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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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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 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 positions 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 3, the second amide bond upstream from the anchor is regioselectively cleaved in all cases when the anchor was "isolated," that is, flanked by noncoordinating 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 a 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

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(1) Croft, L. R. *Handbook of Protein Sequence Analysis*, 2nd ed.; Wiley: Chichester, U.K., 1980.
(2) Thomas, J. J.; Bakhtiar, R.; Siuzdak, G. *Acc. Chem. Res.* **2000**, *33*, 179.
(3) Heyduk, T.; Baichoo, N.; Heyduk, E. *Met. Ions Biol. Syst.* **2001**, *38*, 255.
(4) *Applications of Chimeric Genes and Hybrid Proteins, Part A: Gene Expression and Protein Purification*; Thorner, J., Emr, S. D., Abelson, J. N., Eds.; Methods in Enzymology; Academic Press: San Diego, CA, 2000; Vol. 326.
(5) Wallace, C. J. A. *Protein Engineering by Semisynthesis*; CRC Press: Boca Raton, FL, 2000.

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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, this can be accomplished 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

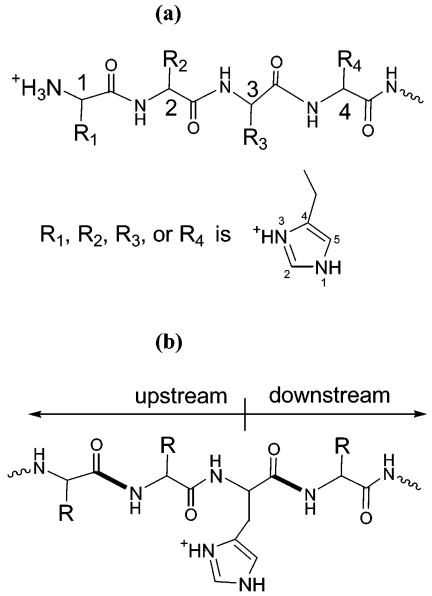


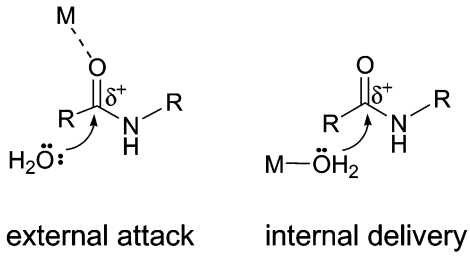
Figure 1. (a) Positions of the anchoring histidine residue with respect to the N-terminus and numbering of the atoms in the imidazole ring. (b) Definition of the upstream and downstream directions from the anchor. The scissile peptide bonds, highlighted, are the second one upstream and the first one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

(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, the Pd(II) ion is readily removed by chelation or precipitation. Because histidine and methionine have a combined average abundance in proteins of only ca. 4.5%, the fragments are relatively long and suitable for bioanalytical applications. In nonaqueous solutions, compatible with hydrophobic proteins, cleavage near the tryptophan residue is possible.[34,35] Potential control of selectivity by the choice of solvent adds 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

