Site-Specific Hydrolytic Cleavage of Cytochrome *c* and of Its Heme Undecapeptide, Promoted by Coordination Complexes of Palladium(II)

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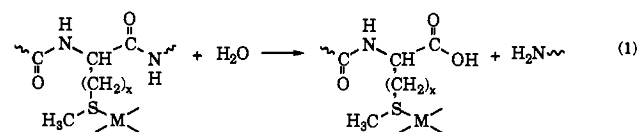
Abstract: Cytochrome *c* is specifically cleaved at the amide bond His18–Thr19 in the presence of an equimolar amount or a small excess of the simple palladium(II) complexes $[Pd(H_2O)_3(OH)]^+$, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$, and *cis*-$[Pd(dtco\text{-}OH)(H_2O)_2]^{2+}$; where dtco is 1,5-dithiacyclooctane and dtco-OH is its 3-hydroxy derivative. Incubation for 2 days at pH 1.7 and 40 °C gives the cleavage yield as high as 80%. A noncoordinating acid is required, presumably to bring cytochrome *c* into the partially unfolded state II. The cleavage site, between His18 and Thr19, is confirmed in experiments with the aforementioned complexes and with the fragment containing residues 11–21 and the heme. Cytochrome *c* and apocytochrome *c* give identical electrophoretograms upon cleavage; evidently, heme does not affect the site and the efficiency of cleavage. The tripeptides AcCys–His–Ala and AcCysMe–His–Gly mimic the important features of the reactive segment Cys17–His18–Thr19 in the protein. Indeed, the palladium(II) complexes promote selective cleavage of the His–Ala and His–Gly bonds in the respective tripeptides, and kinetics of the cleavage of the former tripeptide is reported. This specificity probably is caused by tridentate coordination of the Cys17–His18 fragment to palladium(II) and by the proximity of the imidazolium group to the scissile bond. The notion of tridentate coordination is supported by infrared and ^1H NMR spectra of binary complexes obtained in the reaction between AcCys–His–Ala and palladium(II) complexes. The tripeptide AcCysMe–Ala–Gln mimics the important features of the unreactive segment Cys14–Ala15–Gln16 in the protein, which lacks the special histidine residue (i.e., the imidazolium group). Indeed, *cis*-$[Pd(en)(H_2O)_2]^{2+}$ cleaves the CysMe–Ala bond in this synthetic tripeptide. Stereochemical and mechanistic aspects of cleavage are discussed. To our knowledge, this is the first example of selective hydrolytic cleavage of a protein effected by a metal complex that is directly attached, not tethered, to the protein.

Introduction

Selective cleavage of proteins and peptides is one of the most common tasks in analytical biochemistry. Besides proteolytic enzymes, there are several standard chemical reagents for this purpose.[1] The most useful among them is cyanogen bromide;[2,3] *O*-iodosobenzoate, hydroxylamine, 2-nitro-5-thiocyanobenzoate, and other compounds are also used.[4–6] Their number, however, remains small. New studies of protein structure and function require new chemical reagents for selective cleavage.

Because structure and reactivity of transition-metal complexes can be precisely controlled by the choice of the metal and the ligands, these complexes are well-suited to biochemical applications.[7] Ability of transition metals to promote cleavage of amide bonds in amino-acid derivatives and peptides has been proven in many studies,[8–11] but cleavage of proteins has been achieved only recently, with iron chelates.[12–18] Although some of these chelates are synthesized and attached to substrates in multistep procedures that require additional reagents in solution, they hold promise because cleavage itself, in the presence of hydrogen peroxide, is fast and specific.

Our goal is to achieve hydrolytic cleavage by metal complexes that are easily prepared and that are attached to proteins without any synthetic effort, by mere incubation. Previous studies in this laboratory[19–22] showed that simple complexes of platinum(II) and palladium(II), shown without ligands in eq 1, bind to the



sulfur atom of *S*-methylcysteine ($x = 1$) and methionine ($x = 2$) in peptides and promote, under relatively mild conditions, selective hydrolysis of the adjacent amide bond on the carboxy side of the anchoring side chain. After the initial studies with platinum(II) complexes, which effect reactions with half-lives in the range of 2 days to 2 h at 40 °C,[19] we turned our attention to palladium(II)

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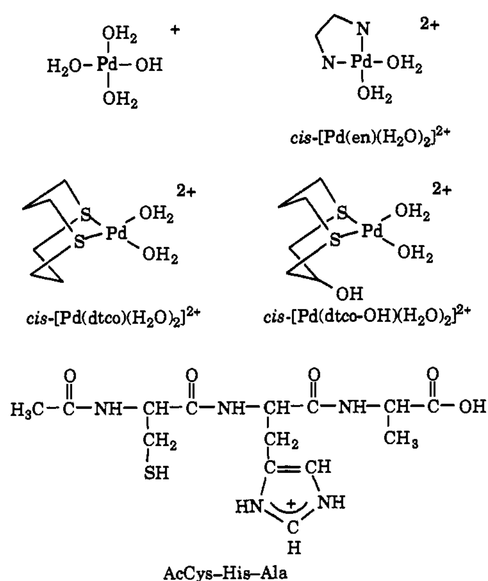
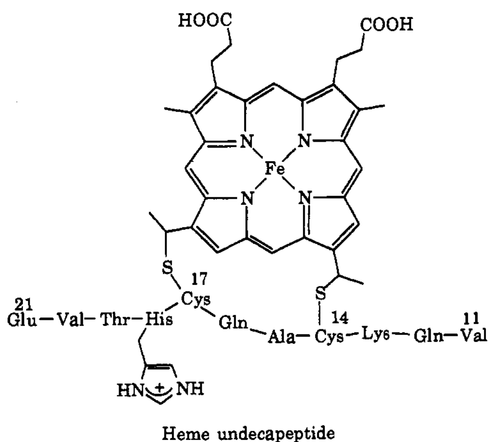
Chart 1



