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Complexes of palladium(II) and platinum(II) as synthetic proteases

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NOTE TO USERS

Complexes of palladium(II) and platinum(II) as synthetic proteases

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Signature was redacted for privacy.

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For the Major Program

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CHAPTER 1. General Introduction

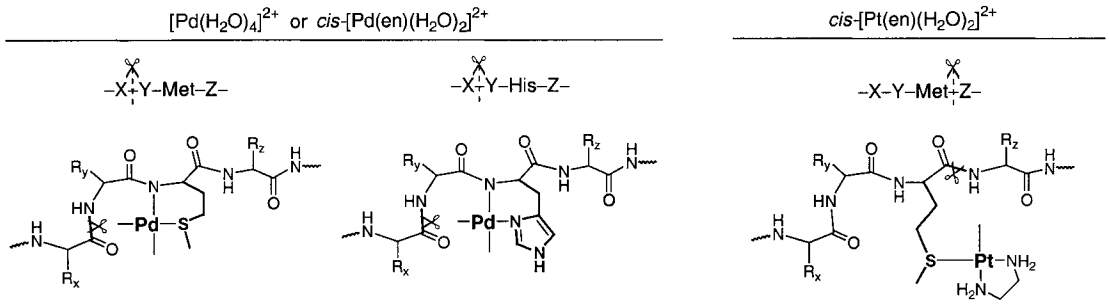
Overview

Selective cleavage of proteins is a common procedure in many biochemical applications ranging from standard protein sequencing to novel methods in proteomics and bioengineering. Few enzymes and synthetic reagents are available for this important task, but new chemical reagents with improved efficiency and adjustable selectivity are highly desired.

Complexes of palladium(II) and platinum(II), two chemically similar transition-metal ions, are emerging as new reagents for selective cleavage of peptides and proteins.[1] The cleavage by Pd(II) complexes, such as $[Pd(H_2O)_4]^{2+}$ or *cis*-$[Pd(en)(H_2O)_2]^{2+}$, consistently occurs in weakly acidic aqueous solutions at the amide bond involving the N-terminus of the residue preceding histidine and methionine residues, i.e., the X-Y bond in the sequence segments X-Y-His-Z and X-Y-Met-Z, where X, Y, and Z are any non-coordinating residues.[2] The cleavage by Pt(II) complexes, such as *cis*-$[Pt(en)(H_2O)_2]^{2+}$, occurs exclusively at the peptide bond involving the C-terminus of methionine residues, i.e. the Met-Z bond.[3]

In our studies with peptide substrates, we explain the exceptional proteolytic selectivity of Pd(II) and Pt(II) complexes by identifying the hydrolytically-active modes in which these metal ions bind to the side chains of the anchoring residues and to the polypeptide backbone. The selectivity of cleavage originates in the selectivity of the coordination – under the reaction conditions, both methionine and histidine residues can bind to the Pd(II) reagents, whereas only methionine residue can bind to the Pt(II) reagent. The mechanism of cleavage originates in the modes of coordination (shown below) – the

anchored Pd(II) or Pt(II) ions can approach the scissile peptide bond and activate it toward hydrolysis.



The Pd(II)-promoted cleavage can be switched from the residue-selective to the sequence-specific mode by simply adjusting the pH of the solution. In neutral solutions, only the X-Pro bond in X-Pro-Met-Z and X-Pro-His-Z sequences is cleaved because of the unique interplay between the anchoring residue and the proline residue preceding it.[4] The Pt(II)-promoted cleavage is catalytic, with small but important turnover of 18 after 7 days.

Our studies with protein substrates[3,5,6] confirmed the cleavage pattern observed with peptides, and demonstrated that the Pd(II) and Pt(II) complexes are well-suited for biochemical applications. The ability of these complexes to cleave proteins at relatively few sites, with explicable selectivity and good yields, bodes well for their growing use in biochemical and bioanalytical practice.

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CHAPTER 2. 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, i.e. 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), 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 4.50 to 0.48. 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, one molar equivalent of the Pd(II) complex distributes itself evenly at both anchors and provides partial cleavage, whereas two 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 towards 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 good 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.

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 towards 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 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, i.e. for either case in Chart 1, the most 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, 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*-$[Pd(en)(H_2O)_2]^{2+}$.[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 1.50 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 sulphone 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. Abbreviations: 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 extinctions 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 eq with respect to the resin loading), HOBt (3.0 eq) and *N*,*N*-diisopropylethylamine (6.0 eq), each added to 3.0 eq 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 hours 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 3 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 sulphone by H_2O_2 and formic acid.[3] 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 one oxygen atom 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. Since 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 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 multiwavelength 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 x 4.6 mm, beads of 5 μm) was used for the analytical runs, and a Vydac C-18 column 218TP101522 (sized 250 x 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) bound to the peptide or its fragment(s) was established not only by the matching molecular mass for peptide plus Pd, but also by the following isotopic distribution, diagnostic for 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 sulphone, 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 vs. 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, i.e., confidence limit greater than 95%.

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 analysis. For NMR spectroscopic study of Met-peptide cleavage, the fragments were separated by the preparative reverse-phase HPLC method.

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 be attached to both anchors. Such macrochelation has never been observed with proteins, either.

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 mixing of each peptide with 1 eq 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 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, γ, and δ-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, a 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.

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 the 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 the 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

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

In the TOCSY ^{1}H 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, i.e., 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 proves 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.

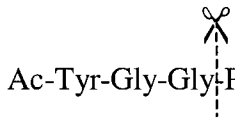
Because the chromatograms and the MALDI mass spectra showed that $[Pd(H_2O)_4]^{2+}$ in acidic solutions forms two complexes with methionine-enkephalin and also with Met-peptide, we can conclude that the coordination modes identified for Met-peptide are general for peptides containing "lone" methionine 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.

In dipeptides of the type Ac-Met-Aa, roughly 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 Enkephaline, 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 vs. 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 minutes of mixing equimolar amounts of Met-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.27. The chromatograms of the samples taken before the addition of $[Pd(H_2O)_4]^{2+}$ and 1, 12, and 24 h after mixing showed gradual disappearance of the peaks of the intact peptide (at 21.0 min) and 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 nm 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 bellow.

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 24 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 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 24 hours. 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) The 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 1.85: $[Pd(H_2O)_4]^{2+}$, $[Pd(NH_3)_4]^{2+}$, *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, within the experimental error, at the same rate. But the relatively strongly bonded chloride anion and the bidentate ethylenediamine ligand slow down 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 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 0.48 to 4.50. 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 the 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 the 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 remains, and peptide bond Gly4-Gly5 gets cleaved. The binding of Pd(II) ion to nitrogen atom is suppressed, 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, the complex ***2*** is the hydrolytically-active species. Because the acid in solution suppresses the formation of the complex ***3*** and thus favors the 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 the 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 1.20 contained additional peaks at 12.2 min and 16.7 min, which grew as pH was lowered to 0.85 and 0.48. The new cleavage fragments were identified by MALDI mass spectrometry experiments as fragments 1-5 and 6-10. Their formation is shown in Scheme 3.

Evidently, at very low pH the first, as well as the second, 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 0.48, the 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 the 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 the 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 $\geq$ 1.50, 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 $\geq$ 1.50, our reagents can be used for practical cleavage of proteins.

Upstream vs. 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 sulphone 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 2.00 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.

Ac-Val-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala

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 occur 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] and 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 Figures 4a and 4b, show, 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 $\geq$ 1.50.

Cleavage of a Peptide Containing Both Histidine and Methionine Residues.

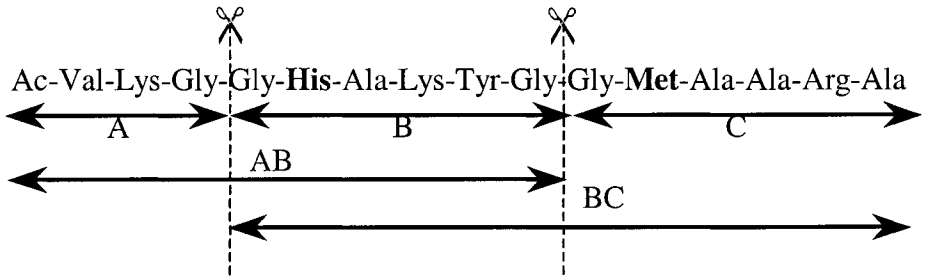
Proteins contain multiple potential anchors for Pd(II) complexes. To examine their interplay in a more realistic substrate, we prepared HisMet-peptide, shown in Chart 2. It contains two possible anchoring residues competing for the Pd(II) reagent, namely His5 and Met11.

The chromatograms of the samples taken from the reaction mixture containing equimolar amounts of HisMet-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 1.80 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. 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 anchor 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 two molar equivalents of $[Pd(H_2O)_4]^{2+}$, under the same conditions as before. The chromatograms recorded within the first three hours 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 confirmed 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, C, and B, respectively.

These results confirm complete, regioselective cleavage of the HisMet-peptide by two equivalents 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 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 solution, these reagents hydrolyze the peptide bond involving the amino group of the residue preceding the methionine or histidine side chain in the sequence, i.e., the second peptide bond upstream of these anchoring residues. In order 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; This material is available free of charge via the Internet at http://pubs.acs.org.

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Graphics

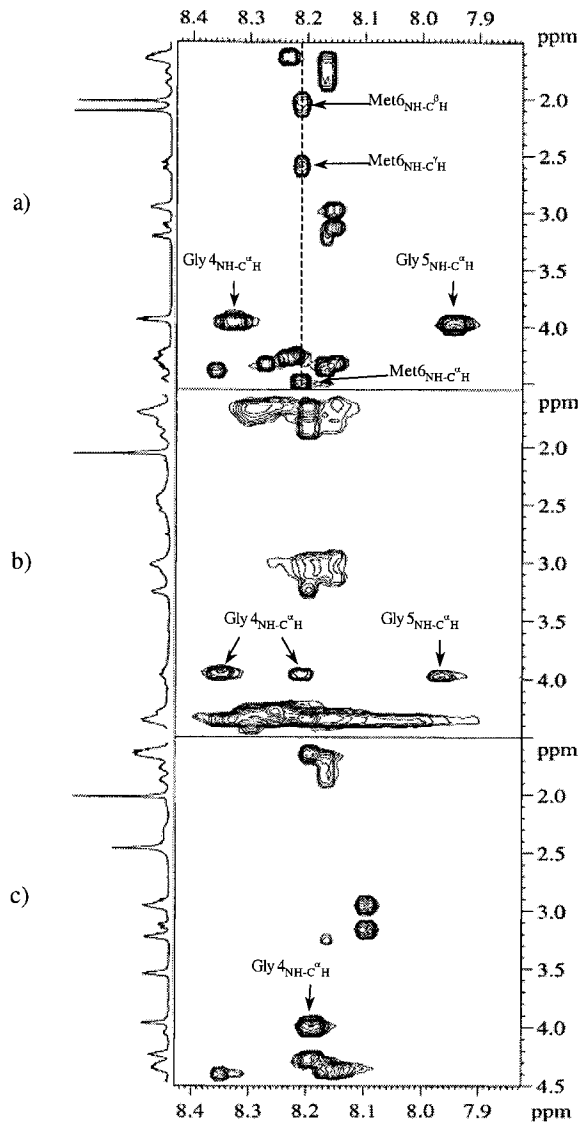


Figure 1. The aliphatic region of one-dimensional ^{1}H NMR spectrum and a part of the NH-to-aliphatic region of TOCSY ^{1}H NMR spectrum of the aqueous solution of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide), at pH 2.3 (a) before addition of $[Pd(H_2O)_4]^{2+}$; (b) after addition of an 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.

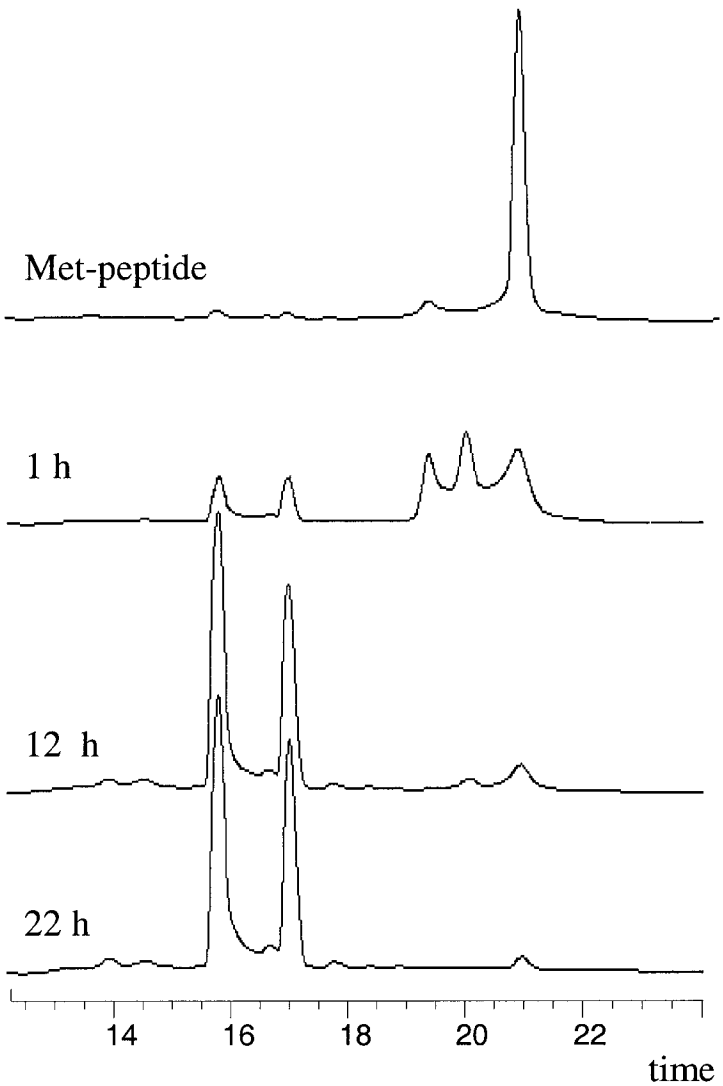


Figure 2. Monitoring the digestion of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) by $[Pd(H_2O)_4]^{2+}$ at pH 2.5 and 60 °C. The chromatograms at 215 nm of Met-peptide and of the samples taken at the specified times after mixing the two reagents. Composition of the fractions: 21.0 min, 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.

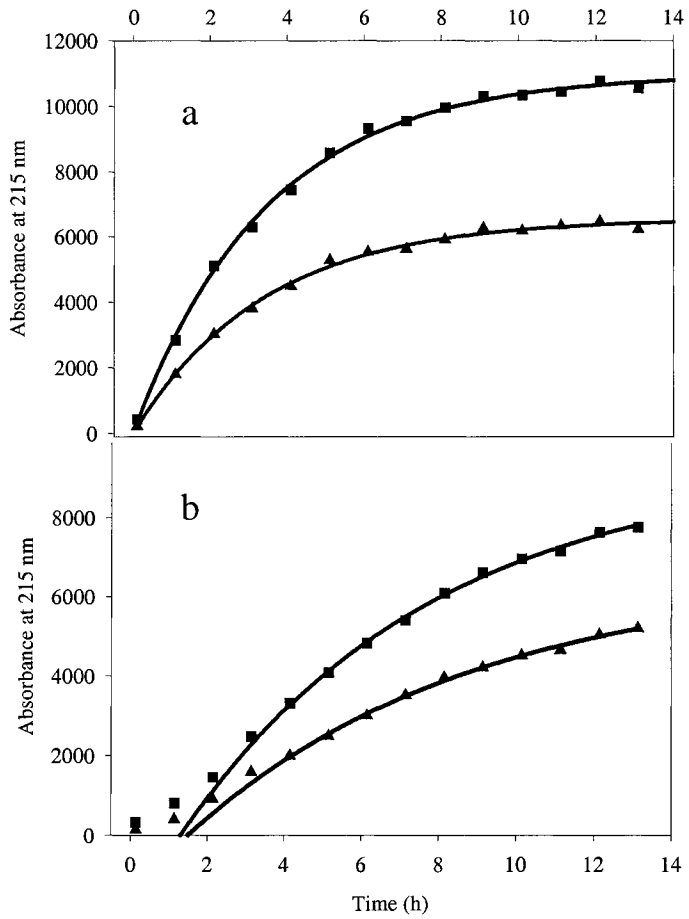


Figure 3. The progress of cleavage of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) at pH 1.85 by (a) $[Pd(H_2O)_4]^{2+}$; and (b) *cis*-$[Pd(en)(H_2O)_2]^{2+}$, followed by HPLC separations of the digests. The data for the peaks eluting at 15.9 min (■) and 17.1 min (▲) are obtained by normalizing 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.

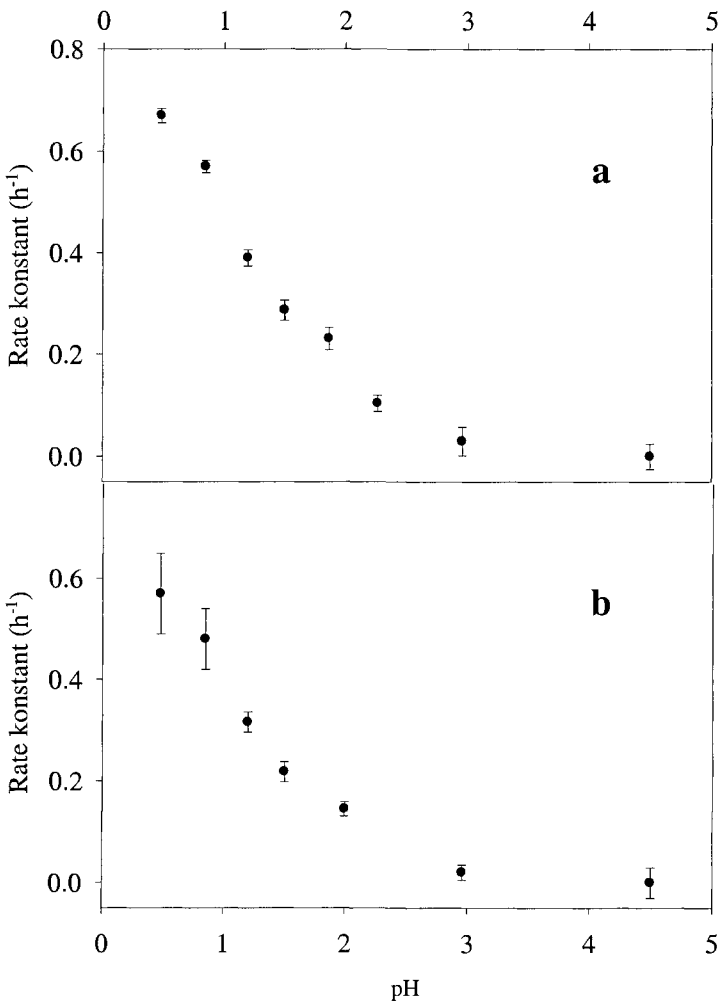


Figure 4. Dependence on pH of the rate constants for cleavage of (a) the Gly4-Gly5 peptide bond in Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) and (b) the Gly3-Gly4 peptide bond in Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala (His-peptide) promoted by $[Pd(H_2O)_4]^{2+}$.

Table 1. Results of HPLC separation and MALDI mass spectroscopic 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

Table 2. Results of HPLC separation and MALDI mass spectroscopic experiments with fragments of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) resulting from the cleavage by $[Pd(H_2O)_4]^{2+}$.

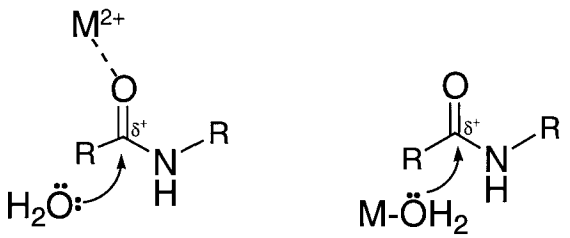
elution time (min)	observed mass (D)	calculated mass (D)	fragment
17.1	576.64	576.68	1-4
15.9	480.7	479.34	5-10

Table 3. Results of HPLC separation and MALDI mass spectroscopic 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

Table 4. Results of HPLC separation and MALDI mass spectroscopic 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 $[Pd(H_2O)_4]^{2+}$. An equimolar amount of the Pd(II) promoter produced all the fragments listed in the table, whereas twofold molar excess over the peptide produced only fragments A, B and C.

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



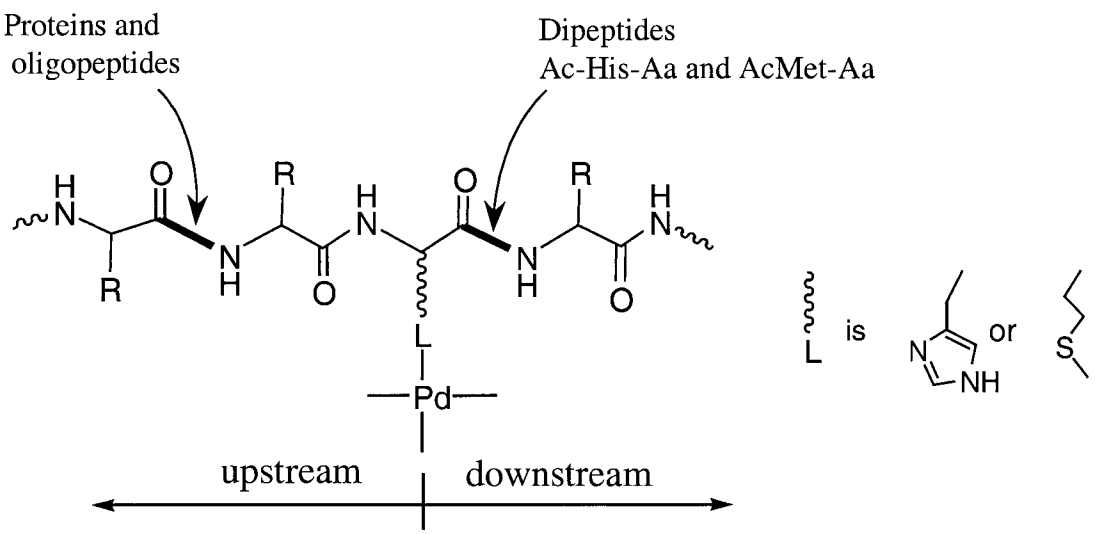
external attack internal delivery

Chart 1. Possible limiting mechanisms for Pd(II)-promoted hydrolysis of peptide bond.

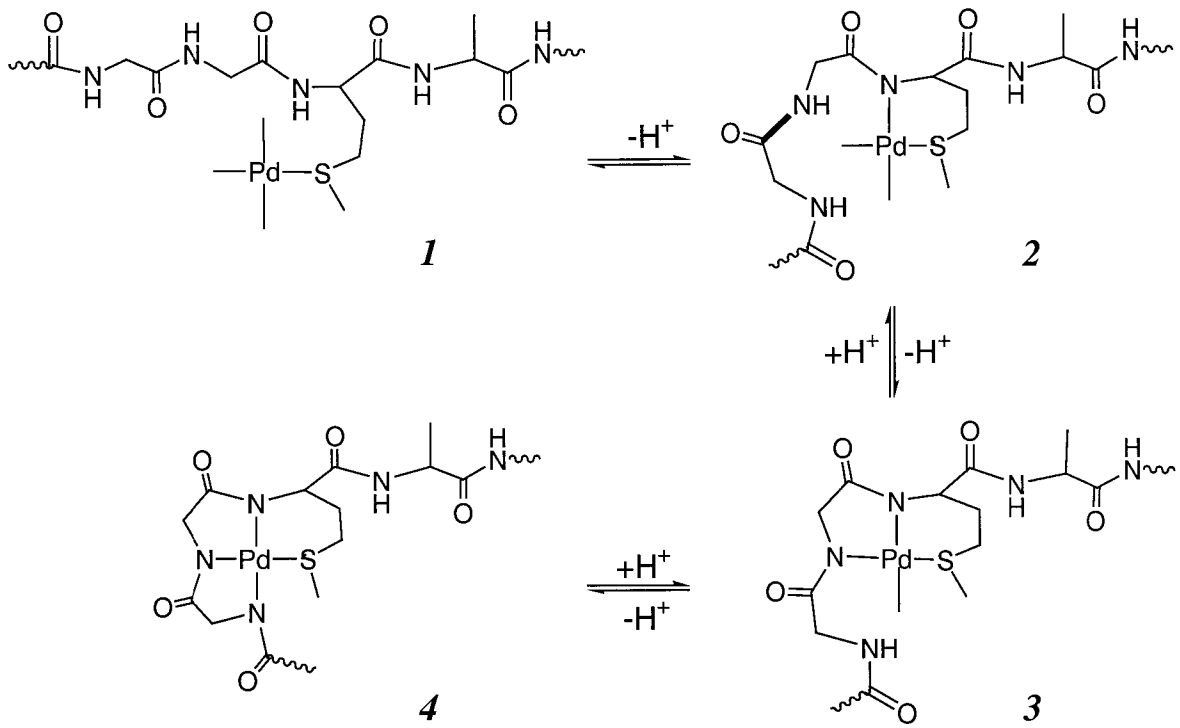
Met-enkephalin:	Ac-Tyr-Gly-Gly-Phe-**Met**
Met-peptide:	Ac-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala
HisMet-peptide:	Ac-Val-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala
His-peptide*:	Ac-Val-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala

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

Chart 2. Peptides cleaved by Pd(II) complexes. The anchoring residues are highlighted and included in the peptide names.

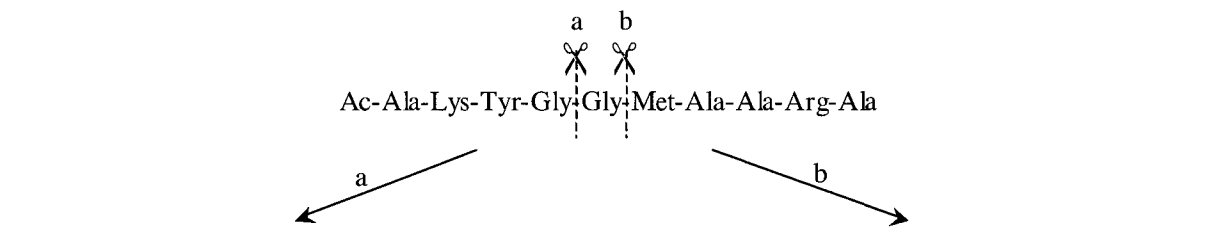


Scheme 1. Palladium(II) complexes act with different regioselectivity in cleaving dipeptides and proteins; the sites of cleavage are highlighted. The anchoring side chain is coordinated to the Pd(II) ion via the group L; the remaining three ligands are not specified. The direction of the cleavage is defined with the anchor as the reference point.



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. The scissile peptide bond is highlighted.

Scheme 3. Dual fragmentation of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide), detected at pH ≤ 1.2. The first, as well as the second peptide bond upstream from the anchoring residue (Met6) is cleaved. The observed and the calculated (in



AcAla-Lys-Tyr-Gly	+	Gly-Met-Ala-Ala-Arg-Ala		AcAla-Lys-Tyr-Gly-Gly	+	Met-Ala-Ala-Arg-Ala	
480.0		576.6		537.7		519.4	
(479.3)		(576.7)		(537.6)		(519.6)	

parentheses) molecular masses in Daltons are shown for each fragment.

Supporting Information

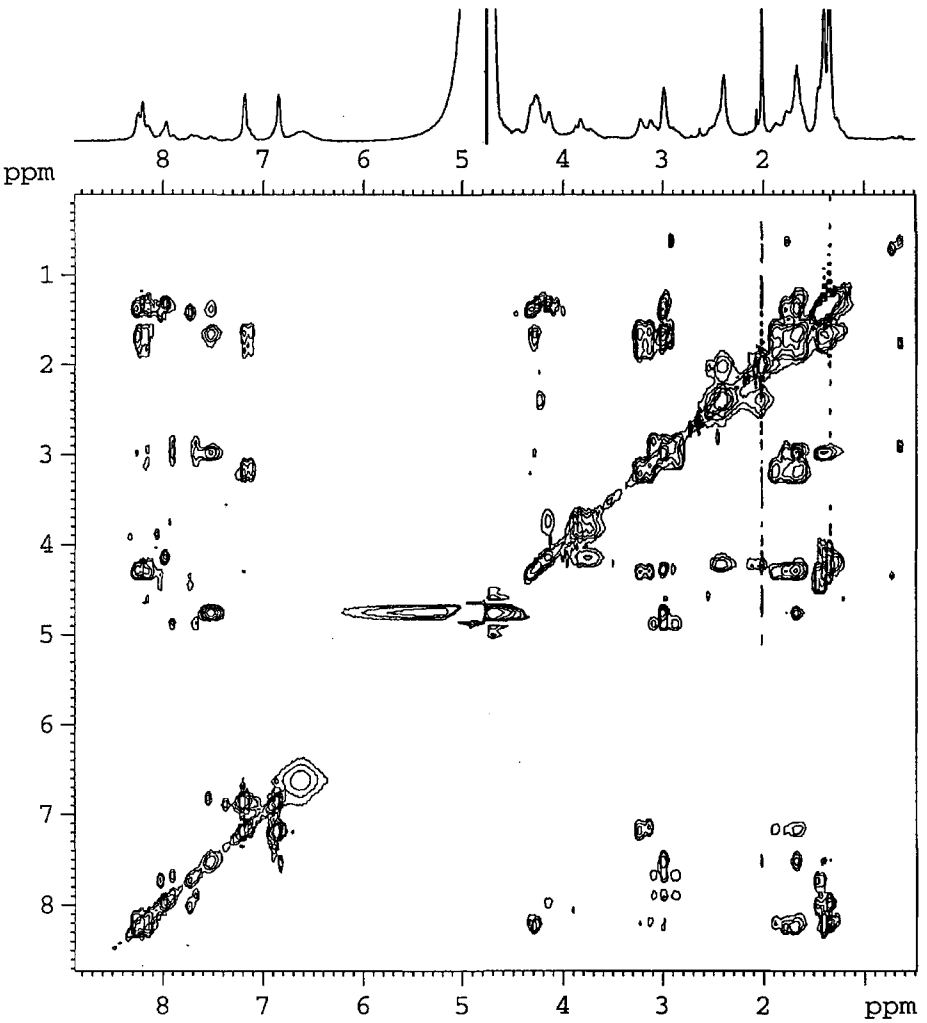


Figure S1. TOCSY ^{1}H-NMR spectrum of a solution containing equimolar amounts of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) and $[Pd(H_2O)_4]^{2+}$at pH 4.5.