(6) Walker, J. M. *The Protein Protocols Handbook*; Humana Press: Totowa, NJ, 2002.
(7) Bryant, R. A. R.; Hansen, D. E. *J. Am. Chem. Soc.* **1996**, *118*, 5498.
(8) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105.
(9) Smith, R. M.; Hansen, D. E. *J. Am. Chem. Soc.* **1998**, *120*, 8910.
(10) Sutton, P. A.; Buckingham, D. A. *Acc. Chem. Res.* **1987**, *20*, 357.
(11) Rana, T. M.; Meares, C. F. *J. Am. Chem. Soc.* **1991**, *113*, 1859.
(12) Rana, T. M.; Meares, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10578.
(13) Suh, J. *Acc. Chem. Res.* **1996**, *25*, 273.
(14) Hegg, E. L.; Burstyn, J. N. *Coord. Chem. Rev.* **1998**, *173*, 133.
(15) Chin, J. *Acc. Chem. Res.* **1991**, *24*, 145.
(16) Kumar, C. V.; Buranaprapuk, A.; Cho, A.; Chaudhari, A. *Chem. Commun.* **2000**, 597.
(17) Kumar, C. V.; Buranaprapuk, A. *J. Am. Chem. Soc.* **1999**, *121*, 4262.
(18) Zhu, L.; Qin, L.; Parac, T. N.; Kostić, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218.
(19) Zhu, L.; Bakhtiar, R.; Kostić, N. M. *J. Biol. Inorg. Chem.* **1998**, *3*, 383.
(20) *Probing of Proteins by Metal Ions and Their Low-Molecular-Weight Complexes*; Sigel, A., Sigel, H., Eds.; Metal Ions in Biological Systems; Dekker: New York, 2001; Vol. 38, Chapters 2−9 and references therein.
(21) Allen, G.; Campbell, R. O. *Int. J. Pept. Protein Res.* **1996**, *48*, 265.
(22) Allen, G. *Met. Ions Biol. Syst.* **2001**, *38*, 197.
(23) Datwyler, S. A.; Meares, C. F. *Met. Ions Biol. Syst.* **2001**, *38*, 213.
(24) Zhu, L.; Kostić, N. M. *Inorg. Chem.* **1992**, *31*, 3994.
(25) Zhu, L.; Kostić, N. M. *J. Am. Chem. Soc.* **1993**, *115*, 4566.
(26) Zhu, L.; Kostić, N. M. *Inorg. Chim. Acta* **1994**, *217*, 21.
(27) Korneeva, E. N.; Ovchinnikov, M. V.; Kostić, N. M. *Inorg. Chim. Acta* **1996**, *243*, 9.
(28) Parac, T. N.; Kostić, N. M. *J. Am. Chem. Soc.* **1996**, *118*, 5946.
(29) Parac, T. N.; Kostić, N. M. *J. Am. Chem. Soc.* **1996**, *118*, 51.
(30) Qiao, F.; Hu, J.; Zhu, H.; Luo, X.; Zhu, L.; Zhu, D. *Polyhedron* **1999**, *8*, 1629.
(31) Luo, X.; He, W.; Zhang, Y.; Guo, Z.; Zhu, L., *Chem. Lett.* **2000**, 1030.
(32) Milović, N. M.; Kostić, N. M. *Met. Ions Biol. Syst.* **2001**, *38*, 145.
(33) Milović, N. M.; Kostić, N. M. *J. Am. Chem. Soc.* **2002**, *124*, 4759.
(34) Kaminskaia, N. V.; Johnson, T. W.; Kostić, N. M. *J. Am. Chem. Soc.* **1999**, *121*, 8663.
(35) Kaminskaia, N. V.; Kostić, N. M. *Inorg. Chem.* **2001**, *40*, 2368.
(36) Chen, X.; Zhu, L.; You, X.; Kostić, N. M. *J. Biol. Inorg. Chem.* **1998**, *3*, 1.
(37) Karet, G. B.; Kostić, N. M. *Inorg. Chem.* **1998**, *37*, 1021.

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[a]



[a] Mechanisms are designated external and internal according to the origin of the nucleophile (water).

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), that is, 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, that is, 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 two kinds of anchors 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 this cleavage directed by the amino-terminus occurred with our Pd(II) proteases, it 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 of cleaving peptides in which the terminal amino group is the only potential anchor for the Pd(II) complex. Next, we investigate the interplay between the N-terminus and a histidine residue in positions 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 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 sulfone 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, an 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; a 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). Solvent B was absent from the eluent for 5 min after the injection of the sample, and then, 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

(38) Zhu, L.; Kostić, N. M. *Inorg. Chim. Acta*, in press.
(39) Buckingham, D. A. *Met. Ions Biol. Syst.* **2001**, *38*, 43.
(40) Elding, L. I. *Inorg. Chim. Acta* **1972**, *6*, 647.
(41) Broennum, B.; Johansen, H. S.; Skibsted, L. H. *Acta Chem. Scand.* **1989**, *43*, 975.
(42) Mehal, G.; Van Eldik, R. *Inorg. Chem.* **1985**, *24*, 4165.
(43) Covington, A. K.; Paabo, M.; Robinson, R. A.; Bates, R. G. *Anal. Chem.* **1968**, *40*, 700.

