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# Combined Use of Platinum(II) Complexes and Palladium(II) Complexes for Selective Cleavage of Peptides and Proteins

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This study shows, for the first time, the advantages of combining two transition-metal complexes as selective proteolytic reagents. In this procedure, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is followed by $[Pd(H_2O)_4]^{2+}$. In the peptide AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala, the Pt(II) reagent cleaves the Met6-Ala7 peptide bond, whereas the Pd(II) reagent cleaves the Gly4-Gly5 bond. In the peptide AcVal-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala, the Pt(II) reagent cleaves the Met11-Ala12 peptide bond, whereas the Pd(II) reagent cleaves the Gly3-Gly4 bond. All cleavage reactions are regioselective and complete at pH 2.0 and 60 °C. Each metal ion binds to an anchoring side chain and then, as a Lewis acid, activates a proximal peptide bond toward hydrolysis by the solvent water. The selectivity in cleavage is a consequence of the selectivity in this initial anchoring. Both Pt(II) and Pd(II) reagents bind to the methionine side chain, whereas only the Pd(II) reagent binds to the histidine side chain under the reaction conditions. Consequently, only methionine residues direct the cleavage by the Pt(II) reagent, whereas both methionine and histidine residues direct the cleavage by the Pd(II) reagent. The Pt(II) reagent cleaves the first bond downstream from the anchor, i.e., the Met-Z bond. The Pd(II) reagent cleaves the second bond upstream from the anchor, i.e., the X–Y bond in the X-Y-Met-Z and in the X-Y-His-Z segments. The diethylenetriamine complex $[Pt(dien)(H_2O)]^{2+}$ cannot promote cleavage. Its prior binding to the Met11 residue in the second peptide prevents the Pd(II) reagents from binding to Met11 and cleaving the Gly9-Gly10 bond and directs the cleavage by the Pd(II) reagent exclusively at the Gly3-Gly4 bond. Our new method was tested on equine myoglobin, which contains 2 methionine residues and 11 histidine residues. The complete methionine-directed cleavage of the Met55-Lys56 and Met131-Thr132 bonds by the Pt(II) reagent produced three fragments, suitable for various biochemical applications because they are relatively long and contain amino and carboxylic terminal groups. The deliberately incomplete histidine-directed cleavage of the long fragments 1···55 and 56···131 at many sites by the Pd(II) reagent produced numerous short fragments, suitable for protein identification by mass spectrometry. The ability of combined Pt(II) and Pd(II) complexes to cleave proteins with explicable and adjustable selectivity and with good yields bodes well for their greater use in biochemical and bioanalytical practice.

## Introduction

Hydrolytic cleavage of proteins is an important and widely used biochemical procedure. Besides the familiar use for protein sequencing, proteolytic cleavage with variable and controllable selectivity is necessary also for several new bioanalytical and bioengineering applications. For example, protein footprinting and studies of protein folding employ limited and relatively nonselective proteolysis of solvent-exposed segments to provide structural information.[1] Proteomics requires selective digestion of the expressed proteins into fragments 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 proteins.[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]

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A small number of enzymes and synthetic reagents are available for selective proteolysis. 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 substrate protein. Despite their catalytic superiority, proteases are sometimes inadequate. They tend to produce short fragments unsuitable for bioanalytical applications, and they are proteinaceous contaminants of the protein digests that they create.

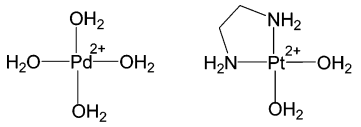
The existing chemical reagents are less effective than the enzymes because they often require harsh conditions and high molar excess and often give only partial selectivity and relatively low yields.[5] For example, cyanogen bromide, a commonly used methionine-selective cleaver, has several shortcomings. It is volatile and toxic, is applied in up to a 100-fold excess over the methionine residues in the substrate, requires 70% formic acid as the solvent, causes various side-reactions, and irreversibly converts the methionine residues to serine lactone.

Emerging biochemical applications would benefit from a broader choice of chemical reagents for protein cleavage, reagents having improved efficiency and selectivity. Hydrolytic cleavage, which leaves the protein fragments pristine, is preferred over oxidative cleavage, which causes irreversible chemical modifications of the fragments. Finding such reagents is a formidable task because the amide bond in peptides and proteins is extremely unreactive toward hydrolysis. Under standard conditions (room temperature and pH 4−8), the half-life for nonselective hydrolysis of a simple peptide is 500−1000 years.[6−8] Controlled and selective cleavage, an 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 sites. 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 reviewed,[12,23,24] but there are few reports of regioselective cleavage of proteins. To our knowledge, combined use of different metal complexes acting with different selectivity has not yet been reported.

Complexes of palladium(II) and of platinum(II), two chemically similar transition-metal ions, are new reagents for selective, hydrolytic cleavage of peptides and proteins.[22,25−27] Palladium(II) complexes cleave His-aa and Met-aa bonds in *N*-acetylated dipeptides of the type AcHis-aa and AcMet-aa, in which aa represents various amino acids.[22] These studies were very useful in explaining the reaction mechanism, but they do not explain the regioselectivity in the cleavage of proteins and longer peptides, in which the Pd(II) ion can interact with the backbone on the amino side, as well as on the carboxy side, of the anchoring side chain. The present study deals with longer peptides and a protein, substrates with which our reagents display their true regioselectivity.[25]

The most effective reagents are $[Pd(H_2O)_4]^{2+}$ and *cis*-$[Pt(en)(H_2O)_2]^{2+}$, whose structures are shown. These metal




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

complexes surprisingly differ in selectivity, as Chart 1 shows. The cleavage by $[Pd(H_2O)_4]^{2+}$ in weakly acidic aqueous solutions consistently occurs at the X-Y bond in the sequence segments X-Y-His-Z and X-Y-Met-Z, in which X, Y, and Z can be any noncoordinating residues. Histidine and methionine residues bind the Pd(II) reagent and direct its action. As the pH of the solution is raised from mildly acidic to neutral, this Pd(II)-promoted selective cleavage becomes specific to X-Pro-His-Z and X-Pro-Met-Z segments, i.e., those in which Y is a prolyl residue.[28] In nonaqueous solutions, Pd(II) reagents act with a different (but explainable) selectivity and promote tryptophan-directed cleavage of amide bonds,[29,30] a reaction potentially useful for cleavage of lipophilic proteins.