	1				5					10					15	
1	GLY	ASP	VAL	GLU	LYS	GLY	LYS	LYS	ILE	PHE	VAL	GLN	LYS	CYS	ALA	15
16	GLN	CYS	HIS	THR	VAL	GLU	LYS	GLY	GLY	LYS	HIS	LYS	THR	GLY	PRO	30
31	ASN	LEU	HIS	GLY	LEU	PHE	GLY	ARG	LYS	THR	GLY	GLN	ALA	PRO	GLY	45
46	PHE	THR	TYR	THR	ASP	ALA	ASN	LYS	ASN	LYS	GLY	ILE	THR	TRP	LYS	60
61	GLU	GLU	THR	LEU	MET	GLU	TYR	LEU	GLU	ASN	PRO	LYS	LYS	TYR	ILE	75
76	PRO	GLY	THR	LYS	MET	ILE	PHE	ALA	GLY	ILE	LYS	LYS	LYS	THR	GLU	90
91	ARG	GLU	ASP	LEU	ILE	ALA	TYR	LEU	LYS	LYS	ALA	THR	ASN	GLU		105

Sequence of cytochrome c



Heme undecapeptide

complexes. With them, half-lives of certain hydrolysis reactions are as short as 13 min at 40 °C.[21] Most interesting, the hydrolysis rate depends on the steric bulk of the leaving fragment in such a way that the reaction is somewhat sequence-selective.[21] This selectivity can be enhanced by further research.

These previous studies, concerning kinetics and mechanism of peptide cleavage, made possible the present study, concerning cleavage of a protein. We cleave cytochrome *c* with the four palladium(II) complexes shown in Chart 1. Because this important protein has been fully characterized and because it has been cleaved before by standard methods, we were able to determine the selectivity of cleavage and to compare our new reagents with existing ones. The results are interesting and promising.

Experimental Procedures

Spectroscopic and Analytical Methods. Proton NMR spectra at 300 MHz of solutions in D_2O were recorded with a Varian VXR300 spectrometer, and DSS was an internal reference. The sample temperature was kept constant within ±0.1 °C. Infrared spectra of Nujol mulls were recorded with an IBM 98 Fourier-transform spectrometer. Ultraviolet–visible spectra were recorded with an IBM 9430 spectrophotometer. The mass spectra were recorded with a Kratos 50 instrument, by the fast-atom-bombardment method.

The pH was measured with a Fischer 925 instrument and a Phoenix Ag/AgCl reference electrode. The uncorrected values in deuterated solvents are designated pH*. The separations of peptides were done with a Beckman Gold HPLC system containing a 166 detector, a 126 AA solvent module, and a Vydac C_{18} column of 5-μm beads sized 0.21 cm × 25 cm. Amino acid analyses were done with an Applied Biosystems 420A/130A autoanalyzer. Amino acid sequences were determined with a 477A protein sequencer and a 120A analyzer, both by Applied Biosystems, Inc. All of these bioanalytical experiments were done by the staff of the Protein Facility at Iowa State University.

Electrophoresis was done with a Bio-Rad Protean II apparatus. An 18% polyacrylamide separating gel was overlaid by a 6.9% stacking gel. Electroblotting on a Bio-Rad polyvinylidene difluoride membrane was done at 295 mA, over 30 min, at room temperature. The gel and the membrane were stained by 0.10% solutions of Coomassie blue R250. The scanning densitometer was Biomed Instruments SL-504-XL, and the estimated relative error of densitometric analysis was ±4%.

Kinetics of AcCys–His–Ala hydrolysis was examined as in our previous studies.[20–22]

Hydrolysis Substrates. Horse-heart cytochrome *c* (types III and VI) and its heme-containing fragment 11–21, called heme undecapeptide or microperoxidase 11, were obtained from Sigma Chemical Co. Apocytochrome *c* was prepared by a published procedure.[23]

The tripeptide *N*-acetyl-L-cysteinyl-L-histidyl-L-alanine (AcCys–His–Ala) was synthesized by the standard solid-state method, and its purity was checked by HPLC; this was done by the staff of the Protein Facility. The infrared spectrum of the tripeptide showed the expected bands at 1728, 1637, and 1541 cm^{-1}, which are assigned to the COOH, COO^-, and CONH groups, respectively. Molecular peak in the mass spectrum: observed $m/z = 372.2$; calculated for $C_{14}H_{21}N_5O_5S$, m/z 371.4. The tripeptides *N*-acetyl-*S*-methyl-L-cysteinyl–L-histidyl–glycine (AcCysMe–His–Gly) and *N*-acetyl-*S*-methyl-L-cysteinyl–L-alanyl–L-glutamine (AcCysMe–Ala–Gln) were synthesized by the published procedure.[24]

Chemicals. Distilled water was further demineralized and purified to a resistivity greater than 17 MΩ cm. The deuterium-containing compounds D_2O, $DClO_4$, and NaOD, the salt $K_2[PdCl_4]$, and the dipeptides L-alanyl–L-glutamine (Ala–Gln) and L-hystidyl–glycine (His–Gly) were obtained from Sigma Chemical Co. All other chemicals were of reagent grade; 1,5-dithiacyclooctan-3-ol (dtco-OH) was obtained from Aldrich Chemical Co. The ethylenediamine complex *cis*-$[Pd(en)Cl_2]$,[25] the 1,5-dithiacyclooctane complex *cis*-$[Pd(dtco)Cl_2]$,[26] and the 1,5-dithiacyclooctan-3-ol complex *cis*-$[Pd(dtco\text{-}OH)Cl_2]$[26] were prepared by published procedures and converted into *cis*-$[Pd(en)(H_2O)_2]^{2+}$, *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$, and *cis*-$[Pd(dtco\text{-}OH)(H_2O)_2]^{2+}$ by treatment with 2 equiv of anhydrous $AgBF_4$ or $AgClO_4$.[27] The complex $[Pd(H_2O)_3(OH)]^+$ as a ClO_4^- salt was prepared by a published procedure.[20] In D_2O solutions, all the exchangeable protons in complexes are actually deuterons, but formulas are written with protons, for consistency.

