Regioselective Cleavage by a Palladium(II) Aqua Complex of a Polypeptide in Different Overall Conformations

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Two molecules of the complex *cis*-$[Pd(en)(H_2O)_2]^{2+}$ lose aqua ligands and bind to His5 and His9 residues in the nonadecapeptide that is the carboxy-terminal segment of the protein myohemerythrin. The known modes of palladium(II)−histidine coordination are detected by ^{1}H NMR spectroscopy. Only the $[Pd(en)(H_2O)]^{2+}$group bound to His5 cleaves the polypeptide backbone; the group bound to His9 does not. Only the amide bond Val3−Pro4 is cleaved. This regioselectivity is attributed to electrostatic repulsion of the $[Pd(en)(H_2O)]^{2+}$group by cationic lysine residues 6, 7, and 10 and the absence of repulsion by the residues "upstream" from His5. The polypeptide in a partially α-helical conformation and the tripeptide AcGly-Gly-His, which adopts many flexible conformations, are both cleaved at the second amide bond "upstream" from the histidine residue bearing the $[Pd(en)(H_2O)]^{2+}$ group. Moreover, the rate constants for the cleavages of these two peptides are virtually the same. Regioselectivity and kinetics of the cleavage of peptides by palladium(II) aqua complexes seems to be affected by the local secondary structure in the vicinity of the scissile bond. This study is a step toward our ultimate goal−design of artificial metallopeptidases.

Introduction

Selective cleavage of proteins and peptides, one of the most important tasks in analytical biochemistry, is usually accomplished with a few proteolytic enzymes (also called proteases or peptidases).[1] So convenient is their immense catalytic power that some of their shortcomings are insufficiently recognized. Because proteases are usually applied to denatured proteins, the cleavage pattern reveals little, if anything, about the three-dimensional structure of the proteins. Even the regioselectivity of cleavage, a desirable property in many applications, becomes a serious shortcoming when the common proteases are applied for so-called footprinting analysis of protein association with other molecules. In these experiments, nonselective cleavage reagents are required.

New reagents, with different characteristics of selectivity in protein cleavage, are needed for many tasks in biochemistry and structural biology. Among them are sequencing, semisynthesis, domain analysis, elucidation of folding, and analysis of non-native states. Very few synthetic reagents are available,[1] and discovery of new ones is hampered by the extreme unreactivity of the amide bond. For example, uncatalyzed hydrolysis of peptides by water in weakly acidic and nearly neutral solutions occurs with half-lives of hundreds of years;[2] even in a 1.0 M HCl solution, the half-life of a simple dipeptide is 5 months.[2]

Transition-metal complexes hold promise as new reagents for protein cleavage by various mechanisms. Several laboratories have used iron−EDTA complexes that are covalently attached to amino acid side chains.[3−12] Although in some cases the cleavage was fast, the synthetic work involved in the attachment and the additional chemicals required for cleavage limit the applicability of this method. Use of untethered chelate complexes eliminates one of the disadvantages.[13−15]

Another method, practiced in our laboratory and elsewhere,[16−18] consists of spontaneous attachment, without tethering, of preformed metal complexes to nucleophilic side chains, followed by cleavage of proximate amide bonds. The initial study with platinum(II) complexes[19] and subsequent investigations with palladium(II) complexes and various peptides[20−27] revealed several aspects of the reaction mechanism. The applications

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to proteins[28,29] showed that cleavage is possible at pH values as high as 6.2 and that different palladium(II) complexes have the same regioselectivity of cleavage.

The main difference between proteins and short peptides is that the former are much more homogeneous than the latter in the secondary and tertiary structures. To investigate possible effects of the secondary structure (conformation) on the cleavage promoted by palladium(II) complexes, we now compare the reactivity of the same nonadecapeptide (so-called 19-mer) in predominantly α-helical and essentially random-coil conformations.

