trityl, Boc is *tert*-butoxycarbonyl, tBu is *tert*-butyl, HBTU is 2-(1-benzotriazole-1-yl)-1,1,3,3-teramethyluronium hexafluorophosphate, and HOBt is *N*-hydroxybenzotriazole.

The stock solution of the complex $[Pd(H_2O)_4]^{2+}$, obtained according to the published procedure,[39] had pH below 0. The complexes $[Pd(NH_3)_4]^{2+}$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were prepared by the published procedures.[40,41] All complexes were prepared as perchlorate salts. The concentrations of the Pd(II) complexes were determined using their published extinction coefficients.

Peptide Synthesis. The peptides Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (termed Met-peptide) and Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (termed HisMet-peptide) were synthesized by a standard, manual Fmoc solid-phase procedure.[3,42] The Fmoc-Ala-Wang resin had a loading of 0.69 mmol/g. The Fmoc group was removed with the 25% (v/v) solution of piperidine in DMF. The reagents for coupling were HBTU (2.9 equiv with respect to the resin loading), HOBt (3.0 equiv), and *N*,*N*-diisopropylethylamine (6.0 equiv), each added to 3.0 equiv of the protected amino acid in DMF. The N-terminus was acetylated by a 20% (v/v) solution of acetic anhydride in DMF. To cleave the peptide from the resin and remove the side-chain protecting groups, the dried peptide resin was kept for 5 h at room temperature in a solution containing by volume 94.0% TFA, 2.5% water, 2.5% 1,2-ethanedithiol, and 1.0% triisopropylsilane. Upon filtration, the supernatant was evaporated to a small volume and added to cold diethyl ether, to precipitate the crude peptide. Upon three washes with diethyl ether, the precipitate was dried, dissolved in water, and filtered. The peptide was purified by reverse-phase HPLC on a C-18 preparative column. The analytical chromatogram of the pure peptide showed purity higher than 99.5%. The MALDI-TOF mass spectrum of the pure peptide contained a single, strong peak that matched the calculated molecular mass of the peptide. For Met-peptide, the found and calculated molecular masses were, respectively, 1036.57 and 1036.51 D; for HisMet-peptide, 1515.08 and 1514.78 D.

The methionine thioether group in the HisMet peptide was oxidized to sulfone by H_2O_2 and formic acid;[3] this oxidized residue is designated Met^{OX}. This modified HisMet peptide, termed His-peptide, was purified by preparative reverse-phase HPLC, and the analytical chromatogram showed purity higher than 99.5%. The MALDI-TOF mass spectrum showed incorporation of exactly two oxygen atoms in HisMet peptide; the found and the calculated masses, respectively, were 1545.76 and 1546.77 D. Evidently, no side reaction had occurred.

Spectroscopic and Analytical Methods. 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 sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in aqueous solutions. Because the peptide cleavage is negligible at room temperature, we safely assumed that the composition of the sample remained unchanged during the NMR measurements.

For the experiments involving Met-peptide, two-dimensional ^{1}H TOCSY (total correlation spectroscopy) spectra in aqueous solution were acquired. The ambiguous assignment of the residues Gly4 and

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Gly5 was resolved by applying ROESY (rotating-frame Overhauser enhancement spectroscopy) to the peptide (data not shown). Each two-dimensional data set consisted of 256 × 2048 complex points. A mixing time of 70 ms was used in TOCSY experiments and 500 ms in the ROESY experiment. The spin-lock field strength during mixing was 2.5 kHz in ROESY experiment and 6.4 kHz in TOCSY experiments. To suppress the water signal, the WATERGATE[43] was incorporated into the pulse sequences. The pH was measured with a Fisher Accumet instrument and an Aldrich Ag/AgCl reference electrode.

The components of the peptide digests were separated by a Hewlett-Packard 1100 HPLC system containing an autosampler and a multi-wavelength detector set to 215, 280, and 350 nm. Absorption at 215 nm is common to all peptides; absorption at 280 nm is specific for peptides containing aromatic residues or bound Pd(II) ion; and absorption at 350 nm is specific for Pd(II) complexes.

A Supelco Discovery C-18 column (sized 250 × 4.6 mm, beads of 5 μm) was used for the analytical runs, and a Vydac C-18 column 218TP101522 (sized 250 × 22 mm, beads of 10 μm) was used for the preparative runs. The eluting solvent A was 0.1% (v/v) TFA in H_2O, and solvent B was 0.08% (v/v) TFA in acetonitrile. In a typical run, the percentage of solvent B in the eluent 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 "fast" analytical run optimized for the kinetic measurements, the fraction of solvent B was initially 10% and was raised to 22% over a 13 min period. The flow rate was 1.0 mL/min for analytical runs and 10 mL/min for preparative runs.

The MALDI-TOF experiments were performed with a Bruker Proflex instrument. The samples containing intact peptide, the reaction mixture of the peptide and the Pd(II) complex (digest), and separate fractions isolated by HPLC runs 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-hydroxycinnamic acid, in solution containing a 2:1 (v/v) mixture of water and acetonitrile. Each spectrum consisted of 100 scans. Solutions of angiotensin II and of oxidized chain B of insulin were used as external standards. The measured molecular mass was compared with the mass expected of a given fragment by PAWS software from ProteoMetrics, LLC.

The presence of Pd(II) ion bound to the peptide or its fragment(s) was established not only by the matching molecular mass for peptide plus Pd atom, but also by the following isotopic distribution, diagnostic of palladium: 1.0% ^{102}Pd, 11.1% ^{104}Pd, 22.3% ^{105}Pd, 27.3% ^{106}Pd, 26.5% ^{108}Pd, and 11.8% ^{110}Pd.

Study of Hydrolysis. Reactions were carried out 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 solution of $[Pd(H_2O)_4]^{2+}$, 20.0 μL of a solution containing 25.0 mg/mL of phenyl methyl sulfone, and 780.0 μL of water. The same conditions were used also for the experiments involving $[PdCl_4]^{2-}$ and $[Pd(NH_3)_4]^{2+}$ complexes. For the cleavage of HisMet-peptide by 2 molar equivalents of Pd(II) complex, the aforementioned mixture contained 4.0 μL of a 500 mM solution of $[Pd(H_2O)_4]^{2+}$. In the experiments with the complex *cis*-$[Pd(en)(H_2O)_2]^{2+}$, 10.0 μL of a 100 mM stock solution was added to the aqueous mixture of the peptide and the internal standard. The pH was adjusted by careful addition of either 1.00 M $HClO_4$ or 1.00 M NaOH, with stirring. The reaction mixture was kept in a dry bath at 60 ± 1 °C. After the reaction was completed, the pH remained within ±0.10 of the initial value. In the control experiments concerning possible "background" cleavage, the conditions were the same, except that the Pd(II) reagent was absent.