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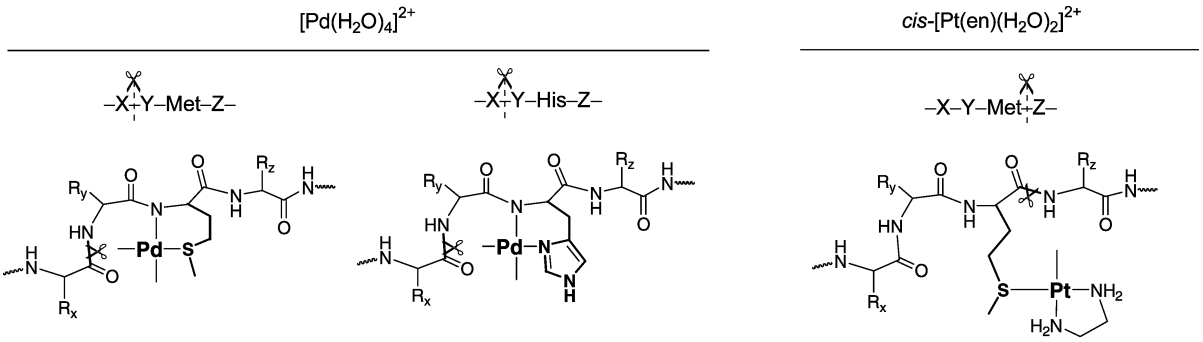
**Chart 1.** Different Cleavage Selectivity of Pd(II) and Pt(II) Complexes, Which Form Different Hydrolytically Active Complexes with the Substrate and Therefore Promote Hydrolytic Cleavage of Different Peptide Bonds.

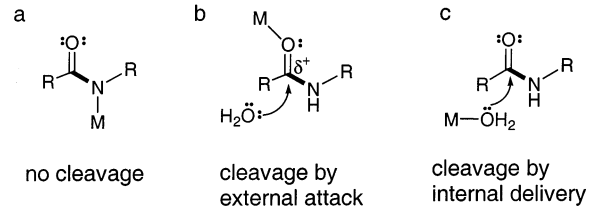



The cleavage of peptides and proteins by *cis*-[Pt(en)-$(H_2O)_2]^{2+}$ in weakly acidic aqueous solutions occurs at the Met-Z bond.[27] We investigated the coordination of histidine-containing and methionine-containing oligopeptides to both Pd(II) and Pt(II) reagents and the dependence of the cleavage rates on solution pH. For both reagents, we identified the hydrolytically active complexes, shown in Chart 1, and realized that the weakly acidic solution is necessary to suppress their conversion to inactive complex(es).[25−27]

Under the reaction conditions, only the side chains of histidine, methionine, and free (reduced) cysteine can bind to Pd(II) ion, and only the last two to Pt(II) ion.[22,31] Fortunately, cysteine residues usually exist as disulfide, a functional group unknown to coordinate to these metal ions. Coordinating residues as X, Y, and Z would likely prevent the formation of the hydrolytically active complexes in Chart 1, thus inhibiting hydrolysis. The patterns of cleavage stated in the preceding two paragraphs were consistently obtained with natural peptides and proteins containing "isolated" anchors and various noncoordinating X, Y, and Z residues: aromatic and aliphatic, polar and nonpolar, charged and neutral.[25−28]

Because the amide group is a poor ligand for transition-metal ions in aqueous solution, this group in peptides and proteins interacts only with the metal ion that is already anchored to a side chain or to the terminal amino group. The anchored metal ion containing at least one accessible coordination site (weakly held by an aqua ligand) can interact with the amide group in different ways, as shown simplistically (without the anchor) in Chart 2. This interaction can either inhibit or promote hydrolytic cleavage. The anchored metal ion can deprotonate the amide NH group and bind the nitrogen atom of the resulting amidate group (Chart 2a).[23,31,32] The coordination of the amidate anion strengthens the C−N bond, protects the amide carbon from nucleophilic attack, and thus inhibits hydrolysis of this peptide bond. The anchored metal ion can also bind to the oxygen atom in the amide group (Chart 2b). This binding 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.[23,31,32] The anchored metal ion can conceivably promote cleavage of a proximal amide group even without binding to it (Chart 2c). In this case, the anchored metal ion can conceivably deliver its aqua ligand to the scissile peptide bond. Both mechanisms b and c in Chart 2 can explain the hydrolytic activity of Pd(II) and Pt(II) reagents in acidic solution, but these two mechanisms cannot be distinguished by purely kinetic methods.[9] We recently gave multiple, albeit indirect, evidence for the external attack, as in Chart 2b.[28]

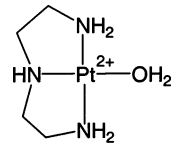
**Chart 2.** Possible Interactions between Anchored Pt(II) or Pd(II) Ion (designated M) and a Proximate Amide Group[a]




[a] For clarity, the anchoring is not shown. (a) Binding to the nitrogen atom requires deprotonation of the NH group and results in the inhibition of the hydrolytic cleavage. (b) Binding to the oxygen atom enhances the electrophilicity of the carbon atom and activates the amide group toward hydrolytic cleavage by solvent water. (c) Close approach by the metal ion aids delivery of its aqua ligand to the carbon atom and thus promotes hydrolytic cleavage of the amide bond.

