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

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Abstract: Complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ promotes selective hydrolytic cleavage of two proteins, horse cytochrome *c* and bovine β-casein. The cleavage is completed in 24 h under relatively mild conditions, at about pH 2.5, and a temperature as low as 40 °C. The results of HPLC and TSDS PAGE separations, MALDI mass spectrometry, and Edman sequencing showed that cleavage occurred exclusively at the peptide bond involving the C-terminus of each methionine residue, both such residues in cytochrome *c* and all six such residues in β-casein. While having the same selectivity as cyanogen bromide (CNBr), the most common chemical protease, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ has several advantages. It is nonvolatile, easy to handle, and recyclable. Its cleavage is residue-selective, the rest of the polypeptide backbone remains intact, and the other side chains remain unmodified. It is applied in approximately equimolar amounts with respect to methionine residues, creates free amino and carboxylic groups, and cleaves even the Met–Pro bond, which is resistant to CNBr and most proteolytic enzymes. Finally the complex also works in the presence of the denaturing reagent sodium dodecyl sulfate. Experiments with the synthetic 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) as substrates, revealed structural and mechanistic features of the proteolytic reactions. We explain why two similar complexes with similar metal ions, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$, differ in selectivity as proteolytic reagents. The selectivity of cleavage is governed by the selectivity of the *cis*-$[Pt(en)(H_2O)_2]^{2+}$ binding to the methionine side chain. The proteolytic activity is governed by the modes of coordination, which control the approach of the anchored Pt^{II} ion to the scissile peptide bond. The cleavage occurs with a small, but significant, catalytic turnover of more than 18 after 7 days. The ability of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to cleave proteins at relatively few sites, with explicable selectivity and catalytic turnover, bodes well for its use in biochemical practice.

Keywords: artificial peptidase · bioinorganic chemistry · platinum · protein cleavage · methionine

Introduction

The hydrolytic cleavage of proteins plays functional and regulatory roles in physiological processes, such as control of the cell cycle, transcription, signal transduction, antigen processing, and apoptosis.[1] The fragmentation of proteins is an important procedure to determine their primary sequence, and is used in several new applications.[2] In proteomics, the expressed proteins are identified from their digests.[3] In protein footprinting and studies of folding, the pattern of proteolytic cleavage provides structural information.[4, 5] In the conversion of engineered proteins to their native form, fusion tags are removed by site-specific cleavage.[6] In protein semisynthesis, the fragments of natural proteins are ligated into new bioengineered proteins.[7]

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

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Supporting information for this article is available on the WWW under http://www.chemeurj.org or from the author.

 DOI: 10.1002/chem.200304772

in them are irreversibly converted to serine lactones. New chemical reagents for protein cleavage, with improved efficiency and selectivity, are desirable for emerging biochemical applications.

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

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

In this study, we propose *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to be a new proteolytic reagent. We show that this complex hydrolytically cleaves proteins in weakly acidic solutions exclusively at the peptide bond that involves the C-terminus of each methionine residue, that is, the Met–Z bond. This simple and readily available Pt^{II} reagent meets the aforementioned requirements of chemical protease. The cleavage is residue-selective with no known side reactions. Because methionine has an average abundance in proteins of only 2.2%, the fragments are relatively long and suitable for biochemical applications.[31] An equimolar amount of the reagent with respect to the methionine residues is sufficient for complete cleavage. Each Met–Z bond is cleaved, even the Met–Pro bond, which is inert to cyanogen bromide. The protein fragments remain intact, because the Pt^{II} reagent can be removed from them by chelation or precipitation.[32–34] The reagent is nonvolatile, easy to handle, and, in principle, recyclable.

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

Results and Discussion

Choice of conditions: Coordinating anions, such as acetate and chloride, are excluded from the reaction mixtures as they bind to the Pt^{II} ion in the reagent, and thus inhibit its binding to the substrate.[35] To ensure unfolding and solubility of the proteins and their fragments, in some cases the protein digests were made in 1.0% (w/v) in SDS, a common denaturant. All experiments were done in mildly acidic solutions, in which the cleavage of proteins was consistently effective. For protein substrates, the Pt^{II}-promoted cleavage was sufficiently fast at pH 2.5 and 40 or 60 °C, while the background cleavage was nearly undetectable. For peptide substrates, the background cleavage was undetectable after 48 h at $1.0 \leq pH \leq 5.0$ and 60 °C.