Cleavage of Cytochrome *c* with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. This procedure is typical of those used also with other promoters, with different acids, and with apocytochrome *c*. Concentration of a solution containing 1.0 mg of cytochrome *c* in 60 μL of solution was determined by reducing an aliquot by a small excess of ascorbate and measuring the absorbance at 550 nm. The pH of another aliquot was adjusted to 1.70 with HBF_4, and an equimolar amount of the complex salt was added. The final concentrations were 1.3 mM each in the protein and the promoter and 100 mM in HBF_4. (In some of the other experiments, the mole ratio of promoter to protein was greater than 1:1.) A 60-μL portion of this solution was incubated at 40 ± 1 °C for 2 days and then divided into 58- and 2-μL portions. The major portion was stored frozen for possible further experiments. The 2-μL portion was prepared for electrophoretic analysis as follows. The reaction was quenched by addition of 0.3 μL of a 200 mM aqueous solution of sodium diethyldithiocarbamate (a 20-fold molar

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excess), pH was adjusted to ca. 6.0 by addition of a 200 mM aqueous solution of Na_2HPO_4 at pH 9.1, and 16 μL of the standard SDS reducing buffer was added. The final solution was kept at 95 °C for 5 min, cooled, and centrifuged to remove a yellow precipitate containing palladium. The clear supernatant was subjected to polyacrylamide gel electrophoresis at 100 V for 3 h.

Site of Cytochrome *c* Cleavage with $[Pd(H_2O)_3(OH)]^+$. The experiment was run as described immediately above through the incubation at 40 ± 1 °C. Then the reaction mixture was lyophilized to dryness, and the solid was dissolved in 200 μL of HPLC-grade water. A 5-μL aliquot was subjected to three cycles of protein sequence analysis by Edman degradation.

Site of Cytochrome *c* Cleavage with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. The experiment was run as described above through the incubation at 40 ± 1 °C. To a 8.0-μL aliquot of the reaction mixture were added 1.2 μL of a 200 mM aqueous solution of sodium diethyldithiocarbamate, 8.0 μL of a 200 mM aqueous solution of Na_2HPO_4, and 64 μL of the SDS reducing buffer. The solution was kept at 95 °C for 5 min, cooled, and centrifuged to remove a yellow precipitate containing palladium. Five 5-μL samples were subjected to electrophoresis, and the bands were transferred to a polyvinylidene difluoride membrane by electroblotting. The fragment band immediately below the whole-protein band in each of the five lanes was cut out, washed with HPLC-grade water three times, and dried in air. The five bands combined were subjected to three cycles of protein sequence analysis by Edman degradation.

Site of Cleavage of Heme Undecapeptide with $[Pd(H_2O)_3(OH)]^+$ and Amino Acid Analysis of a Fragment Peptide. Heme undecapeptide, the segment 11–21 of cytochrome *c* with the heme still covalently attached, is shown in Chart 1. It was treated with $[Pd(H_2O)_3(OH)]^+$ as described above for the whole protein. After the incubation, the solution was lyophilized to dryness to remove HBF_4 and quench the reaction. The residue was redissolved in HPLC-grade water and subjected to a reversed-phase HPLC separation with a gradient. One solvent was a 0.10% aqueous solution of CF_3COOH; the other was a 0.080% solution of CF_3COOH in acetonitrile, and its proportion increased from 0 to 45% over 45 min. The fraction with the retention time of 10.1 min did not contain the heme. It was subjected to amino acid analysis, with norleucine as an internal standard.

Cleavage of AcCys–His–Ala with Various Palladium(II) Complexes. Because the solutions of $[Pd(H_2O)_3(OH)]^+$, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, and *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$ were prepared in D_2O, the exchangeable protons in the H_2O and OH^- ligands were in fact deuterons. Their respective pH* values were ca. 1.0, 2.0, and 2.0. The complexes were prepared fresh, to suppress formation of oligomeric hydroxo-bridged species. In three different experiments, the peptide was mixed with equimolar amounts of the first two complexes and with a 2-fold molar excess of the third; the concentrations of the substrate and the promoter were 10–20 mM each. The total volume was 600 μL. The reactions were followed by ^1H NMR spectroscopy, and 16 scans were taken each time. Although a yellow precipitate was formed in the reaction mixtures containing $[Pd(H_2O)_3(OH)]^+$ and *cis*-$[Pd(en)(H_2O)_2]^{2+}$, appearance of free alanine was easily monitored even in these mixtures. After the hydrolysis reactions the yellow solid was filtered off, washed with water, dried in vacuo, and examined by infrared spectroscopy.

The temperature was kept within ±0.1 °C. The estimated error in peak integration was ±5%. First-order logarithmic plots of substrate concentration or product concentration versus time were linear for 3 half-lives. Further details are given in our previous publications.[20–22]

Cleavage of AcCysMe–His–Gly and of AcCysMe–Ala–Gln with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. The complex was prepared fresh. Each reaction mixture in D_2O was 20 mM in both the peptide and *cis*-$[Pd(en)(H_2O)_2]^{2+}$; as before, the exchangeable protons in the complex were actually deuterons. The pH* was 1.0, the temperature was 40°, and the total volume was 600 μL. The reactions were followed by ^1H NMR spectroscopy, as in the experiments above.