For the kinetic measurements, 40.0 μL samples were periodically taken from the reaction mixture and immediately subjected to reverse-phase HPLC separation. Because the cleavage is negligibly slow at room temperature, the species distribution in the chromatogram reflected that in the digest at the time of sampling. To compensate for the possible error in the injection volume and evaporation, the areas under the chromatographic peaks were integrated and normalized to that of phenyl methyl sulfone, the internal standard. The error of this integration was estimated at 5%. The plots of the peak area of the cleavage fragments versus time were 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 two standard deviations, that is, confidence limit greater than 95.0%.

The cleavage fragments were collected from the analytical runs, dried by blowing the stream of nitrogen, dissolved in 10.0 μL of water, and subjected to MALDI-TOF mass spectrometry. For NMR spectroscopic study of Met-peptide cleavage, the fragments were separated by the preparative reverse-phase HPLC method.

Chart 2. Peptides Cleaved by Pd(II) Complexes[a]

Met-enkephalin:	Ac-Tyr-Gly-Gly-Phe-**Met**
Met-peptide:	Ac-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala
HisMet-peptide:	Ac-Val-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala
His-peptide*:	Ac-Val-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala

* Met^{OX} contains sulfone, a noncoordinating group, in the side chain

[a] The anchoring residues are highlighted and included in the peptide names.

Results and Discussion

Substrates and Reagents for Cleavage. In addition to using methionine enkephalin, an endogenous opioid neurotransmitter,[44] we designed and synthesized three peptides that resemble natural products. All of them are shown in Chart 2. Each of these substrates contains a methionine or a histidine residue as an anchor for the Pd(II) complex. Because the anchoring methionine residue in Met-enkephalin forms the C-terminus, cleavage of this peptide is possible only upstream from the anchor. The anchoring residues in Met-peptide, HisMet-peptide, and His-peptide are internal, that is, removed from the termini. For the purpose of our study, these peptides are realistic mimics of proteins since the cleavage is now possible both upstream and downstream from the anchors. At the same time, these peptides are more convenient than large proteins for structural NMR studies and for chromatographic separations and mass-spectroscopic identification of the cleavage fragments.

Tyrosine residue, an aromatic chromophore, allows specific detection of fragments containing it, besides general detection of amide bond chromophore in all peptides. For accurate kinetic experiments, positively charged lysine or arginine residues on both sides of each anchor ensure that under acidic or neutral conditions the peptide and its fragments will remain charged and thus soluble in water. To preclude unwanted binding of Pd(II) complexes to the N-terminus, all the peptides are acetylated there, and thus made similar to protein segments.

The anchoring side chains are preceded by two Gly residues and followed by an Ala residue. Because these flanking residues are the same for the histidine and methionine anchors, we can directly compare the regioselectivity and rates of cleavage controlled by these two anchors. In the HisMet-peptide the two anchors are separated by five residues, to avoid the formation of macrochelate complexes, in which one Pd(II) complex would

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be attached to both anchors. Such macrochelation is rare in proteins too.

All reactions were done in acidic solutions because in neutral and basic solutions deprotonation of aqua ligands on Pd(II) ion would result in the formation of insoluble hydroxo-bridged Pd(II) species. Although complex $[Pd(H_2O)_4]^{2+}$ deprotonates into $[Pd(H_2O)_3(OH)]^{+}$ with pK_a of ca. 3.0,[45] we always use the former formula for the sake of consistency. Coordinating anions are absent because they would compete with the peptides for the Pd(II) reagent and thus inhibit the cleavage reaction. All of these conditions were also used in cleaving proteins.

In the control experiments, all the conditions were the same, except that the Pd(II) reagent was absent. The chromatograms of these solutions after 14 days showed only the peak of the intact peptide. Evidently, in the absence of Pd(II) complexes, the acid-catalyzed hydrolysis of the peptide substrates was undetectable.

Binding of Pd(II) Complexes to the Peptides. Upon the mixing of each peptide with 1 equiv of $[Pd(H_2O)_4]^{2+}$, rapid color change signaled immediate binding. After incubation for 5 min, a sample was taken and analyzed by HPLC. In each case, the chromatogram contained two new major peaks in addition to the peak of the uncoordinated peptide. These new species showed absorption at 215 and 280 nm, diagnostic of the peptide backbone and the aromatic residues, and also absorption at 350 nm, diagnostic of Pd(II) complexes.

The fractions from the analytical HPLC runs were analyzed by MALDI mass spectrometry. Each new species showed a peak corresponding to the intact peptide and a peak corresponding to its Pd(II) complex. The isotopic distribution within the latter peak confirmed this composition.

The mode(s) of binding of $[Pd(H_2O)_4]^{2+}$ to Met-peptide were determined by one-dimensional ^{1}H NMR and the two-dimensional TOCSY ^{1}H NMR experiments at room temperature. We recorded the spectra of the reaction mixture after 5 min at pH 2.3 and 4.5, and after 14 h at pH 2.3. The aliphatic region in the one-dimensional spectrum and a part of the amide-to-aliphatic region in the TOCSY spectrum are shown in Figure 1.

Upon addition of $[Pd(H_2O)_4]^{2+}$ to Met-peptide at pH 2.3, signals of methionine residue shifted the most. The disappearance of the SCH_3 singlet at 2.12 ppm (Figure 1a) and appearance of a broad signal at 2.45 ppm prove binding of Pd(II) complex to the methionine side chain. The methionine NH signal at 8.05 ppm, and also its cross-peaks to α-, β-, and γ-CH resonances marked in Figure 1a, disappeared upon addition of $[Pd(H_2O)_4]^{2+}$, as shown in Figure 1b. These disappearances prove that the amide nitrogen atom becomes deprotonated, clear evidence of its coordination to the Pd(II) ion. This upstream coordination of the anchored Pd(II) ion is also evident in the movement from 4.45 to 3.96 ppm of the methionine α-CH group resonance (2D signals not shown).[27,46,47] The α-CH resonance of Gly5 at 3.98 ppm broadened considerably, and its cross-peaks to amide NH partially diminished (see Figure 1b). Moreover, two different amide-to-α-CH cross-peaks are observed for Gly4. Clearly, two species are present in the solution. In one species, the nitrogen atom of Gly5 is deprotonated and therefore coordinated to Pd(II) ion, whereas in the other species, this atom is protonated and not coordinated. The nitrogen atom of Gly4 remains protonated in both species.