Cleavage of cytochrome *c* and *β*-casein: Figure 1 shows the absence of cleavage in the absence of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, and the presence of cleavage in the presence of this Pt^{II} reagent. The electrophoretic band corresponding to the intact cytochrome *c* gradually diminishes and vanishes after 24 h at 40 °C, while one narrow band (designated A) and one broad band (designated B), corresponding to lower molecular masses, emerge. Clearly, the cleavage of cytochrome *c* promoted by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ is selective, and is completed within 24 h. The products of cleavage (protein fragments) are identified in Table 1. The fragment 1···65 was undetectable by the Edman method, because the terminal amino group was acetylated; however, it was firmly identified in two ways by the molecular mass of the fragments and as a complement to the other two fragments. The small fragments 66···80 and 81···104 were not resolved by electrophoresis, but were

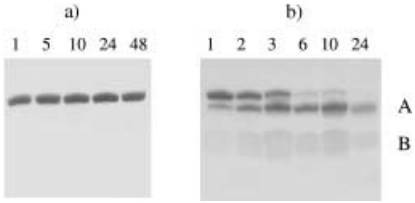


Figure 1. Tricine-SDS PAGE electrophoretogram of equine cytochrome *c* at pH 2.5 and 40 °C, kept for the number of hours shown, in the solution that contained 1.0% (w/v) SDS: a) without *cis*-$[Pt(en)(H_2O)_2]^{2+}$, and b) with 5 equivalents of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The results of MALDI-MS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

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

Electro-phoretic band	N-terminal sequence	Elution time [min]	Molecular mass [D] observed	calculated	Fragment
A	not determinable	18.5	7815.3	7802.6	1 ··· 65
B	EYLEN	28.2	1812.1	1811.1	66 ··· 80
B	IFAGI	23.8	2782.3	2780.3	81 ··· 104

successfully separated by size-exclusion chromatography (see Figure S1 in the Supporting Information).

The results from Table 1 confirm the cleavage pattern in Figure 2. The three fragments are products of the cleavage at two peptide bonds, namely Met65-Glu66 and Met80-Ile81.

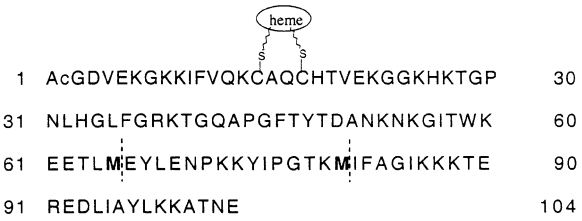


Figure 2. Amino acid sequence of horse cytochrome *c* showing the sites of the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at the C-terminal side of methionine residues, that is, at the first amide bond "downstream" from each methionine residue.

This Pt^{II}-promoted cleavage clearly is selective, and it is directed by each of the two methionine residues. This selectivity, observed in our earlier studies with methionine-containing di- and tripeptides, remained unperturbed by other amino acid residues.[35] These results demonstrate for the first time that complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ works as a proteolytic reagent.

With its longer chain and six methionine residues, bovine β-casein tests more stringently the selectivity of *cis*-[Pt(en)-$(H_2O)_2]^{2+}$ as a chemical protease. Treatment of this protein with ten equivalents of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5 and 60 °C in the absence of SDS was followed by size-exclusion chromatography. The chromatograms of the fresh β-casein solution and control solution (Figure 3a and b) show the intact protein eluting at 14.6 min, and only minor peaks in the control solution—evidence for almost negligible background cleavage. Figure 3c shows that after 24 h, the reaction mixture lacks the intact protein and contains seven protein fragments, produced by the Pt^{II} reagent and eluting between 15.7 and 30.9 min. The results in Table 2 confirm the cleavage pattern in Figure 4. All six Met–Z peptide bonds are present, and no other peptide bonds are cleaved. Moreover, in the absence of SDS, the cleavage of β-casein is completed in 24 h, whereas in the presence of 1.0 % (w/v) SDS the cleavage is completed in 10 h (see Figure S2 in the Supporting Information).

In general, unfolding of protein substrates by detergents, such as SDS, promotes faster and complete cleavage. Proteolytic enzymes, however, are mostly or completely inactivated

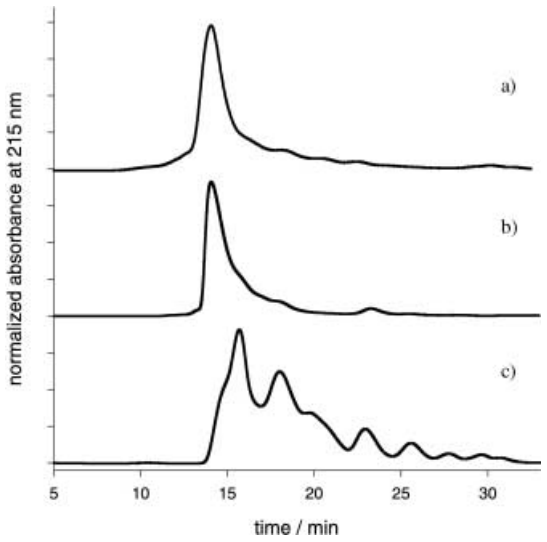


Figure 3. Size-exclusion chromatograms of β-casein: a) fresh solution; b) after 24 h at pH 2.5 and 60 °C; and c) after 24 h at pH 2.5 and 60 °C in the presence of 10 equivalents of *cis*-$[Pt(en)(H_2O)_2]^{2+}$. The identity of the fractions are shown in Table 2.