The selectivity of cleavage, the subject of this study, is determined by the stereochemistry of coordination and can be understood regardless of the actual mechanism of the hydrolytic step. Two conditions must be fulfilled for the activation of an amide group toward cleavage. First, the anchored metal ion must approach this group. Second, the metal ion must retain at least one aqua ligand after anchoring. 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).[25,31,32] If either condition is absent, cleavage does not occur.[26]

In the present study, we use peptide substrates to demonstrate for the first time that Pd(II) and Pt(II) reagents can be used together, to obtain various cleavage patterns. These two reagents can act successively on the same anchor, or



$[Pt(dien)(H_2O)]^{2+}$

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they can be selectively delivered to different anchors. We also introduce the complex $[Pt(dien)(H_2O)]^{2+}$, in which dien is diethylenetriamine. This complex, shown above, can bind to methionine side chain, but it loses the only aqua ligand in this process and therefore cannot cleave the polypeptide backbone. Instead, the bound $Pt(dien)^{2+}$ group prevents binding of the Pd(II) reagent and inhibits the cleavage directed by the blocked methionine residue. The selectivity in cleavage is a consequence of the selectivity in prior coordination. Because both histidine and methionine residues bind to the Pd(II) reagent, both of them direct the Pd(II)-promoted cleavage. Because only methionine residue binds to the Pt(II) reagent under the reaction conditions, only methionine directs the Pt(II)-promoted cleavage. The selectivity of cleavage is a consequence of the modes of anchoring of the reagents to the reactive segments. Only upon this 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 our new reagents. We use *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$ in succession to cleave myoglobin, a protein containing 2 methionine residues and 11 histidine residues. Cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is suitable for biochemical applications that require large fragments. The fragments can be easily separated, and they remain pristine, i.e., unmodified. Further cleavage of these fragments by $[Pd(H_2O)_4]^{2+}$ is suitable for the identification of the original protein because it yields many small fragments, easily detectable by mass spectrometry. The ability of combined 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), human angiotensin II, oxidized chain B of human insulin, equine cytochrome *c*, and equine myoglobin were obtained from Sigma Chemical Co. Palladium sponge, *cis*-$[Pt(en)Cl_2]$, $K_2[PdCl_4]$, piperidine, trifluoroacetic acid (TFA), 3,6-dithia-1,8-octanediol, 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.

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 C-18 preparative column, as described previously.[25] The purity, examined by analytical HPLC, was higher than 99.5%; the molecular masses (in D) were as follows: for Met-peptide, 1036.51 (calcd) and 1036.57 (found); for HisMet-peptide, 1514.78 (calcd) and 1515.08 (found).

The stock solution of the complex $[Pd(H_2O)_4]^{2+}$ and the pure complexes *cis*-$[Pd(en)(H_2O)_2]^{2+}$, *cis*-$[Pt(en)(H_2O)_2]^{2+}$, and $[Pt(dien)(H_2O)]^{2+}$ (dien is diethylenetriamine) were prepared as perchlorate salts by published procedures.[33−36] The concentrations were determined using their published absorptivities (extinction coefficients).

**HPLC Separations.** The components of the digests were separated by a Hewlett-Packard 1100 HPLC system containing an autosampler and a multiwavelength detector set to 215, 270, and 410 nm. Absorption at 215 nm is common to all peptides and proteins, absorption at 270 nm is due to aromatic residues and the bound Pt(II) ion, and absorption at 410 nm is diagnostic of heme. In the reverse-phase separations, an analytical Supelco discovery C-18 column (sized 250 mm × 4.6 mm, beads of 5 μm) and a preparative Vydac C-18 column 218TP101522 (sized 250 mm × 22 mm, beads of 10 μm) were used in the experiments involving peptides; an analytical Vydac C-5 column 214TP54 (sized 150 mm × 4.6 mm, beads of 5 μm) and a preparative Discovery bio wide pore C-5 (sized 250 mm × 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 0.0% for 5 min after the injection of the sample and then raised gradually to 45.0% over a 35-min period. The flow rate was 1.0 mL/min in the analytical runs and 10.0 mL/min in the preparative runs. The Superdex peptide HR 10/30 column, with optimal separation range from 1.0 to 7.0 kD, was used in the size-exclusion separations. 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) or 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 Pt(II) or Pd(II) ions, 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.** 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 as long as 1 day, and 20.0-μL samples were taken periodically. In the experiment with Met-peptide, 200.0 μL of a 5.0 mM substrate solution was mixed with 10.0 μL of a 100.0 mM stock solution of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and 700.0 μL of water. After 12 h, 100 μL of a 100 mM solution (a 10-fold excess) of 3,6-dithia-1,8-octanediol was added to the reaction mixture, and the two peptide fragments were separated by preparative HPLC and lyophilized to dryness. A 1.0 mL aqueous solution of the methionine-containing fragment was mixed with 1.0 μL of a 500 mM stock solution of $[Pd(H_2O)_4]^{2+}$, and the pH was adjusted to 2.0. In a typical experiment with HisMet-peptide, involving equimolar amounts of the Pt(II) reagent and the methionine residue in the substrate, 200.0 μL of a 5.0 mM HisMet-peptide solution was mixed with 10.0 μL of a 100.0 mM stock solution of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and 700.0 μL of water. For the cleavage of HisMet-peptide with combined Pt(II) and Pd(II) reagents, the equimolar mixture of the peptide and *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.0 was kept for 1.0 h at 60 ± 1 °C, and then an equimolar amount of $[Pd(H_2O)_4]^{2+}$ was added. For the cleavage of

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myoglobin by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, 600.0 $\mu$L of a 1.0 mM solution was made 5.0 mM in the cleavage reagent (by adding 30.0 $\mu$L of a 100 mM solution of the reagent) and 0.20 mM in methyl phenyl sulfone (the internal standard), and the mixture was kept for 24 h at pH 2.5. The fragments were separated by preparative reverse-phase HPLC and lyophilized to dryness.

The two of three myoglobin fragments produced by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ cleavage that contained histidine residues were next cleaved by $[Pd(H_2O)_4]^{2+}$. To a solution of 5.0 mg of the fragment in 800.0 $\mu$L of water was added the required volume of a 500 mM solution of $[Pd(H_2O)_4]^{2+}$, so that the Pd(II) reagent was equimolar with histidine residues in the fragment. The mixture was kept at pH 2.0 and 60 ± 1 °C. A 1.0-$\mu$L sample taken from the digest after 12 h 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 reactions were followed by reverse-phase HPLC in the case of peptide substrates and by gel-exclusion combined with reverse-phase HPLC in the case of myoglobin. The separated fragments were lyophilized to dryness, redissolved, and analyzed by MALDI-TOF mass spectrometry and Edman N-terminal sequencing.