Chart 2. Substrates for Binding to Pd(II) Complexes and Possible Hydrolytic Cleavage by These Complexes[a]

substrate	sequence
oxidized[b] 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[b] 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] Anchoring side chains are highlighted. [b] Cys^{OX} is cysteinic acid.

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 and heated for 5 min at 95 °C; 20.0 μL of this mixture was loaded into the well. The gels were blotted applying a 50.0 V potential for 4 h onto a PVDF membrane by a semidry procedure using a Trans-Blot SD system equipped with a 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 sulfone 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 an 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

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. A strongly acidic solution would promote undesirable "background" cleavage. A 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

(44) Schaegger, H.; Von Jagow, G. *Anal. Biochem.* **1987**, *166*, 368.

of the Pd(II) reagent with the area of the signal for the intact peptide showed that 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.

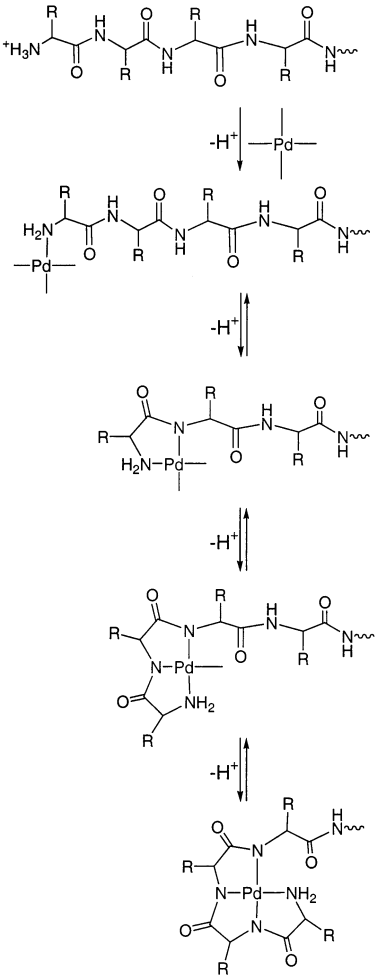
Choice of Substrates. The biologically active, natural peptides in Chart 2 are chosen because they contain methionine or histidine residue at various positions in the sequence. To check whether the same regioselectivity is observed with proteins, we chose also ubiquitin and cytochrome *c*. These two 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 equiv of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were kept at pH 2.0 and 60 °C. The chromatogram after 3 days 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 days 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 chain A of insulin but does not cleave it.

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 the 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.

Anchoring Side Chain at Positions 1 and 2. When the 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 Scheme 2a,b.[50−54] Peptides containing an

Scheme 1[a]



[a] 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 because other amide groups cannot approach the Pd(II) ion.

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 day).

When a peptide containing a histidine or a methionine as the second residue is mixed with the complex *cis*-$[Pd(en)Cl_2]$, tridentate complex **3** is the major product even at pH values

(45) Aswad, D. W. *Deamidation and Isoaspartate Formation in Peptides and Proteins*; CRC Press: Boca Raton, FL, 1995.
(46) Appleton, T. G. *Coord. Chem. Rev.* **1997**, *166*, 313.
(47) Agoston, C. G.; Jankowska, T. K.; Sovago, I. *J. Chem. Soc., Dalton Trans.* **1999**, 3295.
(48) Wilson, E. W., Jr.; Martin, R. B. *Inorg. Chem.* **1970**, *9*, 528.
(49) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385.
(50) Rabenstein, D. L.; Isab, A. A.; Shoukry, M. M. *Inorg. Chem.* **1982**, *21*, 3234.
(51) Wienken, M.; Zangrando, E.; Randaccio, L.; Menzer, S.; Lippert, B. *Dalton Trans.* **1993**, 3349.
(52) Milinković, S. U.; Parac, T. N.; Djuran, M. I.; Kostić, N. M. *J. Chem. Soc., Dalton Trans.* **1997**, 2771.
(53) Tsiveriotis, P.; Hadjiliadis, N.; Stavropoulos, G. *Inorg. Chim. Acta* **1997**, *261*, 83.
(54) Kozlowski, H.; Bal, W.; Dyba, M.; Kowalik-Jankowska, T. *Coord. Chem. Rev.* **1999**, *184*, 319.
(55) Siebert, A. F. M.; Sheldrick, W. S. *J. Chem. Soc., Dalton Trans.* **1997**, 385.