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

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

Figure 4. Amino acid sequence of bovine β-casein showing the sites of the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$, at the C-terminal side of methionine residues, that is, at the first amide bond "downstream" from each methionine residue.

even by 0.1 % SDS, because they themselves get denatured.[1] Unlike proteolytic enzymes, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ remains active in the presence of 1.0 % (w/v) SDS. The ability of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to work as a protease under denaturing condition sets it apart from proteolytic enzymes.

Advantages of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ over cyanogen bromide as a chemical protease: The selectivity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$

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

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

Characteristics	CNBr	*cis*-$[Pt(en)(H_2O)_2]^{2+}$
selectivity of cleavage	Met-X	Met-X
typical reaction time	ca. 24 h	ca. 24 h
typical reaction conditions	70% CF_3COOH or 100% CH_3COOH, room temperature	pH ~2.5, 40 °C
reagent to Met mole ratio	up to 100:1	ca. 1:1
fate of Met	converted to Ser lactone	intact
Met–Pro bond cleaved?	no	yes
side reactions	oxidation of Trp, Tyr, and Cys	unknown

Remarkably, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ even promotes the cleavage of the Met109–Pro110 and Met185–Pro186 peptide bonds in β-casein. The Met–Pro peptide bond is unreactive to CNBr or any other cleavage reagent. Internal X–Pro peptide bonds are resistant even towards enzymatic proteolysis, because of their unique conformational requirements caused by the cyclic structure of the proline residue.[36, 37]

Selectivity of cleavage promoted by *cis*-$[Pt(en)(H_2O)_2]^{2+}$: We explored the mechanism of proteolytic action of the Pt^{II} reagent with two synthetic peptides, namely AcAla-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala (termed Met-peptide) and AcVal-Lys-Gly-Gly-**His**-Ala-Lys-Tyr-Gly-Gly-**Met**-Ala-Ala-Arg-Ala (termed HisMet-peptide). These realistic substrates are more convenient than natural proteins for NMR spectroscopic characterization of Pt^{II}-substrate binding, HPLC separations, mass-spectrometry identification of the cleavage fragments, and kinetic elucidation of the reaction mechanism.

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

The reaction mixture was kept for 12 h at 60 °C and analyzed by HPLC. The initial Pt(en)–peptide complex formed, eluting at 17.4 min, was absent, whereas two new components, eluting at 9.8 and 16.2 min, were present. No other species were found in the reaction mixture, not even after 24 h. The results in Table 4 prove specific cleavage of the Met–Z bond, as shown below.

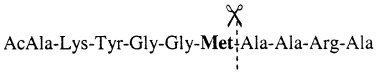


Table 4. Results of HPLC separation, and MALDI mass spectroscopic identification of fragments of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) resulting from the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$.

Elution time [min]	Molecular mass [D] observed	calculated	Fragment
9.8	387.95	388.22	7 ··· 10
16.2	667.30	667.27	1 ··· 6

Chromatograms of the mixtures containing *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and HisMet-peptide in the ratios 1:1 and 2:1, kept for 12 h at 60 °C, were identical, showing only two peaks, which eluted at 9.8 and 17.7 min. The specific cleavage of the Met–Z bond is shown below. The results in Table 5 prove this specific cleavage.

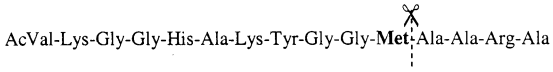


Table 5. Results of HPLC separation and MALDI mass spectroscopic identification of fragments of AcVal-Lys-Gly-Gly-His-Ala-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (HisMet-peptide) resulting from the cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$.

Elution time [min]	Molecular mass [D] observed	Fragment calculated	
9.8	387.95	388.22	12 ··· 15
17.7	1147.30	1146.33	1 ··· 11

The HisMet-peptide contains two potential anchors for the Pt^{II} reagent—His5 and Met11. At pH 2.5, however, only Met11 can coordinate to the Pt^{II} atom, because protonation of the imidazole group inhibits coordination of His5. This coordination would require prior deprotonation of the imidazolium group, which occurs only at pH > 3.5.[38, 39] Since only methionine anchors the Pt^{II} ion, only methionine can direct the cleavage. This claim is backed by an additional control experiment; when the thioether group of Met11 in HisMet-peptide was oxidized to sulfone, a noncoordinating group, the resulting $HisMet^{OX}$-peptide was not cleaved by the *cis*-$[Pt(en)(H_2O)_2]^{2+}$ (data not shown). Evidently, selective cleavage is a result of selective binding of Pt^{II} ion to the cleavage-directing side chain of methionine.