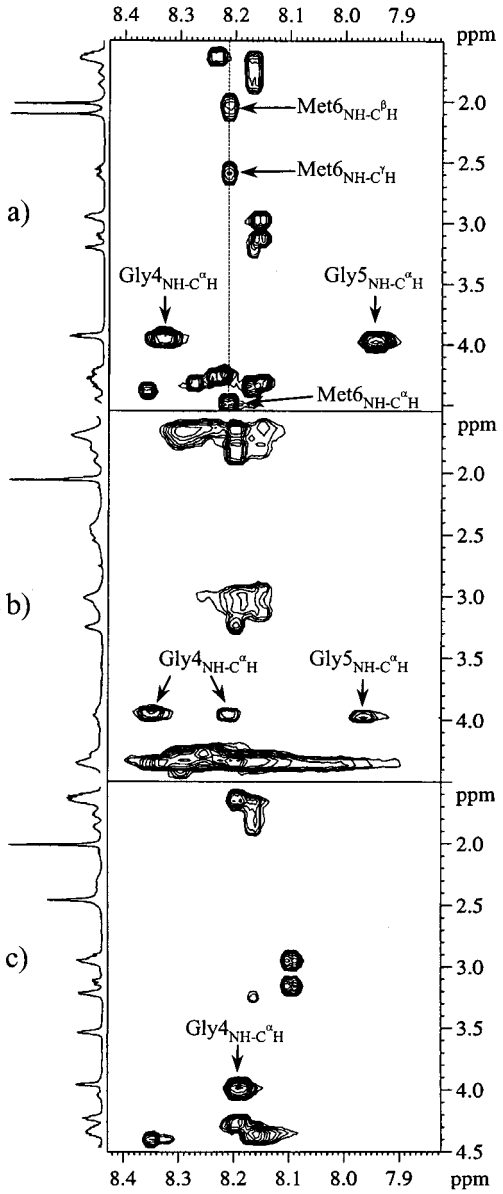

Figure 1. The aliphatic region of one-dimensional ^{1}H NMR spectrum and a part of the NH-to-aliphatic region of TOCSY ^{1}H NMR spectrum of the aqueous solution of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide), at pH 2.3 (a) before addition of $[Pd(H_2O)_4]^{2+}$; (b) after addition of an equimolar amount of $[Pd(H_2O)_4]^{2+}$ and incubation for 5 min at 60 °C; and (c) after the cleavage by the equimolar amount of $[Pd(H_2O)_4]^{2+}$ for 24 h at 60 °C.

The results of HPLC and MALDI mass spectrometry experiments consistently show that two complexes, designated **2** and **3** in Scheme 2, are formed when Met-peptide reacts with $[Pd(H_2O)_4]^{2+}$. These structures are evident from the TOCSY ^{1}H NMR spectrum. In complex **2**, the peptide is bound to Pd(II) ion as a bidentate ligand, via the methionine side chain and the first peptide nitrogen upstream of this anchor. In complex **3**, the peptide is a tridentate ligand, additionally coordinated to Pd(II) ion via the second peptide nitrogen upstream of the methionine anchor. These two complexes are exchanging at pH 2.3, as is evident from broad α-CH resonances and from the

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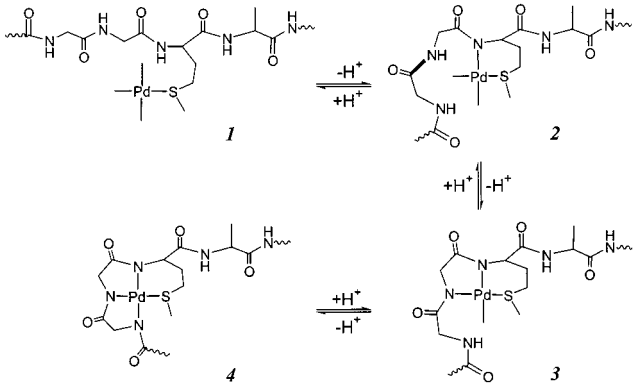
Scheme 2. Coordination to Pd(II) of the Methionine Side Chain (the Anchor) Followed by the Stepwise Coordination of the Deprotonated Nitrogen Atoms in the Peptide Backbone Upstream from the Anchor[a]



[a] The scissile peptide bond is highlighted.

pattern of amide-to-α-CH cross-peaks for Gly4 and Gly5 in the TOCSY ^{1}H NMR spectrum.

In the TOCSY ^{1}H NMR spectrum of the same solution at pH 4.5, the amide-to-α-CH cross-peak for Gly5 completely disappeared, indicating complete deprotonation of the amide nitrogen of Gly5, that is, its complete coordination to Pd(II) ion (see Figure S1 in the Supporting Information). Only a weak amide-to-α-CH cross-peak is found for Gly4, an indication that Pd(II) ion displaced most of the peptide hydrogen atom in Gly4. Complete absence of the amide-to-α-CH cross-peaks for Gly5 and Met6 and minor presence of this peak for Gly4 prove that at pH 4.5 the major species is the tetradentate complex **4**, and the minor species is the tridentate complex **3**. Evidently, the pK_a value for the NH group of Gly5 in the presence of anchored Pd(II) complex is closer to 2.3 than to 4.5.

Because the chromatograms and the MALDI mass spectra showed that $[Pd(H_2O)_4]^{2+}$ in acidic solutions forms two complexes with methionine-enkephalin and also with Met-peptide, we conclude that the coordination modes identified for Met-peptide are general for peptides containing "isolated" methionine residues. The Pd(II) ion anchored to a methionine side chain in a peptide gradually deprotonates the peptide nitrogen atom(s) upstream from the anchor and binds to them. The extent of this process depends on pH, and the displacement of each next proton requires higher pH because the positive charge of the Pd(II) ion and its Lewis acidity are decreasing with each new coordination of a peptide nitrogen atom. Transition-metal ions anchored to a side chain usually require neutral to basic solutions for upstream coordination,[23,48−54] but Pd(II) ion can readily deprotonate upstream peptide nitrogen atoms even in acidic solution. The pK_a for the deprotonation of the first peptide nitrogen upstream by a Pd(II) ion anchored at a histidine residue is estimated at ca. 2.0,[23,50] consistent with our results.