Experimental Procedures

Chemicals. Distilled water was demineralized and purified to a resistivity greater than 18 MΩ cm. The deuterium-containing compounds D_2O, $DClO_4$, and NaOD and the salt $AgClO_4 \cdot H_2O$ (99.999% pure) were obtained from Aldrich Chemical Co. The solvent 2,2,2-trifluoroethanol and the peptide L-glycyl-L-glycyl-L-histidine (Gly-Gly-His) were obtained from Sigma Chemical Co. The terminal amino group in the peptide was acetylated by a standard procedure.[20] All other chemicals were of reagent grade. The deuterium-containing 2,2,2-trifluoroethanol (CF_3CD_2OD) was obtained from Isotec Inc. The peptide designated ECH-3, synthesized by a standard solid-state method and checked for purity by HPLC, was a gift by Professor M. Reza Ghadiri of Scripps Research Institute. The complex *cis*-$[Pd(en)(H_2O)_2]^{2+}$, in which en is ethylenediamine, was prepared by treating the corresponding dichloro complex with 2 equiv of $AgClO_4$ and removing AgCl by centrifugation, all in the dark.[30] The aqua ligands can be H_2O (in circular dichroism samples and cleavage experiments monitored by HPLC) or D_2O (in ^1H NMR spectroscopic experiments); H_2O is written consistently, for simplicity.

Spectroscopic and Analytical Methods. Proton NMR spectra were recorded in D_2O and in a 1:1 v/v mixture of D_2O and CF_3CD_2OD, with a Bruker DRX 400 spectrometer, and with DSS as an internal reference. Temperature was kept within ±0.5 °C. In all NMR studies, the concentration of the polypeptide was 2.0 mM. The pH was measured with a Fisher 925 instrument and a Phoenix Ag/AgCl reference electrode and not corrected for the deuterium effect.

The circular dichroism (CD) measurements were recorded with a JASCO J-710 polarimeter equipped with a PTC-348W Peltier thermostat. Peptide concentrations for the CD experiments were determined by quantitative amino acid analysis, with norleucine as an internal standard. The optical path length was 1 mm. Each spectrum is an average of 10 scans. The mean residue ellipticity, [Θ], has the units deg cm^2 $dmol^{-1}$. Circular dichroism spectroscopy is the method of choice for determining the extent of random-coil and α-helical conformations of peptides.[31] A negative band at wavelengths less than 200 nm is diagnostic of a random-coil conformation, whereas a strong, positive band near 190 nm and negative bands at 208 and 220−222 nm are diagnostic of an α-helical conformation.[31] The fraction of α-helical polypeptide was estimated from the mean residue ellipticity in the region 220−222 nm.[32]

The peptides were separated with a Beckman Gold HPLC system containing a 166 detector, a 126 AA solvent module, and a 2.1 × 250 mm Vydac C18 column of 5-μm beads. Amino acid sequences were determined with a 477A protein sequencer and a 120A analyzer, both by Applied Biosystems Inc. The first three residues from the amino terminus in each of the peptides separated by HPLC were determined by Edman degradation. All of these bioanalytical experiments were done by the staff of the Protein Facility at Iowa State University.

Cleavage of Peptides with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. Kinetics of AcGly-Gly-His hydrolysis was examined as in our previous studies.[24,25] The nonadecapeptide, designated simply the polypeptide, was cleaved by two procedures, in aqueous solutions. In the first procedure, 5 μL of a 100 mM solution of *cis*-$[Pd(en)(H_2O)_2]^{2+}$in H_2O was added to 100 μL of a 1 mM solution of the polypeptide in H_2O. The reaction mixture was kept at 40 °C. In the second procedure, 50 μL of CF_3CH_2OH and 5 μL of a 100 mM solution of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ in H_2O were added to 50 μL of a 1 mM solution of the polypeptide in H_2O. The reaction mixture was kept at 22 °C. Both reactions were quenched by adding 5 μL of a 100 mM aqueous solution of sodium diethyldithiocarbamate, Na(ddtc), to a 20-μL aliquot of the reaction mixture. The insoluble $[Pd(ddtc)_2]$ was removed by centrifugation, and the clear solution was analyzed by HPLC. The reactions at 22 °C were followed over 36 days, and the rate constants for the cleavage were calculated from the initial rate.

Results and Discussion

The Polypeptide. The main substrate for cleavage is the carboxy-terminal segment of the monomeric protein myohemerythrin, acetylated and amidated at the ends in order to prevent binding of the amino and carboxylic groups to palladium(II). Previous studies have shown that the side chains of aspartic acid, glutamic acid, and lysine have too low affinity to cause detectable binding to palladium(II); the acidic solutions used in our experiments further suppress this binding.

Ac-E-V-V-P-**H**-K-K-Nle-**H**-K-D-F-L-E-K-I-G-G-L-NH_2

In the preceding sequence only His5 and His9 are potential ligands for palladium(II).