Studies of peptide coordination to Pt^{II} ion and to other transition-metal ions, showed that the coordination mode is unaffected by the noncoordinating side chains surrounding the methionine anchor.[39–50] These neighbors may provide additional interactions, and thus may affect the overall stability of the complex, but not its structure. Therefore, the selectivity of the backbone cleavage should be independent of the side chains surrounding the methionine anchors. On this basis, and from our consistent results with two proteins and two synthetic peptides, we can conclude that all peptide or protein sequences containing an "isolated" methionine resi-

due are expected to be cleaved by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at the Met–Z bond, that is, at the carboxylic side of the anchoring methionine residue. Free cysteine residues can also bind the Pt^{II} ion, but they usually exist in proteins as disulfides, which are poor ligands for this ion. Although thiol and thioether ligands have similar nucleophilicities towards the Pt^{II} ion,[51] *cis*-$[Pt(NH_3)_2Cl_2]$ and *cis*-$[Pt(en)Cl_2]$ prefer to bind to the methionine side-chains at about pH 7.[52, 53] The proteins used in this study do not contain free cysteine residues, and more studies are necessary to determine how these residues will interact with *cis*-$[Pt(en)(H_2O)_2]^{2+}$ under the reaction conditions. If free sulfhydryl groups molecules interfere with the cleavage, they can be blocked prior to cleavage.[8]

Hydrolytic activity of *cis*-$[Pt(en)(H_2O)_2]^{2+}$: Two conditions must be fulfilled for the Met-Z peptide group to be activated for cleavage by the Pt^{II} complex. First, the anchored Pt^{II} complex must approach this group. Second, after anchoring, the Pt^{II} ion must contain at least one aqua ligand. This loosely-bound ligand can either be displaced by the carbonyl oxygen atom (in external attack) or delivered to the carbonyl carbon atom (in internal delivery).[28, 44, 54] If either condition is absent, cleavage does not occur.[29] We recently gave multiple, albeit indirect, evidence for the external attack by solvent water on the carbonyl carbon atom, whose electrophilicity is enhanced by binding of the metal ion, a Lewis acid, to the carbonyl oxygen atom.[30] Regardless of the mechanism of the actual hydrolytic step, the selectivity of cleavage depends on the stereochemistry of coordination. In this study, we focus on structural requirements for the cleavage. The results are valid whether the cleavage step involves external attack or internal delivery.

We followed the kinetics of Met-peptide cleavage by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ in the pH interval from 1.2 to 3.1. The appearance in time of both fragments always obeyed the first-order rate law, as exemplified in Figure S3 in the Supporting Information. Because the binding of the Pt^{II} ion to the sulfur atom of methionine occurs within minutes, and because the subsequent reactions are intramolecular and take hours, fitting of the kinetic results to the first-order rate law is justified. Figure 5 shows that the rate constant for cleavage increases with increasing acidity. To explain how the methionine-anchored Pt^{II} reagent promotes the hydrolysis of the Met–Z peptide bond, we relate these kinetic data to the NMR spectroscopic determination of the coordination modes.

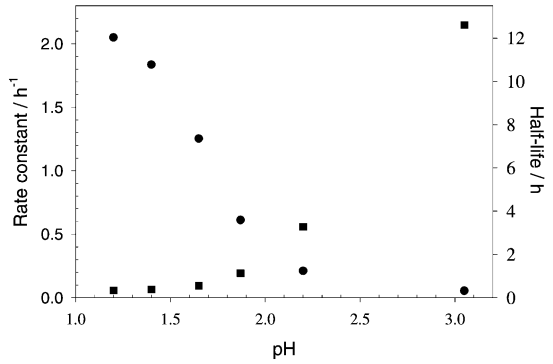


Figure 5. Effects of pH on the first-order rate constant (circles), and half-life (squares) for the cleavage ofAcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) by *cis*-$[Pt(en)(H_2O)_2]^{2+}$.

^{1}H NMR TOCSY spectra were recorded at room temperature and pH 2.5, before and 1 h after the adddition of one equivalent of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ to the Met-peptide. Signals of the methionine residue shifted the most. The disappearance of the SCH_3 singlet at 2.12 ppm and appearance of a broad signal at 2.45 ppm (see Figure S4 in the Supporting Information) confirm binding of the Pt^{II} complex to the methionine side-chain. The methionine NH signal at 8.22 ppm, and also its cross-peaks to α-CH, β-CH, and γ-CH resonances marked in Figure 6a, almost completely disappeared upon addition of