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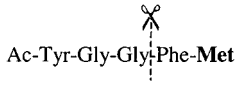
Table 1. Results of HPLC Separation and MALDI Mass Spectrometric Experiments with Fragments of N-Acetylated Tyr-Gly-Gly-Phe-Met (Met-enkephalin) Resulting from the Cleavage by $[Pd(H_2O)_4]^{2+}$

elution time (min)	observed mass (D)	calculated mass (D)	fragment
16.1	337.87	337.30	1-3
20.6	297.27	296.39	4-5

In dipeptides of the type Ac-Met-Aa, studied in our laboratory earlier, only one peptide nitrogen atom upstream from the anchor is available for coordination to Pd(II) ion, but this process was largely suppressed by acid. Instead, the anchored Pd(II) ion interacts with the downstream peptide bond and promotes its cleavage. In longer peptides that we study now, several peptide bonds are available upstream from the anchor, and the anchored Pd(II) ion readily binds upstream, even in acidic solutions. This interaction may promote hydrolysis of an upstream peptide bond. Studies with dipeptides were useful for understanding the kinetics and stereochemistry of the cleavage reactions, but only the present study, with longer peptides, explains the regioselectivity of cleavage found in proteins.

Cleavage of Methionine Enkephalin, in Which Only Cleavage Upstream Is Possible. The samples of the reaction mixture containing equimolar amounts of N-acetylated Met-enkephalin and $[Pd(H_2O)_4]^{2+}$ at pH 2.5 were collected every hour. The two broad chromatographic peaks with retention times of 24.1 and 26.8 min, corresponding to the initially formed Pd-peptide complexes, gradually decreased, while new peaks at 16.1 and 20.6 min gradually emerged. The fraction at 16.1 min absorbed at 215 and 280 nm but not at 350 nm, an indication that this fraction contains tyrosine residue but does not carry Pd(II) ion. The fraction at 20.6 min was detectable at all three wavelengths, evidence that this fraction contains Pd(II) ions. The reaction was followed for 24 h, after which time the peaks at 24.1 and 26.8 min disappeared.

As Table 1 and the diagram below show, the fraction eluting at 16.1 min is the fragment 1-3, and the fraction eluting at 20.6 min is the Pd(II)-carrying fragment 4-5.



This assignment based on the MALDI mass spectra corroborates our previous conclusion from the monitoring of the chromatographic fractions. *With methionine enkephalin, for the first time we observed the regioselective cleavage of the second peptide bond upstream from methionine residue.*

Upstream versus Downstream Cleavage of a Methionine-Containing Peptide. The interesting finding with Met-enkephalin raised this question: Will this regioselectivity persist in peptides containing an internal (not C-terminal) methionine residue? In other words, will both upstream and downstream cleavage occur if peptide bonds are available on both sides of the anchoring residue?

(1) Regioselectivity of Cleavage. As described above, complexes **2** and **3** are formed within 5 min of mixing equimolar amounts of Met-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.3. The chromatograms of the samples taken before the addition of $[Pd(H_2O)_4]^{2+}$ and 1, 12, and 22 h after mixing showed gradual

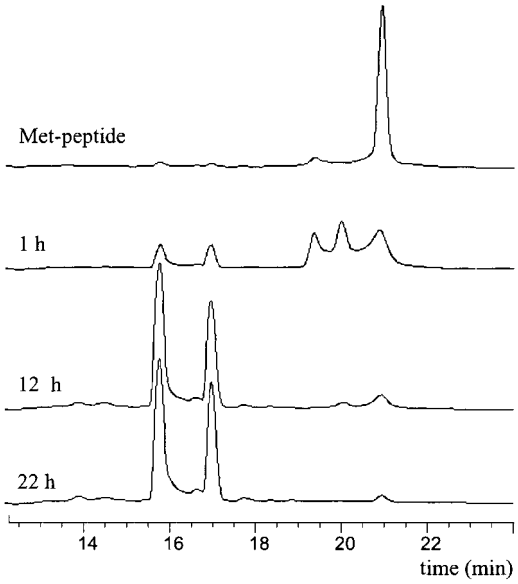


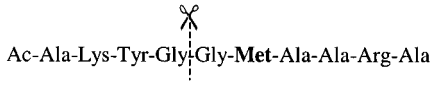
Figure 2. Monitoring the digestion of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) by $[Pd(H_2O)_4]^{2+}$ at pH 2.3 and 60 °C. The chromatograms at 215 nm of Met-peptide and of the samples taken at the specified times after mixing the two reagents. Composition of the fractions: 21.0 min, whole peptide; 19.4 and 20.1 min, Pd(II)-complexes of the whole peptide; 15.9 min, fragment Gly-Met-Ala-Ala-Arg-Ala bearing one Pd(II) atom; and 17.1 min, fragment Ac-Ala-Lys-Tyr-Gly.

Table 2. Results of HPLC Separation and MALDI Mass Spectrometric Experiments with Fragments of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) Resulting from the Cleavage by $[Pd(H_2O)_4]^{2+}$

elution time (min)	observed mass (D)	calculated mass (D)	fragment
17.1	576.64	576.68	1−4
15.9	480.70	479.34	5−10

disappearance of the peaks due to the intact peptide (at 21.0 min) and to two Pd(II)-peptide complexes (at 19.4 and 20.1 min), and appearance of new peaks at 15.9 and 17.1 min; see Figure 2. The fraction eluting at 15.9 min absorbs at 215, 280, and 350 nm, evidence for a Pd(II) complex. The fraction eluting at 17.1 min absorbs at 215 and 280 nm but not at 350 nm (data not shown), evidence for a fragment containing tyrosine residue and no bound Pd(II) ions.

The results of the MALDI mass spectrometric characterization are summarized in Table 2 and diagrammatically shown below.



The fraction eluting at 15.9 min is the Pd(II)-containing fragment 5-10, and the fraction eluting at 17.1 min is the pristine fragment 1-4. Again, this assignment agrees with the chromatograms recorded at different wavelengths. No other species was present in the digest after 22 h. That only the intact Met-peptide was observed in the chromatogram of the control solution in the absence of Pd(II) complex rules out background cleavage by the acidic solution.

