New Selectivity and Turnover in Peptide Hydrolysis by Metal Complexes. A Palladium(II) Aqua Complex Catalyzes Cleavage of Peptides Next to the Histidine Residue

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Abstract: This seems to be the first report that a transition-metal complex bonded to a histidine residue effects hydrolytic cleavage of a peptide next to this residue. Dipeptides AcHis-Aa in which the C-terminal amino acid designated Aa is Gly, Ala, Ser, Thr, Leu, Phe, and Tyr are completely hydrolyzed at 60 °C and $1.46 \leq pD \leq 2.61$ in the presence of *cis*-$[Pd(en)(H_2O)_2]^{2+}$. The reaction is conveniently monitored by ^1H NMR spectroscopy, and we report the kinetics. The reaction is unimolecular with respect to the palladium(II)−peptide complex. The cleavage is regioselective. In all the aforementioned dipeptides and in the tripeptide AcGly-His-Gly only the amide bond involving the carboxylic group of histidine is cleaved; the amide bond involving the amino group of histidine is not cleaved. When the carboxylic group of histidine is free, as in AcGly-His, *cis*-$[Pd(en)(H_2O)_2]^{2+}$ does not effect hydrolysis. Lability of palladium(II) complexes and the acidic solution make possible a modest turnover in hydrolysis; the catalyst can cleave several equivalents of the dipeptide. The dipeptides AcHis-Aa, and also one product of their cleavage, AcHis, exist free and bound to the catalyst. They form similar palladium(II) complexes, five types of which are distinguishable by ^1H NMR spectroscopy. The other products of cleavage, the amino acids Aa, exist free and in chelate complexes *cis*-$[Pd(en)(N,O\text{-Aa})]^+$. Partial binding of the catalyst to the peptide and to its cleavage products gives rise to an extended and complex equilibrium. Increase in pH favors catalytically-inactive palladium(II)−peptide complexes, inhibits their conversion into catalytically-active complexes, and lowers the observed rate constant for hydrolysis. Because the equilibria are reversible, even the peptide bound in inactive complexes eventually becomes hydrolyzed. When palladium is removed as a diethyldithiocarbamate complex, the equilibria are abolished and only ethylenediamine, AcHis, and Aa remain. The rate constant for cleavage decreases as the steric bulk of the amino acid Aa increases and as intrapeptide hydrogen bonds mediated by water restrict the access of the palladium(II) catalyst to the His-Aa bond. This hydrogen bonding is possible only when the amino acid Aa contains a hydroxyl group in a flexible side chain, as in Ser and Thr. The intrapeptide hydrogen bonding is impossible when a hydroxyl group is held relatively rigidly, as in Tyr, and, of course, when the hydroxyl group is absent, as in the other four amino acids. The kinetic effects of steric bulk and of specific hydrogen bonding may allow sequence selectivity in cleavage of peptides with palladium(II) complexes. This study points the way toward artificial metallopeptidases, coordination complexes with enzyme-like properties.

Introduction

Selective cleavage of peptides and proteins is one of the most common and the most important procedures in biochemistry. Although several proteolytic enzymes are available for this purpose, only trypsin is highly regioselective.[1] Because these enzymes are usually applied to denatured proteins, the pattern of cleavage reveals little, if anything, about the structure and function of the protein of interest. Moreover, the particular conditions (pH, temperature, etc.) under which the enzymes are active may be incompatible with the proteins or processes under investigation.

Modern methods of automatic sequencing of proteins work better with relatively long peptides than with the short ones that are usually obtained from proteolytic digestion. As the requirements for these traditional applications are changing, new challenges are emerging. Conceptually new methods of cleavage are needed for many tasks in biochemistry and molecular biology: semisynthesis of proteins; sequencing of large or blocked proteins; structural and functional analysis of protein domains; study of protein association with other proteins, with nucleic acids, and with membranes; investigation of protein folding and of incompletely-folded intermediates; development of new drugs; and so on. Chemical (nonenzymatic) reagents are well suited for many of these tasks. While the need for them is growing, only one, cyanogen bromide, is available for routine use.[1] Although cyanogen bromide is very useful, it has several shortcomings. It is volatile and toxic, requires harsh conditions, and often produces incomplete cleavage.

Transition-metal complexes are ideally suited for hydrolytic cleavage of regular (unactivated) amide bonds in proteins.[2−8] Clever studies with cobalt(III) complexes[2] revealed some mechanistic features of these interesting reactions, but they did not result in practical methods for analytical biochemistry. Because cobalt(III) forms an inert complex with the N-terminal amino acid in the peptide, only the N-terminal amide bond is cleaved. Practical applications, however, usually require cleavage of internal amide bonds.