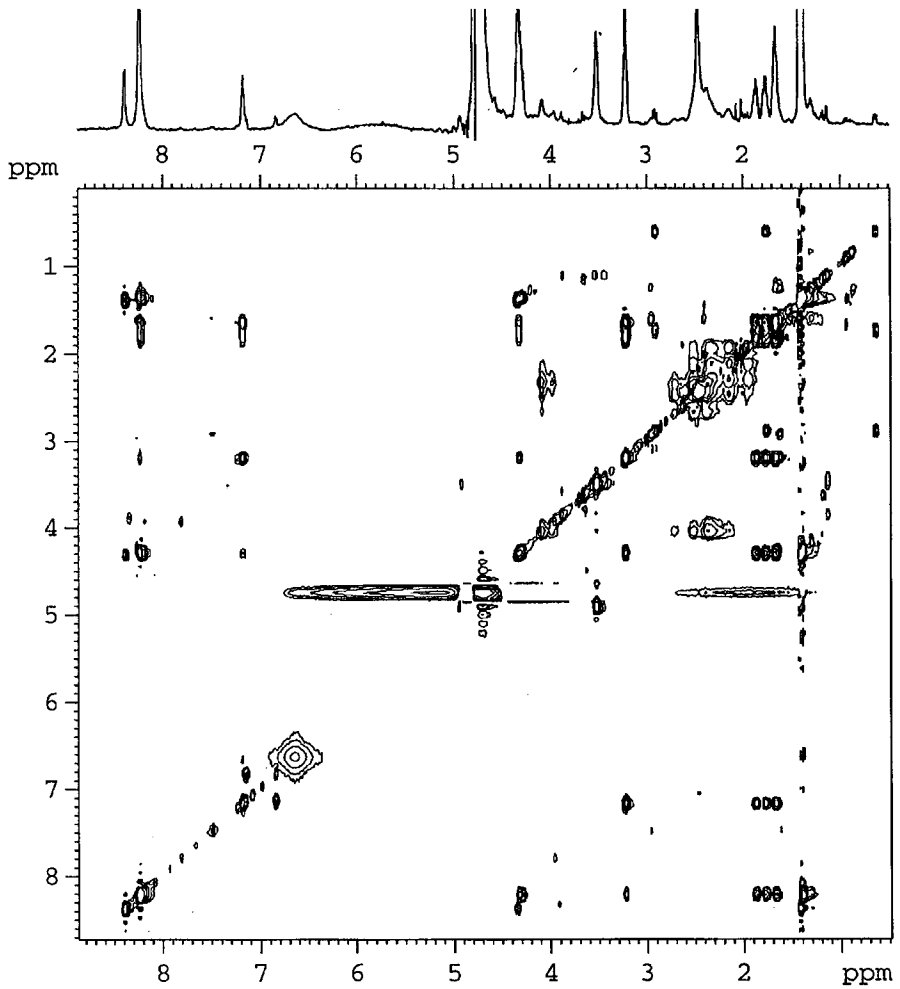


Figure S2. TOCSY ^{1}H-NMR spectrum of Pd(II)-bearing peptide Gly-Met-Ala-Ala-Arg-Ala, the fragment of the cleavage of Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) by $[Pd(H_2O)_4]^{2+}$.

Table S1. Rate constants for cleavage of the Gly4-Gly5 peptide bond in Ac-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) promoted by $[Pd(H_2O)_4]^{2+}$ at different pH values. At pH ≤ 1.20, the Gly5-Met6 bond is also cleaved

pH	rate constant (h^{-1})
4.50	$2x10^{-6}$
2.96	0.03
2.27	0.10
1.87	0.23
1.50	0.29
1.20	0.39
0.85	0.57
0.48	0.67

Table S2. Rate constants for appearance of the Pd(II)-carrying fragment Gly-His-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala upon cleavage of the Gly4-Gly5 peptide bond in Ac-Val-Lys-Gly- Gly-His-Ala-Lys-Tyr-Gly-Gly-Met^{OX}-Ala-Ala-Arg-Ala (His-peptide) promoted by $[Pd(H_2O)_4]^{2+}$ at different pH values.

pH	rate constant (h^{-1})
4.50	$1x10^{-6}$
2.96	0.02
2.00	0.14
1.50	0.22
1.20	0.32
0.85	0.48
0.48	0.58

CHAPTER 3. Interplay of Terminal Amino Group and Coordinating Side Chains in Directing Regioselective Cleavage of Natural Peptides and Proteins with Palladium(II) Complexes

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Abstract

Palladium(II) ions anchored to side chains of histidine and methionine residues in peptides and proteins in weakly-acidic aqueous solutions promote hydrolytic cleavage of proximate amide bonds in the backbone. In this study, we determine how attachment of Pd(II) ions to histidine and methionine anchors and also to the terminal amino group in six natural peptides (chains A and B of insulin, segment 11-14 of angiotensinogen, pentagastrin, angiotensin II, and segment 3-8 of angiotensin II) and two proteins (ubiquitin and cytochrome *c*) affects regioselectivity and rate of the backbone cleavage. These Pd(II)-promoted reactions follow a clear pattern of regioselectivity, directed by the anchoring side chains. When the Pd(II) reagent is nonspecifically anchored to the terminal amino group, the

ligating site that is present in almost all proteins, the cleavage is fortunately absent. When the reagent is anchored to a residue in the positions nos. 1, 2, or 3, cleavage is absent, because the terminal amino group and deprotonated amide nitrogen atom(s) interposed between it and the anchor "lock" the Pd(II) ion in hydrolytically-inactive chelate complexes. When the reagent is anchored to residues in positions beyond no. 3, the second amide bond upstream from the anchor is regioselectively cleaved in all cases when the anchor was "isolated," i.e., flanked by non-coordinating side chains. Segment 3-8 of angiotensin II undergoes additional cleavage, which we explain by determining the rate constants for the cleavage, identifying the rate-limiting displacement of ethylenediamine ligand from the Pd(II) ion, and detecting several intermediates. Experiments with cytochrome *c* demonstrate that the number of cleavage sites can be controlled by adjusting the mole ratio of the Pd(II) reagent to the substrate. Our inorganic peptidases are useful for biochemical applications because their regioselectivity and reactivity set them apart from proteolytic enzymes and organic chemical reagents.

Introduction

Hydrolytic cleavage of proteins is an important procedure in biochemical and bioengineering practice. Fragmentation of proteins is necessary for the standard determination of their primary sequence,[1] and also for several recently-developed applications. In proteomics, the expressed proteins are identified from their digests.[2] In protein footprinting and folding studies, the pattern of proteolytic cleavage yields structural information.[3] In conversion of engineered proteins to their native form, fusion tags are removed by site-specific cleavage.[4] In protein semisynthesis, the fragments of natural

proteins are recombined with synthetic peptides by chemical ligation to obtain new bioengineered proteins.[5]

The key step in all of the aforementioned procedures is selective cleavage of the polyamide backbone. This controlled fragmentation can be achieved with proteolytic enzymes and synthetic reagents. The enzymes effect fast and catalytic cleavage, but only few of them are usable in practice, and they often produce short fragments, undesirable for bioanalytical applications. Synthetic reagents, such as cyanogen bromide, BNPS-skatole, and N-bromosuccinimide also are few.[6] They often require harsh conditions, must be applied in high excess, and yet often give incomplete selectivity and relatively low yields. Even cyanogen bromide, the most common chemical reagent for fragmentation of proteins, has 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.

A broader choice of chemical reagents, having improved efficiency and regioselectivity in protein cleavage, is desired. Finding new reagents, however, is a challenging task because the amide bond (so-called peptide bond) is extremely unreactive toward hydrolysis under standard conditions. The half-life for cleavage of N-acetylated dipeptide AcGly-Gly is 250-600 years at pH 4.0-8.0 and room temperature.[7-9] Nonselective hydrolysis of peptides and proteins requires incubation with strong acids or strong bases, but even then the half-lives at room temperature are measured in months and years.

Some transition-metal complexes have emerged as new synthetic reagents for cleavage of amide bonds.[10-20] Because of their small size, these complexes can be useful

probes in structural studies of conformation or accessibility of protein regions.[3] These "inorganic proteases" are inexpensive and may be recyclable. They can cleave proteins terminally or internally. Most important, the internal cleavage can be made regioselective or even site-selective,[21,22] providing large protein fragments suitable for sequencing and other tasks.

The regioselectivity in the attachment of the metal complex to the protein, and thus regioselectivity in subsequent cleavage, can be achieved in two ways. First, by tethering the metal ion to a side chain of cysteine.[23] Because this tether is flexible, the anchored metal ion can promote cleavage of multiple amide bonds within its reach. In the second method, introduced in our laboratory,[24-33] the heteroatoms in the side chains of methionine (sulfur) and histidine (nitrogen) spontaneously coordinate to palladium(II) aqua complexes directly, without the tether. The Pd(II) ion so attached in aqueous solution promotes selective hydrolysis of only those amide bonds that are proximate to the anchoring side chain. Cleavage occurs under acidic conditions, which are required also by some proteolytic enzymes.[6] After the cleavage, Pd(II) ion is readily removed by chelation or precipitation. Because histidine and methionine have a combined average abundance in proteins of only ca. 5.5%, the fragments are relatively long and suitable for bioanalytical applications. In non-aqueous solutions, compatible with hydrophobic proteins, cleavage near tryptophan residue is possible.[34,35] Potential control of selectivity by the choice of solvent ads to the versatility of our Pd(II) reagents.

Dipeptides AcMet-X and AcHis-X, in which the amino-terminus is protected by acetylation, are usually cleaved at the Met-X and His-X bonds. Cleavage of the dipeptides AcHis-X can be made catalytic,[29,36,37] with a modest but significant turnover. The studies

with dipeptides clarified the kinetics, stereochemistry, and mechanism of these new reactions but contributed little to our understanding of the pattern that we found in cleaving proteins.[18,19,30,38] We recently clarified this pattern by showing that the $[Pd(H_2O)_4]^{2+}$ complex consistently cleaves synthetic polypeptides exclusively at the second peptide bond upstream from the histidine and methionine anchor(s), i.e., the peptide bond involving the N-terminus of the residue that precedes the anchor.[33] See Figure 1.

The amide bond can be hydrolyzed by two kinetically-indistinguishable limiting mechanisms, shown in Chart 1. A transition-metal 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, thus cleaving it. Regardless of the hydrolytic mechanism, i.e., for either case in Chart 1, two conditions must be met. First, the metal complex must approach the scissile amide bond. Second, the metal complex must contain at least one aqua ligand, to be either displaced by the carbonyl oxygen atom or delivered to the carbonyl carbon atom. If either of these conditions is absent, cleavage does not occur. Because the first requirement is a structural one, this study deals with stereochemical aspects of the cleavage reactions.

In this work, we explore for the first time the interplay between twp kinds of anchor for transition-metal ions: the side chains of methionine and histidine on one hand, and the terminal amino group on the other. Cobalt(III) complexes bind to the amino-terminus and promote cleavage of only the first amide bond.[39] If present with our Pd(II) proteases, this cleavage directed by the amino-terminus would diminish the regioselectivity of the desired backbone cleavage directed by the side chains of methionine and histidine. We now address this question by systematically investigating cleavage of eight natural peptides and proteins.

Overview of This Study. First, we check the possibility for cleaving peptides in which the terminal amino group is the only potential anchor for Pd(II) complex. Next, we investigate the interplay between the N-terminus and a histidine residue in positions nos. 1, 2, 3, and beyond 3 (see Figure 1a). On the basis of the new results and the known coordination modes of Pd(II) ion, we explain the absence of cleavage in three natural peptides (chain A of insulin, segment 11-14 of angiotensinogen, and pentagastrin) and also the occurrence of regioselective, Pd(II)-promoted cleavage of two natural peptides (chain B of insulin and angiotensin II). We confirm this regioselectivity in cleaving two proteins (ubiquitin and cytochrome *c*). In all cases studied, whenever the anchoring residue occupies a position beyond no. 3 in the sequence and is not adjacent to another potential anchoring residue, the second amide bond upstream from the anchor is cleaved. This study demonstrates that the new Pd(II) reagents are fit for biochemical applications.

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), human angiotensin II, human pentagastrin, bovine ubiquitin, equine cytochrome *c*, oxidized chain A of bovine insulin (in which thiol groups are converted to sulfonate groups), and similarly oxidized chain B of bovine insulin were obtained from Sigma Chemical Co. The segment 3-8 of angiotensin II was obtained from Bachem Bioscience Inc. Methyl phenyl sulphone was obtained from Lancaster Synthesis Inc. The reagents $[Pd(H_2O)_4]^{2+}$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were synthesized as described previously.[29,40] The reagent $[Pd(H_2O)_4]^{2+}$ was kept in a stock solution that was > 1.0 M in $HClO_4$, and thus its pH was negative. All complexes were

prepared as perchlorate salts. The concentrations of the Pd(II) complexes were determined using their published extinction coefficients.[41,42]

Spectroscopic and Analytical Methods. Proton NMR spectra were recorded with Bruker DRX 300 and Bruker DRX 500 spectrometers in D_2O at 298 K. The pH was measured with a Fisher Accumet instrument and an Aldrich Ag/AgCl reference electrode. The pD values were calculated by the standard formula pD = pH + 0.4.[43]

The components of the peptide digests were separated by either a Hewlett Packard 1100 HPLC system containing a multiwavelength detector or an HPLC system containing 110A pumps from Beckman, AS4000 autosampler from Hitachi, and a V4 detector from Isco set at 215 nm. A Vydac C18 column 218TP54 was used for the analytical separations of peptides; Vydac C4 column 214TP54, for analytical separations of ubiquitin digests; and a Vydac C-18 column 218TP101522, for preparative separations. The eluting solvents were 0.1% trifluoroacetic acid in water (A) and 0.08% trifluoroacetic acid in acetonitrile (B). The solvent B was absent from the eluent for 5 min after the injection of the sample, and than it increased gradually to 45% over a 35-min period. The flow rate was 1.00 mL/min for analytical runs and 10.0 mL/min for preparative runs.

Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with a Bruker Proflex™ instrument. The samples of the reaction mixture and the fractions isolated by HPLC were prepared by a standard dried-droplet procedure. Solutions of angiotensin II, oxidized chain B of insulin, and cytochrome *c* were used as external standards. The measured molecular mass was compared with the expected mass of a given peptide, calculated by PAWS software from ProteoMetrics, LLC.

Each MALDI mass spectrum contained the signals for the substrate or its fragments free of Pd(II) ion, and also the signals for the corresponding Pd(II)-bound species. The presence of Pd(II) ion bound to the substrate or its fragments was established from the correct molecular mass and from the isotopic distribution diagnostic of palladium. For the sake of clarity, the molecular masses reported in tables are those for the fragments free of Pd(II) ions.

Electrophoresis. A Protean II electrophoretic cell was combined with a 3000 Xi power supply (both produced by Bio-Rad Inc.) in experiments at 150 V lasting for 1.5 h. A standard procedure for tricine-(sodium dodecyl sulfate) polyacrylamide gel electrophoresis (TSDS-PAGE) was used.[44] The gels were stained for 1 h by an aqueous solution containing 40% methanol, 10% acetic acid, and 0.1% (w/w) Coomassie blue R-250 dye, and destained with nearly the same solution that lacked the dye.

A 10.0-μL sample of the cytochrome *c* digest was mixed with 40.0 μL of the sample buffer, heated for 5 min at 95 °C; 20.0 μL of this mixture was loaded into the well. The gels were blotted applying 50.0 V-potential for 4 h onto a PVDF membrane by a semi-dry procedure using a Trans-Blot SD system equipped with Power Pac 300 power supply (both obtained from Bio-Rad, Inc.). After the membrane was stained, destained, and rinsed with water, the bands were cut and subjected to N-terminal Edman analysis with a 494 Procise Protein Sequencer/140C analyzer, from Applied Biosystems. The acetylated N-terminus in horse cytochrome *c* is undetectable by the standard Edman method. All of these analyses were done at the Protein Facility.

Study of Hydrolysis. A solution containing 2.0 μmol of the substrate was mixed with 20.0, 40.0, or 100.0 μL of a 0.10 M solution (1, 2, or 5 molar equiv) of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$, and water was added to the final volume of 2.00 mL. The pH was adjusted by careful addition of either $HClO_4$ or NaOH; it remained within ±0.1 of the initial value after the reactions were completed. The reaction mixture was kept at 60±1 °C for 4 d, and 10.0-μL samples were taken periodically. In the control experiments for detecting possible background cleavage the conditions were the same, except that the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ was absent. The time profiles for cleavage reactions were determined from the analytical reverse-phase HPLC of the samples. The fragments were then separated by preparative reverse-phase HPLC, lyophilized to dryness, redissolved, and identified by MALDI-TOF mass spectrometry, and, in some cases, by ^{1}H-NMR spectroscopy, N-terminal sequencing, and amino-acid analysis. At the end of the incubation, the Pd(II) ion was removed by the addition of 10-fold molar excess of cysteine, a strong chelating reagent.

Precipitation during the hydrolysis of angiotensin II prevented exact quantitation of the products. To be accurate, we followed the kinetics of cleavage by using its segment 3-8, the peptide Val-Tyr-Ile-His-Pro-Phe, which remained soluble during the reaction. In the kinetic experiments with this segment, methyl phenyl sulphone was added to the reaction mixture as an internal standard. The 10-μL samples were periodically taken from the reaction mixture and immediately separated by HPLC at room temperature. In the short time required for this separation and at the lowered temperature the cleavage did not advance significantly, so that the chromatographic fractions correctly represented the composition of the reaction mixture at the time of the sampling. The chromatograms were integrated by a Shimatzu C-

R3A integrator, with ah estimated error of ±5%. The areas under peaks were normalized to that of the internal standard, to compensate for the error in the injection volume and for evaporation.

Results and Discussion

The Choice of Conditions. Coordinating anions, such as acetate and chloride, are excluded from the reaction mixtures lest they bind to the Pd(II) ion in the reagent and thus inhibit the substrate cleavage.[24] All experiments were done at 60 °C and at $1.8 \leq pH \leq 3.0$, conditions that consistently allowed effective cleavage of the peptides and proteins. Strongly-acidic solution would promote undesirable "background" cleavage. Weakly-acidic solution suppresses deprotonation of aqua ligands and consequent formation of insoluble, and thus hydrolytically-inactive, hydroxo-bridged Pd(II) species. In the chosen pH range, the Pd(II)-promoted cleavage is sufficiently fast, while the background cleavage is nearly or completely absent. When observed, this minor background cleavage occurred at the first amide bond downstream from aspartate and glutamate residues. The propensity of these amide bonds to hydrolysis in acidic solutions is well known;[45] The comparison of the total area of the chromatogram for the control reaction mixture free of the Pd(II) reagent with the area of the signal for the intact peptide showed that the this background cleavage, when present, is less than 5.0% after 24 h. Clearly, the selective cleavage of the substrates is caused by the Pd(II) complex.

The Choice of Substrates. The biologically-active, natural peptides in Chart 2 are chosen because they contain methionine or histidine residue at various positions in the

sequence. To check whether the same regioselectivity is observed with proteins, we chose also ubiquitin and cytochrome *c*. These two proteins are realistic, stringent test-cases for our Pd(II) reagents.

Terminal Amino Group as an Anchor. Solutions containing oxidized chain A of insulin and 1, 2, or 5 molar equivalents of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were kept at pH 2.0 and 60 °C. The chromatogram after 3 d showed that the peak for the intact substrate at 19.0 min was replaced by a new peak at 17.1 min. The MALDI mass spectrum of each reaction mixture after 3 d showed only the peaks for the intact substrate (observed, 2530.8 D; calculated, 2531.6 D) and for the substrate-Pd(II) complex (observed, 2635.7 D; calculated, 2636.6 D). Evidently, the Pd(II) complex binds to the chain A of insulin, but does not cleave it.

The chain A of insulin lacks histidine and methionine residues, and has only the terminal amino group as a possible anchor. Binding of Pd(II) and other transition-metal ions to peptides free of coordinating side chains involves anchoring to terminal amino group followed by deprotonation of the amide NH group(s) that follow the N-terminus, and Pd(II) coordination to the resulting anionic nitrogen atom(s);[46,47] see Scheme 1. Coordination of each additional amidate nitrogen atom creates a new five-membered ring. The Pd(II) ion is the most effective metal ion in promoting deprotonation of amide nitrogen atoms:[48,49] the estimated pK_a values of triglycine in the presence of Pd(II) ion are ca. 2 for the first NH group and ca. 4 for the second,[46] instead of ca. 15 for the free NH group in acetamide. Ultimately, a stable square-planar complex can be formed in which the peptide wraps around the Pd(II) ion as a tetradentate ligand, bonded via the terminal amino group and the first three amidate nitrogen atoms in the sequence. Because binding to deprotonated amide nitrogen

strengthens the C–N bond, none of these three amide bonds undergoes hydrolytic cleavage. *Their unreactivity is beneficial because it ensures that Pd(II)-promoted cleavage is guided only by histidine and methionine residues.*

The Anchoring Side Chain at Positions 1 and 2. When histidine or methionine anchor is the first or the second residue (from the N-terminus), the anchoring of the Pd(II) ion is followed by the coordination upstream from the anchor, as shown in Schemes 2a and 2b.[50-54] Peptides containing N-terminal histidine or methionine residue, such as His-Gly and Met-Gly, bind to the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ as bidentate ligands.[24,53,55] The anchored Pd(II) ion displaces a proton from the terminal ammonium group with $pK_a < 1.0$. The resulting bis(bidentate) complex *2* is unproductive for the hydrolysis of the peptide because the Pd(II) ion lacks aqua ligands and is held away from the downstream peptide bond. *Either "shortcoming" renders the complex hydrolytically-inactive.* Consequently, His-Gly peptide cannot be cleaved (at pH 3.0 and 60 °C during 1 d).

When a peptide containing a histidine or a methionine as the second residue is mixed with the complex *cis*-$[Pd(en)Cl_2]$, the tridentate complex ***3*** is the major product even at pH as low as 1.5.[52,54] The ethylenediamine ligand is completely displaced by the substrate backbone. Again, *Pd(II) ion "locked" in the tridentate complex cannot promote hydrolytic cleavage because it cannot approach the scissile bond.*

The Anchoring Side Chain at Position 3. Because the segment 11-14 of angiotensinogen, Val-Ile-His-Asn, contains a histidine residue at position 3, cleavage is in principle conceivable both downstream and upstream from this anchor. The ^1H NMR spectrum of the equimolar mixture of Val-Ile-His-Asn and the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$

at pH 2.0 and 60 °C recorded after a 18-min incubation showed coordination of the peptide to the Pd(II) reagent. The imidazole H-2 and H-5 singlets for the free peptide (at 8.61 ppm and 7.33 ppm, respectively) had already decreased, as shown in Figure 2. In this spectral region, six peptide-containing complexes were detected in addition to the uncoordinated peptide. In the period of 10 h, the signals for five of them decreased, while the aforementioned signals for the uncoordinated (free) peptide and the corresponding signals at 7.65 and 6.97 ppm for the final complex gradually increased. After 20 h, these were the only two species detected in solution.

The reaction between Val-Ile-His-Asn and five molar equiv of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0 was followed by HPLC. The reaction mixture after 24 h contained a single species, eluting at 27.9 min. The ^1H NMR spectrum of this product was identical to that of the final complex from the equimolar mixture. Clearly, the Pd(II) ion binds to Val-Ile-His-Asn, but this binding does not result in cleavage.

Various complexes of Pd(II) and histidine-containing peptides have been reported in the literature. For their identity, see previous studies.[29,56] Imidazole atoms N-3 and N-1 in the anchor, and also the (deprotonated) nitrogen atoms of the Ile-His and Val-Ile amide bonds upstream from the anchor, can all act as ligands. The chemical shifts of imidazole atoms H-2 and H-5[29,56] show that the final product is the complex designated ***4*** in Scheme 2c. This complex, too, is hydrolytically inactive. The amidate groups coordinated to the Pd(II) ion are actually protected by this coordination against hydrolytic cleavage. *Because the Pd(II) ion "wrapped" in the peptide backbone lacks an aqua ligand and cannot approach other, potentially scissile, amide bonds in the substrate, complex* ***4*** *is hydrolytically-inactive.*

The growth with time of the imidazole H-5 resonances for both the uncoordinated peptide and the final complex was successfully fitted to the first-order rate law, with the same rate constant of $(1.0 \pm 0.1)\cdot 10^{-3}$ min^{-1}. The ethylenediamine singlet for *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at 2.63 ppm gradually decreased while the singlet for $[Pd(en)_2]^{2+}$ at 2.74 ppm increased, both with that same rate constant (the results are not shown).

Evidently, the tetradentate complex ***4*** and $[Pd(en)_2]^{2+}$ are formed simultaneously, in the process in which the displacement of ethylenediamine is the rate-limiting step. The removal of this bidentate ligand from the complex ***2*** is required for the formation of the complex ***4*** by two-step deprotonation of the NH group in the Val-Ile amide bond and the terminal ammonium group of the valine residue, as shown in eq 1. Displaced ethylenediamine then reacts with the free cleavage reagent according to eq 2.

$$\underset{\textbf{2}}{\textit{cis}\text{-}[Pd(en)(Val\text{-}Ile\text{-}His_{-2H}\text{-}Asn)]^{2+}} \rightarrow enH_2^{2+} + \underset{\textbf{4}}{[Pd(Val_{-H}\text{-}Ile_{-H}\text{-}His_{-2H}\text{-}Asn)]} \quad (1)$$

$$enH_2^{2+} + \textit{cis}\text{-}[Pd(en)(H_2O)_2]^{2+} \rightarrow [Pd(en)_2]^{2+} + 2\,H_3O^+ \quad (2)$$

The stability of a tetradentate complex of type ***4*** formed between a transition-metal ion and the sequence containing His3 is important because such complexes are present in metal-transporting proteins, e.g., serum albumin. Bioactive peptides such as histatines and neuromedins[57] require a metal ion in order to be recognized by the receptor. The peptide conformation seems to be random in the absence of the metal ion, but well-defined in its presence.

Pentagastrin, βAla-Trp-Met-Asp-Phe-NH_2, contains Met3 as an anchor for the Pd(II) ion. The MALDI mass spectrum of the mixture of this peptide and 5 molar equiv of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ after 48 h at pH 1.3 or 3.0 contained only peaks at 668.0 and

773.0 D, corresponding, respectively, to the intact peptide (calculated, 668.2 D) and peptide+Pd (calculated, 773.2 D). Evidently, as with Val-Ile-His-Asn, a Pd(II) ion is bound to pentagastrin but does not cleave it.

Studies in our and other laboratories of Pd(II)-peptide complexes showed that peptides containing histidine and methionine residues at corresponding positions in the sequence behave similarly. Upon anchoring to the sulfur atom of methionine, Pd(II) ion deprotonates and binds the nitrogen atoms of upstream amide groups.[33] Therefore, pentagastrin likely forms the methionine analog of complex ***4***, the only difference being that the amino nitrogen atom and the first amide nitrogen atom (both of them in the βAla residue) form with the Pd(II) ion a six-membered ring, rather than a five-membered ring.

The results in this and the preceding subsections show that *if the anchoring residue is first, second, or third in the sequence, the Pd(II) reagent becomes "locked" in a hydrolytically-inactive polydentate complex.* This generalization is important for understanding how Pd(II) complexes function as artificial peptidases.

The Anchoring Side Chain in Positions Beyond 3: Cleavage of Chain B of Insulin. MALDI mass spectrum of the reaction mixture at pH 2.0 containing oxidized chain B of insulin and 5 molar equiv of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ after a 15-min incubation showed peaks corresponding to the intact peptide (3495.9 D) and to its complexes containing one Pd atom (3600.5 D), one Pd(en) group (3661.0 D), two Pd atoms (3706.3 D), two Pd(en) groups (3766.2 D), and three Pd atoms (3812.8 D). The chromatogram of this mixture contained two major fractions, for Pd(II)-peptide complexes, eluting at 34.9 and 35.1 min, and the minor one, for the intact peptide, eluting at 33.6 min. The three possible anchoring sites are the imidazole groups in the two histidine residues and the terminal amino group; see Chart 2.

The results in Table 1 prove that the Pd(II) reagent promoted cleavage of the Asn-Gln bond, the second one upstream from the His5 anchor, and of the Gly-Ser bond, the second one upstream from the His10 anchor, as shown below.

Phe-Val-Asn-Gln-**His**-Leu-Cys^{OX}-Gly-Ser-**His**-Leu-Val-Glu-Ala-Leu-

-Tyr-Leu-Val-Cys^{OX}-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

Gradual formation of white precipitate during the reaction prevented detailed kinetic analysis. The MALDI mass spectrum of the precipitate redissolved in a 1:1 mixture of water and acetonitrile showed molecular masses of 2547.6 and 3496.2 D, which correspond, respectively, to the fragment Ser9···Ala30 and the intact chain B of insulin. The identity of the precipitate was confirmed by its amino-acid analysis, which showed the expected residues, and by N-terminal sequencing, which showed in first three cycles the expected sequences, namely Phe-Val-Asn and Ser-His-Leu. The chromatogram of the supernatant contained four major products, identified in Table 1. Several minor fractions were also observed and they, resulted from nonselective cleavage after glutamate residues, estimated from the chromatogram to be ca. 5% after 24 h. Each major peak, however, was observed only in the solution containing the Pd(II) reagent. This is clear evidence for selective cleavage promoted by the Pd(II) complex.

The anchored Pd(II) reagent promoted cleavage of the second peptide bond upstream from each of the two anchoring residues – His5 and His10. When the anchoring residue sits beyond the position 3, cleavage follows the regular pattern, understandable from the familiar modes of peptide coordination to the Pd(II) atom.[33,54,58] As Scheme 3 shows, Pd(II) ion anchored to the N-3 atom of imidazole may bind to amidate groups upstream. When the

initial reagent is *cis*-$[Pd(en)(H_2O)_2]^{2+}$, this process involves displacement of the ethylenediamine ligand, as discussed above. The anchored Pd(II) ion deprotonates the first amide group upstream from the anchor relatively easily, with $pK_a < 2.0$.[49] Binding of the negatively-charged amidate ligand lowers the Lewis acidity of the Pd(II) ion and weakens its ability to deprotonate and bind the second and third amide NH groups further upstream in the substrate backbone. At pH 2.3, the major species are the bidentate complex ***2*** and the tridentate complex ***3***. At pH 2.0, the unidentate complex ***1*** and its minor linkage isomer, involving coordination via the N-1 atom of imidazole, are also present.[29] Because the amide group is much less acidic ($pK_a \approx 15$) than the terminal ammonium group ($pK_a \approx 9$), the deprotonation of the third amide group upstream from the anchor and its coordination to the anchored Pd(II) ion is largely suppressed at pH 2.3. Therefore, the tetradentate complex of type ***4*** is absent at pH 2.3. Instead of "wrapping" itself into the peptide and forming the hydrolytically-inactive complex ***4***, the Pd(II) ion forms the hydrolytically-active complex ***2***. This complex is reactive for both reasons discussed in the Introduction. It is oriented toward the scissile bond, and it contains aqua ligands, to be displaced by the carbonyl oxygen atom (in the external mechanism) or to be delivered to the carbonyl carbon atom (in the internal mechanism). For obvious reasons, complex ***2*** promotes hydrolysis of the second peptide bond upstream from the anchor.