Because the cleavage reactions are very slow at room temperature, the species distribution in the chromatographic separations corresponded to those in the digest samples. In the kinetic measurements with myoglobin, 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 for the cleavage products versus time were fitted to the first-order rate law with SigmaPlot v. 5.0, obtained from SPSS Inc.

## Results and Discussion

**Selective Cleavage of Met-Peptide by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and by $[Pd(H_2O)_4]^{2+}$ in Succession.** An equimolar mixture of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) and *cis*-$[Pt(en)(H_2O)_2]^{2+}$ was kept at pH 2.0 for 12 h. The chromatogram of this solution lacked the signal of the intact peptide, eluting at 20.8 min, and contained two fractions, eluting at 4.9 and 14.3 min; see Figure 1a and b. A solution containing 10 equiv of 3,6-dithia-1,8-octanediol was added to the reaction mixture, to displace the Pt(II) reagent from the Met6 anchor during 1 h at 60 °C. The fragments of Met-peptide were chromatographically separated and lyophilized to dryness. The MALDI mass spectra showed the slower fraction to be AcAla-Lys-Tyr-Gly-Gly-Met (obsd 667.30 D and calcd 667.27 D) and the faster fraction to be Ala-Ala-Arg-Ala (obsd 387.95 D and calcd 388.22 D). A solution containing the former (methionine-containing) fragment and an equimolar amount of $[Pd(H_2O)_4]^{2+}$ was kept for 24 h at 60 °C and analyzed as shown in Figure 1c. The two products, eluting at 5.2 and 11.6 min, were identified by MALDI mass spectrometry as fragments Gly-Met (obsd 206.64 D and calcd 206.07 D) and AcAla-Lys-Tyr-Gly (obsd 479.62 D and calcd 479.29 D), respectively.

Scheme 1 shows that both cleavage reactions are directed by the same residue, Met6, but that they occur on the opposite sides of this residue. The Pt(II) reagent promoted

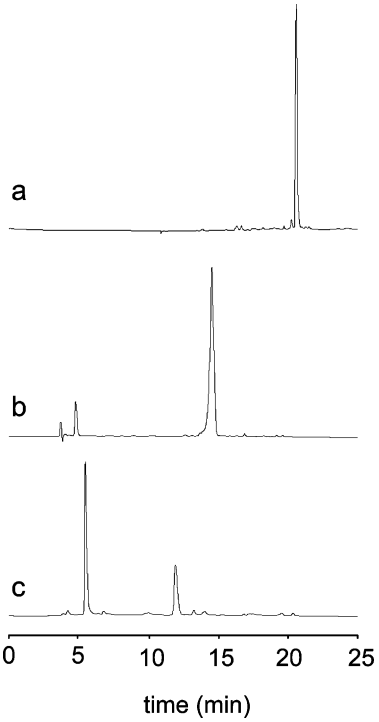
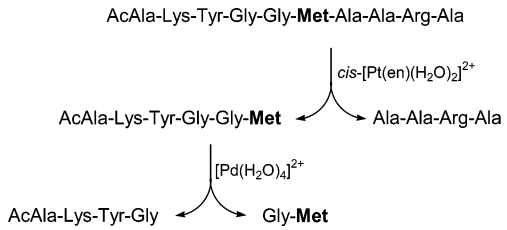



**Figure 1.** Monitoring by HPLC of the successive cleavage of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) by equimolar amounts of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and of $[Pd(H_2O)_4]^{2+}$ at pH 2.0 and 60 °C. Prior to cleavage by $[Pd(H_2O)_4]^{2+}$, the Pt(II) ion was removed by chelation with 3,6-dithia-1,8-octanediol. (a) The chromatogram at 270 nm of the intact peptide, eluting at 20.8 min. (b) The chromatogram at 215 nm of the substrate fragments produced by *cis*-$[Pt(en)(H_2O)_2]^{2+}$; the fractions eluting at 4.9 and 14.3 min are Ala-Ala-Arg-Ala and AcAla-Lys-Tyr-Gly-Gly-Met, respectively. (c) The chromatogram at 270 nm of the secondary fragments of the primary fragment AcAla-Lys-Tyr-Gly-Gly-Met; the fractions eluting at 5.2 and 11.6 min are Gly-Met and AcAla-Lys-Tyr-Gly, respectively.

**Scheme 1.** Two Proteolytic Reactions Directed by the Methionine Residue: Cleavage of the Met-peptide by the Pt(II) Reagent and Subsequent Cleavage of the Methionine-Containing Fragment by the Pd(II) Reagent




the cleavage of the first peptide bond downstream, the Met6-Ala7 bond, and the Pd(II) reagent promoted the cleavage of the second bond upstream, the Gly4-Gly5 bond. As Figure 1 shows, both cleavage reactions are selective and complete. For the first time, we demonstrate such clean cleavage by two different transition-metal complexes.

**Selective Cleavage of HisMet-Peptide Separately by $[Pd(H_2O)_4]^{2+}$ and 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 very recent study[25] showed that the reaction mixture containing only 1 equiv of $[Pd(H_2O)_4]^{2+}$ per substrate contained all possible products of cleavage directed by His5, by Met11, and by both of these anchors simultaneously. Such fragmentation proved that the Pd(II) reagent shows no binding

**Table 1.** Results of HPLC Separation and MALDI Mass-Spectroscopic Identification of Fragments of AcVal-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (HisMet-Peptide)[a]

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

[a] Resulting from the cleavage by an equimolar amount of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ followed by an equimolar amount of $[Pd(H_2O)_4]^{2+}$.

or cleaving preference for either of the two competing anchors and cleavs (incompletely by design) at the second peptide bond upstream from each of these two anchors. When HisMet-peptide was cleaved by 2 molar equiv of $[Pd(H_2O)_4]^{2+}$, under the same conditions, at the end of the reaction only three fragments were present. Now that His5 and Met11 did not have to compete for the Pd(II) reagent, cleavage directed by both anchors was complete. The selectivity remained the same as when the cleavage was set up to be incomplete.