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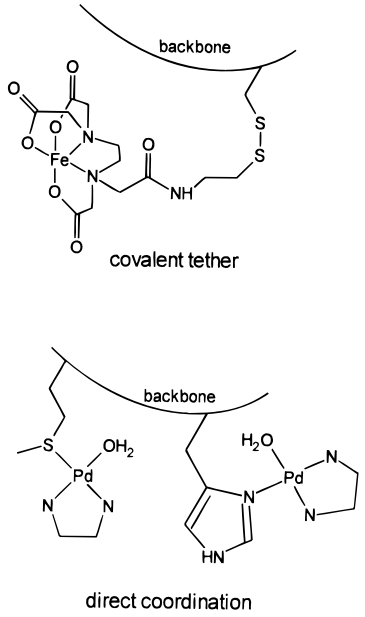
Chart 1. Two Ways of Attaching Transition-Metal Complexes to Proteins



Such cleavage of peptides and proteins promoted by transition-metal complexes has recently been achieved in this[9−14] and other[15−23] laboratories. Regioselectivity of cleavage depends on the site of attachment of the metal complex to the substrate and on the proximity—in sequence or in space—of this site to the scissile amide bond. Regioselectivity of backbone cleavage therefore depends on (but not only on) the regioselectivity of binding of the metal complex to the side chains. This binding can be achieved in two ways, shown schematically in Chart 1—by covalent tethers[15−23] and by direct coordination of the heteroatom in the side chain to the transition metal.[9−14] The former method involves multi-step chemical synthesis that sometimes, but not always, results in permanent attachment favorable for fast and complete cleavage. The latter method amounts to simply mixing the substrate and the metal complex. This spontaneous binding is governed by familiar principles of coordination chemistry, such as the trans effect and the affinity of the soft sulfur-containing ligands (methionine, cysteine, or *S*-methylcysteine) for the soft platinum(II) and palladium(II) atoms.[24−26] Cleavage by tethered complexes requires additional reagents, such as hydrogen peroxide and ascorbic acid. Cleavage by directly-attached complexes, however, requires only water (at the required pH value) and is highly regioselective. In all peptides but one studied in this laboratory the amide bond involving the carboxylic group of the anchoring amino acid was selectively hydrolyzed; the one exceptional case has been explained.[14]

To our knowledge, all the cases of peptide and protein cleavage by transition-metal complexes involve stoichiometric reactions, those in which the complex effects cleavage of no more than an equimolar amount of the substrate. This limitation stems from the promoter design (covalent tethering) or from the relative inertness of the complexes that cobalt(III) forms with any ligands and that platinum(II) and palladium(II) form with sulfur ligands. Now we report a truly catalytic cleavage, with a modest turnover of the peptide on the palladium(II) complex. This advance is made possible by the use of the imidazole group of histidine as the new anchor for the palladium(II) catalyst. Since this ambidentate ligand can coordinate through either nitrogen atom and also make hydrogen bonds, histidine presents new possibilities for control of selectivity.

Experimental Procedures

Chemicals. Distilled water was demineralized and purified further. The compounds D_2O, $DClO_4$, NaOD, and $K_2[PdCl_4]$ were obtained from Aldrich Chemical Co. Anhydrous $AgClO_4$ was obtained from G. Frederic Smith Chemical Co. All the common chemicals were of reagent grade. Dipeptides Gly-His, His-Gly, His-Ala, His-Ser, His-Leu, His-Phe, and His-Tyr, the tripeptide Gly-His-Gly, and the amino acids 1-methylhistidine and 3-methylhistidine were obtained from Sigma Chemical Co. The terminal amino group in each peptide and amino acid was acetylated by a standard procedure.[9,10] The dipeptide AcHis-Thr was synthesized by a standard solid-state method, and its purity was checked by HPLC; this was done by the Protein Facility at this university.

The complex *cis*-$[Pd(en)Cl_2]$ was converted into *cis*-$[Pd(en)(H_2O)_2]^{2+}$ by a treatment with 2 equiv of $AgClO_4$ (explosive substance!) at pH 2.0 and by removal of AgCl by centrifugation, all in the dark. The UV−vis absorption band is at 340−345 nm, as in a previous report.[27a] The stock solution of the aqua complex in D_2O was stable for as long as 6 months at 4 °C. Since the solvent in all the reaction mixtures was D_2O, the formulas H_2O, H^+, and OH^- actually mean D_2O, D^+, and OD^-.

Measurements. Routine ^1H NMR spectra, in D_2O and with DSS as an internal reference, were recorded with Varian VXR 300, Nicolet NT 300, and Varian Unity 500 spectrometers. Temperature was kept at 60 ± 0.5 °C. The pH values were measured with a Fischer 925 instrument and a Phoenix Ag/AgCl reference electrode, standardized in H_2O solutions. They were converted to pD values by the standard formula:[27b] pD = pH + 0.41. But in conceptual references to acidity and basicity the common symbol pH is used. Ultraviolet−visible spectra were recorded with an IBM 9420 spectrophotometer.

Proton NMR spectra with water suppression, whether one-dimensional or NOESY, were obtained with a Varian Unity 500 spectrometer. The 1−1 echo sequence, consisting of an excitation pulse $90°_x-\tau_1-90°_{-x}$ and a refocusing pulse $90°_\phi-\tau_2-90°_{-\phi}$, does not introduce any phase errors.[28] For good suppression, we recorded 64−320 scans. The solvent was a 9:1 mixture by volume of H_2O and D_2O, the concentration of the amino acid or the peptide was 20−50 mM, the internal reference was DSS, and the temperature was 21 ± 0.5 °C.