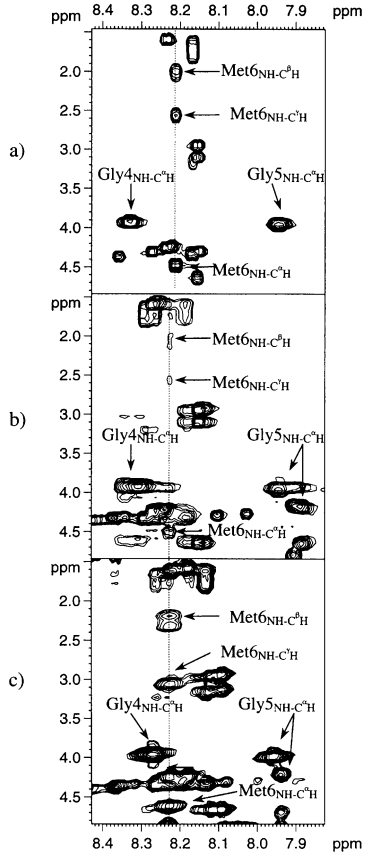
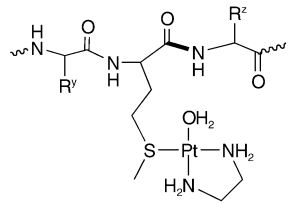


Figure 6. The NH-to-aliphatic region of the two-dimensional TOCSY ^{1}H NMR spectrum of the aqueous solution of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) at room temperature: a) before addition of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.5; b) after addition of an equimolar amount of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and 1 h at pH 2.5; and c) after addition of an equimolar amount of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and 1 h at pH 1.0.

cis-$[Pt(en)(H_2O)_2]^{2+}$, as shown in Figure 6b, because the amide nitrogen atom became mostly deprotonated. This is evidence for its almost complete coordination to the Pt^{II} ion. Clearly, two species are present. In the major one, the nitrogen atom of Met6 is deprotonated and coordinated to Pt^{II} ion, whereas in the minor one this atom remains protonated and is not coordinated. The α-CH to amide NH cross-peaks between Gly4 and Gly5 (marked in Figure 6) remained,

indicating that the amide group connecting these two residues remained protonated and not bound to the Pt^{II} ion.

These two species are designated **1** and **2** in Scheme 1. Such complexes of Pt^{II} ion with short peptides are known.[39, 50, 55] In complex **1**, the minor species at pH 2.5, the anchoring



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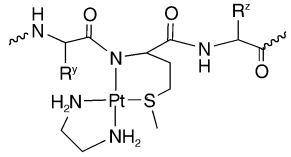


2

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

methionine side-chain displaced an aqua ligand from *cis*-$[Pt(en)(H_2O)_2]^{2+}$. In complex **2**, the predominant species at pH 2.5, the peptide is additionally coordinated to the Pt^{II} ion by the first peptide nitrogen atom upstream from the anchor. The coordinated sulfur atom is a chiral center in both complexes **1** and **2**. The existence of two diastereomers caused the splitting of the α-CH to amide NH resonance of the nearby Gly5.[56]

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

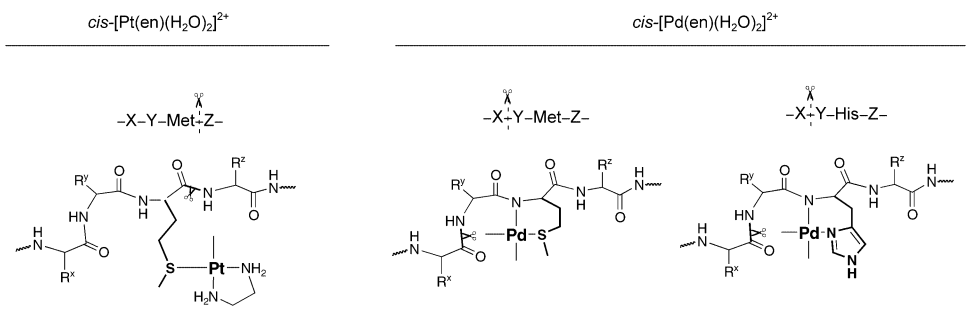
At pH 2.5, complex **2** is a major species, but increasing the acidity would suppress its formation from complex **1** because the H^+ ion would compete with the Pt^{II} ion for the amidate nitrogen of Met6. Indeed, the TOCSY spectrum at pH 1 (see Figure 6c) showed the Met6 NH signal at 8.22 ppm with its cross-peaks to aliphatic CH resonances; this is clear evidence that complex **1** is the predominant species. A minor shift of all NH resonances at this pH is caused by the change in the acidity.