Regioselective cleavage is evidently a result of the Pd(II) complex binding to the anchoring (histidine or methionine) side chain and to the peptide backbone preceding the anchor (upstream from it). Studies in our and other laboratories showed that non-coordinating side chains flanking the anchor do not alter the coordination modes.[46,49,54,59-61] Therefore, *the regioselective cleavage in the hydrolytically-active complex **2** is expected to*

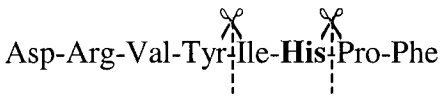
be a general property of peptides containing an "isolated" methionine or histidine residue beyond position 3 in the sequence.

After the cleavage is completed, the Pd(II) reagent remains bound to the fragment downstream from the cleaved bond. Further hydrolysis of this fragment is impossible because the anchoring residue is the second one from the newly-created N-terminus. The anchored Pd(II) reagent becomes "locked" in the hydrolytically-inactive complex ***3*** and cannot promote further cleavage of the peptide involved in this complex. This result is important for the understanding and the use of our Pd(II) reagents, because it shows that the regioselectivity directed by histidine and methionine residue persists, without unwanted additional cleavage. At the end of the incubation, the Pd(II) atom can be easily removed by an excess of a chelating reagent, and the protein fragments are obtained pristine.

Regioselectivity and Kinetics of Primary and Secondary Cleavage of Angiotensin II. The MALDI mass spectrum of the mixture containing angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and 5 molar equiv of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ after a 15-min incubation showed prominent peaks corresponding to the intact peptide (1046.8 D) and its complexes with one Pd atom (1152.4 D), one Pd(en) group (1213.2 D), two Pd atoms (1260.0 D), and two Pd(en) groups (1319.5 D). The chromatogram contained several broad peaks for the various Pd(II)-peptide complexes and a diminished peak, eluting at 24.7 min, for the intact peptide. Our results point at two binding sites in the peptide, namely His6 and the terminal amino group.

The products after a 24-h period were identified from the MALDI mass spectrum of the reaction mixture; the results are summarized in Table 2. The expected signal of the

fragment Ile-His in the MALDI mass spectrum was covered by a signal of the matrix. The observed fragments correspond to two cleavage sites near the histidine anchor: the His-Pro bond, the first downstream, and the Tyr-Ile bond, the second one upstream, as shown below.



We followed the kinetics of this dual cleavage in experiments with the segment 3-8 of angiotensin II, Val-Tyr-Ile-His-Pro-Phe. The chromatogram of a mixture of this substrate and 5 molar equiv of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ after 24 h showed four products, eluting at 3.3, 15.0, 21.2, and 23.1 min. Upon preparative HPLC separation, each product was identified by the characteristic ^{1}H-NMR chemical shifts; the results are given in Table 3 and below.

Background cleavage of Val-Tyr-Ile-His-Pro-Phe was not observed in the absence of Pd(II) reagent after two weeks. As with the whole angiotensin II, the Pd(II) reagent promoted selective cleavage of the segment 3-8 at the first amide bond downstream and the second amide bond upstream from the histidine anchor. Figure 3 shows the kinetics of this dual cleavage, monitored through the substrate (Figure 3a), all three fragments (Figure 3b and 3c), and several intermediates (Figure 3d). The His-Pro bond is cleaved with the first-order rate constant $k_1=2.0\cdot10^{-3}$ min^{-1}. This process occurs without a lag period, and we call it primary cleavage. That concentrations of the intermediates vary at different rates and reach their maxima at different times are signs of their intricate interconversions. The MALDI mass spectrum of the reaction mixture after 3 h showed the following peaks: 263.3 D, for the

fragment Pro-Phe; 775.0, 880.0, and 940.0 D, for the intact peptide and its complexes with Pd and Pd(en); and 530.8 D, 635.8 D, and 696.0 D, for the fragment Val-Tyr-Ile-His and its complexes with Pd and Pd(en). Evidently, the intermediates are various Pd(II) complexes with the intact hexapeptide and with the Val-Tyr-Ile-His fragment, a product of the primary cleavage.

Unlike the cleavage of the His-Pro bond, which started immediately upon mixing of the substrate and the Pd(II) reagent, the cleavage of the Tyr-Ile bond was delayed. The first six data points in Figure 3c show a lag time and were justifiably omitted from the fitting to the first-order kinetic low. This fitting gave the same rate constant for the formation of Val-Tyr and Ile-His fragments, $k_2=1.1\cdot10^{-3}$ min^{-1}. Evidently, these fragments arise in the secondary cleavage of fragment Val-Tyr-Ile-His, a product of the primary cleavage. These kinetic results are consistent with the reaction mechanism in Scheme 4. The Pd(II) complex binds to the imidazole N-3 atom in the side chain of His 4 immediately upon mixing, and forms complex of type ***1*** in which the downstream His-Pro bond is cleaved with the rate constant k_1. The bidentate ligand ethylenediamine does not interfere with this primary cleavage because the Pd(II) ion has an accessible coordination site, loosely occupied by an aqua ligand.[25,29]

The secondary cleavage upstream from the histidine anchor involves the formation of the hydrolytically-active complex of type ***2***, a process that requires the rate-limiting displacement of the ethylenediamine ligand. Indeed, the rate constants for the cleavage and displacement are the same. The observed lag period is the time required for this displacement.

The uncommon downstream cleavage of angiotensin II can be attributed to the presence of a proline residue next downstream from the anchoring histidine residue. Proline

is unique among common amino acids in forming a tertiary amide group. Dipeptides of the type X-Pro bind to transition-metal ions such as Ni(II) and Cu(II) more strongly than do similar dipeptides lacking proline.[49,62] This much-enhanced affinity was attributed to the fact that the amide nitrogen atom of proline is intrinsically the most basic atom in the polypeptide (or protein) backbone. Indeed, the rate constant for the downstream cleavage of dipeptides AcHis-X is ca. 10^4 times higher if X is sarcosine (N-methyl glycine), which forms a tertiary amide bond, than if X is glycine, which forms a common secondary amide bond.[26] We will continue to study the role of proline in the hydrolytic cleavage of peptides and proteins.

Confirmation of Regioselectivity: Cleavage of Ubiquitin. The chromatogram of fresh ubiquitin contained a single peak, eluting at 15.3 min, whereas the chromatogram of the mixture containing ubiquitin and 2 molar equiv of the reagent $[Pd(H_2O)_4]^{2+}$ at pH 2.5 after 30 min showed a single broad peak eluting at 12.9 min and absorbing at 215, 280, and 350 nm. The first two wavelengths are diagnostic of the protein; the third, of Pd(II)-protein complexes. Evidently, the Pd(II) reagent is bound to the protein. Because the protein is fully unfolded under the reaction conditions, both Met1 and His68, potential anchors, are expected to be accessible to the Pd(II) reagent.

The peak for the intact protein was absent in both the chromatogram and the MALDI mass spectrum of the digest after 24 h, indicating that the cleavage was complete. The chromatogram of this digest showed a small, sharp peak eluting at 9.1 min and a large, broad peak eluting at 12.8 min. Minor background cleavage, estimated at < 5%, was evident from the presence of few other minor peaks in the chromatograms of both the reaction mixture and the control solution free of the Pd(II) reagent. The MALDI mass spectrometric results in

Table 4 prove that only the Thr66-Leu67 bond is cleaved, as shown in the ubiquitin sequence below.

1 MQIFVKTLTGKTITLEVEPSDTIENVKAKI 30

31 QDKEGIPPDQQRLIFAGKQLEDGRTLSDYN 60

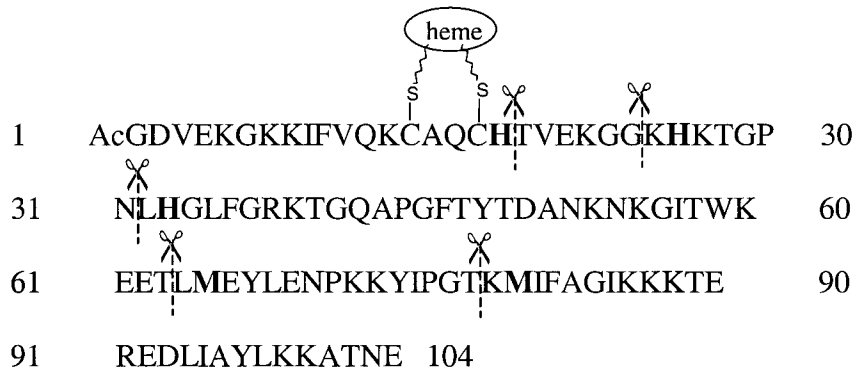
61 IQKEST↓LHLVLRLRGG 76

We can readily understand these results. Coordination of the N-terminal methionine residue does not result in proteolytic cleavage, as explained above. The Pd(II) reagent binds to the only internal anchor available, His68, and cleaves the second bond upstream from this anchor. Ubiquitin confirms the general pattern of regioselectivity of our Pd(II)-containing peptidases.

Control of Regioselectivity by the Reagent-to-Protein Ratio: Cleavage of Cytochrome *c*. The reaction mixture containing cytochrome *c* and 10 mole equiv of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0 remained clear after incubation for 24 h. Electrophoretograms of this mixture and of the control solution free of Pd(II) regent are shown in Figure S1 in the Supporting Information. Minor cleavage of the Asp2-Val3 and Asp50-Ala51 bonds was also observed in both the reaction mixture and the control solution. Five bands, designated A-E, were subjected to N-terminal analysis, and the reaction mixture that gave rise to these bands was analyzed by MALDI mass spectrometry. The results are correlated in Table 5.

Because the terminal amino group in equine cytochrome *c* is acetylated, the N-terminal fragments were undetectable by the Edman method. Fortunately, these fragments

were identified in the MALDI mass spectrum of the digest. The results in Table 5 prove the pattern of cleavage shown in the sequence of cytochrome *c* below.



Evidently, the Pd(II)-promoted cleavage is regioselective. It is directed by the two methionine and three histidine internal residues, which are capable of anchoring the reagent. In the case of four anchors, namely His26, His33, Met65, and Met80, the cleaved amide bond is the second one upstream from the anchor. Only in the case of His18 does the cleavage occur downstream from that anchor, at the His18-Thr19 bond.

The His26, His33, Met65, and Met80 anchors are all "isolated", i.e., flanked by non-coordinating side chains, which cannot alter the binding of the Pd(II) reagent to the anchor and to the backbone. Regardless of the identity of the neighboring non-coordinating residues, the regioselectivity observed with peptides persisted in this protein. The His18, however, is not an "isolated" anchor since it is preceded by the S-alkylated residue Cys17, another potential anchor for the Pd(II) reagent. This S-alkylation is a consequence of covalent linkage between the protein backbone and heme, and is unique for cytochromes of type *c*. This special cysteine side chain contains a thio ether group, a feature shown in the simplistic drawing in the protein sequence above. This thio ether group, too, can bind to the Pd(II) reagent. It is likely that both adjacent residues, His 18 and Cys17, anchor the Pd(II) ion and

prevent its binding to the upstream amide nitrogen atom and formation of the hydrolytically-active complex of type ***2***. Because this requirement for the upstream cleavage is not fulfilled, cleavage occurs on the other side of the His18 anchor, at the next amide bond downstream.

The His18-Thr19 bond was the only one cleaved in the early experiments, in which equimolar amounts of cytochrome *c* and the Pd(II) reagent were used.[18,30] In the present experiment, in which the Pd(II) reagent is present in tenfold molar excess over the protein and in twofold molar excess over the five potential anchors, cleavage at additional sites is to be expected; indeed, we detected it. Regioselectivity of this additional cleavage obeys the established pattern, however. *Evidently, the number of cleavage sites can be controlled by adjusting the molar ratio of the substrate and the Pd(II) reagent.*

Conclusions

This study explores the reactivity of Pd(II) complexes as reagents for regioselective hydrolysis of eight natural peptides and proteins. The regioselectivity of cleavage is directed by the binding of the Pd(II) reagent to the side chains of internal histidine and methionine residues and to the deprotonated amide groups in the peptide backbone. A clear pattern emerged for occurrence and nonoccurrence of cleavage. Substrates lacking an anchoring residue cannot be cleaved. When the anchoring residue is present in position 1, 2, or 3 from the N-terminus, the amide nitrogen atoms upstream from the anchor become deprotonated and bind the anchored Pd(II) ion into a stable bidentate (position 1), tridentate (position 2), or tetradentate (position 3) chelate, thus "locking" this ion and preventing its approach to the scissile amide bond. When the anchoring residue is present in a position beyond 3 in the sequence and no other coordinating side chain interferes, the cleavage occurs at the second

amide bond upstream from the anchor. If the complex *cis*-$[Pd(en)(H_2O)_2]^{2+}$ is used as the reagent, this upstream cleavage is preceded by the rate-limiting displacement of the ethylenediamine ligand. In exceptional cases, such as one of the sites in angiotensin II and one of those in cytochrome *c*, cleavage can occur also at the first peptide bond downstream from the anchor. The ability of Pd(II) complexes to cleave proteins at relatively few sites, with explicable selectivity, and with good yields bodes well for their wider use in biochemical and bioanalytical practice.

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GRAPHICS

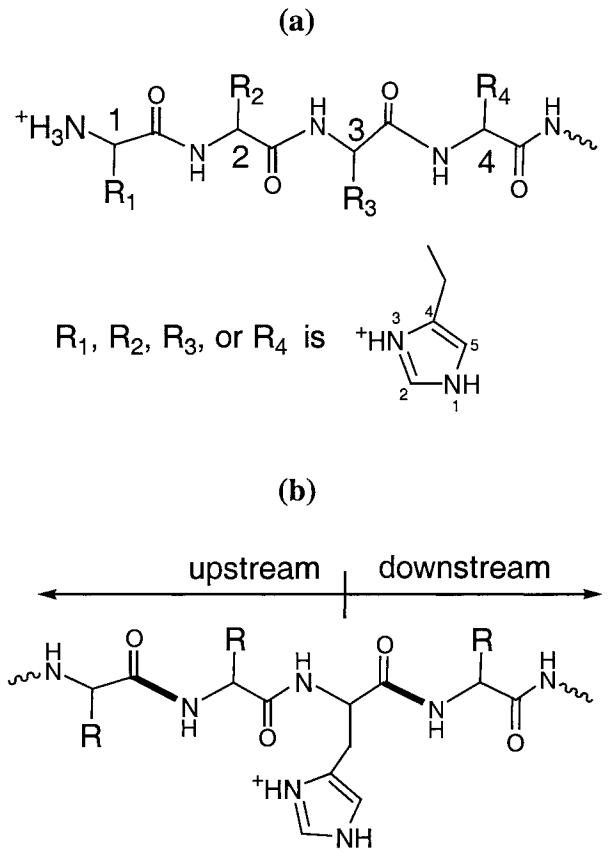


Figure 1. (a) Positions of the anchoring histidine residue with respect to the N-terminus and numbering of the atoms in the imidazole ring. (b) Definition of the upstream and downstream directions from the anchor. The scissile peptide bonds, highlighted, are the second one upstream and the first one downstream from the anchor.

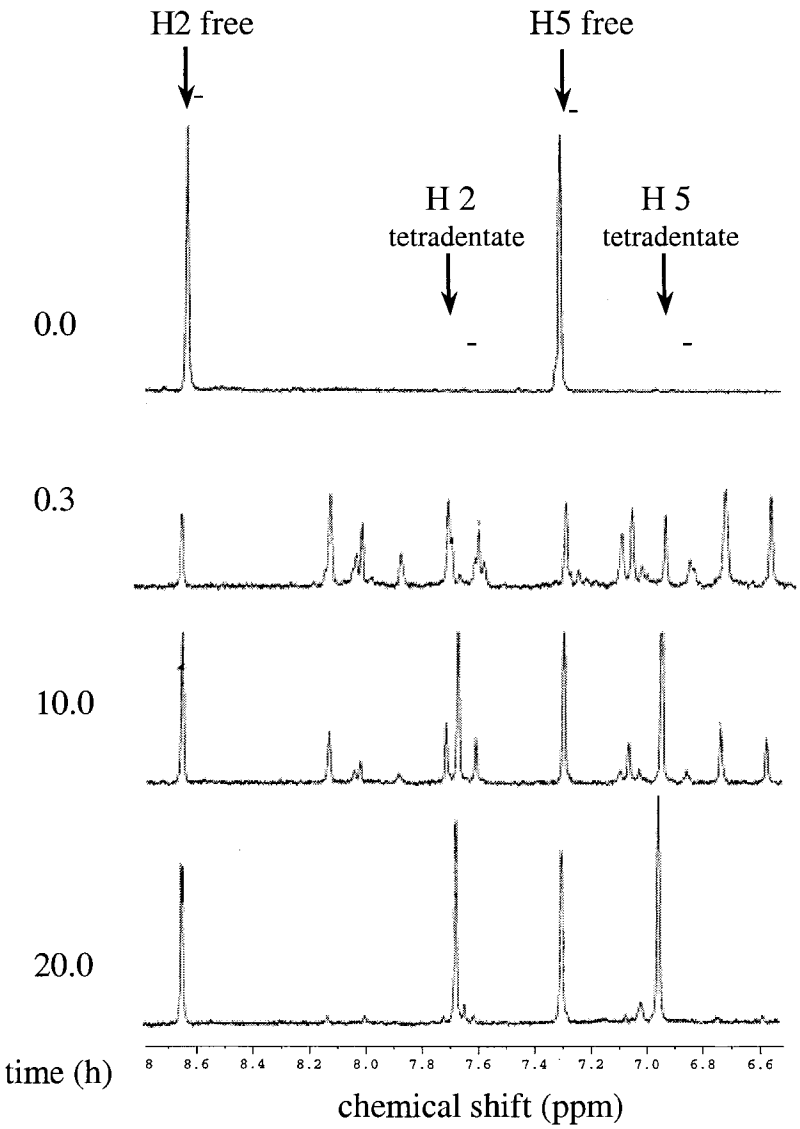


Figure 2. Proton NMR spectra of the peptide Val-Ile-His-Asn before, and at three times after, the addition of an equimolar amount of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0 and 60 °C

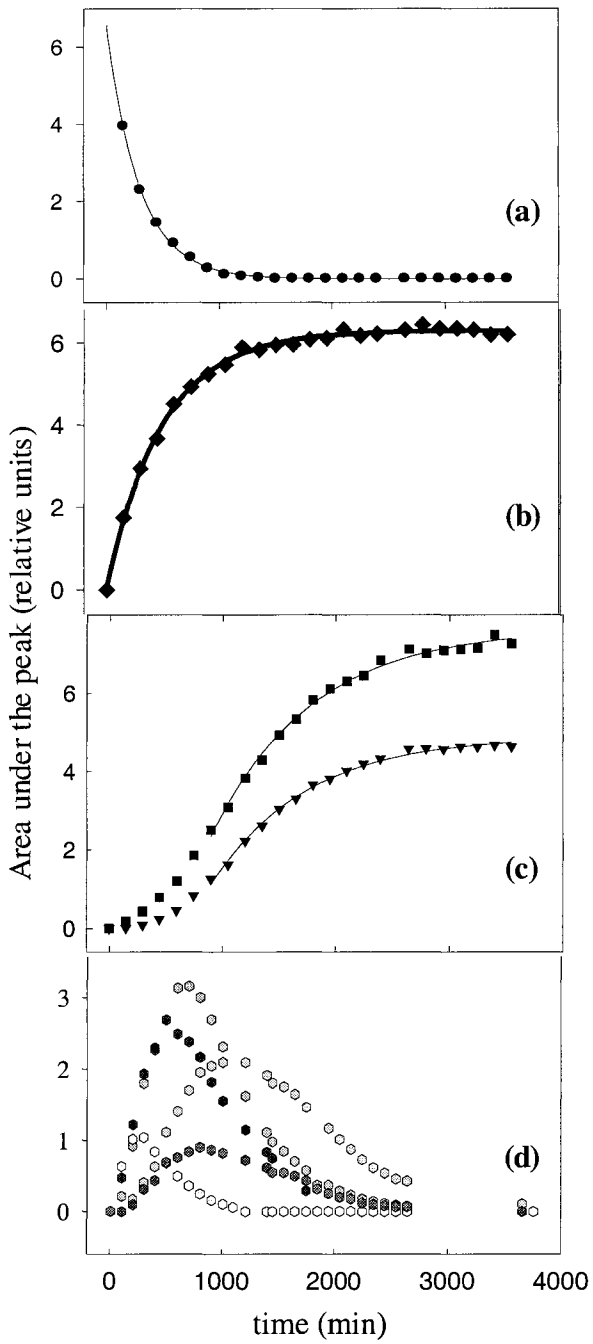


Figure 3. The progress of cleavage of the peptide Val-Tyr-Ile-His-Pro-Phe (segment 3-8 of angiotensin II), by the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ followed by HPLC. Solid lines are exponential fittings to first-order kinetic law. The mechanism is shown in Scheme 4. (a) Disappearance of the intact peptide. (b) Appearance of the fraction eluting at 23 min, which contains the fragment Pro-Phe, the product of the primary cleavage.(c) Appearance of fractions eluting at 15 min (■) and 21 min (▲), which contains the fragments Val-Tyr and Ile-His, respectively, the products of the secondary cleavage. (d) The intermediates formed and consumed during the cleavage (note the very last points).

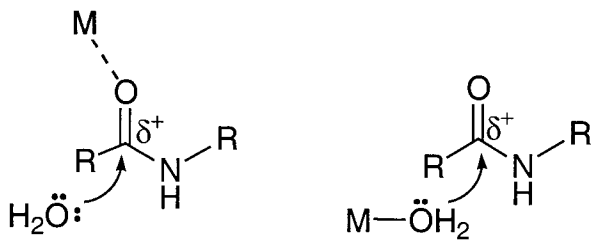


Chart 1. Possible limiting mechanisms for hydrolysis of carboxylic amide, promoted by a transition-metal ion acting as a Lewis acid or as a carrier of the nucleophile. The mechanisms are designated external and internal according to the origin of the nucleophile (water).

substrate	sequence
oxidized[a] chain A of bovine insulin	Gly-Ile-Val-Glu-Gln-Cys^{OX}-Cys^{OX}-Thr-Ser-Ile-Cys^{OX}-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys^{OX}-Asn
segment 11-14 of human angiotensinogen	Val-Ile-**His**-Asn
human pentagastrin	βAla-Trp-**Met**-Asp-Phe-NH_2
human angiotensin II	Asp-Arg-Val-Tyr-Ile-**His**-Pro-Phe
segment 3-8 of human angiotensin II	Val-Tyr-Ile-**His**-Pro-Phe
oxidized[a] chain B of bovine insulin	Phe-Val-Asn-Gln-**His**-Leu-Cys^{OX}-Gly-Ser-**His**-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys^{OX}-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala
bovine ubiquitin	76 amino-acid residues; see Results and Discussion
equine cytochrome *c*	104 amino-acid residues; see Results and Discussion

[a] Cys^{OX} is cysteinic acid

Chart 2. The substrates for binding to Pd(II) complexes and possible hydrolytic cleavage by these complexes. The anchoring side chains are highlighted.

Table 1. Results of HPLC separation and MALDI mass spectrometric identification of the fragments obtained when the oxidized chain B of insulin was cleaved by the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
31.2	2547.6	2547.2	Ser9···Ala30
27.1	964.2	964.4	Phe1···Gly8
18.8	obscured	378.2	Phe1···Asn3
18.2	604.6	604.2	Gln4···Gly8

Table 2. Results of HPLC separation and MALDI mass spectrometric identification of the fragments obtained when angiotensin II was cleaved by the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
26.3	551.3	551.3	Asp1···Tyr4
15.1	obscured	268.1	Ile5-His6
23.1	263.3	263.1	Pro7-Phe8

Table 3. Results of HPLC separation and ^{1}H-NMR spectroscopic identification of the fragments obtained when Val-Tyr-Ile-His-Pro-Phe (segment 3-8 of angiotensin II) was cleaved by the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$

elution time (min)	characteristic ^{1}H-NMR signals (ppm)		identity
3.3	en	2.74	$[Pd(en)_2]^{2+}$
15.0	Ile His	H_ε 0.88, H_γ 0.98 H-2 7.62, H-5 6.96	Ile-His
21.2	Val Tyr	H_γ 0.76, 0.82 H_δ 6.68, H_ε7.05	Val-Tyr
23.1	Pro Phe	H_β 1.80, H_γ 2.30 $H_{\delta,\varepsilon,\zeta}$ 7.23	Pro-Phe

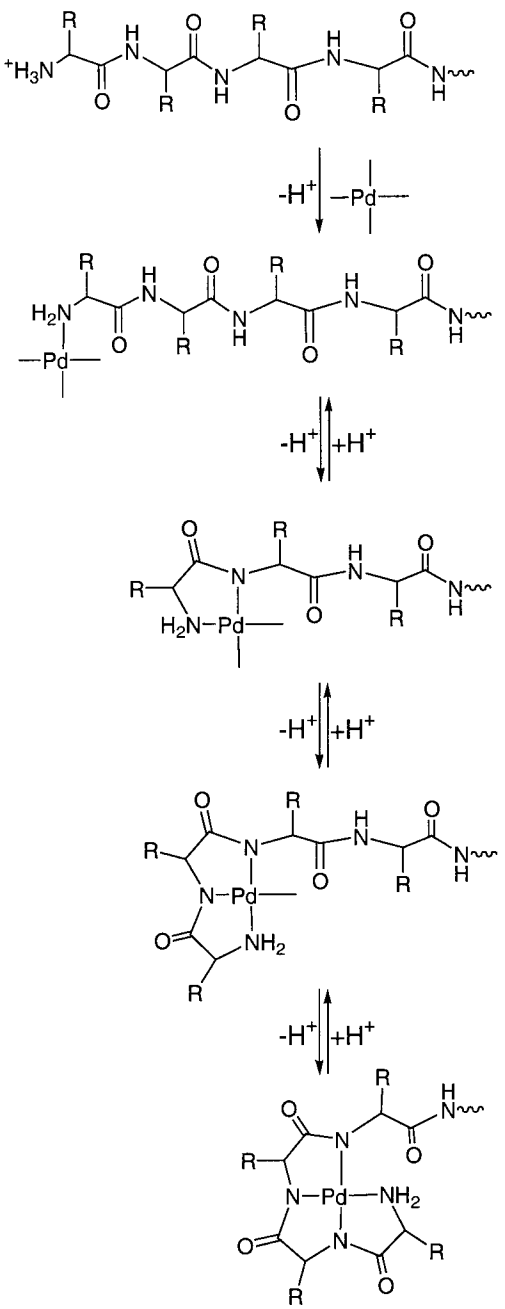
Table 4. Results of HPLC separation and MALDI mass spectrometric identification of the fragments obtained when ubiquitin was cleaved by the reagent $[Pd(H_2O)_4]^{2+}$

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
9.9	1133.2	1133.4	Leu67···Gly76
12.8	7436.0	7449.4	Met1···Thr66

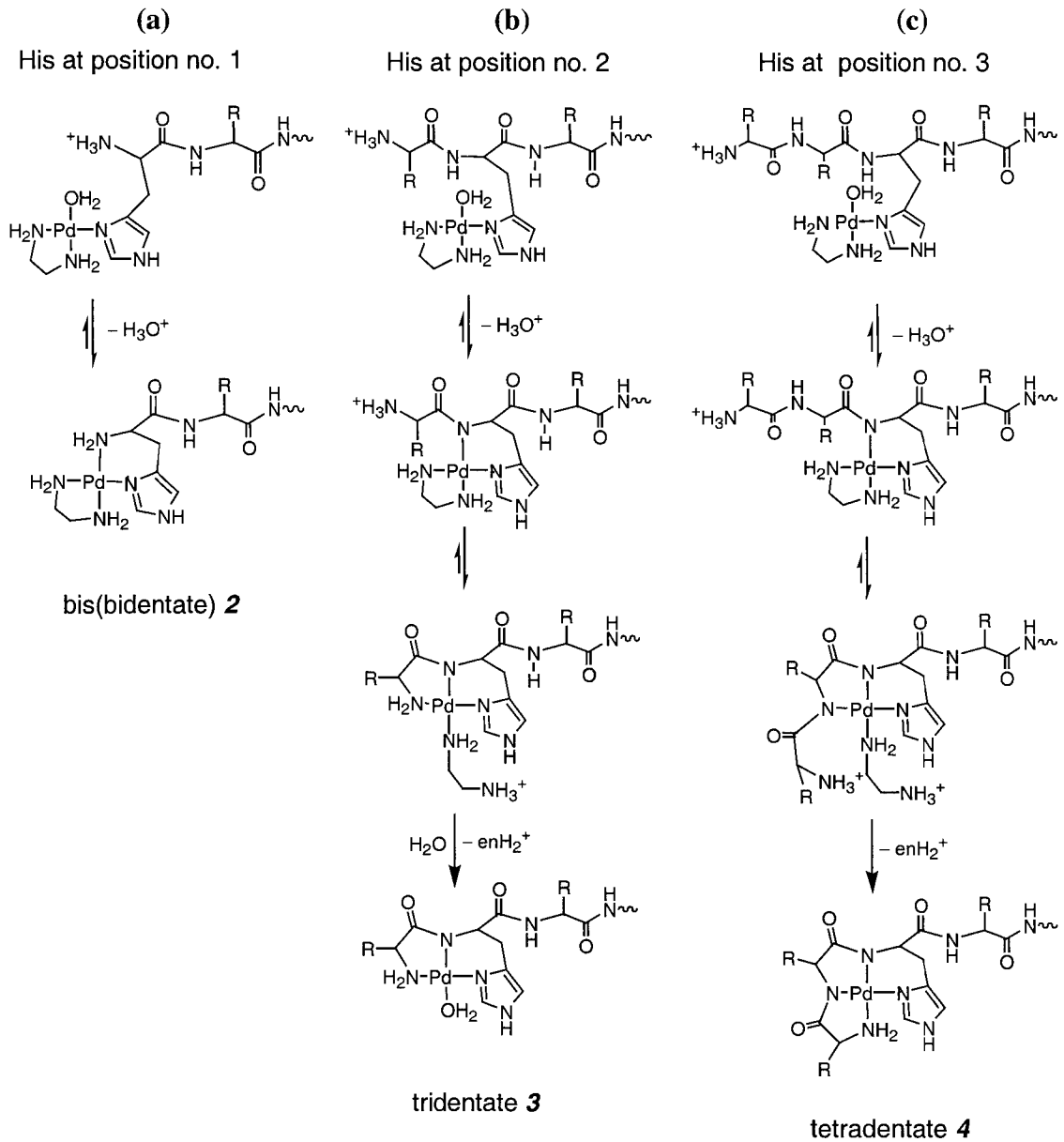
Table 5. The fragments of cytochrome *c* obtained by cleavage with the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$, separated by TSDS-PAGE, and identified by MALDI-MS and N-terminal sequencing.