Molecular Modeling. Desktop Molecular Modeler, version 1.2, by Oxford University Press was used for minimization of total energies of the dipeptides AcHis-Ser, AcHis-Thr, and AcHis-Tyr. Rotations around single bonds were performed in order to optimize hydrogen

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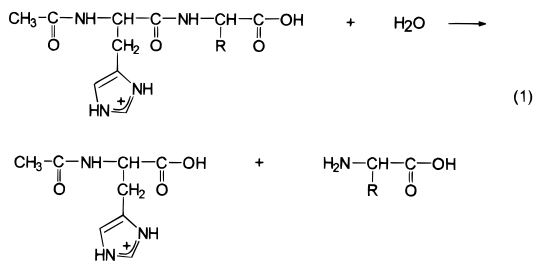
bonding, mediated by one or two water molecules, between the NH groups in imidazole on the one side and the OH group in serine, threonine, or tyrosine on the other.

Kinetics of Hydrolysis. The following solutions in D_2O were mixed in an NMR tube: 200 μL of a freshly-prepared 100 mM solution of *cis*-$[Pd(en)(H_2O)_2]^{2+}$, 200 μL of a 100 mM solution of the peptide, and 100 μL of a 100 mM solution of DSS. The volume was adjusted by adding 200 μL of D_2O, and acidity was adjusted with a 2.0 M solution of $DClO_4$. The final mixture was 26−30 mM in both the palladium(II) complex and the peptide. Acidity was varied in the interval $1.46 < pD < 2.61$; the pD values at the beginning and at the end of the experiment differed by less than 0.10. Acquisition of the ^{1}H NMR spectra began as soon as possible after the mixing, and 16 scans were taken each time. The temperature was kept at 60 ± 0.5 °C. A typical kinetic plot consisted of 20 points taken over two to three half-lives, but the cleavage of AcHis-Gly was monitored for 4 half-lives. Concentrations of the peptide and of the hydrolysis products were determined, with an estimated error of ±5%, from the known initial concentrations of AcHis-Aa and from integrated resonances of the leaving group (Aa) and the internal reference (DSS). In control experiments, peptide solutions without *cis*-$[Pd(en)(H_2O)_2]^{2+}$ were kept at pD 1.4 at 60 °C and occasionally examined by ^{1}H NMR spectroscopy.

Products of Hydrolysis. Free amino acids were identified by their ^{1}H NMR chemical shifts and by enhancement of their resonances upon spiking of the reaction mixtures with authentic samples. Coordinated amino acids were identified in two ways: first, by comparing the spectra of the reaction mixtures with the spectra of solutions prepared by mixing equimolar amounts of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and the amino acid of interest, at the same pD values; second, by adding 2 equiv of sodium diethyldithiocarbamate (Na_2DDTC) to the solution after the hydrolysis was complete; separating by centrifugation the yellow precipitate, presumably $[Pd(DDTC)_2]$, and recording the ^{1}H NMR spectrum of the clear supernatant.

Results and Discussion

Catalytic Cleavage. The subject of this study is hydrolytic cleavage of the dipeptides AcHis-Aa into *N*-acetylhistidine and the amino acid Aa, shown in eq 1. (In the acidic medium the



amino group of the free amino acid will be protonated.) This reaction is catalyzed by *cis*-$[Pd(en)(H_2O)_2]^{2+}$. The cleavage was easily monitored by following the ^{1}H NMR resonances listed in Table 1. Typical NMR spectra, showing complete hydrolysis of the His−Aa bond, are presented in Figure 1a. The seven C-terminal amino acids, designated Aa, were Gly, Ala, Ser, Thr, Leu, Phe, and Tyr. They differ in the side chain, designated R. At the end of the dipeptide hydrolysis, the leaving group Aa was mostly free and partly coordinated to palladium(II). Both forms were identified by their ^{1}H NMR resonances, but only the free amino acid is shown in Figure 1a. The resonances of amino acids coordinated to palladium(II) fall outside of the spectral region in Figure 1a; see Table 1. When palladium was completely removed by sodium diethyldithiocarbamate, the solution contained only AcHis, enH_2^{2+}, and the free amino acid Aa. Evidently, the cleavage is "clean" and complete.

Attachment of the Catalyst to the Substrates. When equimolar amounts of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and AcHis-Aa were mixed in solutions having $1.46 \leq pD \leq 2.61$, five NMR-detectable complexes spontaneously formed. Their characteristic ^{1}H NMR resonances are shown in Figure 1b, and their compositions are shown in Scheme 1. In all of the complexes ethylenediamine remains a bidentate ligand. Evidently, the imidazole group and other ligands in the dipeptide AcHis-Aa that displace aqua ligands have relatively weak trans effects and do not cause removal of the ethylenediamine ligand. In this respect imidazole (in the side chain of histidine) differs from the thioether (in the side chain of methionine); the latter promotes rapid displacement of ethylenediamine from the coordination sphere of palladium(II).[29]

Table 1. Monitoring of Dipeptide Hydrolysis in D_2O Solutions, at $1.46 \leq pD \leq 1.51$