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

Coordination of the amidate nitrogen atom of Met6 in complex **2** keeps the Pt^{II} ion away from the Met6–Ala7 peptide bond. Results of both NMR spectroscopy and MALDI mass spectrometry results showed that the ethylenediamine ligand remained bound to the Pt^{II} ion. Therefore, the Pt^{II} ion in complex **2** lacks an aqua ligand necessary for hydrolytic activity. Complex **2** is hydrolytically inactive, because it violates both conditions needed for activity stated above. The anchored Pt^{II} ion in complex **1**, however, can interact with the Met6–Ala7 peptide bond and promote its cleavage. Complex **1** is a hydrolytically-active species, because the anchored Pt^{II} complex can approach the scissile amide bond and the Pt^{II} ion contains an aqua ligand. Because the acidic solution shifts the equilibrium in Scheme 1 towards complex ***1***, the rate constant for the cleavage increases as pH decreases, as Figure 5 shows. We refrain from fitting the results in Figure 5 because we have an inexact value of pK_a; however our estimated value of 1.8 ± 0.4 agrees with the trends in Figure 5. The acid in solution is not a cleavage agent by itself, but is required to prevent the formation of the hydrolytically inactive complex **2**.

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

Complexes *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ as artificial peptidases: Since the Pt^{II} and Pd^{II} ions are alike in electron configuration and other properties, their complexes tend to react similarly. Indeed, both complexes promote selective cleavage of peptides and proteins. Their selectivities, however, are surprizingly different, as Scheme 2 shows. While *cis*-$[Pt(en)(H_2O)_2]^{2+}$ cuts the first peptide bond downstream from methionine anchor (the Met–Z bond), the analogous Pd^{II} complex, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, cuts the second peptide bond upstream from methionine and histidine anchors (the X–Y bond) in the X-Y-Met-Z and X-Y-His-Z sequences.[28] At acidic pH, the soft Lewis acid Pt^{II} binds only the soft Lewis

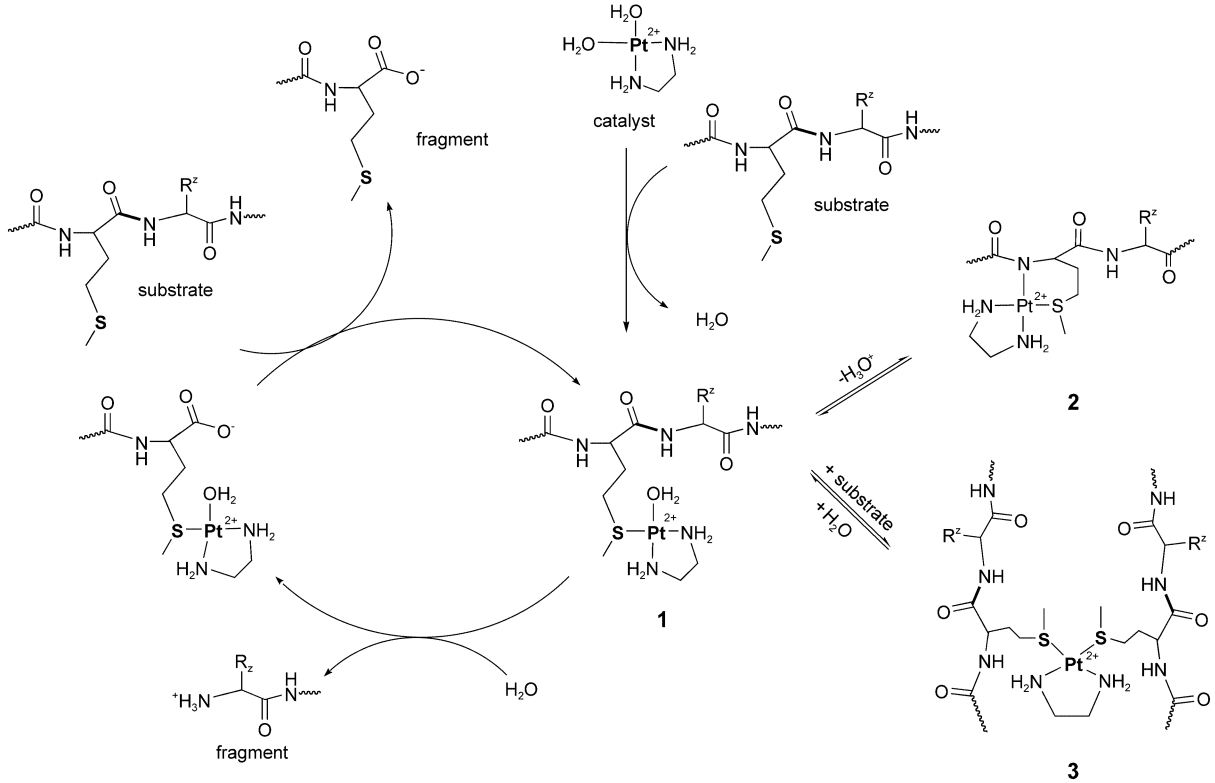


Scheme 2. Different proteolytic selectivity of Pt^{II} and Pd^{II} complexes. These two metal ions form different hydrolytically active complexes with the substrate, and promote hydrolytic cleavage of different peptide bonds. For explanation, see text.