electrophoretic band [a, b]	molecular mass (D)		fragment
	calculated	observed	
und.	12357.9	12370.3	AcGly1···Glu104 [c]
B	9701.2	9701.6	Thr19···Glu104
C	9129.5	9130.1	Lys25···Glu104
n. d.	9336.3	9341.0	AcGly1···Thr78
D	8366.7	8366.6	Leu32···Glu104
E	6679.6	6675.2	Thr19···Thr78
E	6107.9	6112.5	Lys25···Thr78
n. d.	4901.5	4908.2	Thr19···Thr63
n. d.	4817.7	4820.0	Leu64···Glu104
und.	4009.3	4008.8	AcGly1···Asn31
und.	3246.4	3244.4	AcGly1···Gly24
und.	2674.8	2674.1	AcGly1···His18
n. d.	1352.5	1350.6	Thr19···Asn31

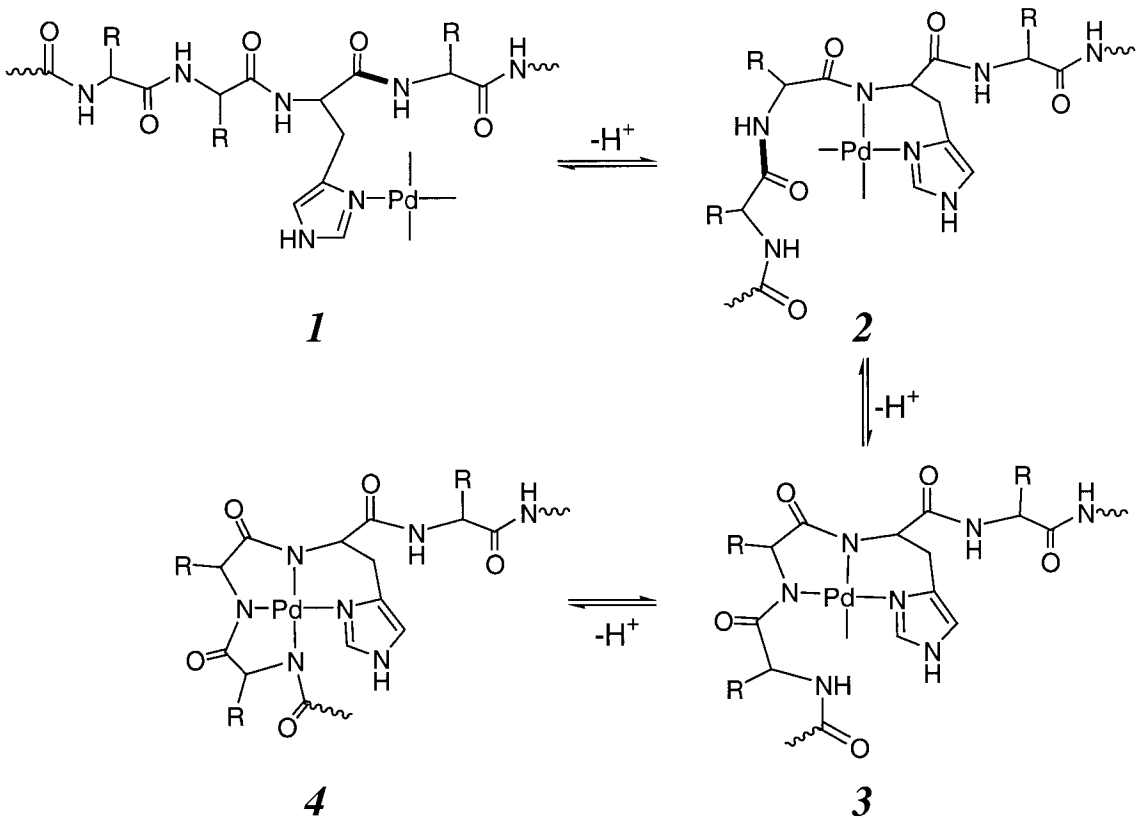
[a] Shown in Figure S1 in the Supporting Information.
[b] Certain fragments are undetectable (und.) or not detected (n. d.) in the electrophoretogram. The acetylated N-terminus is undetectable by the standard Edman method, as explained in the text.
[c] Intact cytochrome *c*.



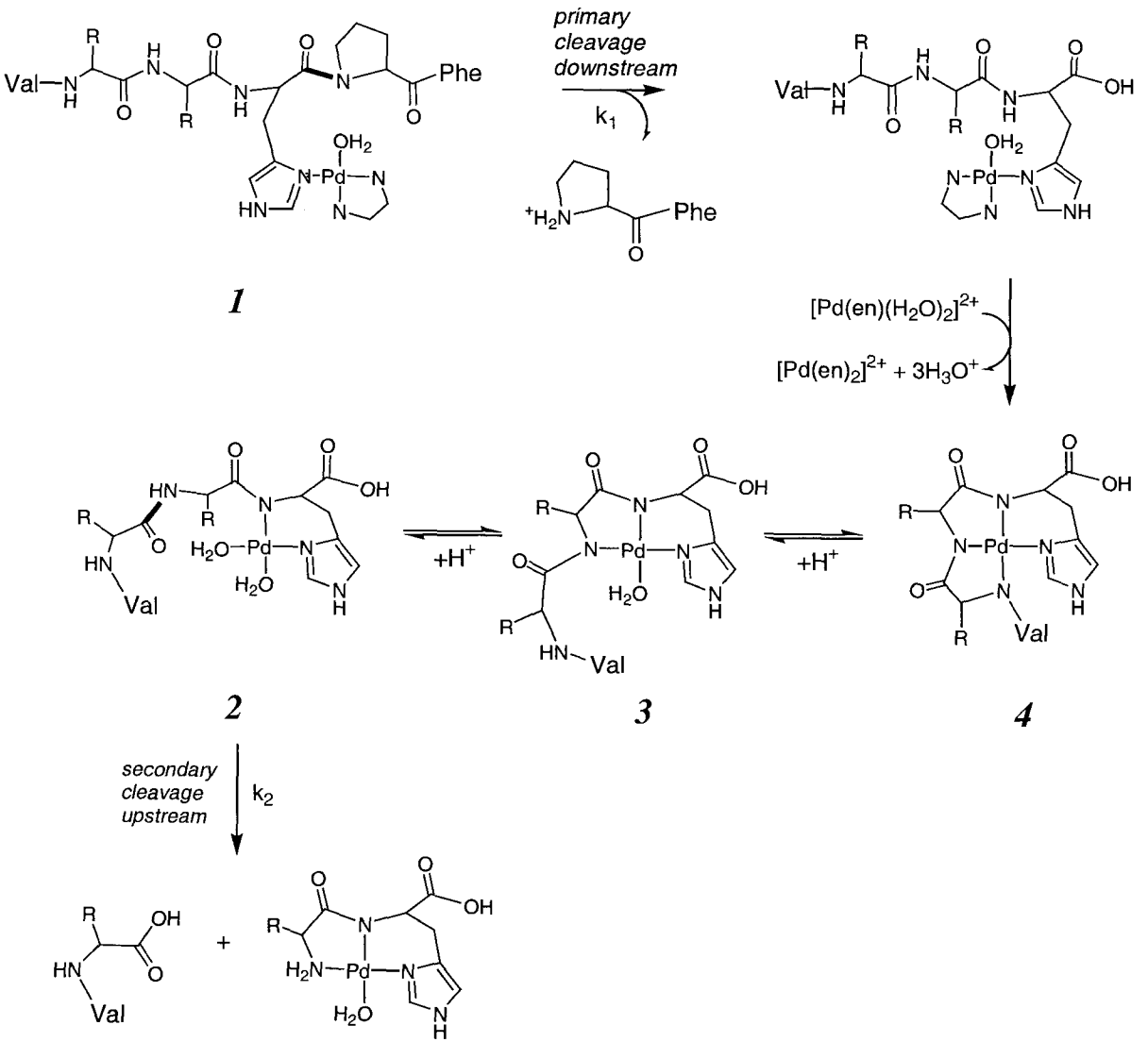
Scheme 1. Peptide or protein that lacks anchoring side chains can bind to a Pd(II) ion via the terminal amino group and three deprotonated amide groups in the backbone, to form a tetradentate chelate complex that is hydrolytically-inactive because the amidate groups coordinated to the Pd(II) ion are actually protected by this coordination against hydrolytic cleavage, and other amide groups cannot approach the Pd(II) ion.



Scheme 2. "Locking" of Pd(II) ion in a hydrolytically-inactive chelate complex inhibits the cleavage of peptides containing a histidine anchor as (a) the first; (b) the second; and (c) the third residue from the N-terminus. A Pd(II) ion anchored to a methionine side chain behaves similarly.



Scheme 3. Equilibrium among complexes that Pd(II) ion forms with peptides having a histidine residue at a position beyond no. 3. The scissile bonds (the second one upstream and the first one downstream from the anchor) are highlighted. For clarity, the cleavage step is not shown. A Pd(II) ion anchored to a methionine side chain behaves similarly.



Scheme 4. The mechanism of cleavage of the peptide Val-Tyr-Ile-His-Pro-Phe (segment 3-8 of angiotensin II), promoted by the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$. Only the middle four residues, those that participate in the reaction, are shown explicitly. Amide bonds both downstream (His-Pro) and upstream (Tyr-Ile) from the histidine anchor are cleaved. Both of these scissile bonds are highlighted.

Supporting Information

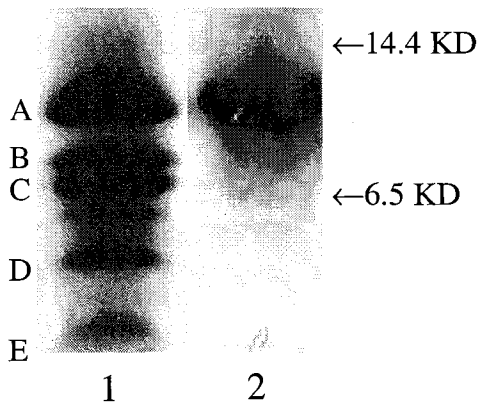


Figure S1. Tricine-SDS PAGE electrophoretogram of equine cytochrome *c* upon cleavage by 10 molar equivalents of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$. Lane 1 shows the digest after 24 h, and lane 2 shows the control mixture free of the Pd(II) reagent. Arrows indicate the positions of molecular-mass markers. The electrobloted bands, designated A-E, were subjected to N-terminal sequencing.

CHAPTER 4. 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 sequences. The hydrolysis requires 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 much 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 it 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]

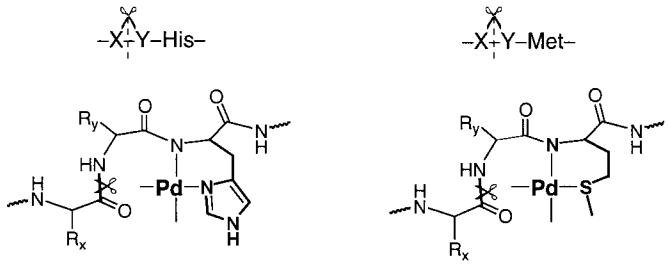
New cleavage reagents having improved efficiency and adjustable selectivity are desired for many emerging applications. Because, however, the peptide bond (i.e. the amide group) is extremely unreactive toward hydrolysis, even nonselective cleavage is hard to achieve. The half-life at room temperature 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, equimolar amount or 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 non-aqueous 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 non-coordinating residues X and Y do not alter the Pd(II) binding modes, indicating that these residues should not alter the cleavage reaction.[25-34] 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 non-coordinating 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 residue[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.

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 sulphone was obtained from Lancaster. A 0.50 M solution of $[Pd(H_2O)_4]^{2+}$ and a 0.10 M solution of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were prepared as perchlorate salts.[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 x 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 x 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 sulphone (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.

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 sulphone, 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 non-coordinating 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 non-coordinating side chains.

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 strong 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.

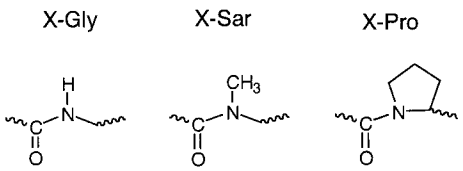
Enhancement by Proline of the Rate of Methionine-Directed Cleavage. The chromatogram of an equimolar mixture of ProMet-peptide and $[Pd(H_2O)_4]^{2+}$ at pH 2.0 and 60 °C after 1.0 h lacked a peak at 18.8 min, due to the intact peptide, and contained peaks at 9.2 and 16.6 min. Both of these peaks were 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 (obs. 431.21 D and calc. 431.22 D) and fragment 6···11 (obs. 601.84 D and calc. 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.

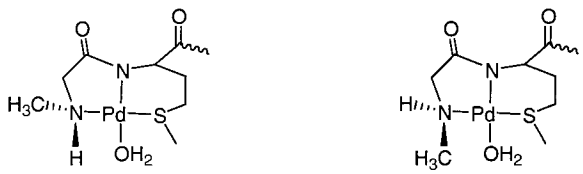
Proline is the only common amino acid to form tertiary peptide bond; it is the only cyclic amino acid; it can adopt the usual trans and also the cis configuration; it has unusual conformational requirements; and of all natural amino acids it has the most basic amino group. Sarcosine forms a tertiary 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 occur at 215 nm, but only the latter two occur 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 (obs. 431.21 D and calc. 431.22 D), whereas both fractions eluting at 9.5 and 10.0 min are the fragment 6···11 (obs. 576.31 D and calc. 576.28 D). These results prove selective cleavage of the Gly5-Sar6 bond, shown below.

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 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 $\geq$ 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 react at pH 7.0 at approximately the same rate at which GlyMet-peptide reacts 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 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-peptide and ProMet-peptide 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 $\geq$ 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 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 its cross-peaks to α-CH, β-CH, and γ-CH signals, 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.

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 $\mathbf{2}^{\mathbf{Pro}}$. The Pd(II) ion in $\mathbf{2}^{\mathbf{Pro}}$, 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 $\mathbf{2}^{\mathbf{Pro}}$ persists at pH 5.0 (and likely also at pH 7.0), unlike the analogous complex **2**, which is only a minor species even at pH as low as 2.3.

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-peptide and ProMet-peptide 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** 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 analogs 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 $\mathbf{2}^{\mathbf{Pro}}$ 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 $\mathbf{2}^{\mathbf{Pro}}$ 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 $\mathbf{2}^{\mathbf{Pro}}$ 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), but 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 $6.0 \cdot 10^{-2}$, $1.4 \cdot 10^{-2}$, and $2.8 \cdot 10^{-3}$ 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 molecule, as in Chart 2b. Parallel correlations in Scheme 3 give additional non-kinetic 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 (obs. 431.21 D and calc. 431.22 D) and fragment 6···11 (obs. 608.19 D and calc. 608.32 D). The cleavage is shown below.

AcLys-Gly-Gly-Ala-Gly-**Pro-His**-Ala-Ala-Arg-Gly

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 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 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). These results confirm that the hydrolytically-active complex is the histidine analogue of $\mathbf{2}^{\mathbf{Pro}}$. 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 tertiary peptide bonds.

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 there were peaks at 12.9, 4.1, and 16.2 min. MALDI mass-spectra showed the following fragments: 1···3 (obs. 409.11 D and calc. 409.20 D), 4···8 (obs. 406.28 D and calc. 406.17 D) and 9···14 (obs. 601.76 D and calc. 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 (obs. 795.20 D and calc. 795.36 D) and 9···14 (obs. 601.76 D and calc. 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 $\mathbf{2}^{\mathbf{Pro}}$ 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 $\mathbf{2}^{\mathbf{Pro}}$ 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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Graphics

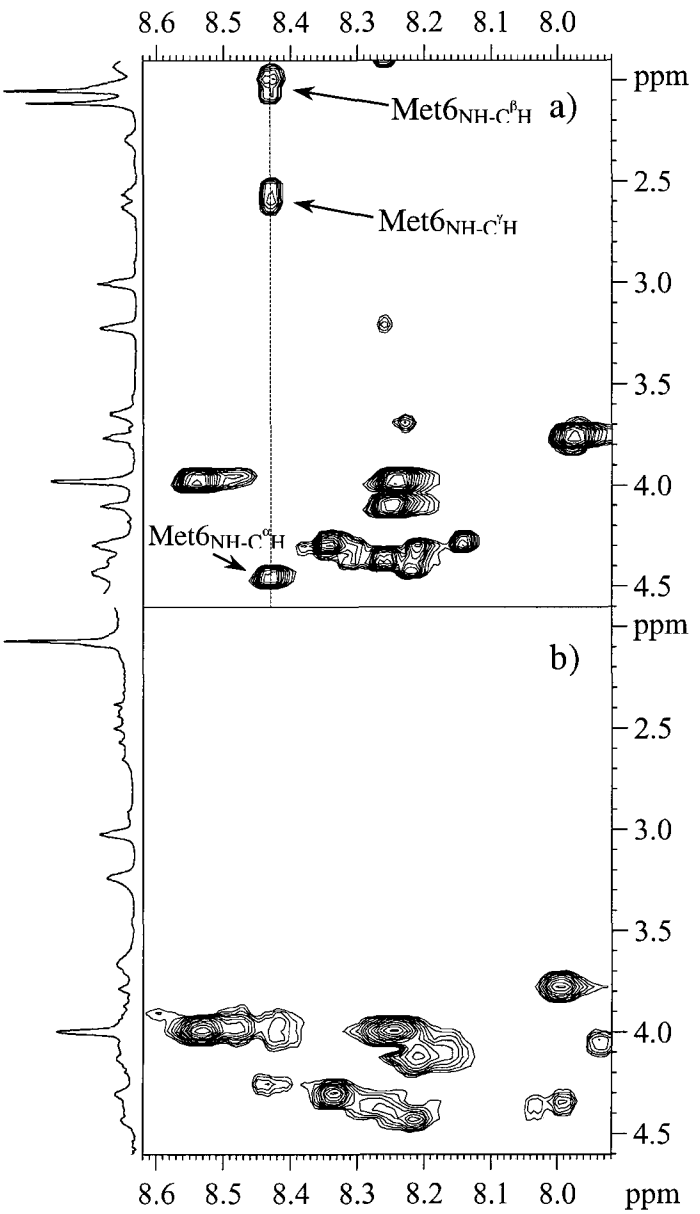


Figure 1. The aliphatic region of the one-dimensional ^{1}H NMR spectrum and a part of the NH-to-aliphatic region of the TOCSY ^{1}H NMR spectrum of the aqueous solution of AcLys-Gly-Gly-Ala-Gly-**Pro-Met**-Ala Ala-Arg-Gly (ProMet-peptide) at room temperature and pH 5.0: (a) before addition of $[Pd(H_2O)_4]^{2+}$;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

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

Chart 1. Synthetic peptides used as substrates for Pd(II)-promoted cleavage

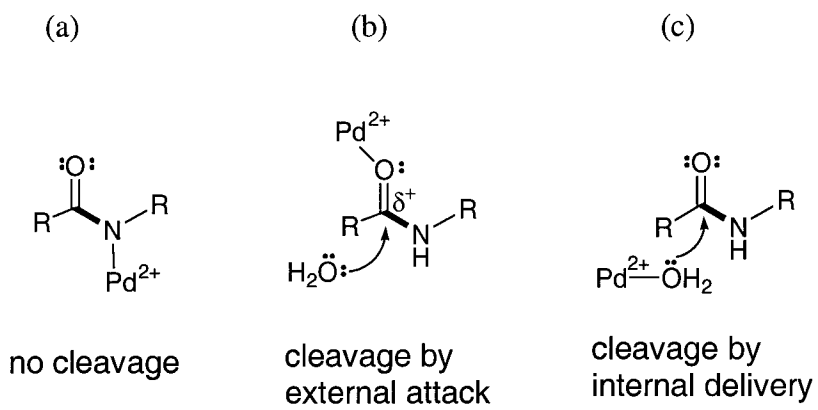
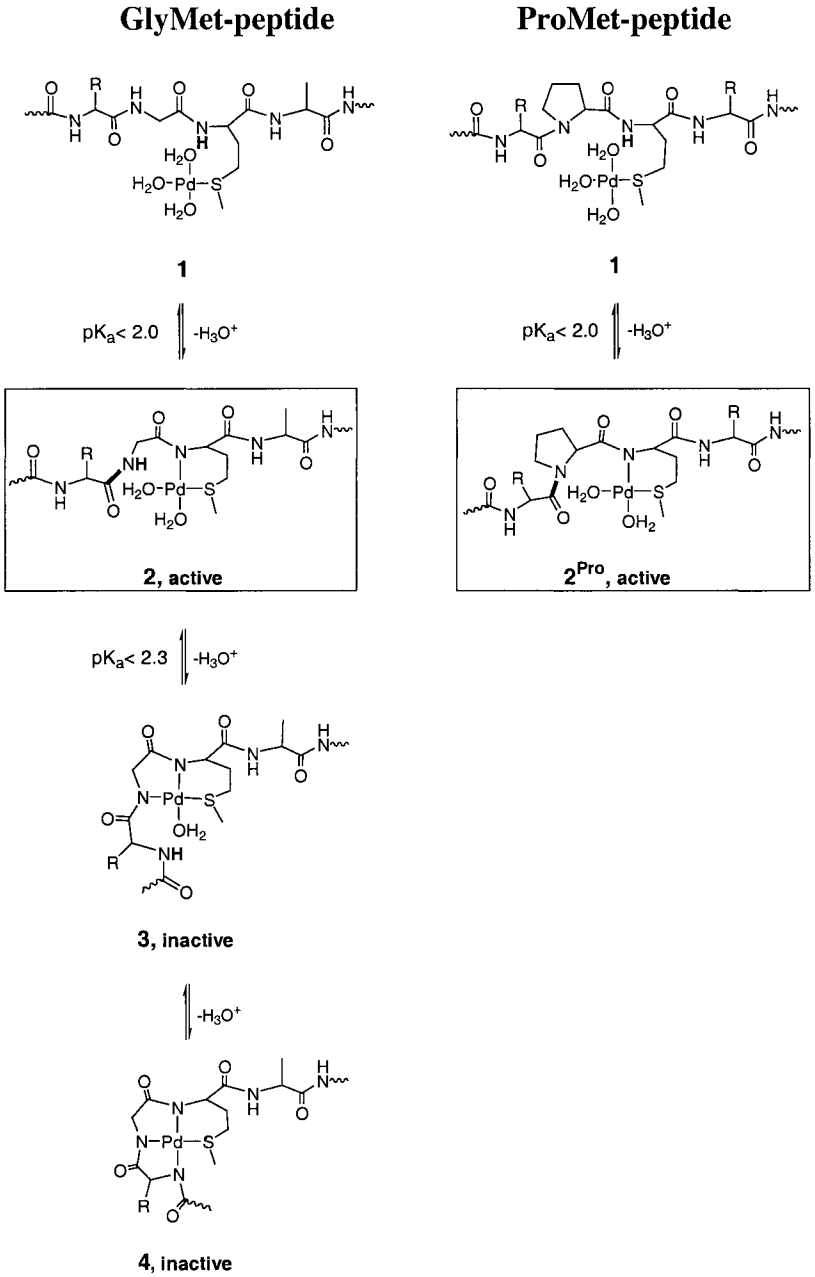
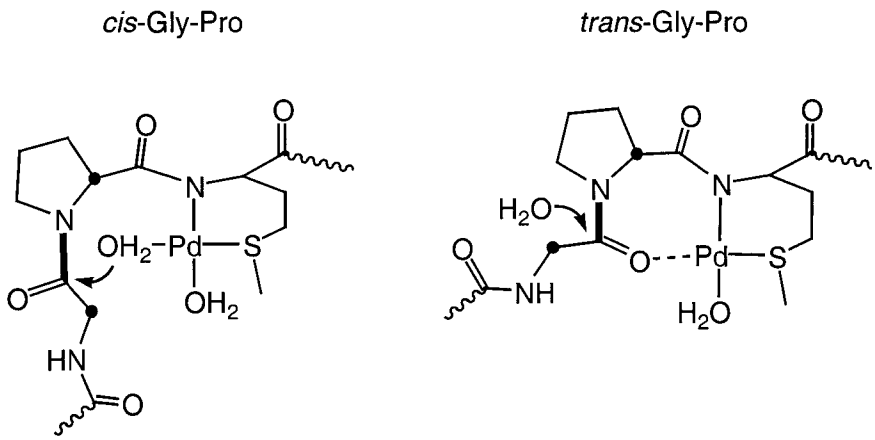


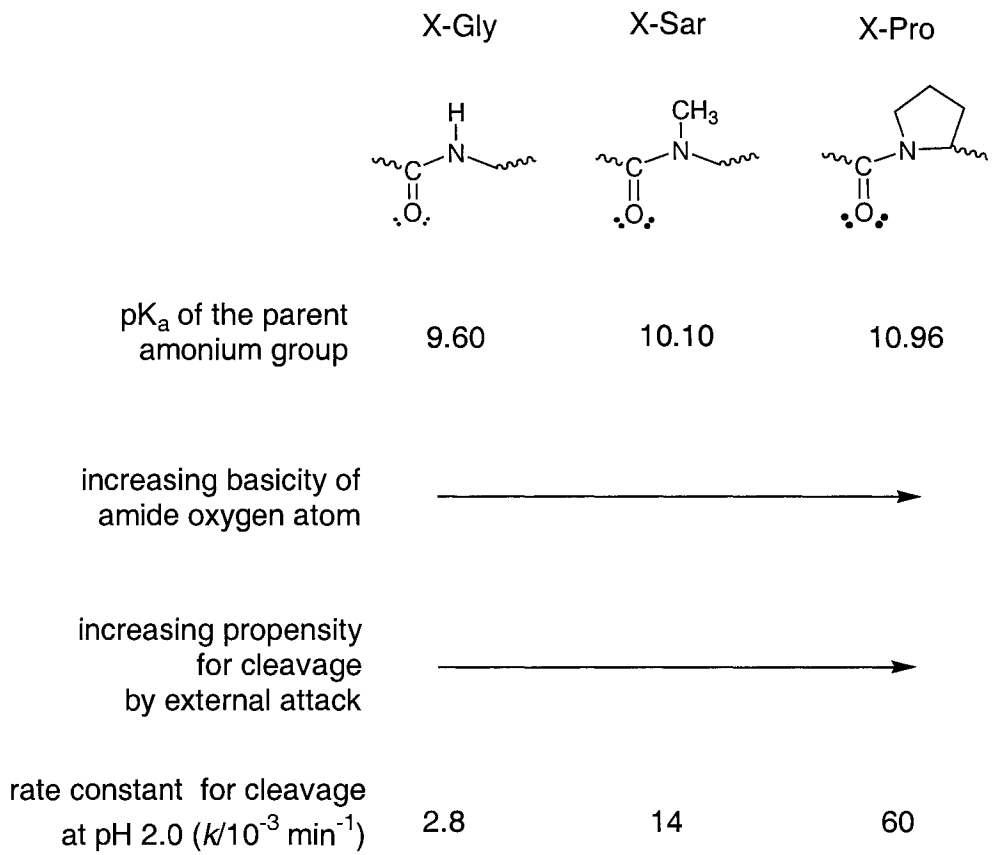
Chart 2. Possible interactions between Pd(II) ion anchored to a side chain of methionine or histidine and a proximate amide group. The anchor is not shown for clarity. (a) Binding to the amidate nitrogen atom requires the deprotonation of the NH group, makes the carbon atom less electrophilic and inhibits the hydrolytic cleavage; (b) Binding to the oxygen atom makes the carbon atom more nucleophilic toward solvent water and thus activates the amide group towards 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.



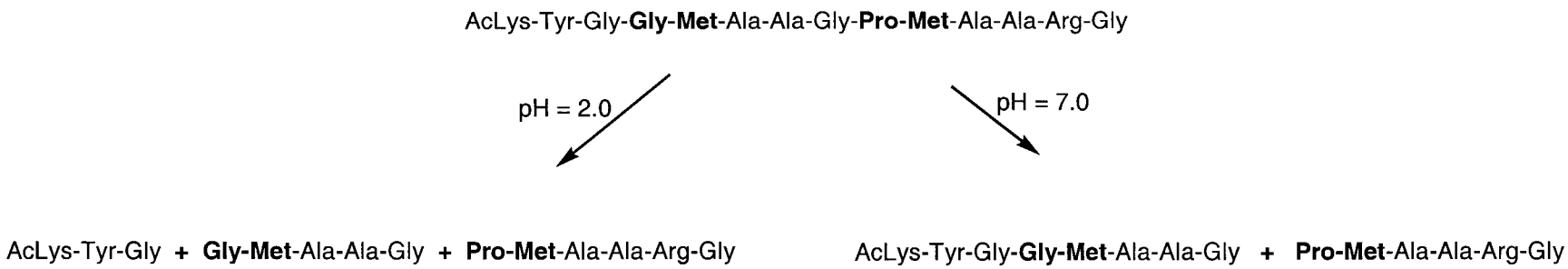
Scheme 1. Binding of Pd(II) ion to the methionine side chain (the anchor) and stepwise coordination of the deprotonated NH groups (amidate anions) in the peptide backbone upstream from the anchor. In GlyMet-peptide, methionine is preceded by an ordinary amino acid (exemplified by glycine), which forms a secondary peptide bond. In ProMet-peptide, methionine is preceded by proline, which uniquely forms a tertiary peptide bond. The displaceable hydrogen atoms and the scissile peptide bond are highlighted. The complexes **2** and $\mathbf{2}^{\mathbf{Pro}}$ 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.