Evidently, the anchored Pd(II) complex again promoted regioselective scission of the second peptide bond upstream from the methionine anchor, in this case the Gly-Gly bond. No cuts occurred downstream from the anchor. This regioselectivity was seen in our cleavage of proteins, but not of dipeptides since they lack the second peptide bond upstream from the anchor. Now that we use appropriate mimics of proteins, we reproduce the regioselectivity seen in proteins.

To understand this regioselectivity, we also investigated the fate of the Pd(II) ion after the cleavage was completed. The TOCSY ^{1}H NMR spectrum of this digest after 24 h (Figure 1c) contains the methionine SCH_3 resonance at 2.46 ppm, indicating thioether coordination to Pd(II) ion.[27,46,47] The α-CH resonance of Gly4 at 3.92 ppm is no longer broad, and a cross-peak relates it to the NH resonance of Gly4 at 8.21 ppm. Evidently, this glycine residue is not involved in coordination to Pd(II) ion. At the same time, the α-CH resonance of Gly5 is shifted upfield to 3.50 ppm, and it shows no cross-peaks in the amide-NH-to-α-CH region, indicating its N-terminal position and coordination to Pd(II) ion.[55,56] These findings confirmed that Met-peptide was cleaved at the Gly4-Gly5 bond.

The Pd(II)-bearing fragment eluting at 15.9 min was isolated from the digest in a preparative HPLC experiment. Its TOCSY ^{1}H NMR spectrum (Figure S2 in the Supporting Information) contains the same set of resonances for Gly5 and Met6 as does the spectrum of the digest after 22 h. The other resonances are assigned to one arginine and three alanine residues, confirming the identity of this peptide as the fragment 5-10.

These results show that Pd(II) ion remains bound to fragment 5-10 of the initial Met-peptide, within the tridentate complex of type **3**. The ligands on Pd(II) ion are the sulfur atom of methionine, the peptide nitrogen atom of methionine, and the newly formed terminal amino nitrogen atom of Gly5 residue.

(2) Effect of the Ligand on the Kinetics of Cleavage. The appearance in time of both fragments of Met-peptide cleaved by $[Pd(H_2O)_4]^{2+}$ was successfully fitted to the first-order rate law. Because the binding of Pd(II) ion to the sulfur atom of methionine occurs fast upon mixing and all the ensuing reactions are intramolecular, fitting of the kinetic results to the first-order rate law is justified. A typical plot is shown in Figure 3a.

We investigated the following four Pd(II) complexes as promoters of the regioselective cleavage of Met-peptide at pH 1.8: $[Pd(H_2O)_4]^{2+}$, $[Pd(NH_3)_4]^{2+}$, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, and $[PdCl_4]^{2-}$. In all cases, only the Gly4-Gly5 bond was cleaved, with the respective first-order rate constants of 0.24(2), 0.25(3), 0.14(3), and 0.13(2) h^{-1}. Although all four complexes show the same regioselectivity, the nature of the ligand affects the rate of the reaction. As the rate constants show, $[Pd(NH_3)_4]^{2+}$ and $[Pd(H_2O)_4]^{2+}$ are more effective than *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and $[PdCl_4]^{2-}$ as promoters of the cleavage.

The coordination of the peptide to the Pd(II) ion is prerequisite for the peptide cleavage. Because the donor atoms in the peptide can easily displace water and ammonia ligands, the first two complexes cleave at the same rate (within the experimental error). The relatively strongly bonded chloride anion and the bidentate ethylenediamine ligand slow the cleavage by competing with the peptide for coordination to Pd(II) ion. The chloride anion additionally inhibits the cleavage reaction by lowering the Lewis acidity of Pd(II) ion.

The complex *cis*-$[Pd(en)(H_2O)_2]^{2+}$ shows a delay, evident in the first part of the plots in Figure 3b, owing to the relatively slow displacement of the bidentate ethylenediamine ligand by

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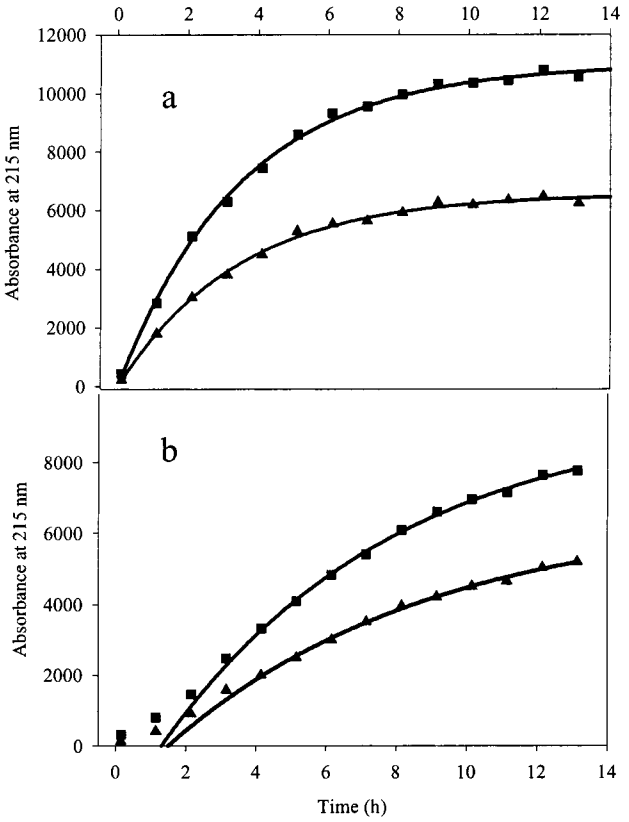


Figure 3. The progress of cleavage of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) at pH 1.85 by (a) $[Pd(H_2O)_4]^{2+}$; and (b) *cis*-$[Pd(en)(H_2O)_2]^{2+}$, followed by HPLC separations of the digests. The data for the peaks eluting at 15.9 min (■) and 17.1 min (▲) are obtained by normalizing the peak area to that of the internal standard phenyl methyl sulfone. The solid lines are fittings to the first-order rate law. The first four data points in the frame (b) were justifiably excluded from the fitting because during this time the ethylenediamine ligand is displaced.