The multiplet at 4.06 ppm of the glycyl residue declined as the singlet at 3.87 ppm of free glycine grew. Addition of glycine to the first reaction mixture enhanced the resonance of the hydrolysis product, and no new resonances appeared.

The doublet at 1.40 ppm of the alanyl methyl group in the starting tripeptide declined as the doublet at 1.55 ppm of this group in the product Ala–Gln grew. Addition of the authentic dipeptide Ala–Gln to the second reaction mixture enhanced the resonance of the hydrolysis product, and no new resonances appeared.

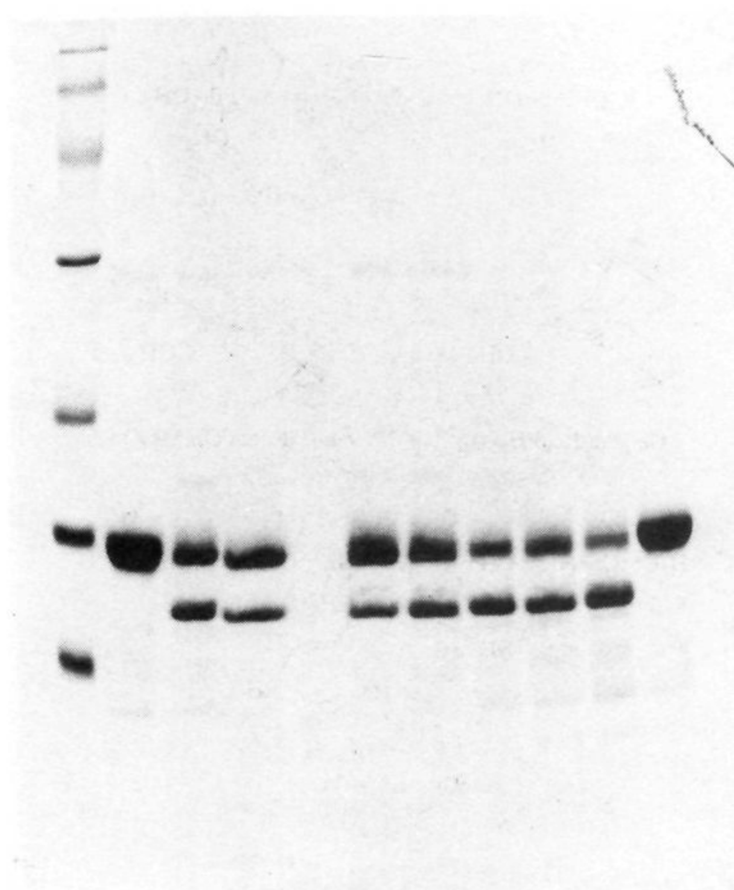

Figure 1. Hydrolytic cleavage of horse-heart cytochrome *c* promoted by *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 1.7. Lanes in the SDS polyacrylamide gel electrophoretogram: (1) Standard markers, with molecular masses of 97.4, 66.0, 45.0, 31.0, 21.5, 14.0, and 6.5 kDa, from top to bottom; (2) fresh solution of cytochrome *c*; (3) equimolar (1.3 mM) mixture of cytochrome *c* and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ in a solution that is 100 mM in HBF_4, after 2 days at 40 °C; (4) equimolar (1.3 mM) mixture of cytochrome *c* and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ in a solution that is 100 mM in CF_3COOH, after 2 days at 40 °C; (5)–(9) solutions as in lane 3, incubation times of 1–5 days, in increments of 1; (10) mixture as in lane 3, but without the palladium(II) complex.

Results and Discussion

Optimal Conditions for Cleavage of Cytochrome *c*. When this protein, whose molecular mass is 12.4 kDa, is cleaved in the presence of palladium(II) reagents, electrophoretograms show one major fragment in addition to the intact protein. Control experiments show no cleavage in the absence of the palladium(II) reagents even after prolonged incubation. As Figures 1–3 show, different palladium(II) complexes in the presence of different acids consistently produce one large fragment, whose molecular mass is approximately 10 kDa. Evidence in the next subsection indicates that this fragment is the same regardless of the complex used for cleavage. The expected complementary fragment, with the molecular mass of approximately 2 kDa, is too small to be seen in the electrophoretograms. If the 10-kDa fragment is one peptide, this pattern is consistent with cleavage at a single site. The appearance in the figures of faint bands at lower molecular masses hints that cleavage is not absolutely "clean". We experimented to optimize efficiency of cleavage. Its apparent yield, estimated by densitometry, is the quotient of the intensity of the band for the large fragment and the combined intensities of the bands for the intact protein and the large fragment.

The complexes *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_3(OH)]^+$, regardless of the counteranions (BF_4^- or ClO_4^-), cleave the protein in the solution that is 100 mM in HBF_4, $HClO_4$ or CF_3COOH or that is 70% v/v in HCOOH. When the mole ratio of the promoter and the protein is 1:1 and the incubation time is 2 days, the cleavage yield is 35–50%. When the solution acidity was maintained by 100 mM HCl, HCOOH, or CH_3COOH, cleavage was not observed. The chloride ion, which is fairly nucleophilic, inactivates the promoter by displacing an aqua ligand from it.[20] The other two acids perhaps are too weak. These experiments show that the acid has to be at least moderately strong and that