	^{1}H NMR chemical shifts (δ, in ppm)		
group	hydrolysis substrate	hydrolysis product	hydrolysis product coordinated to Pd(II)
Gly CH_2	AcHis-Gly 4.01 s	Gly 3.89 s	3.55 s
Ala CH_3	AcHis-Ala 1.40 d	Ala 1.58 d	1.44 d
Ser CH	AcHis-Ser 4.45 t	Ser 4.22 t	3.72 t
Thr CH_3	AcHis-Thr 1.18 d	Thr 1.35 d	1.26 d
Leu CH	AcHis-Leu 4.42 t	Leu 4.08 t	3.61 t
Phe CH	AcHis-Phe 4.60 t	Phe 4.36 t	3.91 t
Tyr CH	AcHis-Tyr 4.61 t	Tyr 4.31 t	3.86 t
Gly CH_2 AcGly-CH_2	AcGly-His-Gly 4.01 s 3.89 s	Gly 3.89 s	3.55 s

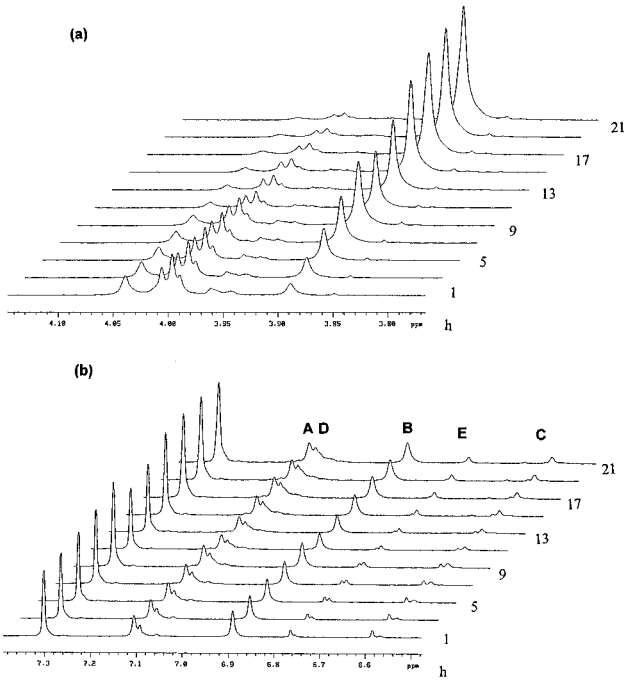
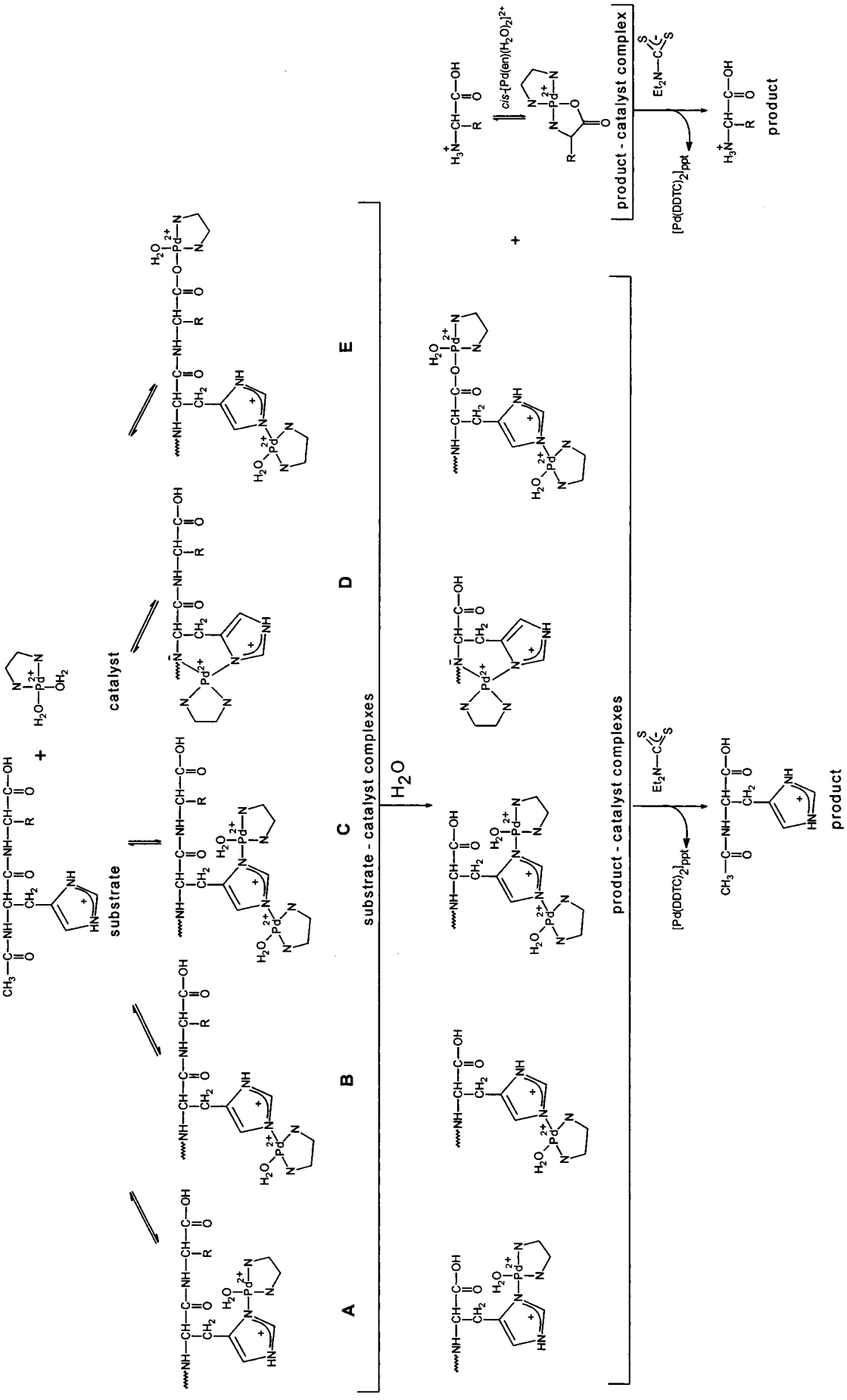
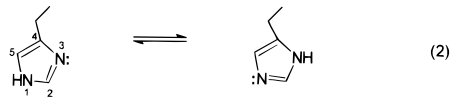