When HisMet-peptide was treated by 1 or 2 molar equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, the result was the same: Only the Met11-Ala12 bond was cleaved. In both cases the reaction was complete, because only Met11 serves as the anchor for the Pt(II) reagent. Clearly, the cleavage selectivity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is different from that of $[Pd(H_2O)_4]^{2+}$. Having shown that these two reagents can be used separately and in succession, we next investigate whether they can be used together.

**Selective Cleavage of HisMet-Peptide by Both *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$.** A reaction mixture was prepared by adding to HisMet-peptide 1 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, keeping this solution for 1 h, and then adding 1 equiv of $[Pd(H_2O)_4]^{2+}$, all at 60 °C. After 1 day, the chromatogram of this mixture contained only three fractions. The MALDI measurements confirmed these three fractions, identified in Table 1. Again, a "clean" pattern of cleavage is obtained.

By adding the reagents in the right order, we selected the anchoring site for each reagent and achieved a variable and selective cleavage. The Pt(II) reagent binds completely to its only anchor, the side chain of Met11, denies this anchor to the Pd(II) reagent, and directs this second reagent to His5. Once the two metal complexes are selectively anchored, they both can promote hydrolytic cleavage of the backbone. The Pt(II) reagent cuts the first bond downstream from Met11, whereas the Pd(II) reagent cuts the second bond upstream from His5 (Chart 3).

**Selective Blocking by $[Pt(dien)(H_2O)]^{2+}$ and Selective Cleavage by $[Pd(H_2O)_4]^{2+}$ of HisMet-Peptide.** To a solution of HisMet-peptide were added an equimolar amount of $[Pt(dien)(H_2O)]^{2+}$ and, after 1 h, an equimolar amount of $[Pd(H_2O)_4]^{2+}$, all at 60 °C and pH 2.0. The chromatogram of this mixture after 1 day showed two fractions, identified in Table 2.

Clearly, the cleavage occurred only at His5, because Met11 was blocked by the $Pt(dien)^{2+}$ group. In a control experiment, an equimolar mixture of HisMet-peptide and

**Chart 3.** Different Patterns of Cleaving HisMet-Peptide by the Pt(II) and Pd(II) Reagents.

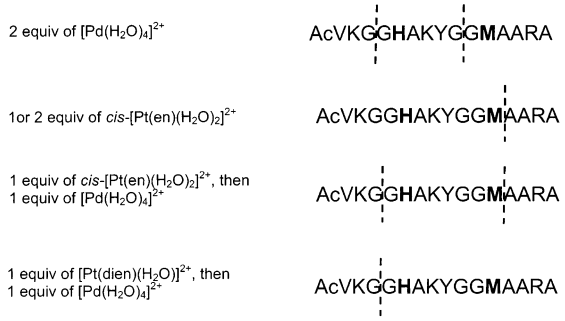



**Table 2.** Results of HPLC Separation and MALDI Mass-Spectroscopic Identification of Fragments of AcVal-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (HisMet-Peptide)[a]

| elution time (min) | molecular mass (D) obsd | molecular mass (D) calcd | fragment |
|---|---|---|---|
| 11.5 | 349.2 | 344.41 | 1···3 |
| 17.21 | 1188.98 | 1188.58 | 4···15 |

[a] Resulting from blocking of Met11 by $[Pt(dien)(H_2O)]^{2+}$, followed by His5 directed cleavage by $[Pd(H_2O)_4]^{2+}$.

$[Pt(dien)(H_2O)]^{2+}$ was 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. Clearly, the complex $[Pt(dien)(H_2O)]^{2+}$ binds to the substrate, but this binding does not result in cleavage. Although the methionine-anchored $Pt(dien)^{2+}$ group is proximal to the scissile peptide bond, the Pt(II) ion lacks an aqua ligand necessary for cleavage.

**Different Selectivities of Palladium(II) and Platinum(II) Reagents.** Although the aqua complexes of Pd(II) and Pt(II) are similar, they exhibit different selectivities and do so even when they act together. Each metal ion activates the peptide bond proximal to it in the hydrolytically active complex, which must contain at least one aqua ligand. This labile ligand can be either displaced by the carbonyl oxygen atom (in the likely mechanism, Chart 2b) or delivered to the carbonyl carbon atom of the scissile peptide bond (in the unlikely mechanism, Chart 2c).[25,27] Our explanation of selectivity in terms of coordination modes is valid, regardless of the actual mechanism in Chart 2.

In acidic solutions, the soft Lewis acid Pt(II) has a high affinity for the soft Lewis base thioether and a lesser affinity for the hard imidazole. The "borderline" Lewis acid Pd(II) shows affinity for both the thioether and the imidazole.[37−40] Significantly, the Pt(II) ion is much more inert than the Pd(II) ion. The rates of ligand substitution in Pt(II) complexes tend to be ca. $10^6$ times smaller than those in similar Pd(II) complexes.[41]

Different coordinations of methionine-containing sequences to Pd(II) and Pt(II) reagents are shown in Scheme