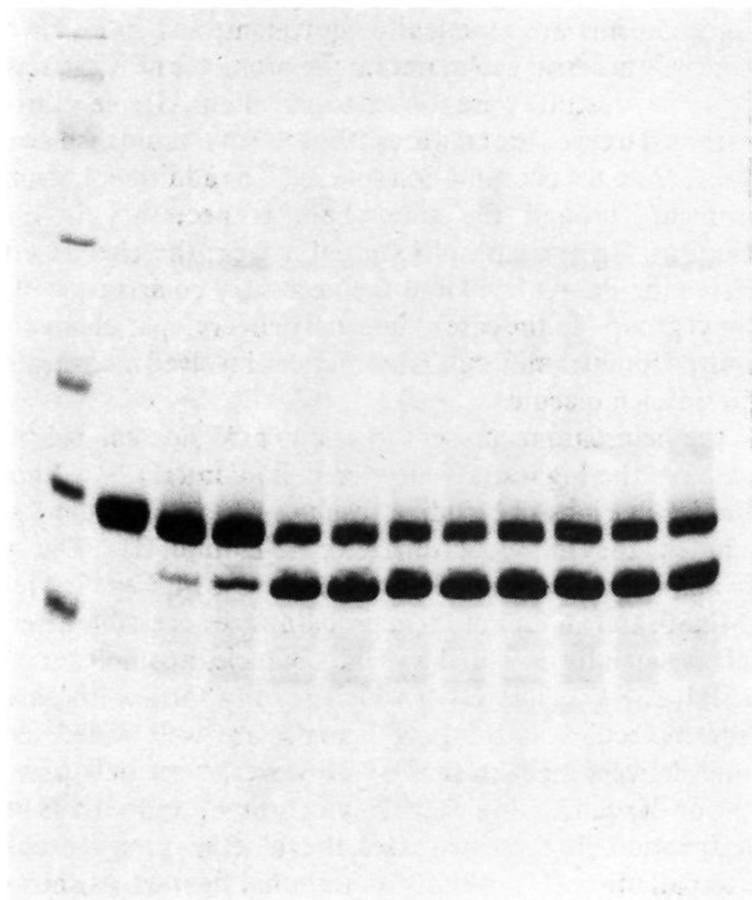

Figure 2. Effect of the mole ratio of *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$ to 1.3 mM horse-heart cytochrome *c* on the yield of hydrolytic cleavage of the protein in solution that is 100 mM in HBF_4, at pH 1.7, after incubation for 2 days at 40 °C. Lanes in the SDS polyacrylamide gel electrophoretogram: (1) molecular-mass standards as in Figure 1; (2) cytochrome *c* without the palladium(II) complex; (3)–(12) mole ratios 1:1 through 10:1, in integral increments.

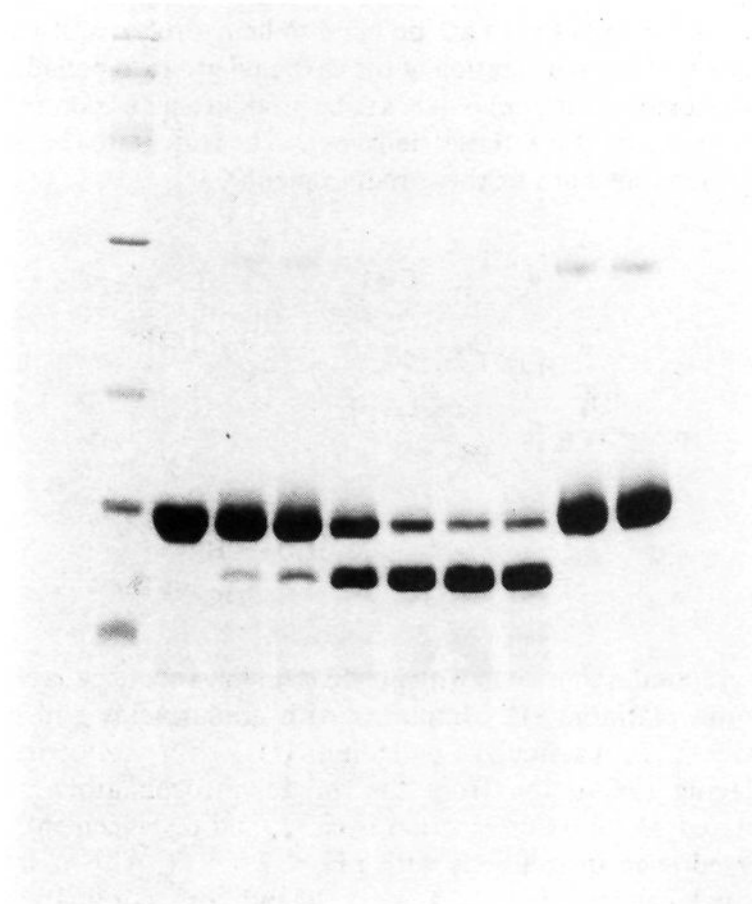

Figure 3. Effect of the mole ratio of *cis*-$[Pd(dtco\text{-}OH)(H_2O)_2]^{2+}$ to 1.3 mM horse-heart cytochrome *c* and of the added glutathione on the yield of hydrolytic cleavage of the protein in solution that is 100 mM in HBF_4, at pH 1.7, after incubation for 2 days at 40 °C. Lanes in the SDS polyacrylamide gel electrophoretogram: (1) molecular-mass standard as in Figures 1 and 2; (2) cytochrome *c* without the palladium(II) complex; (3)–(8) mole ratios of the Pd(II) complex to the protein of 1:1, 2:1, 4:1, 6:1, 8:1, and 10:1, respectively; (9) and (10) mole ratios of the Pd(II) complex to the protein to glutathione of 4:1:16 and 6:1:24, respectively.

its anion must not coordinate to palladium(II); HBF_4, $HClO_4$, and CF_3COOH satisfy both requirements. Many experiments with these three acids showed that the optimal pH values are 1.6–2.0. In this range there is no cleavage in the absence of the promoters. In their presence, cleavage apparently takes place at one site. Cleavage was not observed at $3.0 \leq pH \leq 5.0$.