Figure 1. Proton NMR spectra of a solution in D_2O that was initially 28 mM in both AcHis-Gly and *cis*-$[Pd(en)(H_2O)_2]^{2+}$, at pD 1.46 and 60 °C. (a) Glycine CH_2 resonances in free glycine (3.89 ppm) and in AcHis-Gly that is free and bound to the catalyst (3.98−4.06 ppm). As the dipeptide is consumed, the free amino acid is produced. (b) Imidazole H-5 resonances in free AcHis-Gly (7.31 ppm) and in the complexes designated A through E that this dipeptide forms with the palladium(II) catalyst.

Scheme 1. Hydrolysis of AcHis-Aa Catalyzed by *cis*-$[Pd(en)(H_2O)_2]^{2+}$



The five complexes can be distinguished on the basis of the chemical shifts of the two imidazole protons, H-2 and H-5. Their respective values in the uncoordinated dipeptides AcHis-Aa are 8.60 and 7.31 ppm at pD 1.46 and are not significantly affected by the identity of the amino acid Aa. The two major complexes are linkage isomers of each other, corresponding to two tautomers of the imidazole ring in histidine (eq 2). In the



(2)

complex designated A, unidentate coordination via the N-3 atom moves the H-2 and H-5 resonances to 8.03 and 7.11 ppm, respectively. In the complex designated B, unidentate coordination via the N-1 atom moves these resonances to 7.87 and 6.89 ppm, respectively. These chemical shifts agree with the values for similar complexes recently published by other researchers.[30]

The three minor complexes contain more than one palladium(II) atom per dipeptide or involve more than one donor atom in the dipeptide. In the complex designated C the deprotonated imidazole (imidazolate anion) bridges two palladium(II) atoms. This is a fairly common mode of imidazole binding, because coordination of the first nitrogen atom facilitates deprotonation, and subsequent coordination, of the other.[31] The chemical shift of 6.58 ppm for the H-5 atom agrees with a published value.[30] Further supporting evidence for the bridging imidazolate ligand comes from our experiments with acetylhistidine singly methylated at N-1 and at N-3. Neither of these amino acids in reactions with *cis*-$[Pd(en)(H_2O)_2]^{2+}$ produced the complex designated C.

In the complex designated D the substrate coordinates to palladium(II) as a bidentate ligand, via the N-3 atom of imidazole and the nitrogen atom of the acetylated amino group. The H-5 resonance occurs at 7.10 ppm, as found also in a relevant previous study.[32] Certain transition-metal ions induce deprotonation of the amide nitrogen atom by binding to it. This process is especially favorable when the metal ion is already anchored to a side chain, so that a chelate ring is formed. Palladium(II) is particularly effective in displacing the proton.[33] The estimated pK_a for this reaction is ca. 2.0, and displacement was observed even in solutions with pH < 2.0.[33−39] A study especially relevant to ours found that tridentate coordination of Gly-His to palladium(II) causes the peptide nitrogen atom to lose the proton with the pK_a value estimated at less than 1.5.[32]

In the complex designated E coordination seems to occur via the N-1 atom of imidazole (as indicated by the H-5 resonance at 6.77 ppm) and the carboxylate group. That the N-3 atom of imidazole is not involved was established in experiments with *N*-acetyl-3-methylhistidine, which gave the complex E in the reaction with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. The N-1 atom and the carboxylate group probably coordinate to two palladium(II) atoms; such binuclear complexes are well known.[30,40,41] Mononuclear complexes in which the peptide molecule acts as a bidentate ligand or two peptide molecules act as unidentate ligands are less likely.