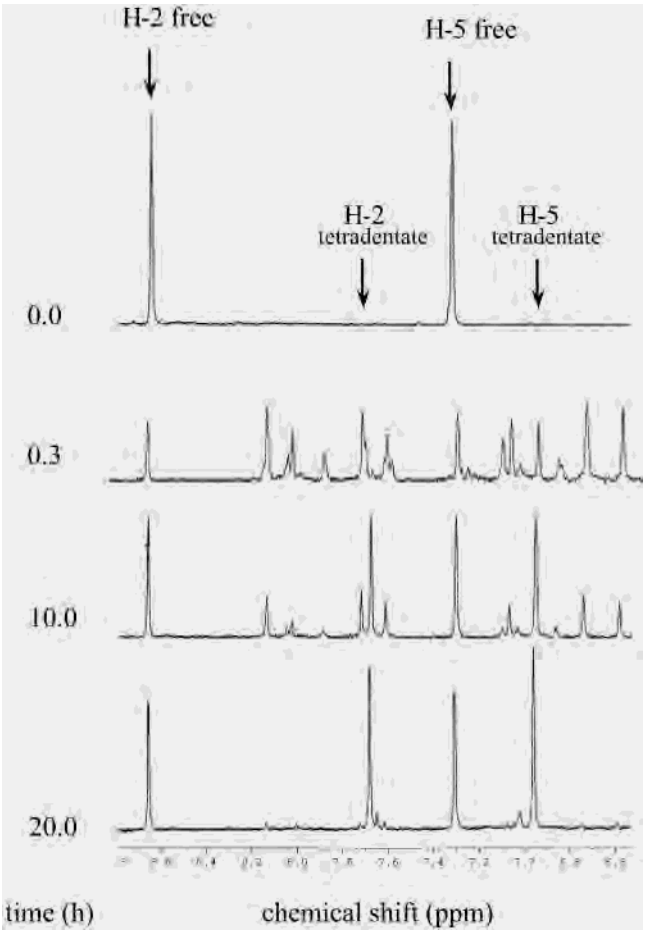


Figure 2. Proton NMR spectra of the peptide Val-Ile-His-Asn before, and at three times after, the addition of an equimolar amount of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0 and 60 °C.

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.

Anchoring Side Chain at Position 3. Because 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 ^{1}H 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 an 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 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 5 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 ^{1}H 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 $(1.0 \pm 0.1) \times 10^{-3}$ 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.