These findings are explicable in terms of the known conformations of cytochrome *c*.[28,29] The so-called acid transition, from the native state III to the partially unfolded state II, has the pK_a of 2.5. This complicated transition involves two or three protons and yields multiple substates in equilibrium.[28]. In the state II the heme crevice is open, and both of the axial ligands to the iron atom (His18 and Met80) are replaced by water molecules. Protonation of His18 also is characterized by the pK_a of 2.5.[28–31] We observed the characteristic shift of the Soret band of ferricytochrome *c* from 410 nm at pH 7.0 to 397 nm at pH 1.8.

As Figure 1 shows, the two-band pattern established after 1 day remains when the equimolar mixture of cytochrome *c* and the palladium(II) complex is incubated for as long as 5 days. There seems to be no advantage in prolonging the incubation beyond 1 or 2 days. The apparent lack of further fragmentation may indicate that cleavage occurs at a single site.

As the mole ratio of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ to cytochrome *c* was raised from 1:1 to 4:1 in incubations that lasted for 6 days, a third band appeared in the electrophoretograms that are not shown. Figure 2 shows that, as the mole ratio of *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$ to cytochrome *c* was raised gradually from 1:1 to 10:1, the cleavage yield first increased and then leveled at 50–60%.

As Chart 1 shows, *cis*-$[Pd(dtco\text{-}OH)(H_2O)_2]^{2+}$ differs from *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$ only in the presence of one hydroxyl group in the bidentate ligand. As Figure 3 shows, the former complex cleaves cytochrome *c* with the apparent yield as high as 80%; the latter complex gave the apparent yield of 60%. In the presence of glutathione, which strongly coordinates to palladium(II), cytochrome *c* remains intact. This control experiment, shown in Figure 3, confirms that attachment of the palladium(II) complex to the protein is required for cleavage.

Site of Cleavage. The cleaved amide bond was located by identifying the first three amino acid residues at the amino terminus of the large fragment resulting from cleavage. Because the terminal amino group in cytochrome *c* is acetylated, this group did not interfere with the sequence determination. The unpurified reaction mixture after incubation of cytochrome *c* with $[Pd(H_2O)_3(OH)]^+$ gave chromatograms in which threonine, valine, and glutamic acid were the most prominent, but some other amino acids were present as contaminants. This is preliminary evidence for the segment Thr19–Val20–Glu21 in the sequence of horse cytochrome *c*, which is shown in Chart 1. We tentatively conclude that it is the His18–Thr19 bond that is specifically cleaved.

In the thorough experiments with cytochrome *c* and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ Edman degradation was done on the purified large fragment of the protein. Now the chromatograms were "clean", and the amino acids could be quantitated. Within the margins of error, 45, 47, and 54 pmol of threonine, valine, and glutamic acid, respectively, correspond to the mole ratio of 1:1:1. These results confirmed the tentative conclusions and also showed that different palladium(II) complexes cleave the same amide bond in the protein, namely His18–Thr19.

Previous studies of peptides consistently showed that palladium(II) complexes effect cleavage of the amide bond involving the carboxylic group of the amino acid (methionine, cysteine, *S*-methylcysteine) to whose sulfur atom the palladium(II) atom is attached. This study of a protein unexpectedly showed cleavage not of the Cys17–His18 bond but of the next bond "downstream" in the sequence. To veryify this fact, we examined the cleavage

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Table 1. Hydrolysis of the Histidine–Alanine Bond in AcCys–His–Ala Promoted by Palladium(II) Complexes

promoter[a]	mole ratio promoter:peptide	pH*[b]	$10^3 k_{obsd}$, min	T, °C
$[Pd(H_2O)_3(OH)]^+$	1:1	1.33	5.4 ± 0.2	40
cis-$[Pd(en)(H_2O)_2]^{2+}$	1:1	1.37	3.3 ± 0.3	40
cis-$[Pd(dtco)(H_2O)_2]^{2+}$	2:1	1.42	7.0 ± 0.5	50

[a] Because the solvent is D_2O, exchangeable H atoms are deuteriated. [b] Uncorrected for isotope effect.

of the fragment 11–21 of cytochrome *c*, called heme undecapeptide or microperoxidase-11 (because of its peroxidase activity). In this fragment the covalent bonds between the backbone and the heme are preserved; see Chart 1. Cleavage of heme undecapeptide yielded one fragment that contains the heme and another that does not. The fragments were separated by HPLC, and the latter one was analyzed. It consists of threonine, valine, and glutamic acid in the mole ratio of 1.04:0.91:1.00. Evidently, heme undecapeptide is cleaved between His18 and Thr19, exactly like the complete protein molecule.

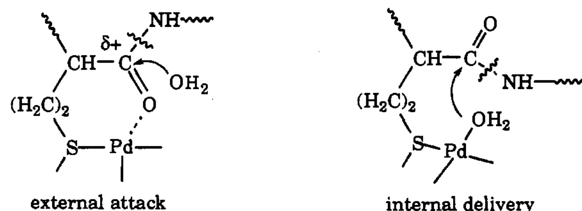
Apocytochrome *c* is cleaved by *cis*-$[Pd(en)(H_2O)_2]^{2+}$ like cytochrome *c*. Electrophoretograms that are not shown again contain two principal bands—the native protein and its larger fragment. Close similarity of these results to those shown in Figure 1 indicates that heme has no effect on the site and yield of cleavage. The cleavage reaction depends only on the polypeptide part of the cytochrome *c* molecule.