Scheme 2. Orientations of the C=O group with respect to the Pd(II) ion in complex $\mathbf{2}^{\mathbf{Pro}}$ in the cis and trans conformations of the scissile X-Pro peptide bond. (For simplicity, the Gly-Pro segment of the Gly-Pro-Met sequence is shown.) The cis conformation, which is not observed for this segment, 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. The 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



Scheme 4. Two levels of regioselectivity in Pd(II)-promoted cleavage of GlyMet···ProMet-peptide. 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.

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.

pH	rate constant $k/10^{-4}$ min^{-1}		
	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

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.

pH	rate constant $k/10^{-4}$ min^{-1}		
	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)

Supporting Information

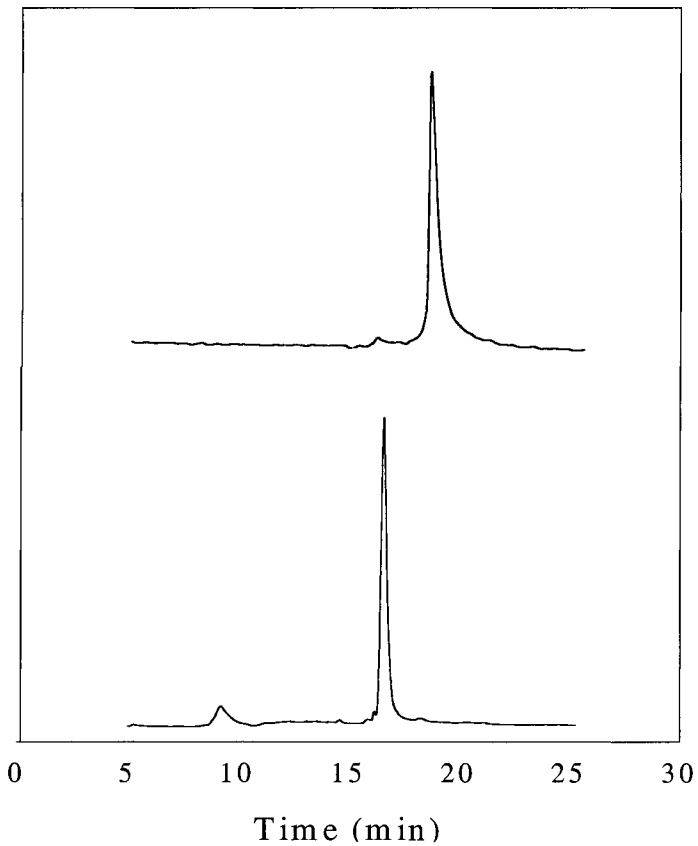


Figure S1. Monitoring the digestion of AcLys-Gly-Gly-Ala-Gly-Pro-Met-Ala Ala-Arg-Gly (ProMet-peptide) by $[Pd(H_2O)_4]^{2+}$ at pH 2.5 and 60 °C. The chromatograms at 215 nm of ProMet-peptide and of the sample taken 1h after mixing the two reagents. Composition of the fractions: 18.8 min, whole peptide; 9.2 fragment AcLys-Gly-Gly-Ala-Gly; and 16.6 min, fragment Pro-Met-Ala-Ala-Arg-Gly bound to Pd(II) reagent. In the runs for the kinetic measurements (see experimental procedures), the respective elution times for the two fragments were 6.1 and 12.5 min.

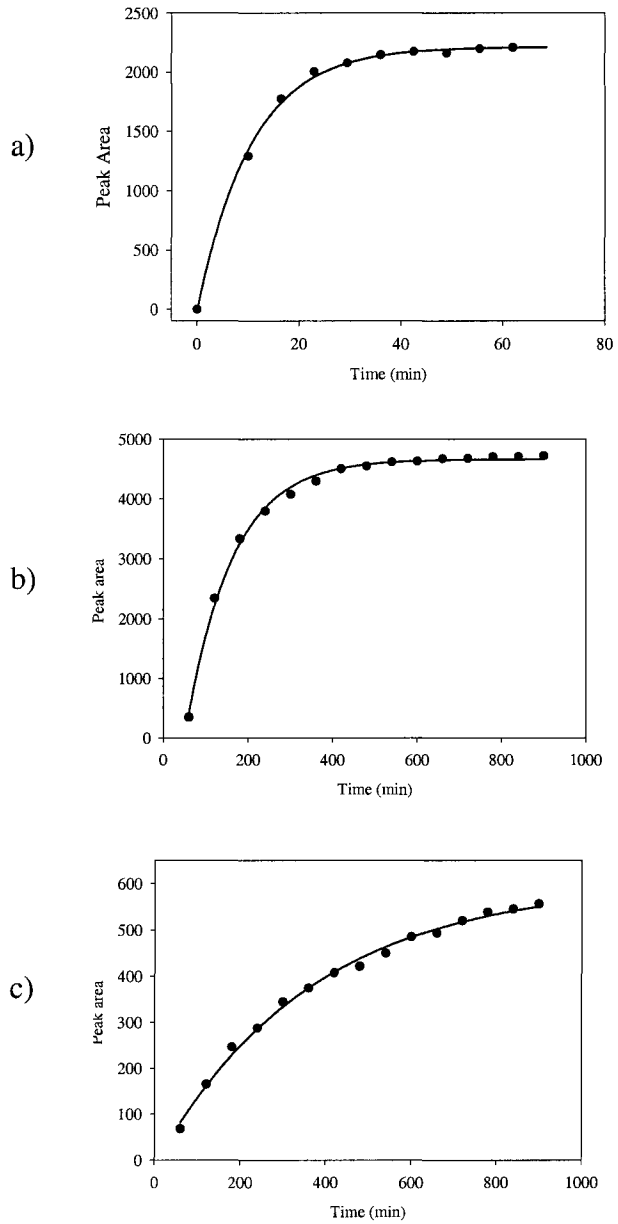
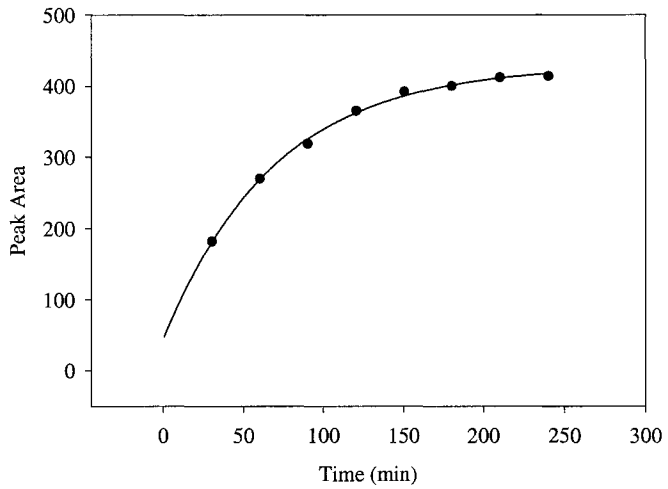


Figure S2. The progress of cleavage by $[Pd(H_2O)_4]^{2+}$ of AcLys-Gly-Gly-Ala-Gly-**Pro-Met**-Ala Ala-Arg-Gly (ProMet-peptide) at a) pH 2.0 and 60 °C; b) pH 4.0 and 60 °C; and c) pH 7.0 and 40 °C followed by HPLC separations of the digests. The data for the peak eluting at 6.1 min are obtained by normalizing the peak area to that of the internal standard methyl phenyl sulphone. The solid lines are fittings to the first-order rate law.

a)



b)

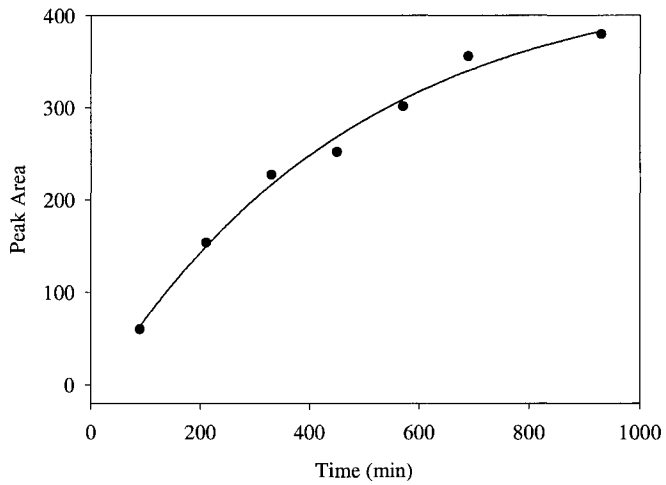
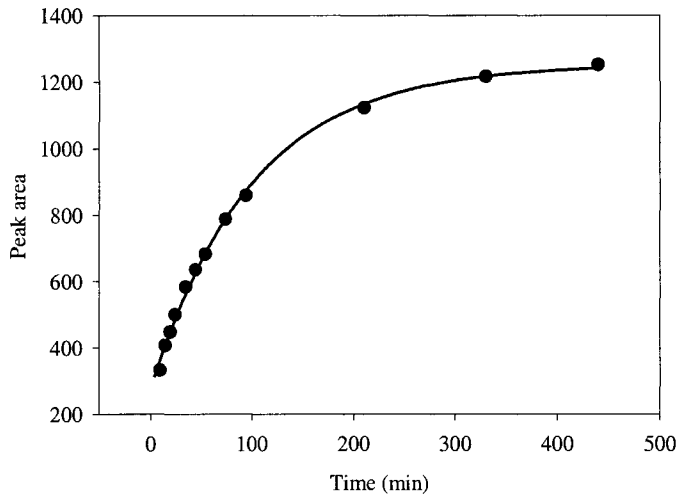


Figure S3. The progress of cleavage by $[Pd(H_2O)_4]^{2+}$ of AcLys-Gly-Gly-Ala-Gly-**Sar-Met**-Ala Ala-Arg-Gly (SarMet-peptide) at a) pH 2.0 and 60 °C; and b) pH 7.0 and 60 °C followed by HPLC separations of the digests. The data for the peak eluting at 6.1 min are obtained by normalizing the peak area to that of the internal standard methyl phenyl sulphone. The solid lines are fittings to the first-order rate law.

a)



b)

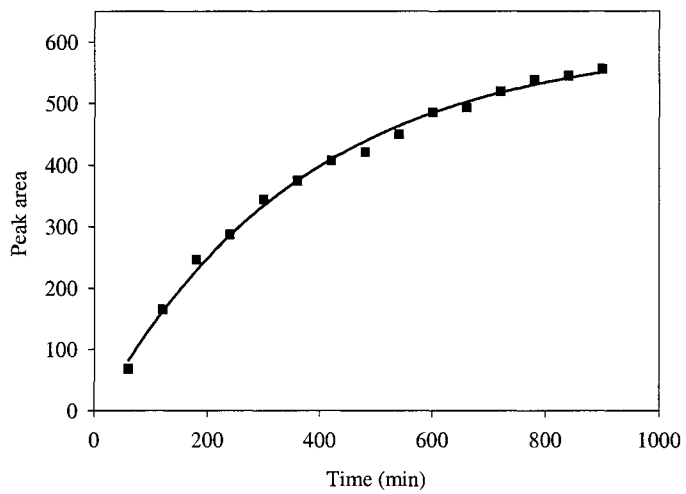


Figure S4. The progress of cleavage by $[Pd(H_2O)_4]^{2+}$ of AcLys-Gly-Gly-Ala-Gly-**Pro-His**-Ala Ala-Arg-Gly (SarMet-peptide) at a) pH 2.0 and 20 °C; and b) pH 7.0 and 40 °C followed by HPLC separations of the digests. The data for the peak eluting at 6.1 min are obtained by normalizing the peak area to that of the internal standard methyl phenyl sulphone. The solid lines are fittings to the first-order rate law.

CHAPTER 5. Transition-Metal Complexes as Enzyme-like Reagents for Protein Cleavage: Complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ as a New Methionine-Specific Protease

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Abstract

Complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ promotes selective hydrolytic cleavage of two proteins, horse cytochrome *c* and bovine β-casein. The cleavage is completed in 24 h under relatively mild conditions, at pH as high as pH 2.5 and temperature as low as 40 °C. The results of HPLC and TSDS PAGE separations, MALDI mass spectrometry, and Edman sequencing showed that cleavage occurred exclusively at the peptide bond involving the C-terminus of each methionine residue – both such residues in cytochrome *c* and all six such residues in β-casein. While having the same selectivity as cyanogen bromide (CNBr), the most common chemical protease, the complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ has several advantages: It is non-volatile, easy to handle, and recyclable; the cleavage by it is residue-selective; the rest of the polypeptide backbone remained intact, and the other side chains remain unmodified; it is applied in approximately equimolar amounts with respect to methionine residues; it leaves the protein fragments pristine; it cleaves even the Met-Pro bond, which is resistant to CNBr

and most proteolytic enzymes; and it works in the presence of the denaturing reagent sodium dodecyl sulfate. Experiments with two synthetic peptide substrates, AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (termed Met-peptide) and AcVal-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (termed HisMet-peptide) revealed structural and mechanistic features of the proteolytic reactions. We explain why two similar complexes of similar metal ions, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ differ in selectivity as proteolytic reagents. The selectivity of cleavage is governed by the selectivity of the *cis*-$[Pt(en)(H_2O)_2]^{2+}$ binding to methionine side chain. The proteolytic activity is governed by the modes of coordination, which control the approach of the anchored Pt(II) ion to the scissile peptide bond. The cleavage occurs with a small but important catalytic turnover of more than 18 after 7 days. The ability of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to cleave proteins at relatively few sites, with explicable selectivity, and with catalytic turnover augurs well for its use in biochemical practice.

Introduction

Hydrolytic cleavage of proteins plays functional and regulatory roles in physiological processes such as control of cell cycle, transcription, signal transduction, antigen processing, and apoptosis.[1] Fragmentation of proteins is an important procedure for the determination of their primary sequence,[2] and also in several new applications. In proteomics, the expressed proteins are identified from their digests.[3] In protein footprinting and studies of folding, the pattern of proteolytic cleavage provides structural information.[4,5] In conversion of engineered proteins to their native form, fusion tags are removed by site-specific cleavage.[6]

In protein semisynthesis, the fragments of natural proteins are ligated into new bioengineered proteins.[7]

Selective proteolysis can be achieved with enzymes and synthetic reagents. Many proteolytic enzymes are known, but only few of them are commonly used. The available proteases usually effect selective and catalytic cleavage under mild conditions, but they are sometimes inapplicable because they cleave at too many sites and produce fragments that are too short. Moreover, enzymes are proteinaceous contaminants of the protein digests that they create. The existing chemical proteolytic reagents are inferior to enzymes because they usually require harsh conditions and high molar excess, and yet often give incomplete selectivity and relatively low yields.[8] Cyanogen bromide, a methionine-selective cleaver, is common in biochemical practice despite its shortcomings. It is volatile and toxic, is usually applied in a 100-fold excess over the methionine residues in the substrate, requires 70% formic acid as the solvent, and causes various side reactions. The protein fragments created by CNBr are no longer native because the methionine residues in them are irreversibly converted to serine lactones. New chemical reagents for protein cleavage, having improved efficiency and selectivity, are desirable for emerging biochemical applications.

Because the peptide bond is extremely unreactive toward hydrolysis, even nonselective cleavage is hard to achieve. Under standard conditions, (room temperature, pH 4-8) the half-life for hydrolysis of a simple peptide is 500-1000 years.[9-11] For controlled and selective cleavage, a formidable task, a chemical reagent must bind to a specific residue and promote cleavage of a peptide bond near the binding site. Relatively mild conditions, equimolar amount or small molar excess over the substrate, and easy removal of the reagent after cleavage are desirable features. Hydrolytic cleavage, which renders the protein

fragments pristine, is preferred over oxidative cleavage, which results in irreversible chemical modifications of the fragments.

Some transition-metal complexes are emerging as new chemical proteases.[12-25] The mechanisms of their action on small substrates have been investigated,[15,26,27] but there are only few reports of regioselective cleavage of proteins. Our recent studies demonstrated the unprecedented selectivity of Pd(II) complexes in cleaving peptides and proteins.[28,29] The cleavage in weakly-acidic aqueous solutions consistently occurred at the second amide bond upstream from histidine and methionine residues, i.e., the X-Y bond in the sequence segments X-Y-His-Z and X-Y-Met-Z, where X, Y, and Z are any non-coordinating residues. We explained this unprecedented selectivity by identifying the hydrolytically-active mode in which the Pd(II) ion binds to the side chains of methionine and histidine residues and to the polypeptide backbone. We also demonstrated that as the pH of the solution is raised from mildly-acidic to neutral, this selective cleavage becomes specific to only X-Pro-His-Z and X-Pro-Met-Z sequences, in which the Y residue is proline.[30]

In this study, we introduce the complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ as a new proteolytic reagent. We show that this complex hydrolytically cleaves proteins in weakly-acidic solutions exclusively at the peptide bond involving the C-terminus of each methionine residue, that is, the Met-Z bond. This simple and readily-available Pt(II) reagent meets the aforementioned requirements of an optimal chemical protease. The cleavage is residue-selective, with no known side reactions. Because methionine has an average abundance in proteins of only 2.2%,[31] the fragments are relatively long and suitable for biochemical applications. An equimolar amount of the reagent with respect to the methionine residues is sufficient for complete cleavage. Each Met-Z bond is cleaved, even the Met-Pro bond, which

is inert to cyanogen bromide. The protein fragments remain pristine because the Pt(II) reagent can be easily removed from them by chelation or precipitation. The reagent is non-volatile, easy to handle, and, in principle, recyclable.

The success in cleaving proteins by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is important for biochemical applications, and also for our understanding of structural and mechanistic requirements for hydrolytic activity of transition-metal complexes. We explain a surprising difference in selectivity between very similar Pd(II) and Pt(II) complexes as cleavage reagents. We investigate the coordination of methionine-containing oligopeptides to *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and the dependence of the cleavage rate on solution pH. The selectivity of cleavage is a consequence of the selectivity of coordination – because only methionine residue can bind to the Pt(II) reagent under the reaction conditions, only the Met-Z bonds are cleaved. The mechanism of cleavage is a consequence of the modes of coordination – methionine-anchored Pt(II) ion can approach the scissile peptide bond and activate it toward hydrolysis. Our results demonstrate that the complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is a new reagent for selective proteolysis, superior to cyanogen bromide.

Experimental Procedures

Chemicals

The complex *cis*-$[Pt(en)Cl_2]$ (in which en is ethylenediamine), sodium dodecylsulfate (SDS), equine cytochrome *c*, and bovine β-casein were obtained from Sigma Chemical Co. Trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid were obtained from Aldrich Chemical Co. Methyl phenyl sulphone was obtained from Lancaster Synthesis Inc. Acetonitrile of HPLC grade was obtained from Fisher Scientific Co. The salts *cis*-

$[Pt(en)(H_2O)_2]^{2+}$ and $[Pt(dien)(H_2O)]^{2+}$ (in which dien is diethylene-triamine) were prepared as perchlorate salts and quantified according to published procedures.[32,33]

The peptides AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (termed Met-peptide) and AcVal-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 and purified by reverse-phase HPLC on a C18 preparative column, as described previously.[28] The purity, examined by analytical HPLC, was higher than 99.5%. For the Met-peptide, the found and calculated molecular masses were, respectively, 1036.57 and 1036.51 D; for the HisMet-peptide, 1515.08 and 1514.78 D.

The methionine thioether group in the HisMet peptide was oxidized to sulphone by H_2O_2 and formic acid,[8] and the resulting $HisMet^{OX}$ peptide was chromatographically purified. The found and the calculated molecular masses, respectively, were 1545.76 and 1546.77 D. Evidently, exactly two oxygen atoms were incorporated in HisMet peptide, without side-reactions.

Spectroscopic and Analytical Methods

Electrophoresis. A Protean II electrophoretic cell was combined with a 3000 Xi power supply, both produced by Bio-Rad, for standard tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (TSDS-PAGE) at 150 V for 1.5 h.[34] The running buffer contained 12.10 g of Tris base, 17.90 g of tricine, and 1.00 g of SDS in 1.00 L. The sample buffer contained 1.0 mL of glycerol, 2.0 mL of 10% SDS (w/v) in water, 1.25 mL 1.00 M Tris-HCl at pH 6.8, 1.0 mg of bromophenol blue, and water up to 10.0 mL. The 16.5% running gels contained 2.0 mL of gel buffer (a solution 3.00 M in Tris-HCl and 0.30% in

SDS, pH=8.45), 2.0 mL of a solution containing 48.0 g of acrylamide and 3.0 g of bis-acrylamide in 100.0 mL, 2.0 mL of water, 5.0 μL of TEMED, and 100.0 μL of 10% (w/v) ammonium persulfate. The stacking gel was made by polymerization of a mixture that contained 100.0 μL of the aforementioned solution of acrylamide and bis-acrylamide, 310.0 μL of gel buffer, 840.0 μL of water, 3.0 μL of TEMED, and 3.0 μL of 10% ammonium persulfate. The gels were stained for 1 h by an aqueous solution containing 40% (v/v) methanol, 10% (v/v) acetic acid, and 1.0% (w/w) Coomassie Blue R-250 dye, and destained with a similar solution without the dye.

A 10.0 μL sample of the cytochrome *c* digest was mixed with 40.0 μL of the sample buffer; 20.0 μL of the mixture was heated for 5 min at 95 °C, allowed to cool, and loaded into the well in the stacking gel. The gels were blotted onto a PVDF membrane by a semi-dry procedure, using Trans-Blot SD system and Power Pac 300 power supply (both by Bio-Rad). The potential was 50.0 V, and the blotting was completed in 4 h. After the membrane was stained, destained, and rinsed with distilled water, the bands were cut and subjected to N-terminal sequencing by Edman degradation, with a 494 Procise Protein Sequencer/140C analyzer from Applied Biosystems, operated by the staff of Protein Facility.

NMR Spectroscopy. 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. The Met-peptide was examined by ^{1}H TOCSY (total correlation spectroscopy) with mixing time of 70 ms. The ambiguous assignment of the residues Gly4 and Gly5 was resolved by ROESY (rotating-frame Overhauser enhancement spectroscopy) with a mixing time of 500 ms. Each two-dimensional data set consisted of 256 × 2048 complex points. The spin-lock field strength during mixing was 6.4 kHz in TOCSY experiments and 2 .5 kHz in

the ROESY experiment. The water signal was suppressed by the WATERGATE [35] method. The pH was measured with a Fisher Accumet instrument and an Aldrich Ag/AgCl reference electrode. The measurements at pH 2.5 and 1.0 were informative, but an accurate study of the effect of pH on the Pt(II)-peptide coordination modes was precluded by the Pt(II)-promoted peptide cleavage, the main subject of this study.

HPLC Separations. The components of the peptide digests were separated by a Hewlett Packard 1100 HPLC system containing an autosampler and a multiwavelength detector set to 215, 280, and 410 nm. Absorption at 215 nm is common to all peptides and proteins; absorption at 280 nm is due to aromatic residues and bound Pt(II) ion; and absorption at 410 nm is diagnostic of heme. In the reverse-phase separations, analytical Supelco Discovery C18 column (sized 250 x 4.6 mm, beads of 5 μm), analytical Vydac C5 column 214TP54 (sized 150 x 4.6 mm, beads of 5 μm), and a preparative Vydac C18 column 218TP101522 (sized 250 x 22 mm, beads of 10 μm) were used. The eluting solvent A was 0.10% (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 then 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 in analytical runs and 10.0 mL/min in preparative runs. In the size-exclusion separations, the Superdex peptide HR 10/30 column, with optimal separation range from 1000 to 7000 D, was used. The solvent was 0.10% (v/v) TFA in H_2O, and the flow rate was 0.50 mL/min.

Mass Spectrometry. The MALDI-TOF experiments were done with a Bruker Proflex™ instrument. The samples containing intact peptide, the reaction mixture of the

peptide and the complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$, and separate fractions isolated by HPLC 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 a 2:1 (v/v) mixture of water and acetonitrile. Each spectrum consisted of 100 scans. For the sake of clarity, molecular masses are reported only for the fragments free of the Pt(en) groups, although the Pt(en)-carrying species were also observed in the MALDI spectra. Solutions of angiotensin II, oxidized chain B of insulin, and cytochrome *c* were used as external standards. The measured molecular mass of a given fragment was compared with the value calculated by PAWS software, obtained from ProteoMetrics, LLC.

Study of Hydrolysis

Aqueous solutions were held in 2.0-mL glass vials. Stock solutions were 5.0 mM in each substrate (cytochrome *c*, β-casein, Met-peptide, or HisMet-peptide). In a typical experiment, involving equimolar amounts of the Pt(II) reagent and the methionine residue in the substrate, 200.0 μL of the substrate solution was mixed with 10.0 μL of a 100 mM stock solution of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, 10 μL of a 20 mM solution of methyl phenyl sulfone (the internal standard), and 700.0 μL of water. The pH was adjusted by $HClO_4$ or NaOH. After the reactions were completed, the pH remained within ±0.1 of the initial value. The mixture was kept at 40±1 or 60±1 °C for 1 day, and 20.0-μL samples were taken periodically. In the control experiments for possible background cleavage, the conditions were the same except that *cis*-$[Pt(en)(H_2O)_2]^{2+}$ was absent. The cleavage was followed by TSDS-PAGE (to resolve large fragments) and by size-exclusion chromatography (to resolve small fragments) for the

protein substrates and by reverse-phase HPLC for the peptide substrates. The separated fragments were lyophilized to dryness, re-dissolved, and analyzed by MALDI-TOF mass spectrometry and Edman N-terminal sequencing.

Because cleavage is very slow at room temperature, the species distribution in the chromatographic runs corresponds to that in the digest sample. In kinetic measurements with Met-peptide, the areas under the chromatographic peaks were normalized to that of methyl phenyl sulphone, to compensate for possible errors in the injection volume and for evaporation. The error of this integration was estimated at 5%. The plots of the peak areas versus time for the cleavage products were fitted to the first-order rate law with SigmaPlot v. 5.0, obtained from SPSS Inc. The fitting to the first-order rate law is correct because the binding of the Pt(II) reagent to the substrate is much faster than the subsequent intramolecular cleavage of the substrate. Each rate constant is the average of two consistent values, obtained by monitoring both fragments of cleavage. The stated errors in the rate constants correspond to two standard deviations, i.e., confidence limit greater than 95.0%.

Study of the Catalytic Turnover

Three reaction mixtures differed from those used for the kinetic experiments only in the amount of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The first mixture contained 10.0 μL of a 10.0 mM solution of the Pt(II) reagent; the second contained 1.0 μL of this solution; and the third contained 1.0 μL of this solution and also 1.80 μL of a 500 mM $Hg(ClO_4)_2$ solution. Each mixture was kept at 60±1 °C and pH 2.0±0.1 for 14 days, and the 40.0-μL samples were periodically analyzed by HPLC. In the control experiments for possible background cleavage by acid or by Hg(II) ions, the conditions were the same except that *cis*-$[Pt(en)(H_2O)_2]^{2+}$ was absent.

Catalytic turnover was calculated as the number of equivalents of the product (fragment Ala-Ala-Arg-Ala) per one equiv of the reagent. To obtain this value, the normalized area under the chromatographic peak of the product was compared to that of this product in the equimolar mixture after the reaction is completed. The error was estimated at 5%.

Results and Discussion

Choice of Conditions. Coordinating anions, such as acetate and chloride, are excluded from the reaction mixtures lest they bind to the Pt(II) ion in the reagent and thus inhibit its binding to the substrate.[36] To ensure unfolding and solubility of the proteins and their fragments, the protein digests in some cases were made 1.0% (w/w) in SDS, a common denaturant. All experiments were done in mildly-acidic solutions, in which cleavage of proteins was consistently effective. For protein substrates, the Pt(II)-promoted cleavage was sufficiently fast at pH 2.5 and 40 or 60 °C, while the background cleavage was nearly undetectable. For peptide substrates, the background cleavage was undetectable after 48 h at $1.0 \leq \text{pH} \leq 5.0$ and 60 °C.

Cleavage of Cytochrome *c* and β-Casein. Figures 1a and 1b show the absence of cleavage in the absence of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and the presence of cleavage in the presence of this Pt(II) reagent. The electrophoretic band corresponding to the intact cytochrome *c* gradually diminishes and vanishes after 24 h at 40 °C, while one narrow band (designated A) and one broad band (designated B), corresponding to lower molecular masses, emerge. Clearly, the cleavage of cytochrome *c* promoted by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is selective, and is completed within 24 h. The products of cleavage (protein fragments) are identified in Table

1. The fragment 1···65 was undetectable by the Edman method because its terminal amino group is acetylated, but it was firmly identified in two ways – by its molecular mass and as a complement to the other two fragments. The small fragments 66···80 and 81···104 were not resolved by electrophoresis but were successfully separated by size-exclusion chromatography (see Figure S1 in Supporting Information).

Table 1 proves the cleavage pattern in Chart 1. The three fragments are products of the cleavage at two peptide bonds, namely Met65-Glu66 and Met80-Ile81. This Pt(II)-promoted cleavage is clearly selective, and it is directed by each of the two methionine residues. This selectivity, observed in our earlier studies with methionine-containing di- and tri-peptides peptides,[36] remained unperturbed by other amino acid residues. *These results demonstrate for the first time that complex cis-$[Pt(en)(H_2O)_2]^{2+}$ works as a proteolytic reagent.*

With its longer chain and six methionine residues, bovine β-casein tests the selectivity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ as a chemical protease. Treatment of this protein with 10 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5 and 60 °C in the absence of SDS was followed by size-exclusion chromatography. The chromatograms of the fresh β-casein solution and of the control solution (in Figures 2a and 2b) show the intact protein eluting at 14.6 min and only minor peaks in the latter solution – evidence for almost negligible background cleavage. Figure 2c shows that the reaction mixture after 24 h lacks the intact protein and contains seven protein fragments, produced by the Pt(II) reagent and eluting between 15.7 and 30.9 min. The results in Table 2 prove the cleavage pattern in Chart 2: All six Met-Z peptide bonds present, and no other peptide bonds, are cleaved. Moreover, in the absence of SDS, the cleavage of β-casein is completed in 24 h, whereas in the presence of 1.0% (w/w) SDS the

cleavage is completed in 10 h (see Figure S2 in Supporting Information).