(37) Appleton, T. G. *Coord. Chem. Rev.* **1997**, *166*, 313−359.
(38) Burgeson, I. E.; Kostić, N. M. *Inorg. Chem.* **1991**, *30*, 4299−4305.
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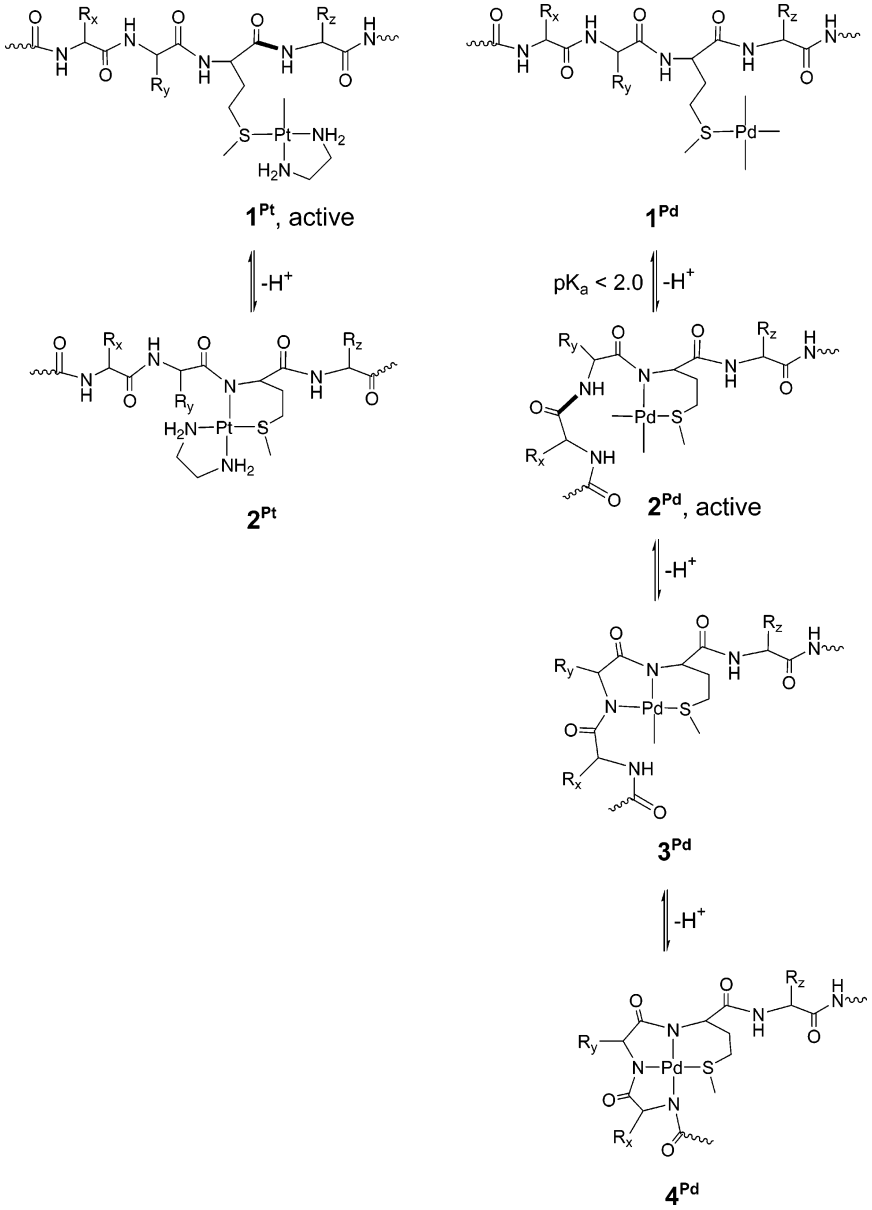
**Scheme 2.** Initial Binding of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and of $[Pd(H_2O)_4]^{2+}$ to a Methionine Anchor Results in Different Complexes of Pt(II) and Pd(II) with the Same Substrate (Peptide or Protein)[a]




[a] All the complexes are hydrolytically inactive, except those marked as active. The scissile bonds in the active complexes are highlighted. The unspecified ligands are $H_2O$ molecules.

2. Once anchored, the two metal ions form different hydrolytically active complexes under the mildly acidic conditions. The substrate (peptide or protein) remains bound to the Pt(II) atom in complex $\mathbf{1^{Pt}}$ as a unidentate ligand in acidic solutions. Increasing pH, however, favors the formation of the complex $\mathbf{2^{Pt}}$.[27] The anchored Pd(II) atom in complex $\mathbf{1^{Pd}}$ additionally binds to the amidate nitrogen atom upstream from the anchor and forms a bidentate complex $\mathbf{2^{Pd}}$.[25] The p$K_a$ for this chelation is less than 2.0.[31,37,42−46] Because Pt(II) is much more inert than Pd(II) to ligand substitution,[41] the ethylenediamine ligand remains coordinated to the former ion[47] but (if initially present) is displaced from the latter,[26] a process assisted by the protonation of ethylenediamine.

At pH 2.0, complexes $\mathbf{1^{Pt}}$ and $\mathbf{2^{Pd}}$ are major species.[25,27] The complex $\mathbf{1^{Pd}}$ is hydrolytically active, but absent under the reaction conditions because it is completely converted into complex $\mathbf{2^{Pd}}$.[25] Both complexes $\mathbf{1^{Pt}}$ and $\mathbf{2^{Pd}}$ are hydrolytically active, but with respect to different amide bonds. The anchored Pt(II) ion in $\mathbf{1^{Pt}}$ remains proximal to the peptide bond downstream from the methionine anchor (the Met-Z bond) and can activate it toward cleavage. The anchored Pd(II) ion in $\mathbf{2^{Pd}}$, however, is kept away from this bond and

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(43) Rabenstein, D. L.; Isab, A. A.; Shoukry, M. M. *Inorg. Chem.* **1982**, *21*, 3234−3236.
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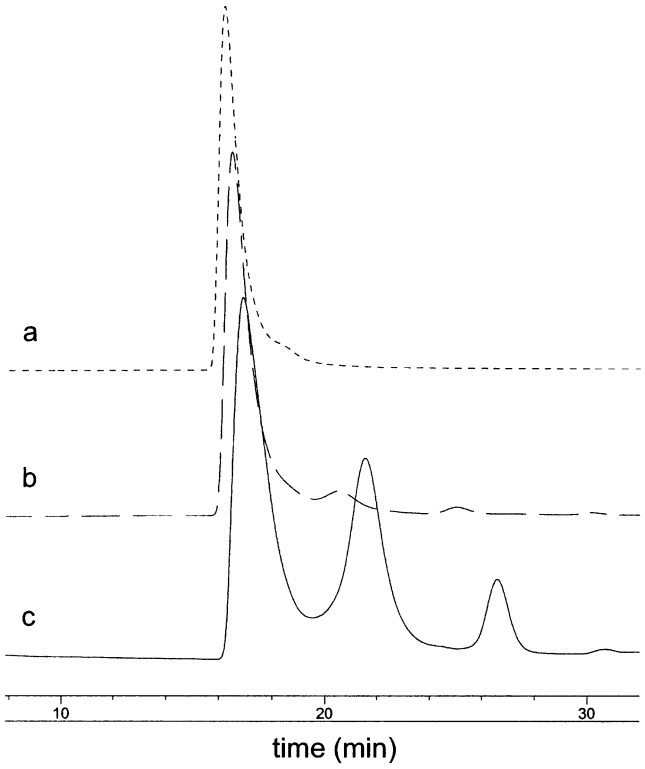