Hydrolysis of AcCys–His–Ala and AcCysMe–His–Gly. These peptides are models of the segment Cys17–His18–Thr19 in cytochrome *c*. Threonine is replaced by alanine or glycine because these two amino acids have simple ^1H NMR spectra that allow easy monitoring of cleavage reactions.

In all reactions, involving either peptide and any palladium(II) complex, only the His–Ala and His–Gly bonds are cleaved. The kinetic results in Table 1 show that the former reaction has half-lives of 2–4 h at 40 °C. Cleavage of the Cys–His and CysMe–His bonds was not observed. These findings further corroborate findings with cytochrome *c* by showing the special reactivity of the amide bond in the -Cys-His-X- sequence. Alkylation of the sulfur atom does not affect the regioselectivity of cleavage.

Hydrolysis of AcCysMe–Ala–Gln. This peptide is a faithful model of the segment Cys14–Ala15–Gln16 in cytochrome *c*. In the presence of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ only the CysMe–Ala bond is cleaved, and cleavage of the Ala–Gln bond was not observed. This regioselectivity is the same as that observed in our previous studies of various peptides—the first amide bond "downstream" from the anchoring side chain is cleaved.[19–22] Clearly, the new regioselectivity observed in cytochrome *c* is a consequence of the histidine residue adjacent to Cys17.

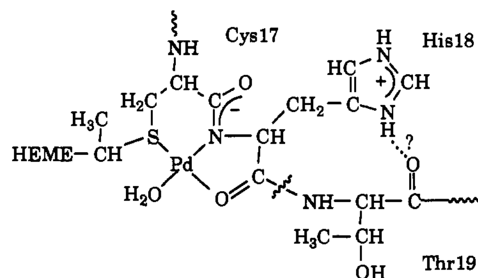
Selectivity of Cleavage. Previous studies showed that palladium(II) complexes promote peptide hydrolysis only if they are attached to an amino acid side chain. This need for anchoring is readily explained in terms of general mechanisms proposed for hydrolysis.[8,9] Briefly, the palladium(II) atom can act in two ways, shown as follows:



As a Lewis acid, it may weakly bind to the carbonyl oxygen atom, further polarize the carbonyl group, and thus facilitate external attack by water. Alternatively, the palladium(II) complex may internally deliver a labile aqua ligand to the amide bond. The two mechanisms are kinetically equivalent, and in either case anchoring is necessary to maintain the promoter near the scissile bond. If the substrate were bonded to palladium(II) solely through the carbonyl oxygen atom, water (the solvent) would displace the substrate from the coordination sphere. The additional, stronger attachment through the side chain is necessary in either mechanism. In the case of external attack, the chelate effect preserves the Pd···O bond and the necessary polarization of the carbonyl group. In the case of internal delivery, spatial proximity is required for intramolecular interactions involved in the transfer of the water molecule.

Of the heteroatoms present in amino acid side chains, sulfur atoms have the highest affinity for palladium(II).[32] Although the imidazole rings in histidine residues nos. 18, 26, and 33 are protonated, they may coordinate to palladium(II). The most likely ligands are the thioether groups in Cys14, Cys17, Met65, and Met80, and all four of them probably are accessible after the protein is partially unfolded by acid. Binuclear complexes of the type $[Pd(\mu_2\text{-}S_{peptide})(H_2O)_2]_2$, which readily form with smaller peptide molecules as bridging ligands, are well suited to the internal-delivery mechanism.[20,21] However, steric bulk prevents formation of such a complex with cytochrome *c* and with its heme undecapeptide. In these two cases, the thioether groups probably bind to palladium(II) initially as terminal ligands, as shown in eq 1. In the absence of the binuclear complex, the external-attack mechanism is more likely. Although the following analysis is developed largely in terms of this mechanism, it may also apply to the internal-delivery mechanism. When the promoter and the substrate form a mononuclear complex, stereochemical factors favoring coordination of the carbonyl oxygen atom (for external attack) also favor approach of the scissile amide bond to a coordinated water molecule (for internal delivery).

For the His18–Thr19 amide bond to be hydrolyzed, it has to be activated by coordination of the carbonyl group to palladium(II) (for external attack) or it has to be brought near a coordinated water molecule (for internal delivery). The tridentate chelation shown satisfies both of these requirements:



This proposal is consistent with previous findings about palladium(II) and platinum(II) complexes with amino acids and peptides.[32–35] In particular, palladium(II) is very effective in displacing the proton from the amide nitrogen atom. The estimated pK_a for this reaction is ca. 2, and displacement was observed even in solutions with pH < 2.0.[36–40] Although the carbonyl oxygen atom is a weak ligand, its coordination is facilitated by the chelate effect. Fused six-membered and five-membered rings impart considerable stability to the proposed

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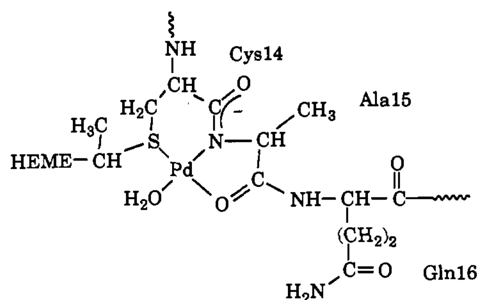
complex. Stereochemical factors seem to favor coordination of the carbonyl oxygen atom and the resulting activation of the amide bond toward hydrolysis.