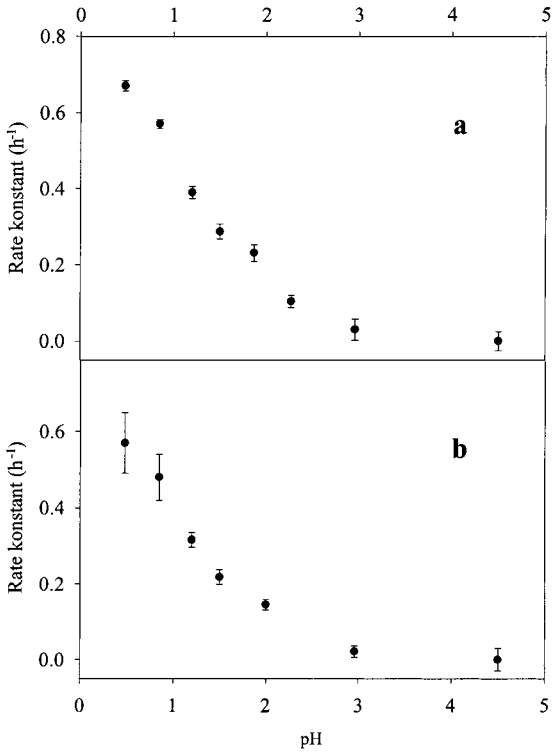


Figure 4. Dependence on pH of the rate constants for cleavage promoted by $[Pd(H_2O)_4]^{2+}$ of (a) the Gly4-Gly5 peptide bond in Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) and (b) the Gly3-Gly4 peptide bond in Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala (His-peptide), in which Met^{OX} contains a sulfone group.

the donor atoms in the peptide.[57] The existence of this delay supports our mechanism of the cleavage. The Pd(II) ion in the complex *cis*-$[Pd(en)(H_2O)_2]^{2+}$ can still effectively bind to the sulfur and the peptide nitrogen atoms of the methionine residue. So bound, however, the Pd(II) ion cannot readily interact with the second peptide bond upstream because the remaining two coordination sites are occupied by ethylenediamine, which is exchanged relatively slowly. Only after this ligand is displaced, in a process assisted by the acidic solvent, can the Pd(II) ion cleave the scissile peptide bond.

The cleavage of the second peptide bond upstream from the anchor is facilitated if Pd(II) ion carries labile ligands. For this reason, we performed further experiments with $[Pd(H_2O)_4]^{2+}$ ensuring that solutions were free of coordinating anions, such as chloride or acetate.

To investigate the dependence of the rate constant for cleavage on the acidity of solution, we ran the reaction at eight pH values ranging from 0.5 to 4.5. The results are summarized in Figure 4a and Table S1 in the Supporting Information. Each rate constant is the average of two consistent values, obtained by monitoring each of the two fragments of cleavage.

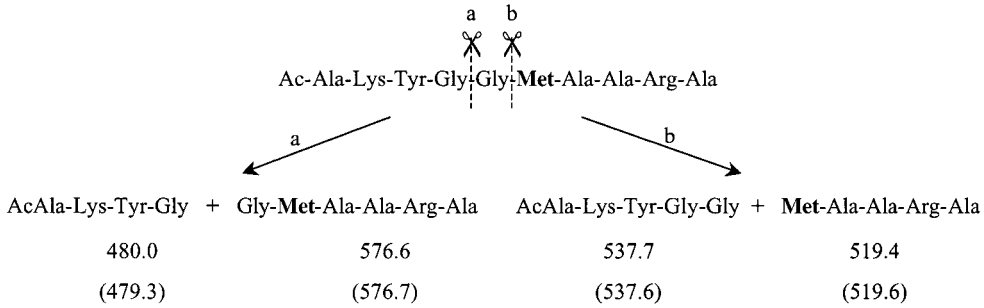
The rate constant for the reaction increases with increasing acidity. The aforementioned NMR experiments demonstrated that at pH 2.3 the only Pd(II)-peptide complexes are **2** and **3**, which undergo exchange. The right part of Scheme 2 explains the regioselectivity of cleavage. In complex **3**, the major species, binding of Pd(II) ion to the amide nitrogens in both Gly5 and Met6 stabilizes both of these peptide bonds.[23] In complex **2**, the minor species at pH 2.3, binding of Pd(II) ion to the peptide nitrogen in Met6 stabilizes only this peptide bond. Because the H^+ ions compete with Pd(II) ion for the amide nitrogen of Gly5, this NH group retains its H^+ ion. The binding of Pd(II) ion to nitrogen atom is suppressed by H^+ ion, and now Pd(II) ion in complex **2** promotes hydrolysis of the amide bond Gly4-Gly5 (by either of the mechanisms mentioned in the Introduction). *Therefore, complex 2 is the hydrolytically active species.* Because the acid in solution suppresses the formation of complex **3** and thus favors complex **2**, the rate constant for the cleavage increases as pH decreases.

At pH 4.5, the dominant species is the tetradentate complex **4**, and the minor species is the tridentate complex **3**. In both complexes, the Gly4-Gly5 bond is stabilized by the binding of Pd(II) ion to the peptide nitrogen. Consequently, cleavage of this bond is extremely slow. Although Pd(II) ion within complex **3** can now conceivably interact with the Tyr3-Gly4 bond, coordination of two anionic nitrogen atoms has quenched the Lewis acidity of Pd(II) ion and rendered it incapable of promoting the cleavage of the third peptide bond upstream from the anchor. If the second peptide bond upstream cannot be cleaved, there is no cleavage at all.

(3) Dual Cleavage at Low pH. The chromatograms of the digests at pH 1.2 contained additional peaks at 12.2 and 16.7 min, which grew as pH was lowered to 0.8 and 0.5. The new cleavage fragments were identified by MALDI mass spectrom-

(57) Milović, N. M.; Kostić, N. M., manuscript in preparation.

Scheme 3. Dual Fragmentation of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide), detected at pH ≤ 1.2[a]



[a] Not only the second, but also the first, peptide bond upstream from the anchoring residue (Met6) is cleaved. The observed and the calculated (in parentheses) molecular masses in daltons are shown for each fragment.

Table 3. Results of HPLC Separation and MALDI Mass Spectrometric Experiments with Fragments of Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala (His-peptide) Resulting from the Cleavage by $[Pd(H_2O)_4]^{2+}$

elution time (min)	observed mass (D)	calculated mass (D)	fragment
4.8	344.91	345.20	1−3
7.9	1220.79	1220.57	4−15

etry experiments as fragments 1-5 and 6-10. Their formation is shown in Scheme 3.