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

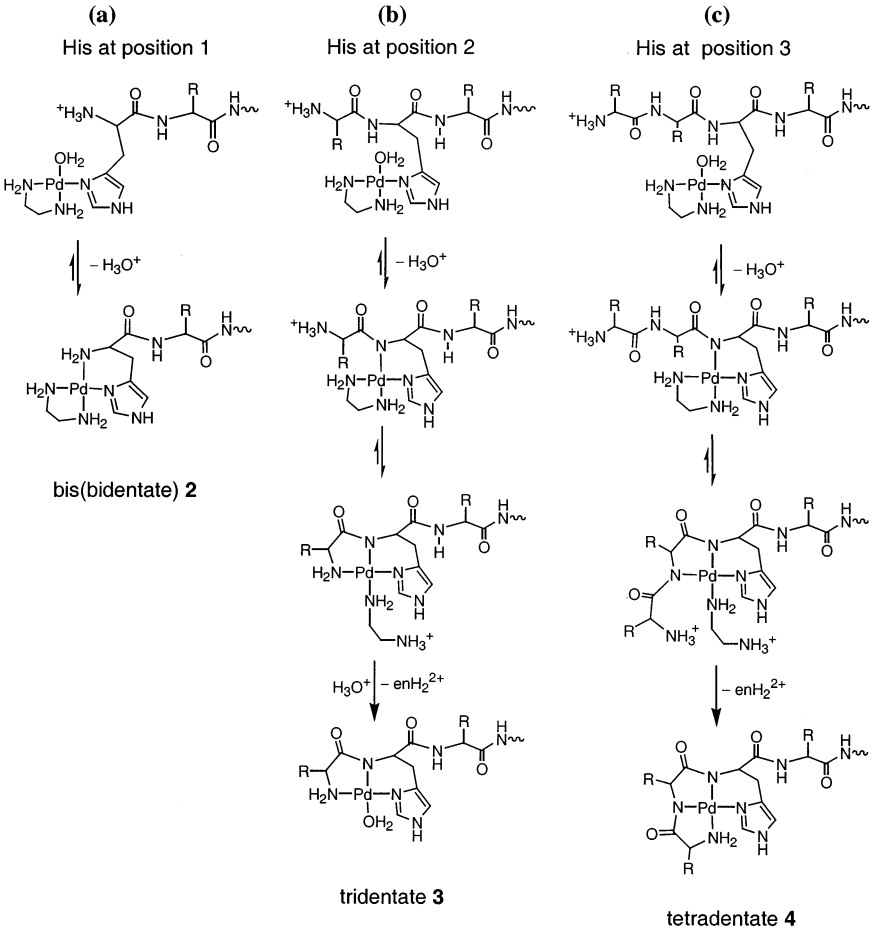
$$enH_2^{2+} + \textit{cis}\text{-}[Pd(en)(H_2O)_2]^{2+} \rightarrow [Pd(en)_2]^{2+} + 2H_3O^+ \quad (2)$$

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 such as 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,

(56) Appleton, T. G.; Ross, F. B. *Inorg. Chim. Acta* **1996**, *252*, 79.
(57) Harford, C.; Sarkar, B. *Acc. Chem. Res.* **1997**, *30*, 123.

Scheme 2[a]



[a] "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. Pd(II) ion anchored to a methionine side chain behaves similarly.

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 analogue 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.

Anchoring Side Chain in Positions Beyond 3: Cleavage of Chain B of Insulin. The 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.

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

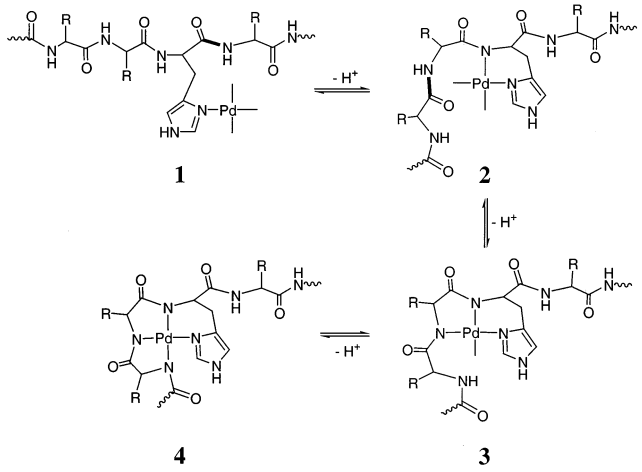
-Tyr-Leu-Val-Cys^{OX}-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

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

Table 1. Results of HPLC Separation and MALDI Mass Spectrometric Identification of the Fragments Obtained When Oxidized Chain B of Insulin Was Cleaved by the Reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$

elution time (min)	molecular mass (D) obsd	molecular mass (D) calcd	fragment
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

Scheme 3. Equilibrium among Complexes that a Pd(II) Ion Forms with Peptides Having a Histidine Residue at a Position beyond 3[a]



[a] The scissile bonds (the first one downstream and the second one upstream 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.