Tridentate coordination of the segment Cys14–Ala15–Gln16



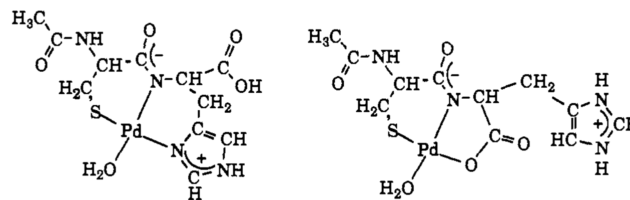
would be similar to the coordination shown above. Since, however, the Ala15–Gln16 amide bond is not cleaved, we reason that this coordination does not occur. The striking difference between the three-residue segments beginning with Cys17 and with Cys 14 is that the former contains an imidazolium ion (in acidic solution) in the position where the latter contains a methyl group. The imidazolium ion may polarize and thus activate the scissile bond; or it may form a hydrogen bond with the carbonyl group of Thr19 so that the resulting large ring helps orient the His18 carbonyl group for coordination to palladium(II); or imidazole may bind palladium(II) and promote cleavage of the next amide bond. We do not yet have evidence for any of these interactions.

Tridentate coordination of the three-residue segments of cytochrome *c* beginning with Met65 and with Met80 is less likely because the fused seven-membered and five-membered rings may be incompatible; we found no precedents for such chelation in relevant complexes. By contrast, there are precedents for the fused six-membered and five-membered rings like those shown above.[41] Although the methionine residues probably form the initial unidentate complexes shown in eq 1, these complexes do not seem to convert into chelate complexes, and cleavage near the methionine anchors is not observed.

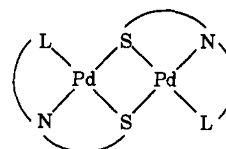
Unidentate attachment of the Pd(II) promoters to the thioether side chains in the protein apparently does not result in cleavage of the next amide bond "downstream", the simple pattern found in our previous studies with short peptides[19–22] and in the experiment with AcCysMe–Ala–Gln described above. In our future work we will carefully search for this simple pattern of cleavage in proteins, and we will study imidazole coordination. But on the basis of the evidence available to date we think that multidentate attachment of the Pd(II) atom to the protein is important for cleavage.

Palladium(II) Complex with AcCys–His. We have some evidence for the tridentate coordination of the peptides to palladium(II) proposed in the preceding subsection. The yellow precipitate remaining after the hydrolysis of AcCys–His–Ala with $[Pd(H_2O)_3(OH)]^+$ gave informative infrared spectra. The bands due to free (at 1730 cm^{-1}) and coordinated (at 1653 cm^{-1}) carboxylic group are both present, and the band at 1558 cm^{-1} is characteristic of the amide group coordinated via the nitrogen atom.[32,42] In the reaction mixture of AcCys–His–Ala and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ the ethylenediamine ligand is readily protonated and displaced; the strong ^1H NMR resonance at 3.40 ppm is due to enH_2^{2+}. After this displacement, the actual promoter in solution most likely is $[Pd(H_2O)_3(OH)]^+$.[20–22] Indeed, when this last complex is added to the peptide, the same yellow precipitate was found. The 1,5-dithiacyclooctane ligand in *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$ is more stable than the ethylenediamine ligand and seems not to be displaced in the reaction mixture with AcCys–His–Ala; there was no precipitate. Because this last complex remained in solution, it could be examined by ^1H NMR spectroscopy. The resonances of the H^2 and H^5 protons showed both free (at 8.61 and 7.30 ppm) and coordinated (at 7.85 and 7.10 ppm) imidazole. Upfield shift of these resonances upon coordination of the N^3 atom to platinum(II) and other metal ions is well-known.[43,44]

The existence of free and coordinated carboxylic groups, evident in the infrared spectra, and the existence of free and coordinated imidazole rings, evident in the ^1H NMR spectra, point to the two geometrical isomers



or to their binuclear counterparts shown schematically as follows:



In this combined formula L represents either the imidazole or the carboxylate ligand. There are precedents for such complexes with cysteinate bridging ligands.[45–48] The tridentate coordination in these isomers is relevant to binding of the palladium(II) complexes to cytochrome *c* and to heme undecapeptide, but the possibility of geometrical isomerism when imidazole is present cannot explain why the His18–Thr19 bond is cleaved and Ala15–Gln16 bond is not. In the future, we will study the special role that the imidazole ring in the side chain of histidine may play in these hydrolysis reactions.

Conclusion. To our knowledge, this study provides the first case of selective hydrolytic cleavage of a protein effected by a directly attached (not tethered) metal complex. Although the reactions are carried out in the pH range between 1.5 and 2.0, this procedure may already be useful. Cyanogen bromide and even hydrolytic enzymes are applied not only in neutral solutions but often in acidic or basic solutions. Many procedures with these established reagents call for 0.10 M HCl or 70% HCOOH as solvents and for heating above room temperature. Cleavage of cytochrome *c* by cyanogen bromide requires 0.10 M HCl,[49–51] greater acidity than that used in our study. As long as "background" cleavage is avoided, the pH and temperature of the solution may be adjusted for optimal reactivity. In fact, acidic conditions are sometimes required to denature the protein and make the anchoring side chains accessible to the reagent. This is the case with cytochrome *c*, which is completely unfolded but whose polypeptide backbone remains intact even at pH ≈ 0.[29] Some of the widely-used proteolytic enzymes are less than fully specific, and cyanogen bromide cleaves proteins with yields well

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below 100%. Our best yield of cleavage, ca. 80%, may already suffice in cases in which specificity may be more important than yield.

This study is an early step toward our ultimate goal—metal complexes as artificial peptidases. Although they are unlikely to surpass proteolytic enzymes in catalytic turnover, these inorganic reagents have several potential advantages. They may prove applicable to folded proteins, their selectivity may be adjustable by choice of ancillary ligands (other than water), they are compatible with a wide range of solution conditions, and they are structurally noninvasive owing to their small size. Still, more studies of the mechanism of cleavage are needed before palladium(II) complexes can be used routinely in biochemistry and structural biology.

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