base, the methionine side chain,[38, 39] whereas the "borderline" acid Pd^{II} ion binds both the soft side chain of methionine and the harder one of histidine.[28] Once anchored, the two metal ions form different hydrolytically active complexes in the mildly acidic solutions. Because Pt^{II} is much more inert than Pd^{II} to ligand substitution,[57] the bidentate ethylenediamine ligand largely persists on the Pt^{II} ion under the reaction conditions and in the time required,[39, 56, 58] but is relatively quickly displaced from the Pd^{II} ion, with the help of H^+ ions in solution.[29] The Pt^{II} binds the substrate as a unidentate ligand. The Pd^{II} ion, free of ethylenediamine, binds to the amidate nitrogen atom upstream from the anchor ($pK_a < 2.0$[39–45]), and forms a bidentate complex similar to **2**, but with available aqua ligands.[28] Both of these complexes are hydrolytically active. Consequently, the anchored Pt^{II} ion remains proximal to the peptide bond downstream from the anchor and can activate it towards cleavage. The anchored Pd^{II} ion, however, is kept away from this bond, but close to the second peptide bond upstream from the anchor, which becomes activated.

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

As Table 6 shows, one equivalent of the Pt^{II} reagent cleaves multiple equivalents of the substrate; this is evidence that the reaction is catalytic. The catalytic cycle is shown in Scheme 3. The catalyst enters the cycle as the substrate displaces one aqua ligand. The hydrolytically active species **1** (in equilibrium with the inactive species **2** and **3**)[56] undergoes hydrolytic cleavage of the highlighted Met–Z bond. One fragment departs, and the methionine-containing fragment remains



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

Table 6. Number of equivalents of AcAla-Lys-Tyr-Gly-Gly-Met-Ala-Ala-Arg-Ala (Met-peptide) cleaved by one equivalent of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ at pH 2.0 and 60 °C, when the mole excess of the peptide (substrate) over the Pt^{II} reagent was 10-fold, 100-fold, and also 100-fold in the substrate and 90-fold in $Hg(ClO_4)_2$.

Time [days]	Number of equivalents cleaved		
	10 equiv	100 equiv	100 equiv + 90 equiv Hg^{II}
0.5	2.1	2.7	3.4
1.0	2.6	3.9	5.8
3.0	3.4	8.4	12.1
7.0	6.9	14.9	18.9

bound to the catalyst. This second fragment is displaced by fresh substrate, in a reaction apparently assisted by the affinity of the soft Hg^{II} ion (when it is present) for the thioether group.[33, 59, 60]

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

Conclusion

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

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

Experimental Section

Chemicals: The complex *cis*-$[Pt(en)Cl_2]$ (en is ethylenediamine), sodium dodecylsulfate (SDS), equine cytochrome *c*, and bovine β-casein were obtained from Sigma Chemical Co. Trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid were obtained from Aldrich Chemical Co. Methyl phenyl sulphone was obtained from Lancaster Synthesis Inc. Acetonitrile of HPLC grade was obtained from Fisher Scientific Co. The complexes *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and $[Pt(dien)(H_2O)]^{2+}$ (in which dien is diethylenetriamine) were prepared as perchlorate salts, and quantified according to published procedures.[61, 62]

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

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

Electrophoresis: A Protean II electrophoretic cell was combined with a 3000 Xi power supply, both produced by Bio-Rad, for standard tricine/sodium dodecyl-sulfate polyacrylamide gel electrophoresis (TSDS-PAGE) at 150 V for 1.5 h.[63] The running buffer contained Tris base (12.10 g), tricine (17.90 g), and SDS (1.00 g), in water (up to 1.00 L). The sample buffer contained glycerol (1.0 mL), 10% SDS (w/v) (2.0 mL) in water, Tris-HCl (1.25 mL, 1.00 M), bromophenol blue (1.0 mg), and water (up to 10.0 mL). The 16.5% of the running gels contained gel buffer (a 3.00 M in Tris-HCl and 0.30% in SDS, pH 8.45), a solution (2.0 mL) containing acrylamide (48.0% w/v) and bis-acrylamide (3.0% w/v), water (2.0 mL), (48.0 g), TEMED (5.0 μL), and 10% (w/v) ammonium persulfate (100.0 μL). The stacking gel was made by polymerization of a mixture that contained 100.0 μL of the aforementioned solution of acrylamide and bis-acrylamide, gel buffer (310.0 μL), water (840.0 μL), TEMED (3.0 μL), and 10% ammonium persulfate (3.0 μL). All the aforementioned solutions were aqueous. The gels were stained for 1 h by an aqueous solution containing 40% (v/v) methanol, 10% (v/v) acetic acid, and 1.0% (w/w) Coomassie Blue R-250 dye, and destained with a similar solution without the dye.