Binding of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ to the Polypeptide. Because previous studies showed that palladium(II) complexes cleave the peptide only if they are attached to the side chain,[19,20,22] we studied the attachment process by ^1H NMR spectroscopy. Both in pure D_2O and in a 1:1 v/v mixture of D_2O and CF_3CD_2OD, at the same pH of 2.5, the free polypeptide showed the singlets for the H-2 protons in the two histidine residues, at 8.63 and 8.64 ppm. Although the signals for the H-5 protons overlapped with those for the aromatic protons in Phe12, complex formation with the histidine residues was clearly evident from the H-2 resonances because chemical shifts of both H-2 and H-5 are very sensitive to coordination of imidazole to palladium(II).[24,25,33] When a 2.0 mM solution of the polypeptide was made 2.0, 4.0, and 10.0 mM in *cis*-$[Pd(en)(H_2O)_2]^{2+}$, the fraction of coordinated histidine residues, determined from the relative intensities of the resonances, increased from 35 to 59 to 100%. The original two singlets were replaced by three pairs of singlets for the H-2 protons in the interval 7.41−8.01 ppm. Evidently, His5 and His9 behave alike in forming three kinds of palladium(II) complexes. They were identified on the basis of the characteristic chemical shifts, as in previous studies by us[24,25] and others.[33] As Chart 1 shows, the unidentate complexes **1** and **2** are linkage isomers of each other; in complex **3**, the palladium(II) atom induced deprotonation of the amide nitrogen, a reaction known to occur even at pH <2.0.[34−40] Our previous

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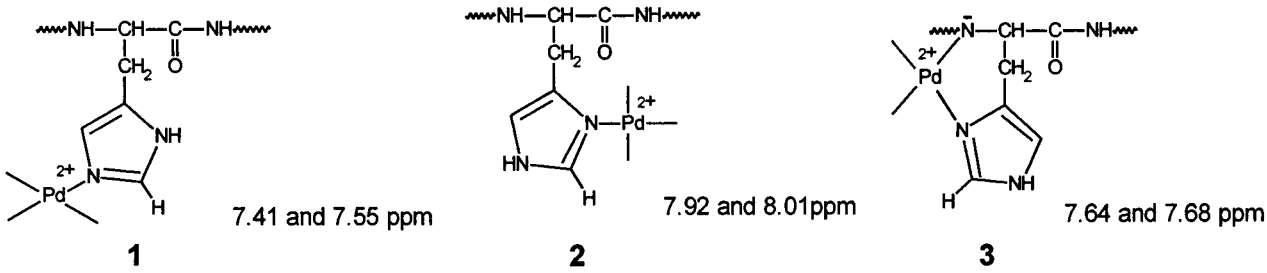
Chart 1. Modes of Binding of $[Pd(en)(H_2O)]^{2+}$ to the Polypeptide and the ^1H NMR Chemical Shifts of the H-2 Protons in His5 and His9 Residues



1 **2** **3**

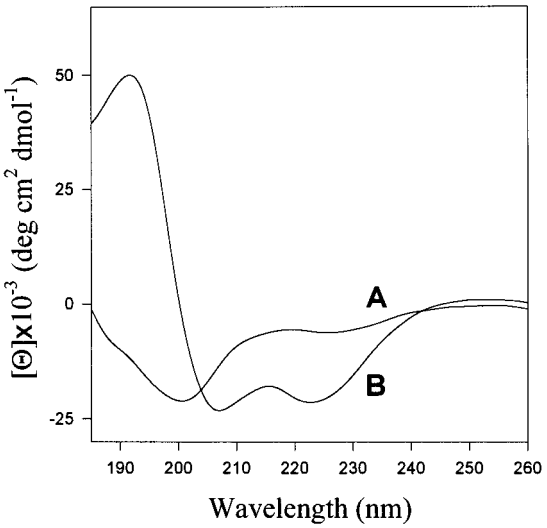


Figure 1. Circular dichroism spectra of solutions that were 8.1×10^{-5} M in the polypeptide and 4.5×10^{-4} M in *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0 in two solvents: (A) water, at 40 °C; (B) 1:1 v/v $H_2O-CF_3CH_2OH$, at 22 °C.