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, resulting from nonselective cleavage after glutamate residues, were also observed. They were 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 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, the 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. 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 are 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 noncoordinating side chains flanking the anchor do not alter the coordination modes.[46,49,54,59−61] Therefore, the regioselective cleavage in 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 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

(58) Pettit, L. D.; Pyburn, S.; Bal, W.; Kozlowski, H.; Bataille, M. *J. Chem. Soc., Dalton Trans.* **1990**, 3565.
(59) Pettit, L. D.; Bezer, M. *Coord. Chem. Rev.* **1985**, *61*, 97.
(60) Kasselouri, S.; Garoufis, A.; Lamera-Hadjiliadis, M.; Hadjiliadis, N., *Coord. Chem. Rev.* **1990**, *104*, 1.
(61) Bal, W.; Chmurny, G. N.; Hilton, B. D.; Sadler, P. J.; Tucker, A. *J. Am. Chem. Soc.* **1996**, *118*, 4727.

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
	obsd	calcd	
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 H_ϵ 0.88, H_γ 0.98 His H-2 7.62, H-5 6.96	Ile-His
21.2	Val H_γ 0.76, 0.82 Tyr H_δ 6.68, H_ϵ7.05	Val-Tyr
23.1	Pro H_β 1.80, H_γ 2.30 Phe $H_{\delta,\epsilon,\xi}$ 7.23	Pro-Phe

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 one downstream, and the Tyr-Ile bond, the second one upstream, as shown.

Asp-Arg-Val-Tyr-Ile-**His**-Pro-Phe

We followed the kinetics of this dual cleavage in experiments with 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 in following paragraphs.

Val-Tyr-Ile-**His**-Pro-Phe

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 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,c), and several intermediates (Figure 3d). The His-Pro bond is cleaved with the first-order rate constant $k_1 = 2.0 \times 10^{-3}$ min^{-1}. This process occurs without a lag period, and we call it primary cleavage. That concentrations of the intermediates vary at different rates

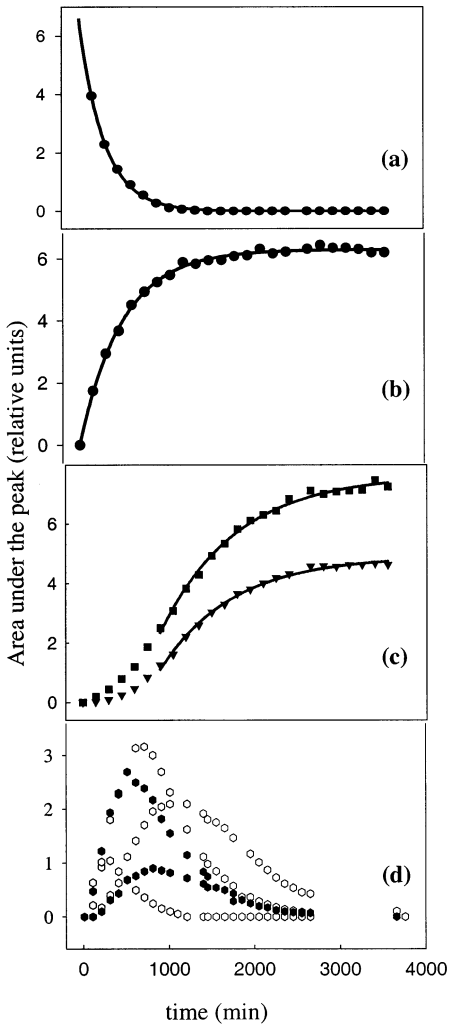
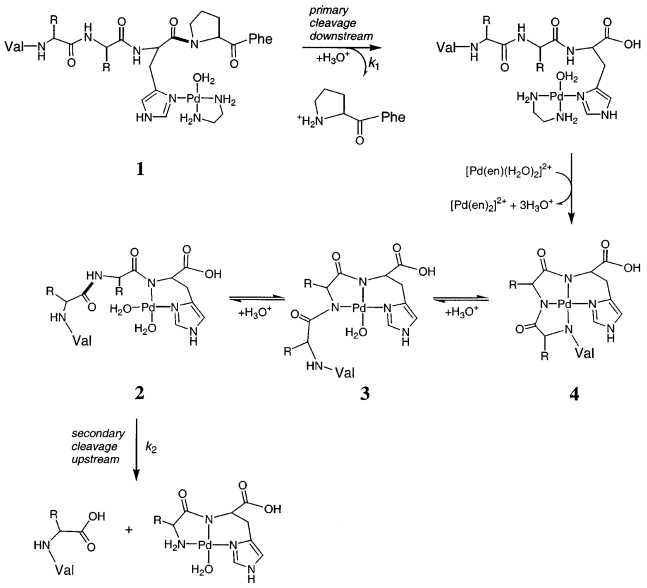