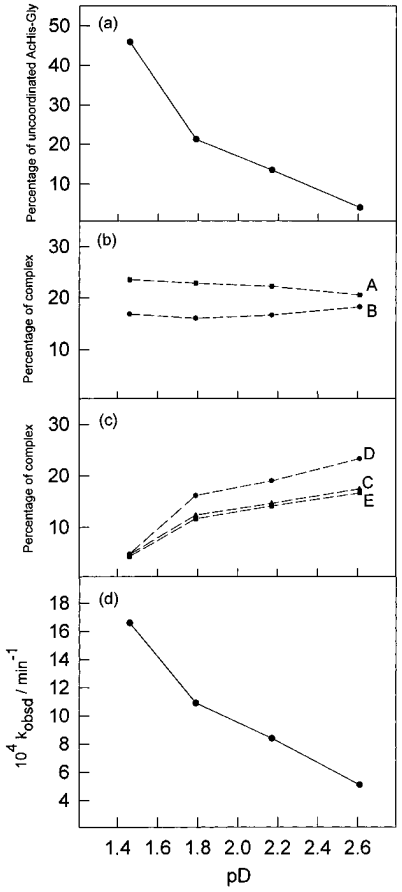


Figure 2. Effects of pD on a solution in D_2O that is initially 28 mM in both AcHis-Gly and *cis*-$[Pd(en)(H_2O)_2]^{2+}$, at 60 °C. (a) Fraction of AcHis-Gly not bound to palladium(II). (b) Fractions of the catalyst−substrate complexes designated A and B at the onset of hydrolysis; see the text. (c) Fractions of the catalyst−substrate complexes designated C, D, and E at the onset of hydrolysis; see the text. (d) The observed rate constant for the hydrolytic cleavage in eq 1.

Addition of sodium diethyldithiocarbamate (Na_2DDTC) to the mixture of five complexes before the hydrolysis has advanced measurably and complete removal of palladium in a precipitate leaves only two NMR-detectable species in solution—enH_2^{2+} and AcHis-Aa. Clearly, all the different species discussed above are products of the dipeptide coordination to palladium(II), and the intact dipeptide can be recovered from all of them.

Reaction between AcHis-Gly and *cis*-$[Pd(en)(H_2O)_2]^{2+}$ was studied at pD values of 1.46, 1.79, 2.17, and 2.61. As Figure 2a shows, palladium(II) and protons compete for the donor atoms in the peptide. At pD 2.61 almost all of the substrate is bound to the catalyst, but in more acidic solutions this binding is only partial. This fact will be important in the discussion of catalytic turnover below.

As Figure 2b shows, unidentate coordination of the imidazole group to palladium(II) in the complexes designated A and B is practically independent of pD in the interval studied. Indeed, the pK_a value of the imidazolium cation falls far outside of this interval. As Figure 2c shows, formation of the substrate−catalyst complexes designated C, D, and E depends on pD in

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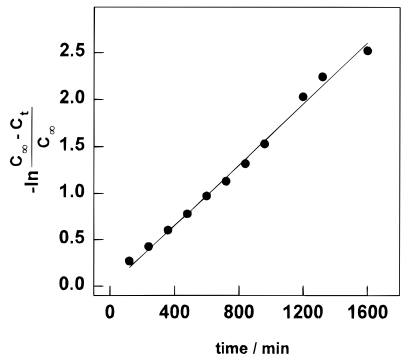


Figure 3. First-order kinetic plot for hydrolytic cleavage (eq 1) of AcHis-Gly in the presence of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ in a D_2O solution that is initially 28 mM in each, at pD 1.46 and 60 °C.

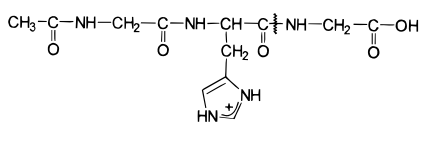
the interval studied. Indeed, the pK_a values of the amide and caboxylic groups in the presence of palladium(II) fall in or near the pD interval studied. The effects and noneffects of pD on the abundances of different substrate-catalyst complexes are consistent with the modes of coordination discussed above.

Noncatalytic Cleavage. Control experiments showed only slight "background" cleavage of peptides in the absence of *cis*-$[Pd(en)(H_2O)_2]^{2+}$. The half-life of the dipeptide AcHis-Gly at pD 1.36 is approximately 50 days; its complete (and nonselective) hydrolysis would take more than half a year. Selective hydrolysis of this dipeptide in the presence of the catalyst was practically complete in <2 days. These control experiments show that the palladium(II) complex, not the acidic solvent, is responsible for the hydrolytic cleavage. Since the protonated carbonyl group of amides has a pK_a value of ca. −1.0,[33] simple acid catalysis cannot significantly contribute to hydrolysis in the range $1.46 \leq pD \leq 2.61$, in which we worked. As in previous studies from this laboratory,[9−14] acidic solutions are needed to suppress formation of hydroxo-bridged, oligomeric palladium(II) complexes, which are catalytically inactive.

Kinetics and Regioselectivity of Cleavage. As Figure 3 shows, the cleavage reaction obeys the first-order rate law for 4 half-lives. The mechanism evidently requires that the peptide be coordinated to palladium(II), so that the reaction is intramolecular with respect to this substrate−catalyst complex.

In N-acetylated dipeptides AcHis-Aa histidine forms two amide bonds, one via the amino group and the other via the carboxylic group. In all of our experiments only the latter bond was cleaved, as shown in eq 1; we never detected acetic acid, a product of hydrolysis of the former bond. Since the acetyl group differs from an amino acid residue, we did additional control experiments with the dipeptide AcGly-His, in which the amino group of histidine forms a genuine peptide bond and the carboxylic group is free. This substrate did not detectably hydrolyze in the presence of *cis*-$[Pd(en)(H_2O)_2]^{2+}$, under the usual conditions. We did not observe the cleavage of either the peptide bond Gly−His or the amide bond $CH_3C(O)$−Gly. When there is no scissile bond on the carboxylic side of the anchoring histidine residue, there is no hydrolysis.