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

NMR spectroscopy: The ^1H NMR spectra were recorded in a mixture that contained 95% (v/v) H_2O and 5% (v/v) D_2O at 25.0 °C with a Bruker DRX500 spectrometer, and referenced to the methyl signal of DSS in aqueous solutions. The Met-peptide was examined by ^1H TOCSY (total correlation spectroscopy) with mixing time of 70 ms. The ambiguous assignment of the residues Gly4 and Gly5 was resolved by ROESY (rotating-frame Overhauser enhancement spectroscopy) with a mixing time of 500 ms. Each two-dimensional data set consisted of 256 × 2048 complex points. The spin-lock field strength during mixing was 6.4 kHz in TOCSY experiments and 2.5 kHz in the ROESY experiment. The water signal was suppressed by the WATERGATE method.[64] The pH was measured with a Fisher Accumet instrument and an Aldrich Ag/AgCl reference electrode. The measurements at pH 2.5 and 1.0 were informative, but an accurate study of the effect of pH on the Pt^{II}–peptide coordination modes was precluded by the Pt^{II}-promoted peptide cleavage, the main subject of this study.

HPLC separations: The components of the peptide digests were separated using a Hewlett–Packard 1100 HPLC system containing an autosampler and a multiwavelength detector set to 215, 280, and 410 nm. Absorption at 215 nm is common for all peptides and proteins; absorption at 280 nm is due to aromatic residues and a bound Pt^{II} ion; and absorption at 410 nm is diagnostic of heme. In the reverse-phase separations, analytical Supelco Discovery C18 column (sized 250 × 4.6 mm, beads of 5 μm), analytical Vydac C5 column 214TP54 (sized 150 × 4.6 mm, beads of 5 μm), and a preparative Vydac C18 column 218TP101522 (sized 250 × 22 mm, beads of 10 μm) were used. The eluting solvent A was 0.10% (v/v) TFA in H_2O, and

solvent B was 0.08% (v/v) TFA in acetonitrile. In a typical run, the percentage of solvent B in the eluent was kept at 0% for 5 min after the injection of the sample, and then raised gradually to 45% over a 35 min period. In the "fast" analytical run optimized for the kinetic measurements, the fraction of solvent B was initially 10%, and was raised to 22% over a 13 min period. The flow rate was 1.0 mL min^{-1} in analytical runs and 10.0 mL min^{-1} in preparative runs. In the size-exclusion separations, the Superdex peptide HR 10/30 column, with an optimal separation range from 1000 to 7000 D, was used. The solvent was 0.10% (v/v) TFA in H_2O, and the flow rate was 0.50 mL min^{-1}.

Mass spectrometry: The MALDI-TOF experiments were done with a Bruker Proflex® instrument. The samples containing intact peptide, the reaction mixture of the peptide and the complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$, and separate fractions isolated by HPLC, were prepared by a standard dried-droplet procedure: 1.0 μL of the sample was mixed with 9.0 μL of a saturated solution of the matrix (α-cyano-4-hydroxycinnamic acid) in a 2:1 (v/v) mixture of water and acetonitrile. Each spectrum consisted of 100 scans. For the sake of clarity, molecular masses are reported only for fragments free of the Pt(en) groups, although the Pt(en)-carrying species were also observed in the MALDI spectra. Solutions of angiotensin II, oxidized chain B of insulin, and cytochrome *c* were used as external standards. The measured molecular mass of a given fragment was compared to the value calculated by PAWS software, obtained from ProteoMetrics, LLC.

Study of hydrolysis: Aqueous solutions were held in 2.0 mL glass vials. Stock solutions were 5.0 mM in each substrate (cytochrome *c*, β-casein, Met-peptide, or HisMet-peptide). In a typical experiment, involving equimolar amounts of the Pt^{II} reagent and the methionine residue in the substrate, the substrate solution (200.0 μL) was mixed with stock solution of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ (10.0 μL, 100 mM), a solution of methyl phenyl sulfone (10 μL, 20 mM) as the internal standard, and water (up to 1.000 mL). The pH was adjusted by $HClO_4$ or NaOH. After the reactions were completed, the pH remained within ±0.1 of the initial value. The mixture was kept at 40 ± 1 or 60 ± 1 °C for 1 day, and 20.0 μL samples were taken periodically. In the control experiments for possible background cleavage, the conditions were the same except that *cis*-$[Pt(en)(H_2O)_2]^{2+}$ was absent. The cleavage was followed by TSDS-PAGE (to resolve large fragments), and by size-exclusion chromatography (to resolve small fragments) for the protein substrates; and, by reverse-phase HPLC for the peptide substrates. The separated fragments were lyophilized to dryness, re-dissolved, and analyzed by MALDI-TOF mass spectrometry and Edman N-terminal sequencing.

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

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

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

Acknowledgement

This work was funded by the National Science Foundation through grant CHE-9816522. We thank D. Bruce Fulton for help with recording and interpreting the NMR spectra.

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Received: January 27, 2003
Revised: May 9, 2003 [F4772]