In general, unfolding of protein substrates by detergents such as SDS promotes faster and complete cleavage. Proteolytic enzymes, however, are mostly or completely inactivated even by 0.1% SDS because they themselves get denatured.[1] Unlike proteolytic enzymes, complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ remains active in the presence of 1.0% (w/w) SDS. *This ability of complex cis-$[Pt(en)(H_2O)_2]^{2+}$ to work as a protease under denaturing condition sets it apart from proteolytic enzymes.*

Advantages of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ over Cyanogen Bromide as a Chemical Protease. The selectivity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is the same as that claimed for the most commonly used chemical protease, cyanogen bromide. Chart 3 shows several advantages of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, notably its ability to cleave all Met-Z bonds selectively and to leave the methionine residue intact. This noninvasiveness, in principle, allows the protein fragment to form a new peptide bond, in a desired semisynthetic protein.[7]

Remarkably, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ promotes the cleavage even of the Met109-Pro110 and Met185-Pro186 peptide bonds in β-casein. The Met-Pro peptide bond is unreactive to CNBr or any other cleavage reagent. Internal X-Pro peptide bonds are resistant even toward enzymatic proteolysis because of their unique conformational requirements, caused by the cyclic structure of proline residue.[37,38]

Selectivity of Cleavage Promoted by *cis*-$[Pt(en)(H_2O)_2]^{2+}$. We explored the mechanism of proteolytic action of the Pt(II) reagent with two synthetic peptides, namely AcAla-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala (termed Met-peptide) and AcVal-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala (termed HisMet-peptide). These realistic substrates are more convenient than natural proteins for NMR spectroscopic

characterization of Pt(II)-substrate binding, HPLC separations and mass-spectrometric identification of the cleavage fragments, and kinetic elucidation of the reaction mechanism.

The equimolar mixture of Met-peptide and *cis*-$[Pt(en)(H_2O)_2]^{2+}$ already after 1h at pH 2.5 and room temperature lacked the free peptide, eluting at 20.7 min, and the free *cis*-$[Pt(en)(H_2O)_2]^{2+}$ reagent, eluting at 3.8 min, but it contained a new component, eluting at 17.4 min. The MALDI mass spectrum of this new HPLC fraction showed a single peak, corresponding to the peptide + Pt(en) group (observed mass 1318.56 D, calculated 1318.62 D). Evidently, the Pt(en) group is bound to the methionine side-chain.

The reaction mixture was kept for 12 h at 60 °C and analyzed by HPLC. The initially-formed Pt(en)-peptide complex, eluting at 17.4 min, was absent, whereas two new components, eluting at 9.8 and 16.2 min, were present. No other species were found in the reaction mixture, not even after 24 h. The results in Table 3 prove specific cleavage of the Met-Z bond, as shown below.

Ac-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala

Chromatograms of the mixtures containing *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and HisMet-peptide in the ratios 1:1 and 2:1 and kept for 12 h at 60 °C were identical showing only two peaks, which eluted at 9.8 and 17.7 min. Again, the results in Table 4 prove specific cleavage of the Met-Z bond, as shown bellow.

Ac-Val-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala

The HisMet-peptide contains two potential anchors for the Pt(II) reagent – His5 and Met11. At pH 2.5 however, only Met11 can coordinate to the Pt(II) atom, because

protonation of the imidazole group inhibits its coordination. This coordination would require prior deprotonation of imidazolium group, which occurs only at pH > 3.5.[39,40] Because only methionine anchors the Pt(II) ion, only methionine can direct the cleavage. This claim is backed by an additional control experiment: when the thioether group of Met11 in HisMet-peptide was oxidized to sulfone, a noncoordinating group, the resulting $HisMet^{OX}$-peptide was not cleaved by the *cis*-$[Pt(en)(H_2O)_2]^{2+}$ (data not shown). *Evidently, selective cleavage is a result of selective binding of Pt(II) ion to the cleavage-directing side chain of methionine.*

Studies of peptide coordination to Pt(II) ion and other transition-metal ions showed that the coordination mode is unaffected by the noncoordinating side chains surrounding the methionine anchor.[40-51] These neighbors may provide additional interactions and thus affect the overall stability of the complex, but not its structure. Therefore, the selectivity of the backbone cleavage should be independent of the side chains surrounding the methionine anchors. Based on this conclusion, and on our consistent results with two proteins and two synthetic peptides we can conclude that all peptide or protein sequences containing an "isolated" methionine residue are expected to be cleaved by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at the Met-Z bond, i.e., at the carboxylic side of the anchoring methionine residue. Although free cysteine residues also can bind the Pt(II) ion, they usually exist in proteins as disulfides, which are poor ligands for this ion. If present, the binding of the Pt(II) reagent to free cysteines can be easily blocked by their prior covalent modification.[8]

Hydrolytic Activity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. Two conditions must be fulfilled for the Met-Z peptide group to be activated for cleavage by the Pt(II) complex. First, the anchored Pt(II) complex must approach this group. Second, the Pt(II) ion after anchoring must contain at least one aqua ligand. This loosely-bound ligand can be either displaced by

the carbonyl oxygen atom (in external attack) or delivered to the carbonyl carbon atom (in internal delivery).[28,45,52] If either condition is absent, cleavage does not occur.[29] We recently gave multiple, albeit indirect, evidence for the external attack by solvent water on the carbonyl carbon atom whose electrophilicity is enhanced by binding of the metal ion, a Lewis acid, to the carbonyl oxygen atom.[30] Regardless of the mechanism of the actual hydrolytic step, the selectivity of cleavage depends on the stereochemistry of coordination. In this study, we focus on structural requirements for the cleavage. The results are valid whether the cleavage step involves external attack or internal delivery.

We followed the kinetics of Met-peptide cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ in the pH interval from 1.2 to 3.1. The appearance in time of both fragments always obeyed the first-order rate law, as exemplified in Figure S3 in the Supporting Information. Because the binding of Pt(II) ion to the sulfur atom of methionine occurs within minutes, and because the subsequent reactions are intramolecular and take hours, fitting of the kinetic results to the first-order rate law is justified. Figure 3 shows that the rate constant for cleavage increases with increasing acidity. To explain how the methionine-anchored Pt(II) reagent promotes the hydrolysis of the Met-Z peptide bond, we relate these kinetic data to the NMR spectroscopic determination of the coordination modes.

Room-temperature ^{1}H NMR TOCSY spectra were recorded at pH 2.5 before, and 1 h after, the addition of 1 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to Met-peptide. Signals of the methionine residue shifted most. The disappearance of the SCH_3 singlet at 2.12 ppm and appearance of a broad signal at 2.45 ppm (see Figure S4 in the Supporting Information) prove binding of the Pt(II) complex to the methionine side-chain. The methionine NH signal at 8.05 ppm, and also its cross-peaks to α-CH, β-CH, and γ-CH resonances, marked in Figure

4a, almost completely disappeared upon addition of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, as shown in Figure 4b, because the amide nitrogen atom became mostly deprotonated. This is evidence for its almost complete coordination to the Pt(II) ion. Clearly, two species are present. In the major one, the nitrogen atom of Met6 is deprotonated and coordinated to Pt(II) ion, whereas in the minor one this atom remains protonated and is not coordinated. The α-CH-to-amide-NH cross-peaks between Gly4 and Gly5 (marked in Figure 4) remained, indicating that the amide group connecting these two residues remained protonated and not bound to Pt(II) ion.

These two species are designated ***1*** and ***2*** in Scheme 1. They are consistent with the known complexes of Pt(II) ion with short peptides.[40,53-55] In the complex ***1***, the minor species at the pH 2.5, the anchoring methionine side chain displaced an aqua ligand from *cis*-$[Pt(en)(H_2O)_2]^{2+}$. In complex ***2***, the predominant species at pH 2.5, the peptide is additionally coordinated to the Pt(II) ion via the first peptide nitrogen atom upstream of the anchor. The coordinated sulfur atom is a chiral center in the six-membered chelate ring of ***2***. Existence of two diastereomers caused the splitting of the α-CH-to-amide-NH resonance of the nearby Gly5.[5,6]

The complexes ***1*** and ***2*** are exchanging at pH 2.5, as evident from broadened α-CH resonances and the partial loss of amide-to-CH cross-peaks for Met6 in the TOCSY spectrum. The extent of this deprotonation increases with increasing pH. Further coordination of the upstream peptide nitrogen of Gly5 is inhibited by both the acidic solvent and the tightly-bound ethylenediamine ligand.

At pH 2.5 the complex ***2*** is a major species, but the increasing acidity suppresses its formation from complex ***1*** because the H^+ ion competes with Pt(II) ion for the amidate nitrogen of Met6. Indeed, the TOCSY spectrum at pH 1.0 (see Figure 4c) showed the Met6

NH signal at 8.05 ppm with its cross-peaks to aliphatic CH resonances, clear evidence for the complex ***1*** as the predominant species. Minor shift of all NH resonances at this pH is caused by the change in the acidity.

The accurate determination of the pK_a for the conversion of ***1*** to ***2*** was obscured by the Pt(II)-promoted cleavage, the reaction of interest in this study. Because Pt(II) ion is relatively inert, it requires long equilibration time (tens of minutes). During this time, the Pt(II)-promoted cleavage proceeds even at room temperature, preventing the precise measurements. Based on the relative intensities of the relevant peaks at pH 1.0 and 2.5, we can only estimate that the process in Scheme 1 has pK_a value of ca. 1.8±0.4.

Coordination of the amidate nitrogen of Met6 in complex ***2*** keeps the Pt(II) ion away from the Met6-Ala7 peptide bond. Both the NMR and the MALDI mass spectrometric results showed that the ethylenediamine ligand remained bound to Pt(II) ion. Therefore, the Pt(II) ion in the complex ***2*** lacks an aqua ligand necessary for the hydrolytic activity. The complex ***2*** is hydrolytically-inactive because it violates both conditions for activity stated above. The anchored Pt(II) ion in the complex ***1***, however, can interact with the Met6-Ala7 peptide bond and promote its cleavage. *The complex **1** is the hydrolytically-active species because the anchored Pt(II) complex can approach the scissile amide bond and because the Pt(II) ion contains an aqua ligand.* Because the acidic solution shifts the equilibrium in Scheme 1 toward the complex ***1***, the rate constant for the cleavage increases as pH decreases, as Figure 3 shows. We refrain from fitting the results in Figure 3 because we have an inexact value of pK_a, but our estimated value of 1.8±0.4 agrees with the trends in Figure 3. The acidic solution is not a cleavage agent by itself, but is required to prevent the formation of the hydrolytically-inactive complex ***2***.

Both aqua ligands in *cis*-$[Pt(en)(H_2O)_2]^{2+}$ are required for its hydrolytic activity. One aqua ligand becomes displaced by anchoring to the substrate, to form the hydrolytically-active complex ***1***. The remaining aqua ligand is required because, in complex ***1***, it can be either displaced by the carbonyl oxygen atom (in the external-attack mechanism) or delivered to the carbonyl carbon atom of the scissile peptide bond (in the internal-delivery mechanism). Indeed, cleavage was absent even after 24 h at 60 °C and pH 2.0 in the equimolar mixture of HisMet-peptide and $[Pt(dien)(H_2O)]^{2+}$, which contains a tridentate ligand. This solution contains a single species, eluting at 17.3 min and showing in the MALDI mass spectrum a single peak for the substrate-reagent complex. Clearly, this monoaqua complex binds the substrate. After having lost the only aqua ligand in this binding, the complex lacks the required aqua ligand and therefore cannot promote peptide cleavage.

Complexes *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ as Artificial Peptidases. Because Pt(II) and Pd(II) ions are alike in the electron configuration and other properties, their complexes tend to react similarly. Indeed, both title complexes promote selective cleavage of peptides and proteins. Their selectivities, however, are surprisingly different, as Scheme 2 shows. While *cis*-$[Pt(en)(H_2O)_2]^{2+}$ cuts the first peptide bond downstream from methionine anchor (the Met-Z bond), the analogous Pd(II) complex, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, cuts the second peptide bond upstream from methionine and histidine anchors (the X-Y bond) in the X-Y-Met-Z and X-Y-His-Z sequences.[28] At acidic pH, the soft Lewis acid Pt(II) binds only the soft Lewis base in the methionine side chain,[39,40] whereas the "borderline" acid Pd(II)binds both the soft methionine side chain and the histidine side chain.[28] Once anchored, the two metal ions form different hydrolytically-active complexes in the mildly-acidic solutions. Because Pt(II) is much more inert than Pd(II) to ligand substitution,[57]

ethylenediamine ligand persists on the former ion under the reaction conditions,[56] but is displaced from the latter,[29] with the help of the H^+ ions in solution. The Pt(II) reagent remains as a unidentate complex. The Pd(II) reagent, free of ethylenediamine, binds to the amidate nitrogen atom upstream from the anchor ($pK_a < 2.0$[40-46]) and forms a bidentate complex similar to ***2***, but with available aqua ligands.[28] Both of these complexes are hydrolytically active. Consequently, the anchored Pt(II) ion remains proximal to the peptide bond downstream from the anchor, and can activate it toward cleavage. The anchored Pd(II) ion, however, is kept away from this bond but close to the second peptide bond upstream from the anchor, which becomes activated.

Catalytic Turnover. Useful distinction between mere promoter (of stoichiometric reactions) and catalyst (of catalytic reactions) is becoming blurred in the literature, as some synthetic reagents are dubbed catalysts or even artificial enzymes although they act without a catalytic turnover. We explored the catalytic activity of the Pt(II) reagent in the excess of the substrate, and also in the excess of the substrate and Hg(II) ion, a labile and soft metal ion that binds methionine side chain, but does not promote peptide cleavage.

As Table 5 shows, one equivalent of the Pt(II) reagent cleaves multiple equivalents of the substrate, evidence that the reaction is catalytic. The catalytic cycle is shown in Scheme 3. First, the substrate displaces one aqua ligand. The hydrolytically active species ***1*** forms an equilibrium with the inactive species ***2*** and ***3***,[56] and undergoes hydrolytic cleavage of the highlighted Met-Z bond. The N-terminal fragment departs, whereas the methionine-containing fragment remains bound to the catalyst. Its departure is assisted by water and by the affinity of the soft Hg(II) ion (when it is present) for the thioether group. The catalyst is recovered and recycled.

The catalytic turnover of 18.9 after 7 days is small but significant. The formation of complexes ***2*** (favored by increasing pH) and the bis monodentate ***3*** (favored by the excess of the substrate)[56] impedes catalysis. The competition between added Hg(II) ion and inert Pt(II) ion for both the substrate and the product helps the displacement of Pt(II) reagent from the product, thus increasing the turnover. Improvement of turnover remains a goal for future studies.

Conclusions

The simple complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ acts with useful regioselectivity in promoting hydrolytic cleavage of proteins. In weakly acidic solution, this reagent cleaves the Met-Z bonds and no others. Even the Met-Pro bond, which is usually resistant to proteolytic enzymes, is cleaved by this new reagent. It has several advantages over cyanogen bromide: It is non-volatile and easy to handle; it is recyclable; the cleavage by it is residue-selective, with no side reactions; it leaves the protein fragments pristine and capable of forming new peptide bonds; and it can be applied in approximately equimolar amounts with respect to the methionine residues.

The cleavage selectivity is determined by the selectivity of the Pt(II) reagent, a soft acid, for methionine side chain, a soft base, under the reaction conditions. The proteolytic activity is governed by the modes of coordination, which control the approach of the $Pt(H_2O)_2^{2+}$ group to the first peptide bond downstream and its activation toward hydrolysis. The ability of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to cleave proteins at relatively few sites, with explicable selectivity, and with good yields augurs well for its use in biochemical practice.

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Graphics

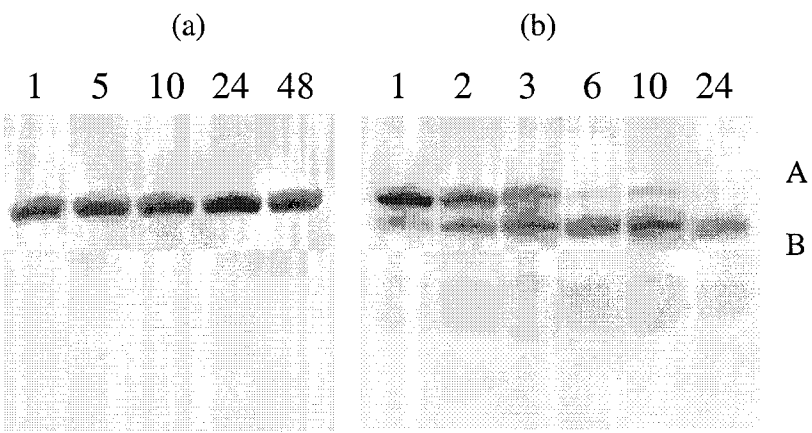


Figure 1. Tricine-SDS PAGE electrophoretogram of equine cytochrome *c* at pH 2.5 and 40 °C, kept for the number of hours shown, in the solution that contained 1.0% (w/w) SDS (a) without *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and (b) with 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The results of MALDI-MS and Edman sequencing in Table 1 showed that the band A contains the fragment 1···65, and the band B contains fragments 81···104 and 66···80.

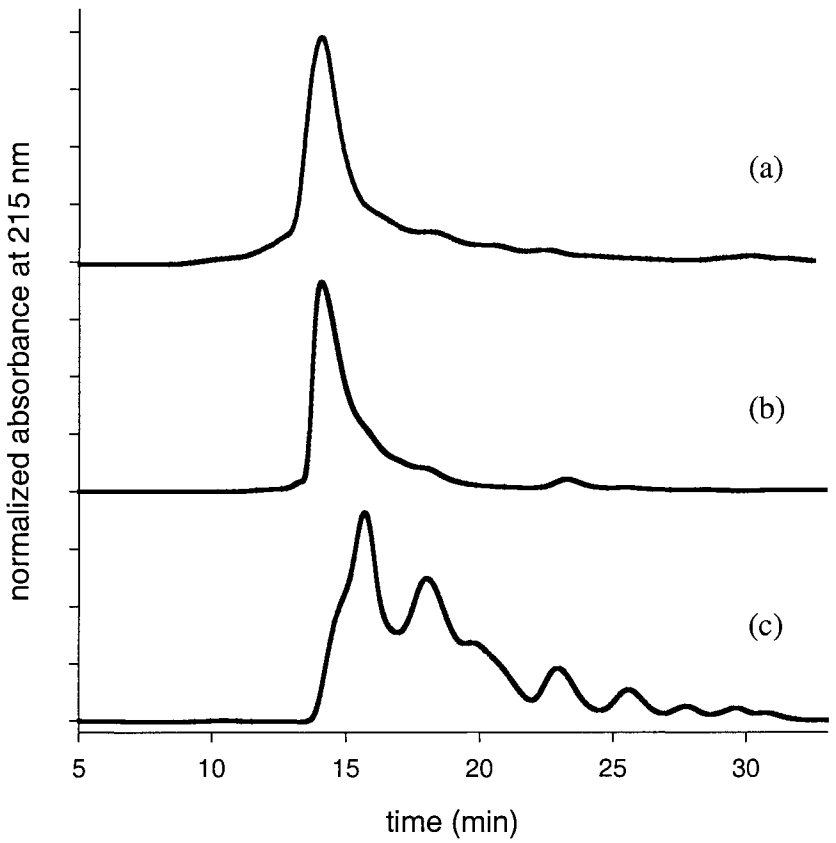


Figure 2. Size-exclusion chromatograms of β-casein: a) fresh solution; b) after 24 at pH 2.5 and 60 °C; and c) after 24 h at pH 2.5 and 60 °C in the presence of 10 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The identity of the fractions is shown in Table 2.

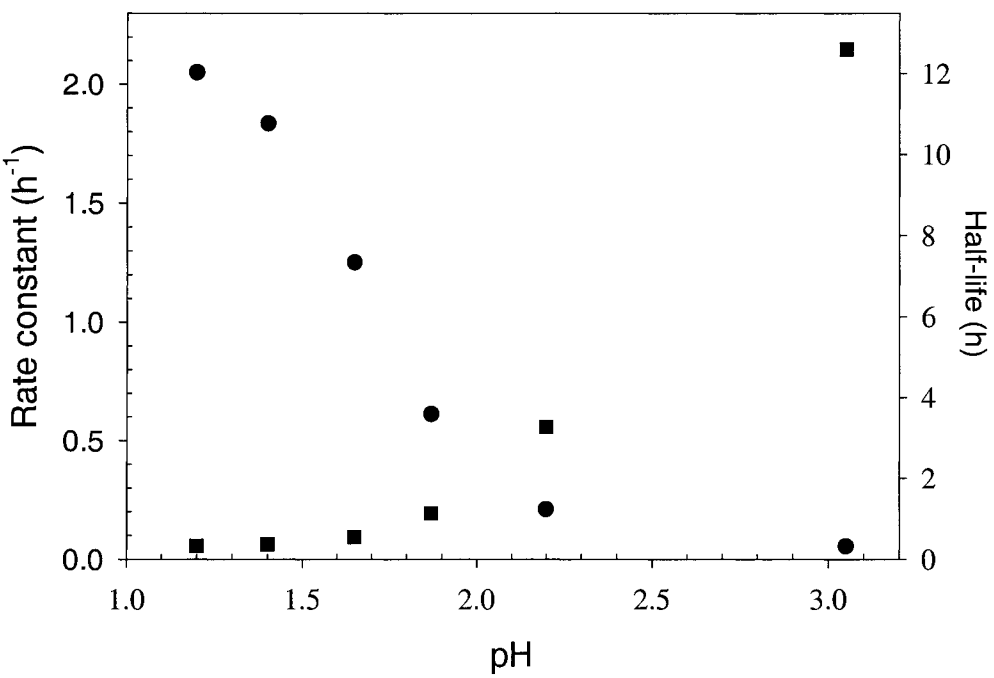


Figure 3. The dependence on pH of the first-order rate constant (circles) and half life (squares) for the cleavage of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) by *cis*-$[Pt(en)(H_2O)_2]^{2+}$.

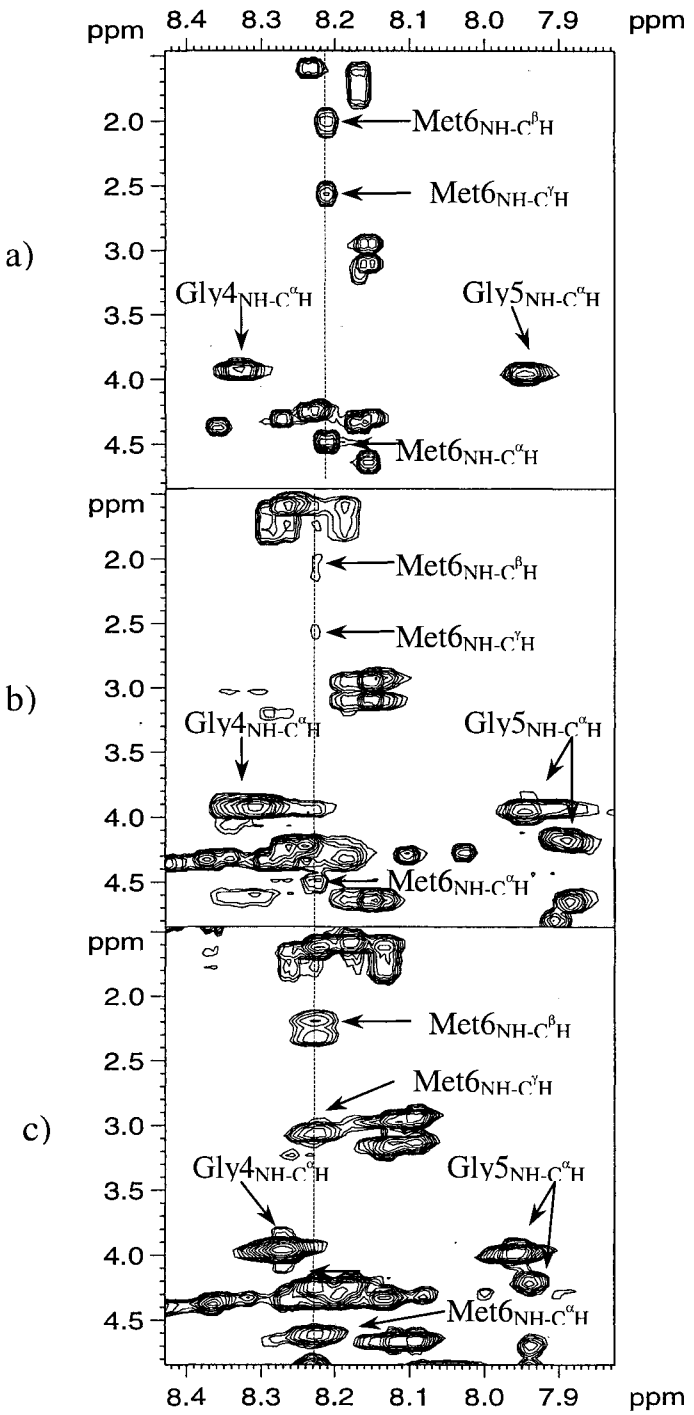


Figure 4. The NH-to-aliphatic region of the two-dimensional TOCSY ^{1}H NMR spectrum of the aqueous solution of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (designated Met-peptide) at room temperature (a) before addition of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5; (b) after addition of an equimolar amount of *cis*-$[Pt(en)(H_2O)_2]^{2+}$and 1 h at room temperature and pH 2.5; and c) after addition of an equimolar amount of *cis*-$[Pt(en)(H_2O)_2]^{2+}$and 1 h at room temperature and pH 1.0.

Table 1. Cleavage of horse cytochrome *c* by 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5. The fragments are separated by gel electrophoresis and by size-exclusion HPLC and identified by MALDI mass spectrometry and Edman N-terminal sequencing.

electrophoretic band	N-terminal sequence	elution time (min)	molecular mass (D)		fragment
			observed	calculated	
A	not determinable	18.5	7815.3	7802.6	1···65
B	EYLEE	23.8	1812.1	1811.1	66···80
B	IFAGI	28.2	2782.3	2780.3	81···104

Table 2. Cleavage of bovine β-casein by 10 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5. The fragments are separated by size-exclusion HPLC and identified by MALDI mass spectrometry and Edman N-terminal sequencing.

elution time (min)	molecular mass (D)		N-terminal sequence	fragment
	observed	calculated		
15.7	10951.86	10950.75	RELEE	1···93
18.0	4069.70	4068.34	PFPKY	110···144
22.9	3238.81	3238.83	FPPQS	157···185
25.5	2665.2	2665.82	PIQAF	186···209
27.8	1382.62	1382.89	HQPHQ	145···156
29.8	949.16	949.61	GVSKV	94···102
30.9	841.02	841.13	APKHK	103···109

Table 3. Results of HPLC separation and MALDI mass spectroscopic identification of fragments of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) resulting from the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$.

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
9.8	387.95	388.22	7···10
16.2	667.30	667.27	1···6

Table 4. Results of HPLC separation and MALDI mass spectroscopic identification of 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 *cis*-$[Pt(en)(H_2O)_2]^{2+}$.

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
9.8	387.95	388.22	12···15
17.7	1147.30	1146.33	1···11

Table 5. Number of equivalents of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) cleaved by one equivalent of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.0 and 60 °C, when the mole excess of the substrate over the reagent was 10-fold, 100-fold, and also 100-fold in the substrate and 90-fold in $Hg(ClO_4)_2$.

time (days)	number of equivalents cleaved		
	10 equiv	100 equiv	100 equiv + 90 equiv Hg(II)
0.5	2.1	2.7	3.4
1.0	2.6	3.9	5.8
3.0	3.4	8.4	12.1
7.0	6.9	14.9	18.9

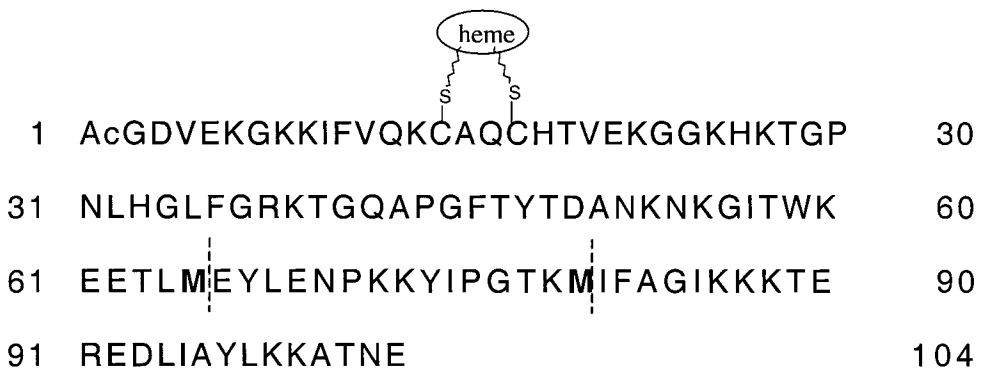
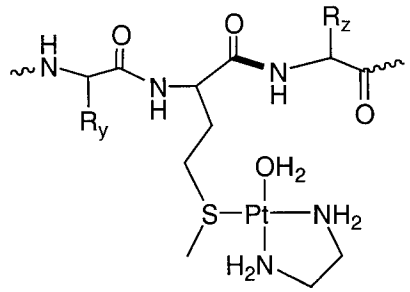


Chart 1. Amino acid sequence of horse cytochrome *c* showing the sites of the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, at the C-terminal side of methionine residues, i.e., at the first amide bond "downstream" from each methionine residue.

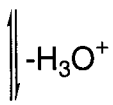
Chart 2. Amino acid sequence of bovine β-casein showing the sites of the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, at the C-terminal side of methionine residues, i.e., at the first amide bond "downstream" from each methionine residue.

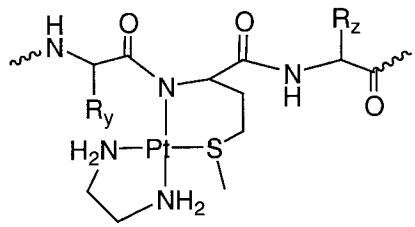
	CNBr	*cis*-$[Pt(en)(H_2O)_2]^{2+}$
selectivity of cleavage	Met-X	Met-X
typical reaction time	ca. 24 h	ca. 24 h
typical reaction conditions	70% CF_3COOH or 100% CH_3COOH, room temperature	pH ≈ 2 40 °C
reagent to Met mole ratio	up to 100:1	ca. 1:1
fate of Met	converted to Ser lactone	intact
reaction completed?	not when X is Ser, Thr, or Cys	yes
Met-Pro bond cleaved?	no	yes
side reactions	oxidation of Trp, Tyr, and Cys	none known

Chart 3. Comparison between cyanogen bromide and *cis*-$[Pt(en)(H_2O)_2]^{2+}$ as reagents for cleavage of proteins after methionine residues.