The last test of regioselectivity was made with the tripeptide AcGly-His-Gly, which contains a total of three amide bonds,



AcGly-His-Gly

two of which are peptide bonds flanking the anchoring histidine residue. Since the free glycine cleaved from the C-terminus and acetylglycine bound at the N-terminus give rise to overlapping ^1H NMR resonances at 3.89 ppm, in this case monitoring of cleavage was not straightforward. In this case we followed the disappearance of the resonance at 4.01 ppm, due to the C-terminal glycine residue, and enhancement of the resonance at 3.89 pm. Monitoring of these two resonances gave consistent results and proved cleavage of the bond marked in the formula of the AcGly-His-Gly. Again, we did not observe the resonances of AcGly and of acetic acid at 3.98 and 2.08 ppm, respectively. Evidently, none of the other amide bonds is cleaved. Presumably they are too distant from the palladium(II) complex anchored to the imidazole group.

Table 2. Hydrolysis of the Histidine−Aa Bond in Dipeptides, Promoted by *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pD 1.46 and 60 °C

Aa in AcHis-Aa	α-CHR in Aa	V_{CHR}, Å^3	$10^4 k_{obsd}$, min^{-1}
Gly	CH_2	18	16.6
Ala	$CHCH_3$	38	9.2
Ser	$CHCH_2OH$	44	4.8
Thr	$CHCH(CH_3)OH$	64	3.5
Leu	$CHCH_2CH(CH_3)_2$	94	4.2
Phe	$CHCH_2C_6H_5$	106	3.6
Tyr	$CHCH_2C_6H_4OH$	112	3.0

In a previous study[14] cytochrome *c* and peptides containing the −Cys-His− segment characteristic of this class of protein were cleaved with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. There was NMR spectroscopic evidence for palladium(II) binding to the cysteine side chain but not for its binding to the imidazole group of histidine. The His−Ala bond in AcCys-His-Ala was cleaved stoichiometrically, with the observed rate constant of 3.3×10^{-3} min^{-1} at 40 °C. As Table 2 shows, the His−Ala bond in AcHis-Ala is cleaved catalytically, with the observed rate constant of 9.2×10^{-4} min^{-1} at 60 °C; the latter reaction is considerably slower than the former. The marked difference in the rates of cleavage and the difference between the stoichiometric and catalytic reactions indicate that the two peptides interact differently with *cis*-$[Pd(en)(H_2O)_2]^{2+}$. Indeed, the protein finding can be explained without invoking palladium(II) binding to the imidazole group.[14] This group in the peptides AcHis-Aa, the subjects of this study, clearly is coordinated to palladium(II).

Dependence of the Hydrolysis Rate on pH. The dependence shown in Figure 2d is relatively small but interesting. It cannot be due to acid−base properties of the aqua ligand, because this ligand bound to palladium(II) deprotonates to a significant extent only at $pH \geq 5$.[27a]

Many previous experiments showed that peptides devoid of anchoring side chains are not cleaved by palladium(II) complexes present in solution.[9−13] Since the catalyst must be attached to the substrate in order to effect hydrolytic cleavage, a comparison between Figures 2a and 2d is puzzling at first—the greater the extent of attachment, the slower the reaction. These two figures are actually consistent, because not all modes of attachment are favorable for catalysis. Coordination via the amide nitrogen atom and via the carboxylic group, interactions favored by the decreasing acidity in the interval studied (see Figure 2c), are unproductive. The complexes designated C, D, and E probably exist outside of the catalytic cycle and tie the catalyst. The complexes designated A through E exist in an extended equilibrium with one another, with the free peptide AcHis-Aa, and with the catalyst. (All of these equilibria could not practically be shown in Scheme 1.) Because the interconversion is somewhat inhibited by the increase of pD from 1.46 to 2.61, the overall rate of hydrolysis decreases somewhat in this interval. Because of the reversibility of these processes,

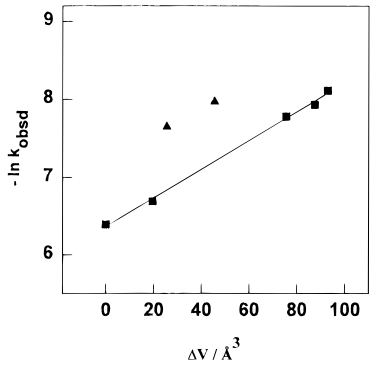


Figure 4. Hydrolytic cleavage (eq 1) of the His−Aa bond in AcHis-Aa catalyzed by *cis*-$[Pd(en)(H_2O)_2]^{2+}$ in D_2O solutions at $1.46 \leq pD \leq 1.51$ and 60 °C. Dependence of the observed rate constant on the steric bulk of the leaving amino acid, Aa. The quantity ΔV is defined in the text.

inactive peptide−catalyst complexes are converted into the active one(s), and all the peptide eventually is cleaved.

Dependence of the Hydrolysis Rate on the Leaving Amino Acid. The seven dipeptides in Table 2 differ in the C-terminal amino acid, which is the leaving group in the hydrolysis reaction (eq 1). Since the strength (thermodynamic stability) and intrinsic hydrolytic reactivity (kinetic stability) of the His−Aa peptide bond should not depend significantly nor systematically on the identity of the amino acid designated Aa, the general trend in the observed rate constants in Table 2 can be attributed to the steric bulk of the leaving amino acid, Aa. This bulk is quantitated as volume of the α-CHR group and calculated on the basis of van der Waals dimensions of functional groups found in amino acids and proteins.[42] The relative volume, ΔV in Figure 4, is the difference between the α-CHR volumes in a given amino acid and in glycine.