**Figure 2.** Size-exclusion chromatograms of myoglobin: (a) fresh solution; (b) after 24 at pH 2.5 and 60 °C; and (c) after 24 h at pH 2.5 and 60 °C in the presence of 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The fractions are identified in Table 3 and Chart 4.

**Table 3.** Cleavage of Equine Myoglobin By 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5[a]

| N-terminal sequence | elution time (min) | molecular mass (D) | | fragment |
|---|---|---|---|---|
| | | obsd | calcd | |
| GLSDG | 21.8 | 6265.81 | 6265.08 | 1···55 |
| KASED | 17.7 | 8213.26 | 8211.53 | 56···131 |
| TKALE | 26.9 | 2511.26 | 2513.89 | 132···153 |

[a] The fragments are separated by size-exclusion HPLC and identified by MALDI mass spectrometry and Edman N-terminal sequencing.

close to the second peptide bond upstream from the methionine anchor (the X-Y bond in X-Y-Met), which therefore becomes activated. As pH is raised, the anchored Pd(II) atom in complex $\mathbf{2^{Pd}}$ additionally binds the amidate nitrogen atoms further upstream, to form the tridentate complex $\mathbf{3^{Pd}}$ and the tetradentate complex $\mathbf{4^{Pd}}$. The complexes $\mathbf{2^{Pt}}$, $\mathbf{3^{Pd}}$, and $\mathbf{4^{Pd}}$ are hydrolytically inactive because the metal ions in them lack the aqua ligands necessary for the cleavage.[25,27]

The hydrolytically inactive complex $[Pt(dien)(H_2O)]^{2+}$ can be used to enhance the selectivity of Pd(II) reagents by restricting its cleavage to histidine residues, as already explained. After this reaction, the $Pt(dien)^{2+}$ group can easily be displaced from the methionine side chains by a superior nucleophile such as $SCN^-$, or $S_2O_3^{2-}$.

**Selective Cleavage of Myoglobin by *cis*-$[Pt(en)(H_2O)_2]^{2+}$.** The reaction of equine myoglobin with 5 equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5 and 60 °C was followed as in Figure 2. Intact myoglobin elutes at 16.6 min. After 1 day, the intact protein is replaced by three fragments, identified in Table 3. They prove that the cleavage is directed exclusively by the methionine residues, namely Met55 and

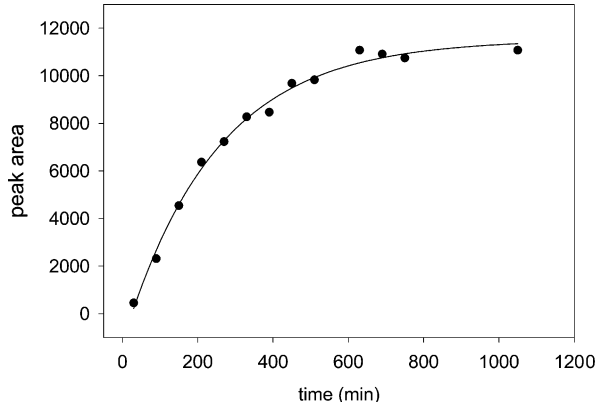


**Figure 3.** Progress of myoglobin cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.0 and 60 °C, followed by HPLC separations. The appearance of fragment 132···153, a product of cleavage, is shown. The solid line is a fitting to the first-order rate law.

Met131. Minor background cleavage, evident both in the absence and in the presence of the Pt(II) reagent, gave minor fractions eluting at 20.7 and 25.3 min. Fortunately, this background reaction accounted for less than 5% of total cleavage, as estimated from the chromatograms.

We followed the kinetics of myoglobin cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.0 by observing the growth of the peak corresponding to the fragment 132···153. This growth obeyed the first-order rate law, as shown in Figure 3. Because the binding of Pt(II) ion to the methionine side chain occurs within minutes and the subsequent intramolecular cleavage reactions take hours, fitting of the kinetic results to the first-order rate law is justified. The obtained rate constant of $4.1 \times 10^{-3}$ $min^{-1}$ corresponds to a half-life of 170 min.

The fragments of myoglobin were chromatographically separated and lyophilized. Evidently, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ can cleave proteins on a preparative scale and produce long fragments. Methionine residues rarely cluster in protein sequences, and their average abundance is only about 2.2%.[48] Therefore, the fragments obtained by methionine-specific cleavage of most proteins are, in principle, expected to be long. They can be used further for sequencing or for recombination into semisynthetic proteins. Because the cleavage is hydrolytic, the newly created amino and carboxylic termini can be coupled again, to form new peptide bonds.