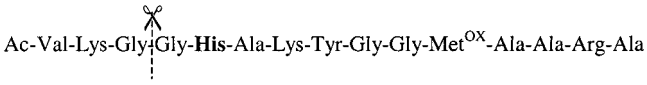
Evidently, at very low pH, not only the second, but also the first, peptide bond upstream from the anchor is cleaved. As mentioned earlier, the pK_a value for the first NH group upstream in the presence of histidine-anchored Pd(II) ion is estimated at ca. 2.0.[23,50] Therefore, as the pH of the reaction mixture is lowered from 2.3 to 0.5, complex **2** is gradually replaced by complex **1**, in which the Pd(II) ion can approach the first peptide bond upstream from the anchor. At these very low pH values, both complexes **1** and **2** are present, and they are hydrolytically active. The anchored Pd(II) ion in complex **1** can interact with the Gly5-Met6 bond, the first one upstream from the anchor, and promote its cleavage. At the same time, the Gly4-Gly5 bond, the second one upstream from the anchor, still undergoes cleavage at this very low pH, in complex **2**.

Now that we understand these side reactions, we know how to suppress them. Practical reactions with our synthetic peptidases should be carried out at pH ≥ 1.5, to avoid both the acid-promoted background cleavage not observed here but possible with proteins, and Pd(II)-promoted nonselective cleavage at multiple sites. Under these regular conditions, our Pd(II) reagents are expected to cut exclusively the second peptide bond upstream from the anchoring residue in a controlled fashion. At pH ≥ 1.5, our reagents can be used for practical cleavage of proteins.

Upstream versus Downstream Cleavage of a Histidine-Containing Peptide. To study cleavage in the vicinity of histidine as an anchor, we chose as a substrate His-peptide, shown in Chart 2. Because the side chain of Met11 in this peptide is oxidized to a sulfone group, which has no ligating properties, the only anchor for Pd(II) ion is His5 residue.

The reaction mixture containing equimolar amounts of His-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.0 and 60 °C was analyzed by HPLC separations periodically. Two products, eluting at 4.8 and 7.9 min in the "fast" analytical run, appeared gradually. The former fraction was detectable only at 215 nm, while the latter fraction was detectable at 215, 280, and 350 nm. Evidently, the former product does not, while the latter one does, contain a bound Pd(II) ion. No other peaks were observed in the chromatogram of the digest. That only the peak of the intact peptide was observed for the solution of the His-peptide incubated (without the palladium complex) at pH 2.0 after 14 days rules out background cleavage by acidic solution.

As Table 3 and the diagram below show, the faster-eluting product of cleavage is the fragment 1-3, whereas the slower-eluting product is the Pd(II)-carrying fragment 4-15.



The C-terminus of the former and the N-terminus of the latter show that the original His-peptide was cleaved only at the Gly3-Gly4 bond. As with Met-peptide discussed above, the cleavage of the His-peptide promoted by $[Pd(H_2O)_4]^{2+}$ occurs regioselectively at the second peptide bond upstream from the anchor. This regioselectivity differs from that seen with dipeptides of type Ac-His-Aa, in which the (first) peptide bond downstream from the histidine anchor was cleaved because the second peptide bond upstream is unavailable.

Because the cleavage near the histidine and the methionine anchors occurs with the same regioselectivity, this cleavage likely occurs by the same mechanism. Indeed, earlier studies from our and other laboratories of the peptide coordination to Pd(II) complexes showed that peptides containing histidine and methionine anchors behave similarly. The Pd(II) ion anchored to the N3 atom of imidazole in the histidine side chain readily deprotonates the first peptide nitrogen upstream.[23,50] Coordination of this deprotonated nitrogen atom forms a six-membered ring, and a complex of type **2** is formed. Complete deprotonation of the second peptide nitrogen upstream, to form a complex of type **3**, requires higher pH. At pH 2.0, complexes of types **2** and **3** coexist in solution, in addition to complex in which Pd(II) ion is bound to N-1 of imidazole.[57] In the complex of type **2** the second peptide bond upstream from the histidine residue is cleaved.

Evidently, the regioselective cleavage is a result of binding of Pd(II) ion to histidine or methionine anchor and to the peptide backbone. Ample precedents of peptide coordination to Pd(II) ion and other transition-metal ions show that the coordination mode is independent of the nature of the noncoordinating side chains surrounding the anchor.[23,48−54,58−62] The neighboring side chains only provide additional interactions that can affect the overall stability of these metal-peptide complexes. Because the neighboring noncoordinating side chains will not alter the

binding of the anchored Pd(II) ion to the upstream backbone, the regioselectivity of the backbone cleavage will also be independent of the nature of the side chains surrounding the anchor. *Therefore, the regioselectivity observed in our model peptides is expected to be general for all peptide or protein sequences containing an "isolated" histidine or methionine residue.*

To investigate the dependence of the rate constant for cleavage of His-peptide on the acidity of solution, we ran the reaction at seven pH values ranging from 0.48 to 4.50. The results are summarized in Figure 4b and Table S2 in the Supporting Information. Each rate constant is the average of two consistent values, obtained by monitoring each of the two fragments of cleavage. As Figure 4 shows, the rate constants for cleavage of the Met-peptide and His-peptide at a certain pH are similar, and their pH-dependencies are also similar. These results show that not only regioselectivity, but also the rate of cleavage promoted by Pd(II) ion, is independent of the anchor.

These similarities are consistent with the equilibrium shown in Scheme 2. Attachment of Pd(II) complex to imidazole nitrogen atom and thioether sulfur atom occurs in seconds, so rapidly that it does not affect the rate of subsequent cleavage, which occurs in hours. After the fast anchoring step, the slow hydrolytic steps are the same for both peptides, regardless of the identity of the anchor—histidine or methionine residue. Consequently, the rate constants for the cleavage of the His-peptide and Met-peptide are similar. The slightly higher rate constants for the latter can be attributed to the relatively strong trans-effect of the coordinated thioether group, which facilitates exchange between the hydrolytically inactive complex **3** and the hydrolytically active complex **2**.

As pH was lowered, the rate constant for methionine-guided cleavage increased faster than that for the histidine-guided cleavage. The added acid has no effect on the coordinating ability of the thioether group, but protonates the N3 atom in the imidazole and inhibits its coordination to the Pd(II) ion. Partial suppression of anchoring slows down the cleavage.[30] To avoid this inhibition, and for other reasons discussed above, cleavage reactions should be run at pH $\geq$ 1.5.