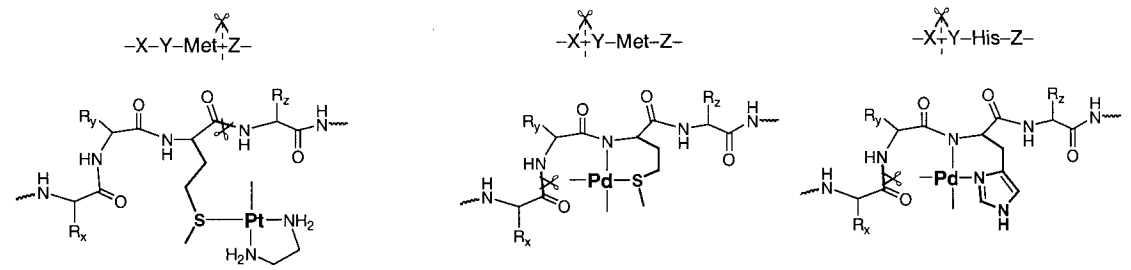
1



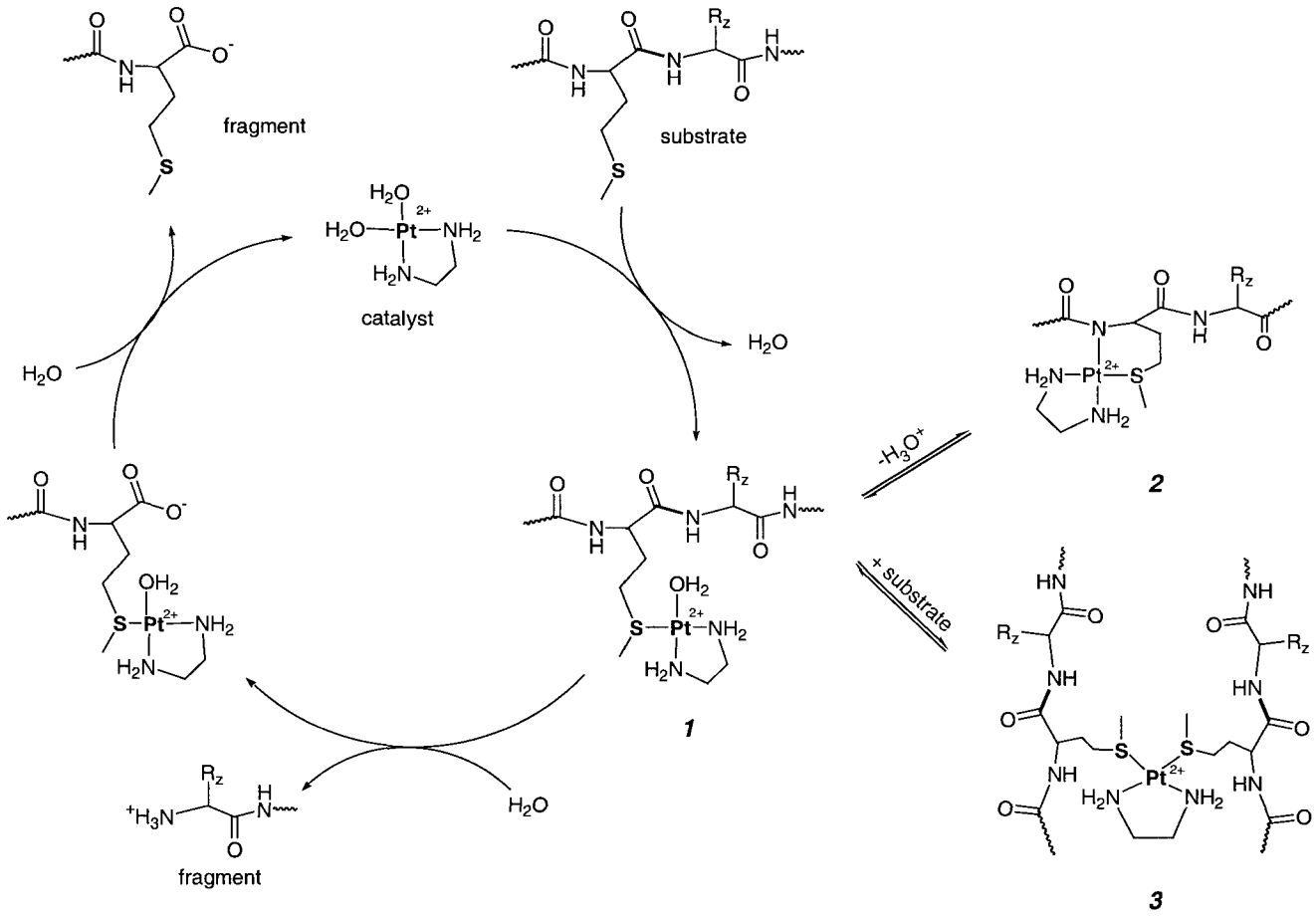


2

Scheme 1. Binding of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to the methionine side chain (the anchor), followed by the deprotonation of the amide group of this methionine residue and coordination of the resulting amidate anion. The scissile peptide bond is highlighted. Only the Y-Met-Z segment of the sequence is shown. Complexes ***1*** and ***2*** exist regardless of the identity of the Y and Z residues. The pK_a for the interconversion lies between pH 1.5 and 2.5.



Scheme 2. Different proteolytic selectivity of Pt(II) and Pd(II) complexes. These two metal ions form different hydrolytically-active complexes with the substrate, and promote hydrolytic cleavage of different peptide bonds. For explanation, see text.



Scheme 3. The catalytic turnover in methionine-directed cleavage of proteins and peptides promoted by *cis*-$[Pt(en)(H_2O)_2]^{2+}$. Only the -Met-Z- sequence is shown because the highlighted Met-Z bond becomes cleaved.

Supporting Information

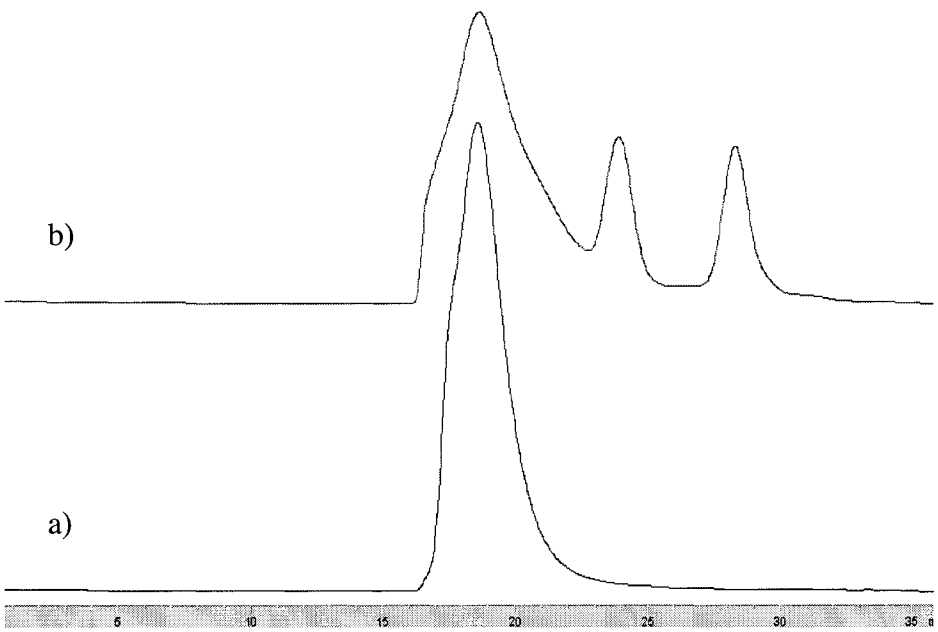


Figure S1. Chromatogram of a) fresh cytochrome *c,* and b) the mixture of cytochrome c and 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ kept for 24 h at pH 2.5 and 60 °C. The results of MALDI-MS and Edman sequencing in Table 1 showed the fractions eluting at 18.5, 23.7, and 28.2 min to contain the fragments 1···65, 81···104, and 66···80, respectively.

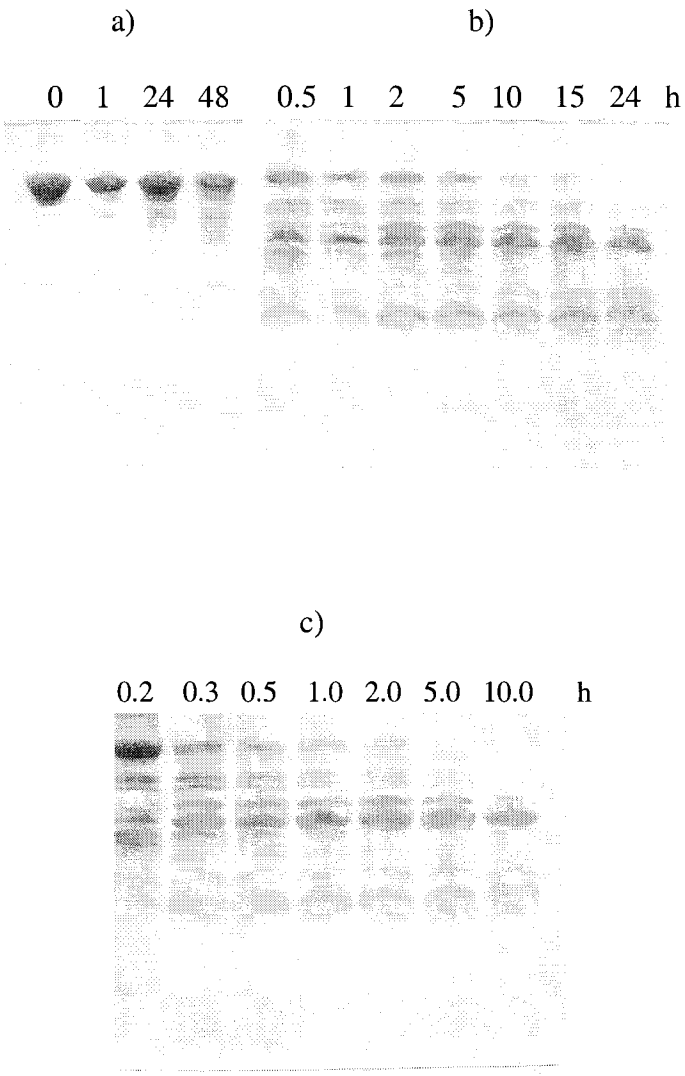


Figure S2. Tricine-SDS PAGE electrophoretogram of bovine β-casein at pH 2.5, kept for the number of hours shown, in the solution that contained 1.0% (w/w) SDS (a) without *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at 40 °C, (b) with 6 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at 40 °C, and c) at 60 °C

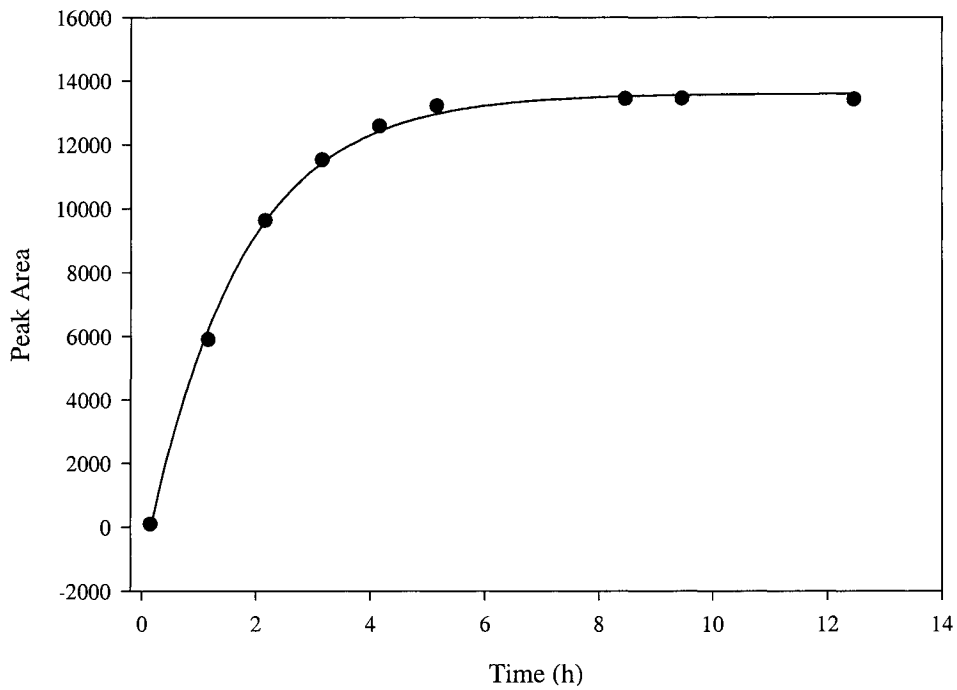


Figure S3. A typical plot showing the progress of cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) at pH 1.8 and 60 °C followed by HPLC separations of the digests. The solid line is the fitting to the first-order rate law.

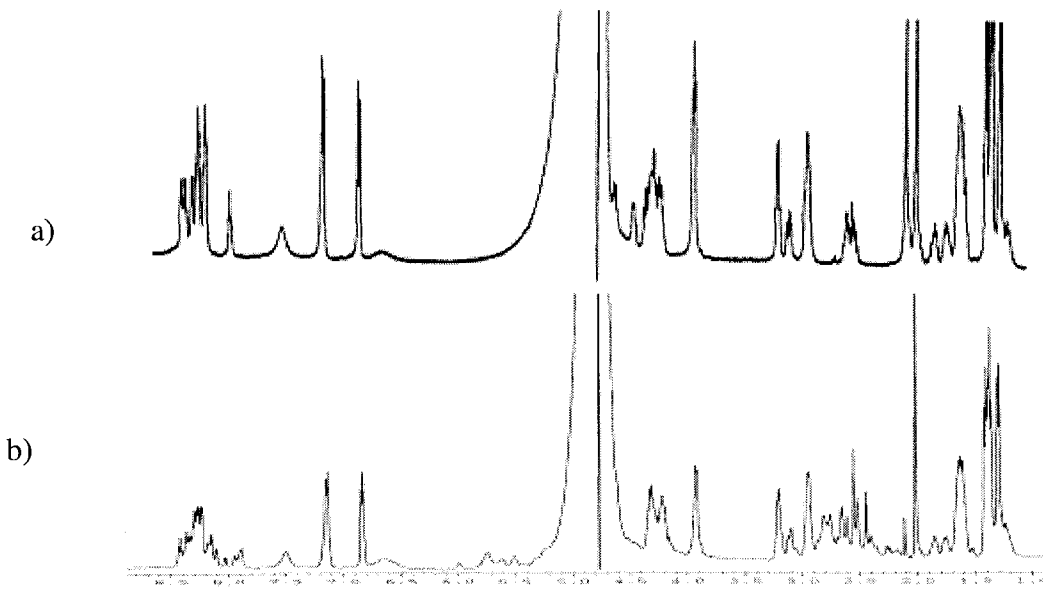


Figure S4. One-dimensional ^{1}H NMR spectrum of the aqueous solution of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) at room temperature and pH 2.5 (a) before addition of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and b) after addition of an equimolar amount of by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5.

CHAPTER 6. Complexes *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$ as Practical Reagents for Selective Cleavage of Peptides and Proteins

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to be submitted to *Inorganic Chemistry*

Introduction

Hydrolytic cleavage of proteins is an important and widely-used biochemical procedure. Besides its standard use for protein sequencing, proteolytic cleavage is necessary also for various newly-developed bioanalytical and bioengineering applications. These new methods require digestion of proteins into small fragments, residue-specific cleavage into larger fragments, and even highly-selective cleavage at a single site in the protein sequence. For example, protein footprinting and studies of folding employ limited and relatively nonselective proteolysis of solvent-exposed protein segments to provide structural information.[1] Proteomics requires selective digestion of the expressed proteins into

fragments whose size is suitable for quick and unambiguous mass-spectrometric detection.[2] Protein semisynthesis involves selective hydrolysis of natural proteins into large fragments, which are then chemically ligated with synthetic peptides to obtain the desired modified protein.[3] Production of bioengineered fusion proteins requires the highest level of proteolytic selectivity – site-specific cleavage that removes the fusion tag from the protein of interest.[4]

A small number of proteolytic enzymes and synthetic reagents are available for selective proteolysis. Very many proteolytic enzymes are known, but only few of them are commonly used. Most of these common proteases are residue-specific, and their selectivity can be adjusted by varying the time of the digestion and the degree of prior unfolding of the protein substrate. Despite their catalytic superiority, proteases are sometimes inadequate because they tend to produce short fragments unsuitable for bioanalytical applications. Moreover, enzymes are proteinaceous contaminants of the protein digests that they create.

The existing chemical reagents are less effective than the enzymes because they often require harsh conditions and high molar excess and often give only partial selectivity and relatively low yields.[5] For example, cyanogen bromide, a methionine-selective cleaver commonly used in biochemical practice has several shortcomings. It is volatile and toxic, is applied in a 100-fold excess over the methionine residues in the substrate, requires 70% formic acid as the solvent, and causes various side reactions. Moreover, the protein fragments created by CNBr are no longer native because the methionine residues in them are irreversibly converted to serine lactones.

Emerging biochemical and applications would benefit from a broader choice of chemical reagents for protein cleavage, having improved efficiency and selectivity. Finding such reagents is a very difficult task because even nonselective cleavage is hard to achieve.

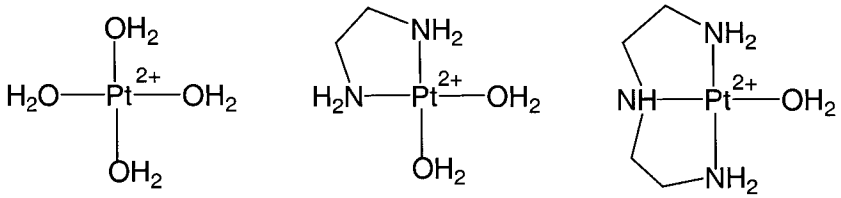
The amide bond in peptides and proteins is extremely unreactive toward hydrolysis. Under standard conditions, (room temperature, pH 4-8) the half-life for hydrolysis of a simple peptide is 500-1000 years.[6-8] Controlled and selective cleavage, even more formidable task, requires a chemical reagent that can selectively bind to certain residues and promote selective cleavage of a peptide bond near the binding site. Hydrolytic cleavage, which renders the protein fragments pristine, is preferred over oxidative cleavage, which results in irreversible chemical modifications of the fragments. Relatively mild conditions, equimolar amount or small molar excess over the substrate, and easy removal of the reagent after cleavage are desirable features.

Some transition-metal complexes are emerging as new chemical proteases.[9-22] The mechanisms of their action on small substrates have been investigated,[12,23,24] but there are only few reports of regioselective cleavage of proteins.

Complexes of palladium(II) and platinum(II), two chemically similar transition-metal ions, are emerging as new reagents for selective cleavage of peptides and proteins.[25,26] Both these metal ions are active as synthetic proteases, but their hydrolytic selectivities are different. The cleavage by Pd(II) complexes, such as $[Pd(H_2O)_4]^{2+}$, (shown below) consistently occurs in weakly acidic aqueous solutions at the peptide bond involving the N-terminus of the residue preceding histidine and methionine residues, i.e., the X-Y bond in the sequence segments X-Y-His-Z and X-Y-Met-Z, where X, Y, and Z are any non-coordinating residues. We also demonstrated that as the pH of the solution is raised from mildly-acidic to neutral, the Pd(II)-promoted selective cleavage becomes specific to only X-Pro-His-Z and X-Pro-Met-Z sequences, in which the Y residue is proline.[27] In non-aqueous solutions, Pd(II)

reagents act with a different (but explainable) selectivity and promote tryptophan-directed cleavage of amide bonds,[28,29] reaction potentially useful for cleavage of lipophillic proteins.

On the other hand, the cleavage by Pt(II) complexes, such as *cis*-$[Pt(en)(H_2O)_2]^{2+}$ occurs in weakly acidic aqueous solutions exclusively at the peptide bond involving the C-terminus of each methionine residue, that is, the Met-Z bond. Cleavage directed by histidine or any other residue is absent. We investigated the coordination of histidine- and methionine-containing oligopeptides to both Pd(II) and Pt(II) reagents and the dependence of the cleavage rates on solution pH. For both reagents, we identified the hydrolytically-active complexes and realized that the acidic solution is necessary to suppress its conversion to inactive complex(es).



$[Pd(H_2O)_4]^{2+}$ *cis*-$[Pt(en)(H_2O)_2]^{2+}$ $[Pt(dien)(H_2O)]^{2+}$

In the present study, we use peptide substrate to demonstrate that Pd(II) and Pt(II) reagents can be used together to obtain different cleavage patterns. We also introduce complex $[Pt(dien)(H_2O)]^{2+}$, which binds to methionine residue but without cleaving it. Instead, this residue get blocked from the Pd(II)-promoted cleavage. We explain the surprising difference in selectivity between the Pd(II) and Pt(II) reagents as synthetic proteases. The selectivity of cleavage is a consequence of the selectivity of coordination. Because histidine and methionine residues can specifically bind the Pd(II) reagent, they also direct the Pd(II)-promoted cleavage. The cleavage by Pt(II) reagent is directed by methionine residues because only methionine residue can bind to the Pt(II) reagent under the reaction

conditions. The mechanism of cleavage is a consequence of the modes of coordination of oligopeptide to the reagents. Only upon anchoring can the Pd(II) and Pt(II) reagent approach the scissile peptide bond and activate it toward hydrolysis.

We also demonstrate two bioanalytical applications of Pd(II) and Pt(II) reagents. We use *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$ to cleave a well-known protein myoglobin, containing 2 methionine residues and 11 histidine residues. Cleavage by $[Pt(en)(H_2O)_2]^{2+}$ is suitable for biochemical applications that require large fragments. The fragments can be easily separated and they remain pristine, without covalent modification. Cleavage of these fragments by Pd(II) reagent is suitable for peptide mapping, because many small peptide fragments are obtained, which can be easily detected by mass spectrometric methods. The ability of the Pt(II) and Pd(II) complexes to cleave proteins with explicable and variable selectivity, and with good yields bodes well for their wider use in biochemical and bioanalytical practice.

Experimental Procedures

Chemicals

The complex *cis*-$[Pt(en)Cl_2]$ (in which en is ethylenediamine) and equine myoglobin were obtained from Sigma Chemical Co. Palladium sponge, *cis*-$[Pt(en)Cl_2]$, $K_2[PdCl_4]$, piperidine, trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid were obtained from Aldrich Chemical Co. Methyl phenyl sulfone was obtained from Lancaster Synthesis Inc. Acetonitrile of HPLC grade was obtained from Fisher Scientific Co. The salts *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pt(dien)(H_2O)]^{2+}$ (in which dien is diethylenetriamine) were prepared as perchlorate salts and quantified according to published procedures.[30,31]

The peptide AcVal-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (termed HisMet-peptide) was synthesized by a standard manual Fmoc solid-phase procedure and purified by reverse-phase HPLC on a C18 preparative column, as described previously.[25] The purity, examined by analytical HPLC, was higher than 99.5% and the found and calculated molecular masses were, respectively, 1515.08 and 1514.78 D.

The stock solution of the complex $[Pd(H_2O)_4]^{2+}$ was obtained according to the published procedure.[32] The complexes *cis*-$[Pd(en)(H_2O)_2]^{2+}$, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pt(dien)(H_2O)]^{2+}$ (in which dien is diethylenetriamine) were prepared by the published procedures.[33,34] All complexes were prepared as perchlorate salts. The concentrations were determined using their published extinctions coefficients.

Spectroscopic and Analytical Methods

HPLC Separations. The components of the peptide digests were separated by a Hewlett Packard 1100 HPLC system containing an autosampler and a multiwavelength detector set to 215, 280, and 410 nm. Absorption at 215 nm is common to all peptides and proteins; absorption at 280 nm is due to aromatic residues and bound Pt(II) ion; and absorption at 410 nm is diagnostic of heme. In the reverse-phase separations, analytical Supelco Discovery C18 column (sized 250 x 4.6 mm, beads of 5 μm), and a preparative Vydac C18 column 218TP101522 (sized 250 x 22 mm, beads of 10 μm) were used in the experiments involving HisMet peptide; analytical Vydac C5 column 214TP54 (sized 150 x 4.6 mm, beads of 5 μm), and a preparative Discovery BIO Wide Pore C5 (sized 250 x 21.2 mm, beads of 10 μm) were used in the experiments involving myoglobin. The eluting solvent A was 0.10% (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 then 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 in analytical runs and 10.0 mL/min in preparative runs. In the size-exclusion separations, the Superdex peptide HR 10/30 column, with optimal separation range from 1000 to 7000 D, was used. The solvent was 0.10% (v/v) TFA in H_2O, and the flow rate was 0.50 mL/min.

Mass Spectrometry. The MALDI-TOF experiments were done with a Bruker Proflex™ instrument. The samples containing intact peptide, the reaction mixture of the peptide and the Pd(II) and Pt(II) complexes, and separate fractions isolated by HPLC 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 a 2:1 (v/v) mixture of water and acetonitrile. Each spectrum consisted of 100 scans. For the sake of clarity, molecular masses are reported only for the fragments free of the Pt(en) or Pd(II) groups, although the species carrying the metal ions were also observed in the MALDI spectra. Solutions of angiotensin II, oxidized chain B of insulin, and cytochrome *c* were used as external standards. The measured molecular mass of a given fragment was compared with the value calculated by PAWS software, obtained from ProteoMetrics, LLC.

Study of Hydrolysis

For the experiments with HisMet-peptide, the aqueous solutions were held in 2.0-mL glass vials. The pH was adjusted by $HClO_4$ or NaOH. After the reactions were completed, the pH remained within ±0.1 of the initial value. The mixture was kept at 40 ± 1 or 60 ± 1 °C for

1 day, and 20.0-μL samples were taken periodically. The stock solution was 5.0 mM. In a typical experiment, involving equimolar amounts of the Pt(II) reagent and the methionine residue in the substrate, and respectively equimolar 200.0 μL of the substrate solution was mixed with 10.0 μL of a 100.0 mM stock solution of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, 10.0 μL of a 20 mM solution of methyl phenyl sulfone (the internal standard), and 700.0 μL of water. For the cleavage of myoglobin by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, 600.0 μL of 1.0 mM solution of protein were incubated with 5 equiv of the reagent (30.0 μL of 0.1 M solution) for 1 day at pH 2.5. The fragments were separated by preparative reverse-phase HPLC, and lyophilized to dryness.

The fragments of myoglobin containing histidine residues were cleaved by $[Pd(H_2O)_4]^{2+}$. 5.0 mg of the fragment is dissolved in 800.0 μL of water and mixed with the corresponding volume of the 0.50 M solution of $[Pd(H_2O)_4]^{2+}$ so that the amount of the reagent is equimolar to histidine residues in the fragment. The mixture was incubated at pH 2.0 and 60 ± 1 °C. A 1 mL sample from the digest after 1 day was analyzed by MALDI-TOF mass spectrometry.

In the control experiments for possible background cleavage, the conditions were the same except that *cis*-$[Pt(en)(H_2O)_2]^{2+}$ was absent. The cleavage was by followed by reverse-phase HPLC for HisMet-peptide and by gel-exclusion combined with reverse-phase HPLC for myoglobin. The separated fragments were lyophilized to dryness, re-dissolved, and analyzed by MALDI-TOF mass spectrometry and Edman N-terminal sequencing.

Because cleavage is very slow at room temperature, the species distribution in the chromatographic runs corresponds to that in the digest sample. In kinetic measurements with HisMet-peptide, the areas under the chromatographic peaks were normalized to that of

methyl phenyl sulfone, to compensate for possible errors in the injection volume and for evaporation. The error of this integration was estimated at 5%. The plots of the peak areas versus time for the cleavage products were fitted to the first-order rate law with SigmaPlot v. 5.0, obtained from SPSS Inc. The fitting to the first-order rate law is correct because the binding of the Pt(II) and Pd(II) reagents to the substrate is much faster than the subsequent intramolecular cleavage of the substrate. Each rate constant is the average of two consistent values, obtained by monitoring both fragments of cleavage. The stated errors in the rate constants correspond to two standard deviations, i.e., confidence limit greater than 95.0%.

Results and Discussion

Selective cleavage of HisMet-peptide

Cleavage by $[Pd(H_2O)_4]^{2+}$ or by *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The HisMet-peptide contains two possible anchoring residues competing for the Pd(II) reagent, namely His5 and Met11. Our previous results showed that the reaction mixture containing equimolar amounts of $[Pd(H_2O)_4]^{2+}$ and the substrate contained all possible products of cleavage guided by His5, by Met11, and by both of these anchor simultaneously.[25] Cleavage occurred at the second peptide bond upstream from these two anchors, and Pd(II) reagent shows no binding or cleaving preference for either anchor. When HisMet-peptide was cleaved by two molar equivalents of $[Pd(H_2O)_4]^{2+}$, under the same conditions, at the end of the reaction only the three fragments resulting by complete cleavage at both anchors His5 and Met11 were present.

When HisMet-peptide is digested by one or two molar equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is the same: only Met11-Ala12 peptide bond is cleaved. Clearly, the cleavage selectivity of

cis-$[Pt(en)(H_2O)_2]^{2+}$ is different than that of $[Pd(H_2O)_4]^{2+}$. It is evident from these experiments that by choosing the reagent, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ or $[Pd(H_2O)_4]^{2+}$, different selectivity can be achieved. To go one step further, we investigate whether these two reagents can be used together.

Cleavage by both *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$. An equimolar reaction mixture made from solutions of HisMet-peptide, *cis*-$[Pt(en)(H_2O)_2]^{2+}$, and $[Pd(H_2O)_4]^{2+}$ was kept at 60 °C and pH 2.0. The chromatogram of this mixture after 1 day contained several fractions, whose identities, determined from MALDI mass spectrometric measurements, are shown in Table 1. Clearly, the two cleavage reagents compete for the common anchor Met11, but only Pd(II) anchors to His5. Depending of the anchored metal ions, Pd(II) or Pt(II), Met11 directs cleavage of the second bond upstream or the first bond downstream, respectively, whereas His5 directs only the Pd(II)-promoted cleavage of the second bond upstream.

In another experiment, the same reaction mixture is prepared by first mixing the substrate with 1 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, keeping this mixture for 1h, and then adding 1 equiv of $[Pd(H_2O)_4]^{2+}$. After 1 day, the chromatogram of this mixture contained only 3 fractions. The MALDI measurements confirmed the identity of these fractions as the fragments of HisMet-peptide shown in Table 1. Clearly, a "clean" cleavage pattern is obtained. The Pt(II) reagent promoted the Met11-directed cleavage, whereas Pd(II) reagent promoted the His5-directed cleavage.