The selectivity of c*is*-$[Pt(en)(H_2O)_2]^{2+}$ is the same as that claimed for the most commonly used synthetic protease, cyanogen bromide (CNBr), but *cis*-$[Pt(en)(H_2O)_2]^{2+}$ has several advantages.[27] It is nonvolatile, easy to handle, and recyclable; it is applied in approximately equimolar amounts with respect to methionine residues; the cleavage by it is residue-selective, with no side reactions; it leaves the protein fragments pristine, i.e., unmodified; it cleaves even the Met-Pro bond, which is resistant to CNBr and most proteolytic enzymes;[49,50] and it works even in the presence of the

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**Chart 4.** Amino Acid Sequence of Equine Myoglobin Showing Cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at the First Amide Bond "Downstream" from Methionine Residues

```
 1 GLSDGEWQQVLNVWGKVEADIAGHGQEVLI          30
31 RLFTGHPETLEKFDKFKHLKTEAEM|KASED         60
61 LKKHGTVVLTALGGILKKKGHHEAELKPLA          90
91 QSHATKHKIPIKYLEFISDAIIHVLHSKHP         120
121 GDFGADAQGAM|TKALELFRNDIAAKYKELG        150
151 FQG                                    153
```

denaturing reagent sodium dodecyl sulfate. These results demonstrate practical utility of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ for cleavage of proteins on a preparative scale.

**Selective Cleavage of Myoglobin Fragments by $[Pd(H_2O)_4]^{2+}$.** Because histidine residues are more abundant than methionine residues in myoglobin, the histidine-directed cleavage would result in numerous short fragments. Such a digest would be complex, and its separation would be impractical. This type of protein digestion, commonly by trypsin, is practical for unambiguous identification of proteins in proteomics. To demonstrate the suitability of $[Pd(H_2O)_4]^{2+}$ for such applications, we cleave two histidine-containing myoglobin fragments obtained by Pt(II)-promoted cleavage. These fragments are designated $Mb^{1-55}$ and $Mb^{56-131}$ (see Chart 4).

A mixture containing $Mb^{1-55}$ and 3 equiv of $[Pd(H_2O)_4]^{2+}$ was kept at 60 °C and pH 2.0. After 12 h, the fragments in Table 4 were found. Results of a similar cleavage of $Mb^{56-131}$ with 8 equiv of $[Pd(H_2O)_4]^{2+}$ are summarized in Table 5.

Tables 4 and 5 prove the cleavage patterns in Charts 5 and 6. Clearly, the Pd(II) reagent promoted selective cleavage of the X-Y bonds in the X-Y-His segments. Because the reaction was deliberately not finished, the cleavage remained incomplete, and multiple fragments existed in the digest. In proteomics, such incomplete fragmentation is preferred over complete digestion because it provides higher "coverage" of sequence, more information, and therefore more reliable protein identification. Charts 5 and 6 show that the Pd(II)-promoted cleavage completely "covered" the sequences 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 for Pd(II) atoms are available. Because the reagent is an inorganic compound and not an enzyme, it does not contaminate the sample with proteinaceous impurities and does not add spurious signals to the mass spectrum of the digest. This noninterference simplifies the detection of the protein of interest.

**Table 4.** Deliberately Incomplete Cleavage of the Fragment 1···55 of Equine Myoglobin (Designated $Mb^{1-55}$) by $[Pd(H_2O)_4]^{2+}$ at pH 2.0[a]

| calcd mass | obsd 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 |

[a] The fragments are identified by MALDI mass spectrometry.

**Table 5.** Deliberately Incomplete Cleavage of the Fragment 56···131 of Equine Myoglobin (designated $Mb^{56-131}$) by $[Pd(H_2O)_4]^{2+}$ at pH 2.0[a]

| calcd mass | obsd 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 |

[a] The fragments are identified by MALDI mass spectrometry.

## Conclusions

Complexes *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_4]^{2+}$ are new reagents for selective hydrolysis of peptides and proteins. Although Pt(II) and Pd(II) ions are chemically similar, these two reagents act with different hydrolytic selectivity. We

**Chart 5.** Amino Acid Sequence of the Fragment 1···55 of Equine Myoglobin (Designated $Mb^{1-55}$), a Product of the Prior Protein Cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, Showing the Partial Cleavage by $[Pd(H_2O)_4]^{2+}$ of the Second Amide Bond "upstream" from Each Histidine Residue

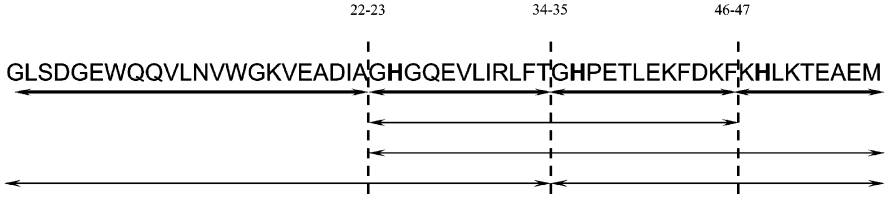
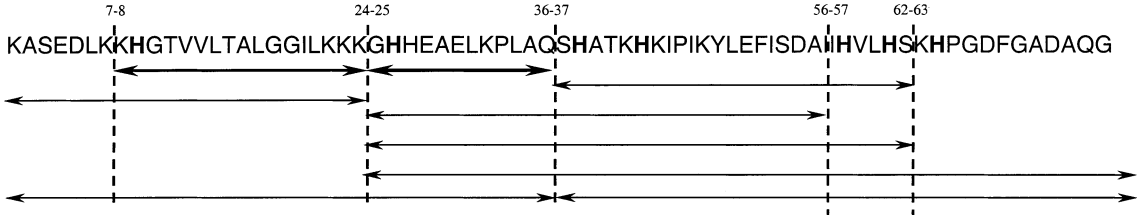



**Chart 6.** Amino Acid Sequence of the Fragment 56···131 of Equine Myoglobin (Designated $Mb^{56-131}$), a Product of the Prior Protein Cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, Showing the Partial Cleavage by $[Pd(H_2O)_4]^{2+}$ of the Second Amide Bond "upstream" from each Histidine Residue



explain this difference by contrasting the modes in which each reagent binds to a specific side chain or to a side chain and the peptide backbone. Protein cleavage by *cis*-[Pt(en)-$(H_2O)_2]^{2+}$ and by $[Pd(H_2O)_4]^{2+}$ is potentially suitable for two bioanalytical applications. The complete cleavage at few sites is useful for preparation and semisynthesis, whereas the incomplete cleavage at many sites is useful for mass-spectrometric analysis in proteomics.

**Acknowledgment.** This work was funded by the National Science Foundation through Grant CHE-9816522.

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