Cleavage of a Peptide Containing Both Histidine and Methionine Residues. Proteins contain multiple potential anchors for Pd(II) complexes. To examine their interplay in a more realistic substrate, we prepared HisMet-peptide, shown in Chart 2. It contains two possible anchoring residues competing for the Pd(II) reagent, His5 and Met11.

The chromatograms of the samples taken from the reaction mixture containing equimolar amounts of HisMet-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 1.8 and 60 °C showed gradual appearance of peaks eluting at 14.8, 15.7, 17.5, 18.1, and 18.7 min. These fragments were identified by MALDI mass spectra, and the results are shown in Table 4. Additional minor peaks in the chromatogram of the digest, which collectively contributed to less than 5% of the total absorption, were not studied further.

Table 4. Results of HPLC Separation and MALDI Mass Spectrometric Experiments with Fragments of Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (HisMet-peptide) Resulting from the Cleavage by $[Pd(H_2O)_4]^{2+}$[a]

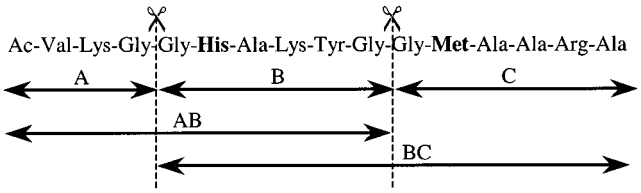
elution time (min)	observed mass (D)	calculated mass (D)	fragment
14.8	344.91	345.20	A
15.7	576.36	576.38	C
17.5	632.47	632.30	B
18.1	958.29	958.50	AB
18.7	1188.98	1188.58	BC

[a] An equimolar amount of the Pd(II) promoter produced all the fragments listed in the table, whereas 2-fold molar excess over the peptide produced only fragments A, B, and C.

The chromatogram of the solution of the free HisMet-peptide at pH 1.8 kept for 14 days showed only the peak of the intact peptide. This control experiment rules out background cleavage in the acidic solution.

The MALDI mass spectrometric assignment in Table 4 of the product eluting at 17.5 min was ambiguous because the observed molecular mass of 632.47 D corresponds to three different fragments of the HisMet-peptide. The ambiguity was removed by C-terminal sequencing using carboxypeptidase Y, combined with MALDI mass spectrometry. The mass spectra of the samples, taken periodically from the digest, showed molecular masses of 575.45, 412.50, and 283.89 D, which correspond to peptides Gly-His-Ala-Lys-Tyr, Gly-His-Ala-Lys, and Gly-His-Ala, respectively. These results identify the fraction eluting at 17.5 min as the fragment B, Gly-His-Ala-Lys-Tyr-Gly.

The fragments in Table 4 resulted from cleavage of peptide bonds Gly3-Gly4 and Gly9-Gly10, as shown in the diagram below. As in Met-peptide and His-peptide, the cleavage was regioselective. Again, it occurred only at the second peptide bond upstream from the histidine and methionine anchors.



In the aforementioned experiments, the substrate and the cleavage reagent were present in equimolar amounts, so that the two anchors in the same substrate had to compete for the Pd(II) complex. Pd(II) complex showed no binding or cleaving preference for either anchor. The digest contained all possible products of cleavage guided by His5, by Met11, and by both of these anchors simultaneously, as expected from the similarity of the rate constants for the cleavage of Met-peptide and His-peptide.

In another series of experiments, HisMet-peptide was digested by 2 molar equivalents of $[Pd(H_2O)_4]^{2+}$, under the same conditions as before. The chromatograms recorded within the first 3 h showed gradual accumulation of five products. They were eluted at 14.8, 15.7, 17.5, 18.1, and 18.7 min, as in the case of the equimolar amount of the complex (see Table 4). After 20 h, the first three peaks were still present in the chromatogram of the digest, whereas the last two were absent. The results of MALDI mass spectrometry experiments con-

(58) Bal, W.; Chmurny, G. N.; Hilton, B. D.; Sadler, P. J.; Tucker, A. *J. Am. Chem. Soc.* **1996**, *118*, 4727.
(59) Bal, W.; Dyba, M.; Kozlowski, H. *Acta Biochim. Pol.* **1997**, *44*, 467.
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(62) Hahn, M.; Kleine, M.; Sheldrick, W. S. *J. Biol. Inorg. Chem.* **2001**, *6*, 556.

firmed the identities of all the observed species, as diagrammed above. The transient intermediates are the fragments designated AB and BC. Their cleavage yields the three final products − peptides designated A, B, and C.

These results confirm complete, regioselective cleavage of the HisMet-peptide by 2 equiv of $[Pd(H_2O)_4]^{2+}$. Now His5 and Met11 each anchor a Pd(II) complex, and the second peptide bond upstream from each anchor becomes cleaved.

The regioselectivity of Pd(II)-promoted cleavage observed in this study for the peptide substrates agrees with that recently observed for protein substrates.[38,57] *Because cleavage near histidine and methionine residues can occur simultaneously, Pd(II) complexes in aqueous solutions can work as synthetic proteolytic reagents specific for these two residues*.

Conclusions

Simple and readily available Pd(II) complexes act with unprecedented and useful regioselectivity in promoting the hydrolytic cleavage of peptides and proteins. In acidic aqueous solution, these reagents hydrolyze the peptide bond involving the amino group of the residue preceding the methionine or histidine side chain in the sequence, that is, the second peptide bond upstream of these anchoring residues. To understand the cleavage of proteins, we investigated this regioselectivity in detail by using natural and synthetic peptides as substrates. Binding of Pd(II) ion to histidine and methionine anchors and to the peptide backbone, familiar from classical studies by coordination chemists, is followed by unexpected and useful cleavage of this backbone. We analyzed the binding to understand the surprising regioselectivity and kinetics of the cleavage. The ability of Pd(II) complexes to cleave proteins at relatively few sites, with explicable selectivity, and with good yields bodes well for their growing use in biochemical and bioanalytical practice.

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Supporting Information Available: Figure S1, showing the TOCSY ^{1}H NMR spectrum of a mixture of Met-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 4.5, and Figure S2, showing the TOCSY ^{1}H NMR spectrum of the Pd(II)-bearing fragment Gly-Met-Ala-Ala-Arg-Ala. Tables S1 and S2, showing the rate constants for cleavage of Met-peptide and His-peptide at different pH values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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