Table 1. Degrees of α-Helicity[a] of the Polypeptide at pH 2.0 (%)

	solvent, temperature (°C)	
polypeptide state	H_2O, 40	1:1 v/v $H_2O-CF_3CH_2OH$, 22
free	<10	≈80
bound to Pd(II)	<10	≈50

[a] Figure 1 and refs 31 and 32.

study showed that these various palladium(II)−peptide complexes exist in an extended equilibrium with one another and with the free peptide and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ but that only complex **2** is active in hydrolysis. Because identical ^1H NMR spectra were obtained in pure D_2O and in a 1:1 v/v mixture of D_2O and CF_3CD_2OD as solvents, we concluded that 2,2,2-trifluoroethanol does not affect binding of palladium(II) to the polypeptide.

Conformation of the Polypeptide. The ability of 2,2,2-trifluoroethanol to stabilize α-helices is well documented and often exploited.[41−43] Results of CD spectroscopic measurements are given in Figure 1 and Table 1. The free polypeptide is essentially completely a random coil in aqueous solutions at 40 °C. The presence of 50% v/v CF_3CH_2OH and cooling to 22 °C induce the conversion to an α-helix. The degree of helicity, however, is less than 100% because the sequence

Table 2. Rate Constants for the Cleavage of Histidine-Containing Peptides Promoted by *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 2.0

	H_2O:CF_3CH_2OH	$10^5 k_{obsd}$ (min^{-1})	
substrate	in the solvent, v/v	22 °C	40 °C
polypeptide	1:1	0.5	
	1:0		15
AcG-G−H	1:1	0.6	
	1:0		19
	1:1		17

contains glycine and proline residues, which locally disrupt this conformation. Because of the presence of Pro4, the region "upstream" of His5 cannot be fully α-helical even in the free polypeptide. Binding of two molecules of the palladium(II) complex has no effect on the random coil (at 40 °C) but causes a decrease in the α-helicity (at 22 °C). Our finding agrees with a previous report[44] that square planar complexes of palladium(II) and platinum(II) somewhat destabilize the α-helical structure of a polypeptide when they bind to histidine side chains.

Cleavage of the Polypeptide. Reaction mixtures that were 1.0 mM in the polypeptide and 5.0 mM in *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were kept at 40 and 22 °C. The latter temperature was a compromise between the requirements for convenient rate of the cleavage (which is enhanced by heating) and the α-helical conformation (which is stabilized by cooling). The outcome of the cleavage reactions was the same at both temperatures. Separations by HPLC yielded only one product, a fragment in which the first three amino-terminal residues were found to be P-H-K. Evidently, only the V3−P4 bond in the complete polypeptide is cleaved. The other product of the polypeptide cleavage, the fragment AcE-V-V, is too short to be retained by the chromatographic column. This nonretention is consistent with our previous findings; tripeptide resulting from cleavage of the heme undecapeptide, a part of cytochrome *c*, was likewise undetectable by HPLC.[28]

Hydrolysis of histidine-containing peptides in the absence of palladium(II) complexes, the so-called background cleavage, was studied at pH 1.0, whereas the cleavage by the palladium(II) complex was studied at pH 2.0. This difference in conditions added rigor to the control experiments and ensured that the cleaving ability of the palladium(II) complex would not be overestimated. Despite the greater acidity, the background cleavage has the half-life of ca. 9 months at 40 °C and is undetectably slow at 22 °C. These conservative control experiments show that the rate constants in Table 2 correspond to hydrolysis promoted by the palladium(II) complex in the reaction mixture. The 30-fold increase (from 5×10^{-6} to 1.5×10^{-4} min^{-1}) in the rate of cleavage of the polypeptide may be due to the unfolding of the α-helix, removal of CF_3CD_2OD, or increase in the temperature. These questions were settled in control experiments with the tripeptide AcG-G-H, which is very

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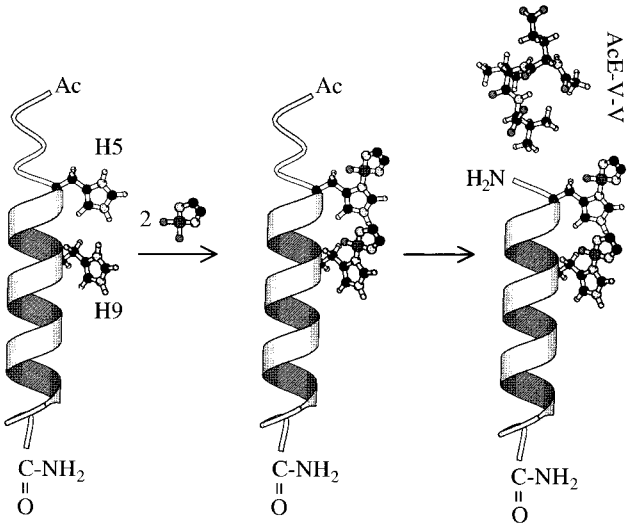