We explore further the "blocking" of Met residues from binding to Pd(II) cleaver by introducing another Pt(II) complex, $[Pt(dien)(H_2O)]^{2+}$. A solution of HisMet-peptide was mixed immediately with a solution containing equimolar amount of $[Pt(dien)(H_2O)]^{2+}$, and

after 1 h with 1 equiv of $[Pd(H_2O)_4]^{2+}$. The chromatogram of this mixture kept at 60 °C and pH 2.0 for 1 d had 2 fractions, and their identities, established from the MALDI mass-spectrometric analysis, are shown in Table 2.

Clearly, only the cleavage at His5 is observed. In a control experiment, a mixture of HisMet-peptide with $[Pt(dien)(H_2O)]^{2+}$ kept under the same conditions for 1 day. The chromatogram contained a single species, eluting at 17.3 min and showing in the MALDI mass spectrum a single peak for the substrate-Pt(dien) complex.

Evidently, both cleavage reagents, Pd(II) and Pt(II) exhibit their unique selectivities and they can act together. By choosing the reagent and the order of addition, the pattern of cleavage can be varied. When both reagents are added in the same time, all possible fragments are obtained, because they compete for their common anchor, methionine. If Pt(II) reagent is added first and allowed to bind completely to the sulfur atom from the side chain of methionine, only three fragments are going to be obtained at the end of the cleavage, resulting from the cleavage at the second bond upstream from His and at the first bond downstream from Met.

Platinum and palladium complexes tend to have similar chemical properties, because Pt(II) and Pd(II) ions are alike in the electron configuration and other characteristics. But there are still some properties in which they differ and that are important for the cleavage of peptide bond. Pt(II) ion is a soft Lewis acid and has high affinity for soft Lewis bases such as thioether sulfur atom, whereas Pd(II) is a "borderline" Lewis acid with high affinity for soft bases like thioether sulfur atom and less soft ones such as imidazole nitrogen atom. Significantly, Pt(II) ion is much more inert than the Pd(II) ion. The ligand substitution rates for Pt(II) are much smaller than those for Pd(II), by a factor of 10^6.[35]

As Chart 1 shows, the selectivities of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$ as cleaving reagents are surprisingly different. While *cis*-$[Pt(en)(H_2O)_2]^{2+}$ cuts the first peptide bond downstream from methionine anchor (the Met-Z bond), the analogous Pd(II) complex, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, cuts the second peptide bond upstream from methionine and histidine anchors (the X-Y bond) in the X-Y-Met-Z and X-Y-His-Z sequences. These selectivities can be explained by the differences mentioned above between Pd(II) and Pt(II) complexes. In this paper, the structural aspects that govern the selectivity and the hydrolytically active complexes are discussed.

The soft Lewis acid Pt(II) binds only the soft Lewis base in the methionine side chain, whereas the "borderline" acid Pd(II)binds both the soft methionine side chain and the histidine side chain. Once anchored, the two metal ions form different hydrolytically-active complexes in the mildly-acidic solutions. Because Pt(II) is much more inert than Pd(II) to ligand substitution,[35] ethylenediamine ligand persists on the former ion under the reaction conditions,[36] but is displaced from the latter,[26] with the help of the H^+ ions in solution. The Pt(II) reagent remains as a unidentate complex. The Pd(II) reagent, free of ethylenediamine, binds to the amidate nitrogen atom upstream from the anchor ($pK_a < 2.0$[37-43]) and forms a bidentate complex.[25] Both of these are hydrolytically active. Consequently, the anchored Pt(II) ion remains proximal to the peptide bond downstream from the anchor, and can activate it toward cleavage. The anchored Pd(II) ion, however, is kept away from this bond but close to the second peptide bond upstream from the anchor, which becomes activated. Evidently, each metal ion will activate the peptide bond proximal to it when bound to the peptide. Because in the case of platinum the ethylenediamine stays bound to it, Pt(II) can not coordinate to the peptide backbone and the only bond accessible to it is the first one

downstream. Pd(II) ion can bind to the peptide backbone, by deprotonating the amide nitrogen atoms and also because the ethylenediamine ligand is displaced. The presence of at least one aqua ligand is required in the hydrolytically-active complexes for the cleavage to take place. The aqua ligand can be either displaced by the carbonyl oxygen atom (in the external-attack mechanism) or delivered to the carbonyl carbon atom of the scissile peptide bond (in the internal-delivery mechanism).[25,44]

$[Pt(dien)(H_2O)]^{2+}$ contains a tridentate ligand and it can bind to the sulfur atom from the side-chain of methionine, but by binding it loses its only aqua ligand. The absence of the aqua ligand makes the resulting complex hydrolytically-inactive, and $[Pt(dien)(H_2O)]^{2+}$ cannot cleave any peptide bond. When $[Pt(dien)(H_2O)]^{2+}$ is bound to the side chain of Met, the anchoring of the Pd(II) reagent is prevented, and the cleavage directed by this residue is inhibited. This property of $[Pt(dien)(H_2O)]^{2+}$ can be used to enhance the selectivity of Pd(II) reagents when cleaving peptides and proteins. If all Met residues are blocked, only the His side chains are available for Pd(II) anchoring and Pd(II)-promoted cleavage.

Selective cleavage of myoglobin

Cleavage of myoglobin by *cis*-$[Pt(en)(H_2O)_2]^{2+}$. Reaction of equine myoglobin with 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5 and 60 °C was followed by size-exclusion chromatography. The chromatograms of the fresh myoglobin solution and of the control solution show only the intact protein eluting at 23.5 min – evidence against background cleavage. Figure 1 shows that the reaction mixture after 1 day lacks the intact protein and contains three peaks, produced by the Pt(II) reagent. The fractions were collected, checked for purity reverse-phase HPLC, and identified by MALDI MS and Edman sequencing. The

results summarized in Table 3, prove the cleavage pattern shown in Chart 3. The reagent promoted selective cleavage of two peptide bonds, Met55-Lys56 and Met131-Thr132. Clearly, the cleavage is directed by the only two methionine residues, Met55 and Met131. Minor background cleavage present in both the control solution and in that containing the Pt(II) reagent, was evident from the two minor fractions eluting at 20.7 and 25.3 min. Fortunately, this background cleavage accounts for less than 3 % of total cleavage, as estimated from the chromatograms.

We followed the kinetics of myoglobin cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ by observing the increase of the peak corresponding to fragment 132···154. The appearance of fragments in time obeyed the first-order rate law, as shown in Figure 2. Because the binding of Pt(II) ion to the sulfur atom of methionine occurs within minutes, and because the subsequent reactions are intramolecular and take hours, fitting of the kinetic results to the first-order rate law is justified. The obtained rate constant is 4.1 10^{-3} min^{-1}, corresponding to a half-life of 170 min.

Clearly, the pattern of myoglobin cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is consistent with that observed with other proteins and peptides[44]. The fragments were easily separated by reverse-phase chromatography, lyophilized, and isolated on a preparative scale. These results demonstrate the applicability of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ for cleaving proteins on a large scale to obtain large fragments. Methionine residues rarely cluster in protein sequence and their average abundance of is only about 2.2%,[45] . Therefore, the fragments obtained by methionine-specific cleavage should be long for most proteins. They can be used further for sequencing or for recombining them into semisynthetic proteins.

The selectivity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is the same as that claimed for the most commonly used chemical protease, cyanogen bromide(CNBr). While having the same

selectivity as CNBr, the complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ has several advantages:[44] It is non-volatile, easy to handle, and recyclable; the cleavage by it is residue-selective, with no side reactions; it is applied in approximately equimolar amounts with respect to methionine residues; it leaves the protein fragments unmodified; it cleaves even the Met-Pro bond, which is resistant to CNBr and most proteolytic enzymes;[46,47] and it works in the presence of the denaturing reagent sodium dodecyl sulfate. These results demonstrate practical utility of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ for preparative-scale cleavage of proteins.

Cleavage of myoglobin fragments by $[Pd(H_2O)_4]^{2+}$. Because histidine residues are more abundant than methionine residues in myoglobin, the cleavage directed by them would result in several short fragments. Such "digest" would be difficult and impractical to separate. This type of protein digestion, commonly used with trypsin as a cleaver, is practical for proteomics, which requires quick and unambiguous identification of proteins by mass-spectrometric methods. To demonstrate the use of $[Pd(H_2O)_4]^{2+}$ as a reagent suitable for the proteomics applications, we cleave two histidine-containing myoglobin fragments obtained by Pt(II)-promoted cleavage, fragments designated Mb^{1-55} and Mb^{56-131} (see Chart 3)

Reaction mixture containing Mb^{1-55} and 3 equiv of $[Pd(H_2O)_4]^{2+}$ was kept at 60 °C and pH 2.0. After 10 h, the sample from this mixture is subjected to MALDI mass spectrometric analysis. The results are summarized in Table 4. The same experiment was done with Mb^{56-131} and 8 equiv of $[Pd(H_2O)_4]^{2+}$, and these results are summarized in Table 5 The fragments listed in Tables 4 and 5 correspond to the cleavage patterns shown in Charts 4 and 5. Clearly, the Pd(II) reagent promoted selective cleavage of X-Y bonds in X-Y-His sequence segments, as observed in our earlier studies with peptides and proteins.[25,26] Because the reaction is not finished, several fragments from incomplete cleavage are

observed in the digest. Such incomplete fragmentation is preferred over complete digestion for the protein identification in proteomics because it provides more information and higher sequence coverage, thus ensuring more reliable protein identification.

The results in Charts 4 and 5 show that the Pd(II)-promoted cleavage provided compete sequence coverage for both Mb^{1-55} and Mb^{56-131}. Clearly, $[Pd(H_2O)_4]^{2+}$ is a suitable reagent for the mass-spectrometric protein identification when numerous anchors are available. Because the reagent has low molecular mass, additional proteinaceous signals in mass spectrum of the digest are absent.

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Conclusions

Two transition-metal complexes, cis-[Pt(en)(H2O)2]2+ and [Pd(H2O)4]2+ are new reagents for selective hydrolysis of peptides and proteins. Although Pt(II) ion and Pd(II) ions are chemically similar, these two reagents act with different hydrolytic selectivity. We explain this difference by comparing different coordination modes in which each reagent anchors to a specific side chain and to the peptide backbone.

Two bioanalytical applications for protein cleavage by cis-[Pt(en)(H2O)2]2+ and by [Pd(H2O)4]2+ are demonstrated: complete cleavage at few sites, suitable for preparative fragmentation, and incomplete cleavage at many sites, suitable for mass-spectrometric analysis in proteomics. With unique and useful selectivities and tunable reactivities, cis-[Pt(en)(H2O)2]2+ and [Pd(H2O)4]2+ are well-suited for wide use in biochemical practice.

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Graphics

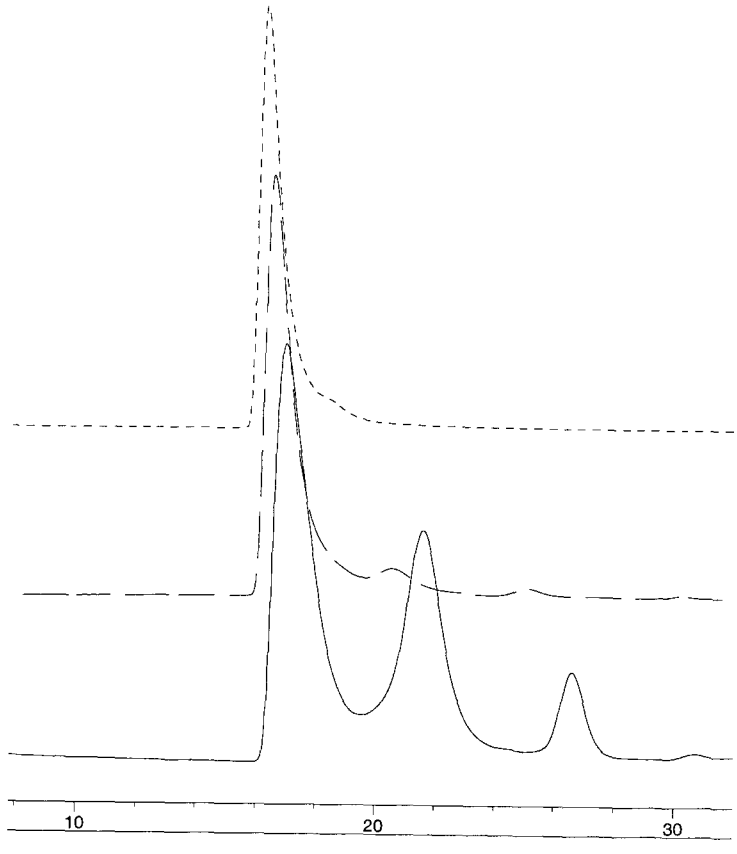


Figure 1. Size-exclusion chromatograms of myoglobin: a) fresh solution; b) after 24 at pH 2.5 and 60 °C; and c) after 24 h at pH 2.5 and 60 °C in the presence of 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The identity of the fractions is shown in Table 3.

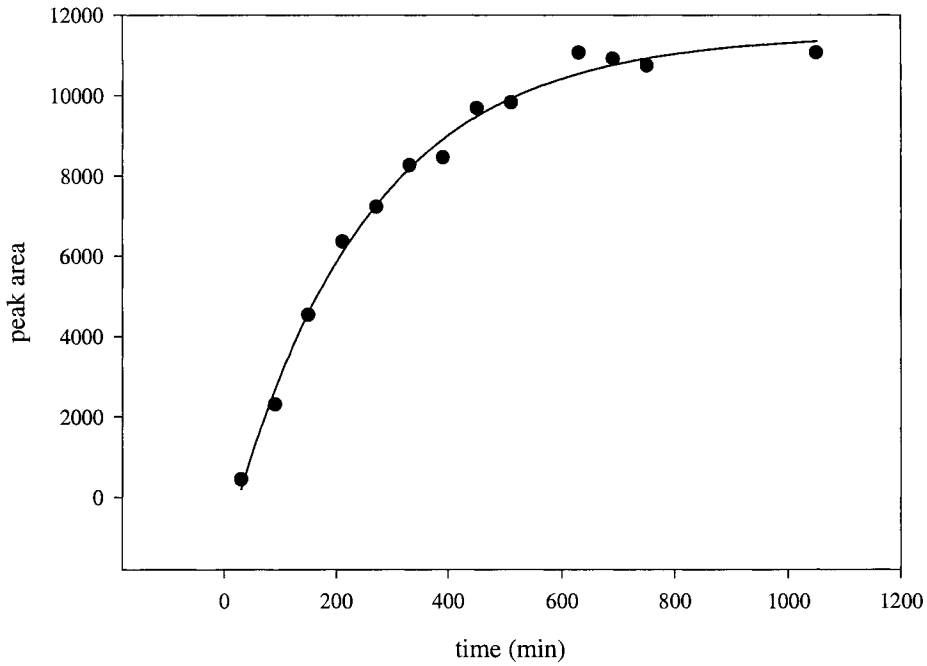


Figure 2. The progress of the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ of myoglobin at pH 1.8 and 60 °C followed by HPLC separations. The formation of fragment 132-154, a product of the cleavage, is shown. The solid line is the fitting to the first-order rate law.

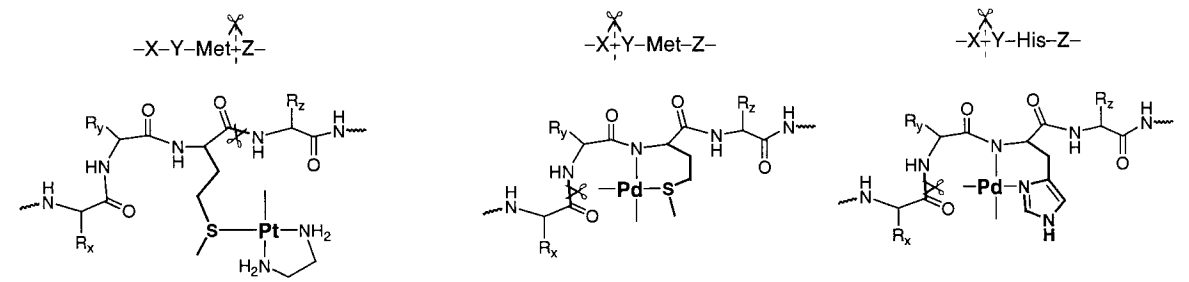


Chart 1. Different proteolytic selectivity of Pt(II) and Pd(II) complexes. These two metal ions form different hydrolytically-active complexes with the substrate, and promote hydrolytic cleavage of different peptide bonds. For explanation see text.

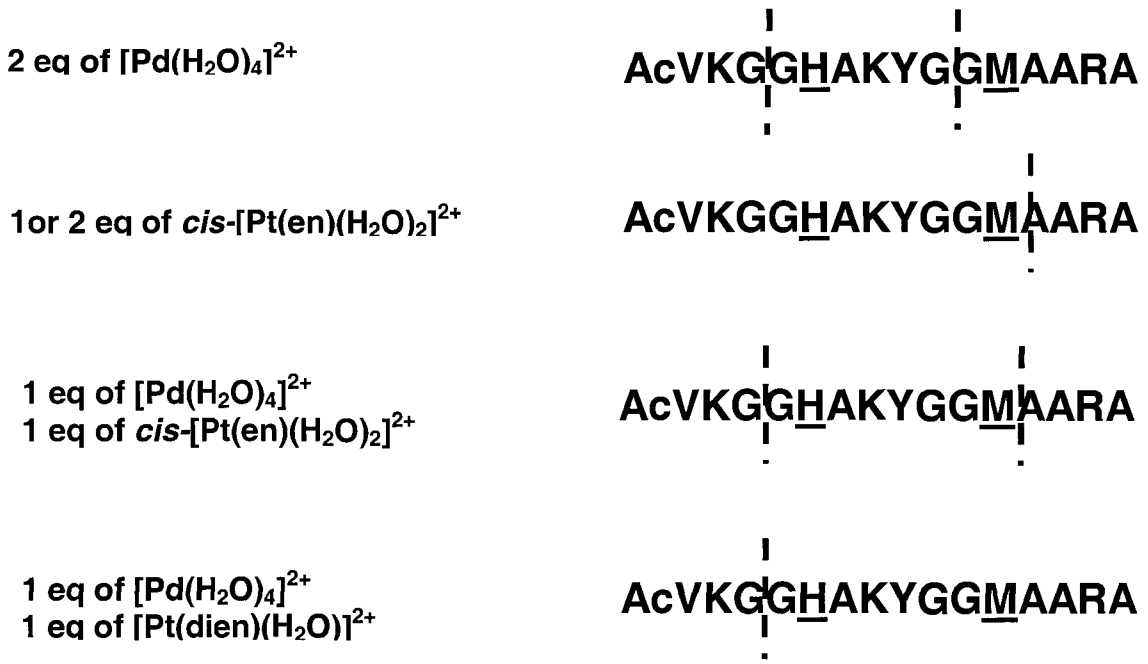


Chart 2. Amino acid sequence of HisMet-peptide showing the sites of the cleavage by the Pt(II) and Pd(II) reagents.

1 GLSDGEWQQVLNVWGKVEADIAGHGQEVLI 30

31 RLFTGHPETLEKFDKFKHLKTEAEM|KASED 60

61 LKKHGTVVLTALGGILKKKGHHEAELKPLA 90

91 QSHATKHKIPIKYLEFISDAIIHVLHSKHP 120

121 GDFGADAQGAM|TKALELFRNDIAAKYKELG 150

151 FQG 153

Chart 3. Amino acid sequence of horse myoglobin showing the sites of the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, at the C-terminal side of methionine residues, i.e., at the first amide bond "downstream" from each methionine residue.

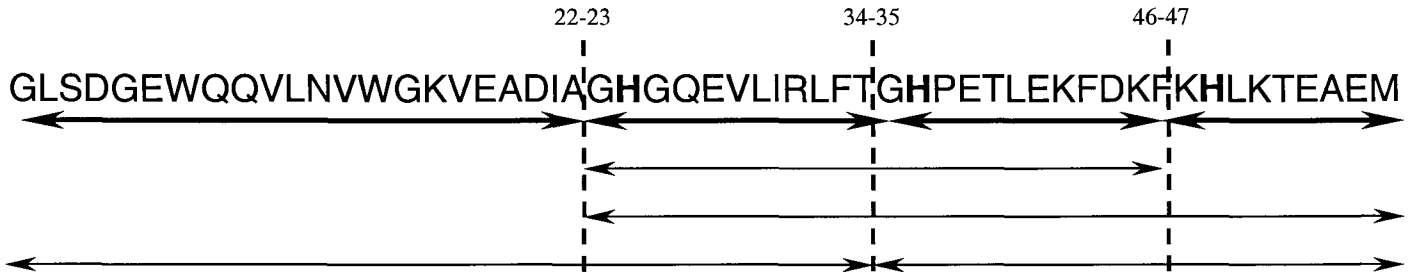


Chart 4. Amino acid sequence of horse myoglobin fragment 1···55 (designated $Mb^{1\text{-}55}$) showing the sites of the cleavage by $[Pd(H_2O)_4]^{2+}$, the second amide bond "upstream" from each histidine residue.

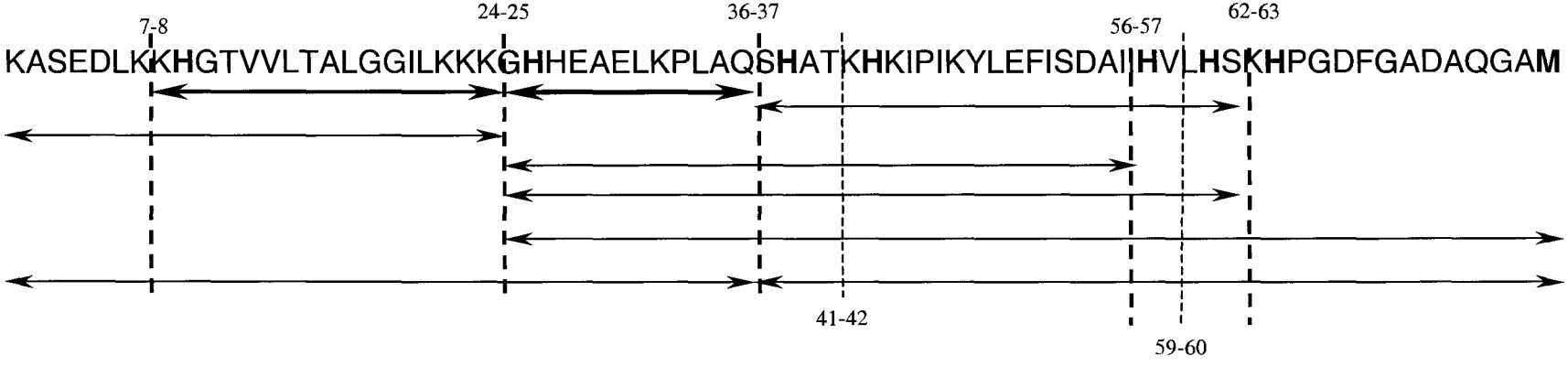


Chart 5. Amino acid sequence of horse myoglobin fragment 56···131 (designated $Mb^{56\text{-}131}$) showing the sites of the cleavage by $[Pd(H_2O)_4]^{2+}$, the second amide bond "upstream" from each histidine residue.

Table 1. Results of HPLC separation and MALDI mass spectroscopic identification of 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 one equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ followed by one equiv of $[Pd(H_2O)_4]^{2+}$.

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
14.4	344.77	344.21	1···3
9.8	387.95	388.22	12···15
17.1	817.92	818.37	4···11

Table 2. Results of HPLC separation and MALDI mass spectroscopic identification of 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 $[Pd(H_2O)_4]^{2+}$, in presence of $[Pt(dien)(H_2O)]^{2+}$.

elution time (min)	molecular mass (D)		fragment
	observed	calculated	
11.5	349.2	344.41	1···3
17.21	1188.98	1188.58	4···15

Table 3. Cleavage of horse myoglobin by 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5. The fragments are separated by size-exclusion HPLC and identified by MALDI mass spectrometry and Edman N-terminal sequencing.

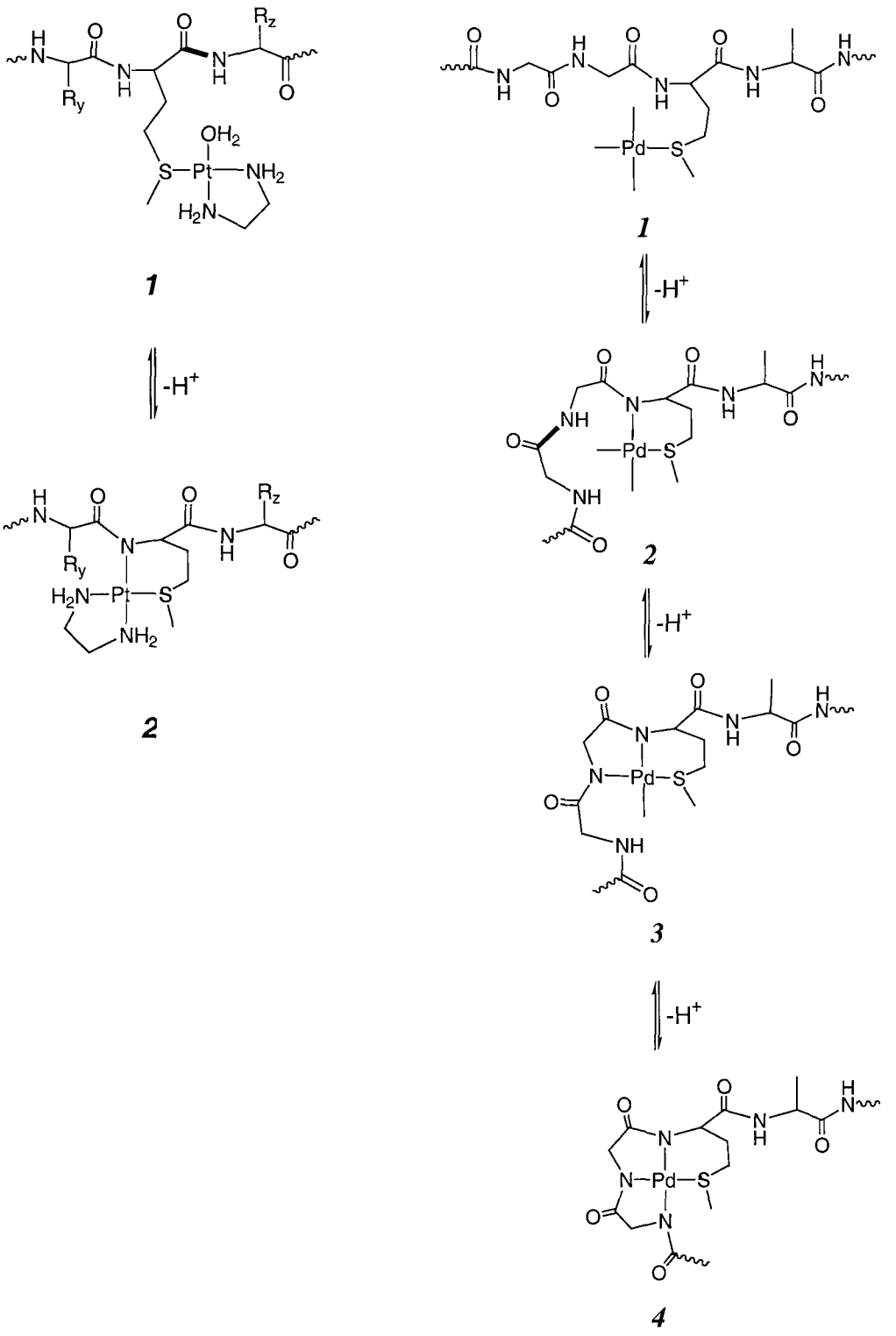
N-terminal sequence	elution time (min)	molecular mass (D)		fragment
		observed	calculated	
GLSDG	21.6	6265.81	6265.08	1···55
KASED	18.2	8213.26	8211.53	56···131
TKALE	26.1	2511.26	2513.89	132···153

Table 4. Cleavage of fragment 1···55 of horse myoglobin (designated Mb^{1-55}) by $[Pd(H_2O)_4]^{2+}$ at pH = 2.0. The fragments are identified by MALDI mass spectrometry.

calculated mass	observed mass	fragment
1086.3	1083.2	47···55
1369.6	1370.0	23···34
1447.6	1442.4	35···46
2414	2413.7	1···22
2514.2	2511.7	35···55
2797.5	2797.6	23···46
3865.0	3865.3	23···55
5192	5192.5	1···46
6265.1	6266.5	1···55

Table 5. Cleavage of fragment 56···131 of horse myoglobin (designated $Mb^{56\text{-}131}$) by $[Pd(H_2O)_4]^{2+}$ at pH = 2.0. The fragments are identified by MALDI mass spectrometry.

calculated mass	observed mass	fragment
1329.5	1329.2	25···36
1763.3	1763.5	8···24
2535.0	2535.4	1···24
2998.0	2996.1	37···62
3623.1	3626.8	25···56
3846.0	3845.3	1···36
4309.9	4306.4	25···62
4382.0	4379.6	37···76
5693.5	5697.6	25···76



Scheme 1. Comparison of the coordination modes formed upon binding of the methionine anchor and of the polypeptide backbone to *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and to $[Pd(H_2O)_4]^{2+}$.

CHAPTER 7. Conclusions

Simple complexes of palladium(II) and platinum(II) act as reagents for selective hydrolytic cleavage of amide bonds in peptides and proteins. The cleavage by Pd(II) complexes, such as $[Pd(H_2O)_4]^{2+}$, consistently occurs in weakly acidic aqueous solutions at the amide bond involving the N-terminus of the residue preceding histidine and methionine residues, that is, the X-Y bond in the sequence segments X-Y-His-Z and X-Y-Met-Z, where X, Y, and Z are any non-coordinating residues. The cleavage by Pt(II) complexes, such as *cis*-$[Pt(en)(H_2O)_2]^{2+}$, occurs exclusively at the peptide bond involving the C-terminus of methionine residues, that is the Met-Z bond.

Structural and kinetic studies with peptide substrates explained the residue-selective proteolytic selectivity of Pd(II) and Pt(II) complexes by recognizing the hydrolytically-active modes in which these metal ions bind to the side chains of the anchoring residues and to the polypeptide backbone. The Pd(II)-promoted cleavage can be made sequence-specific – in neutral solution, only the X-Pro bond in X-Pro-Met and X-Pro-His segments get cleaved. The Pt(II)-promoted cleavage occurs with a catalytic turnover.

Cleavage studies with protein substrates confirmed the cleavage pattern observed with peptide substrates, and demonstrated the suitability of the Pd(II) and Pt(II) reagents for various biochemical applications.

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