Figure 3. Progress of cleavage of the peptide Val-Tyr-Ile-His-Pro-Phe (segment 3−8 of angiotensin II), by the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ followed by HPLC. Solid lines are exponential fittings to first-order kinetic law. The mechanism is shown in Scheme 4. (a) Disappearance of the intact peptide. (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 contain 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).

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, 635.8, 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, $k_2 = 1.1 \times 10^{-3}$ min^{-1}.

Scheme 4. Mechanism of Cleavage of the Peptide Val-Tyr-Ile-His-Pro-Phe (Segment 3−8 of Angiotensin II), Promoted by the Reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ [a]



[a] 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.

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 His14 immediately upon mixing and forms a 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.

(62) Martin, R. B. *Met. Ions Biol. Syst.* **2001**, *38*, 1.

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
	obsd	calcd	
9.9	1133.2	1133.4	Leu67···Gly76
12.8	7436.0	7449.4	Met1···Thr66

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 potential anchors, Met1 and His68, 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 a 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.

1 **M**QIFVKTLTGKTITLEVEPSDTIENVKAKI 30

31 QDKEGIPPDQQRLIFAGKQLEDGRTLSDYN 60

61 IQKEST|L**H**LVLRLRGG 76

We can readily understand these results. Coordination of the N-terminal methionine residue does not result in proteolytic cleavage, as explained previously. 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.

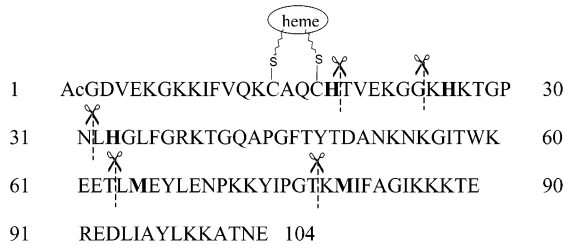
Control of Regioselectivity by Adjustment of the Reagent-to-Protein Ratio: Cleavage of Cytochrome *c*. The reaction mixture containing cytochrome *c* and 10 mol equiv of the reagent *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0 remained clear after incubation for 24 h. Electrophoretograms of this mixture and of the control solution free of Pd(II) reagent 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.

Table 5. 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) obsd	molecular mass (D) calcd	fragment
und	12357.9	12370.3	AcGly1···Glu104[c]
B	9701.2	9701.6	Thr19···Glu104
C	9129.5	9130.1	Lys25···Glu104
nd	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
nd	4901.5	4908.2	Thr19···Thr63
nd	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
nd	1352.5	1350.6	Thr19···Asn31

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

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*.



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", that is, flanked by noncoordinating 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 noncoordinating residues, the regioselectivity observed with peptides persisted in this protein. The His18, however, is not an "isolated" anchor because 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 thioether group, as shown in the simplistic drawing in the protein sequence above. This thioether group, too, can bind to the Pd(II) reagent. It is likely that both adjacent residues, His18 and Cys17, anchor the Pd(II) ion and prevent its binding to the upstream amide nitrogen atom and the 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 10-fold molar excess over the protein and in 2-fold 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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Supporting Information Available: Additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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