Figure 2. Cleavage of the Val3−Pro4 bond in the nonadecapeptide promoted by the $[Pd(en)(H_2O)]^{2+}$ group bonded to the N-3 atom in the imidazole ring of His5. The final products are a tripeptide and a doubly-labeled hexadecapeptide. The $[Pd(en)(H_2O)]^{2+}$ group bonded to the imidazole ring of His9 does not cleave the polypeptide backbone. The peptide is only partially α-helical. The terminal segments, and possibly some internal regions as well, are unfolded. Symbol Ac represents the acetyl group.

flexible and therefore has very heterogeneous configurations. As Table 2 shows, the rate constant for its cleavage at 40 °C is practically unaffected by the presence (or absence) of CF_3CD_2-OD in solution. The similarity of the rate constants for cleavage of the tripeptide and the polypeptide at 40 °C does not rule out conformational effects on the kinetics of the polypeptide cleavage, because at this temperature both peptides have random-coil conformations. But the near equality of the rate constants at 22 °C, at which the polypeptide is partially α-helical while the tripeptide is still random, indicates that the cleavage is unaffected by the overall conformation of the substrate. This is to be expected, because the reaction should be affected only by the structure of the reactive site. Since the conformation in the vicinity of His5 is probably unfolded (see above), we suggest that the similarity of the rate constants reflects the similarity of the (unfolded) conformations in the vicinity of the histidine residues near which the cleavage occurs.

Regioselectivity of Cleavage. As Figure 2 shows schematically, both histidine residues 5 and 9 in the polypeptide are tagged with $[Pd(en)(H_2O)]^{2+}$ groups, but only the group at His5 cleaves the backbone. Experiments with cytochrome *c*[28] and myoglobin[29] likewise indicated that molecules of the palladium(II) complex attached to some amino acid residues do, whereas molecules of the same complex attached to residues of the same kind elsewhere in the protein do not, cause the backbone cleavage. The cleaved peptide bonds tend to be one to three positions removed from the residues capable of anchoring the palladium(II) complexes. Because the polypeptide in this study is structurally simpler than the aforementioned proteins, we can now address the important question of regioselectivity.

The observed pattern of cleavage may be a consequence of the electrostatic interactions between the cationic groups $[Pd(en)(H_2O)]^{2+}$ and proximate side chains. The residue His9 in the polypeptide is surrounded on both sides by cationic lysine residues 6, 7, and 10, which may repel the palladium(II) complex from the backbone. The residue His5 is followed "downstream" by cationic lysine residues 6 and 7 and preceded "upstream" by electroneutral residues 2−4 and Glu1, which is protonated and therefore electroneutral at pH 2.0. The palladium(II) complex, therefore, is repelled from cleaving on the carboxylic side of His5 but not from cleaving on the amino side of this residue. The relatively low rate constants in Table 2 may reflect the lack of negatively charged residues near the histidine anchors, residues that would attract the $[Pd(en)(H_2O)]^{2+}$ group toward the backbone and accelerate its cleavage.

The scissile bonds in myoglobin usually lie upstream, seldom downstream, from the residues capable of anchoring the palladium(II) complexes.[29] Now we find a similar pattern with peptides. Both in the tripeptide AcG-G-H and in the polypeptide, the cleavage occurs at the second amide bond upstream from a histidine residue. The stereochemical and other causes for this emerging pattern will be a subject of future research.

Conclusions

Previous studies in this laboratory showed that palladium(II) complexes hydrolytically cleave peptides and proteins in the vicinity of amino acid side chains to which these complexes are attached. This study indicates that cleavage is probably unaffected by the overall conformation of the substrate, at least in the polypeptide examined. Perhaps the local conformation in the vicinity of the palladium(II) binding site and the scissile bond is important for cleavage; in this polypeptide, this local conformation is not α-helical. Much more remains to be learned about the effects and noneffects of secondary structure on cleavage, but it is already clear that palladium(II) complexes differ in regioselectivity from proteolytic enzymes and from the few other chemical reagents that are used for cleavage of peptides and proteins. This study is yet another step toward our ultimate goal—design of artificial metallopeptidases.

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