Figure 4 shows that for five of the seven peptides the rate constant decreases smoothly as the volume of the leaving amino acid Aa increases (eq 3). A similar trend emerged in a previous study of peptides containing methionine as the anchor for palladium(II) complexes.[11] In the mechanism involving external

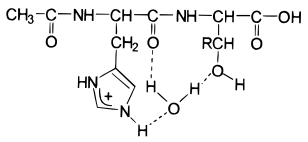
$$-\ln k_{obsd} = 6.4 + 0.019\Delta V \qquad (3)$$

attack by water the palladium(II) atom, as a Lewis acid, polarizes the carbonyl group in the scissile peptide bond. In the mechanism involving internal attack by water the palladium-(II) atom delivers an aqua ligand to the scissile bond. Both mechanisms require a close approach of the pendant catalyst to the adjacent peptide bond, and in both cases the steric bulk of the leaving amino acid hinders the crucial interaction. Although the slope of the line in eq 3 is relatively small, it is expected to become greater when the palladium(II) atom is encumbered with ancillary ligands bulkier than ethylenediamine. In our future studies we will investigate this new approach to sequence-specificity of cleavage.

Effects of Hydrogen Bonding on the Hydrolysis Rate. Of the seven leaving amino acids Aa in Table 2, two clearly deviate from the linear plot in Figure 4. Since both of them contain a hydroxyl group in the side chain, we analyzed possible hydrogen bonds and their effects on the hydrolysis reaction. Hydrogen bonds are essential for substrate recognition and specificity in enzymatic catalysis, and we were intrigued by their possible role also in the catalysis by transition-metal complexes.

Alanine and serine differ little in size, but the latter contains a hydroxyl group. Proton NMR spectra with water suppression of AcHis-Ala, AcHis-Ser, AcHis, and the imidazolium ion at pH 1.50 all contained the same broad resonance at 13.5 ppm, characteristic of the NH group of imidazole involved in hydrogen bonding.[43] The absence of this prominent resonance in the spectra of serine and of alanine at pH 1.50 confirmed the involvement of the imidazole proton. Since the imidazolium ion can be only a donor, not an acceptor, in a hydrogen bond, the acceptor in our cases must be water. Indeed, imidazolium−water hydrogen bond is well known.[44]

A methyl group at the N-3 atom of imidazole in N-acetylhistidine does not affect the resonance at 13.5 ppm, but a methyl group at the N-1 atom causes this resonance to disappear from the ^1H NMR spectrum. Clearly, it is the proton at the N-1 atom that is involved in hydrogen bonding. A simple hydrogen bond between the imidazolium ion in the side chain and water in the bulk solvent is not expected to be specific to only one of the two NH groups. The specificity that we found suggests a precise stereochemical requirement such as is needed in intramolecular hydrogen bonding. Indeed, our molecular modeling showed that a water molecule fits well in a position shown below in the dipeptides AcHis-Ser (R is H) and AcHis-Thr (R is CH_3), the two that deviate from the plot in Figure 4. Hydrogen bonds similar to the one that we propose are well documented.[45]



The NMR resonance for the hydroxyl proton in serine was unobservable, because the rate constant for its exchange with the solvent water in acidic solution is around 3×10^9 min^{-1}.[46] Therefore NOESY spectra were inconclusive on the question of spatial proximity of the OH group of serine and an NH group of the imidazolium side chain in AcHis-Ser. Molecular modeling ruled out this proximity, but we obtained evidence that the resonance at 13.5 ppm is due to an intramolecular hydrogen bond. A ^1H NMR spectrum of AcHis-Ser in the nonaqueous solvent dimethyl sulfoxide-d_6 did not show this resonance. Clearly, the hydrogen atom in the imidazolium ion is not shared with the hydroxyl group of serine; this direct interaction, if possible, would remain in dimethyl sulfoxide. The hydrogen bond probably is mediated by a water molecule, as depicted above.

The deviations from the line in Figure 4 can be nicely explained in terms of hydrogen bonding through a water molecule. When the N-1 atom of imidazole is engaged in this bonding, the palladium(II) catalyst binds preferrentially (although not exclusively) to the N-3 atom. Presence of the resonance at 13.5 ppm in the ^1H NMR spectrum of an equimolar mixture of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and AcHis-Ser shows that the hydrogen bond involving the imidazolium group remains after the dipeptide becomes coordinated to palladium(II) in the catalyst. Since hydrogen bonding hinders the rotation of the histidine side chain, the catalyst spends more time on the far side of the anchor, away from the potential leaving amino acid. The net effect of hydrogen bonding is to lessen, although not

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eliminate, the probability of palladium(II) approaching the scissile bond.

Tyrosine, the last amino acid in Table 2 and Figure 4, also contains a hydroxyl group, but it falls on the same plot with amino acids containing simple alkyl and aryl side chains. Molecular modeling shows that, because of the rigidity of the aromatic ring, a water molecule cannot simultaneously form hydrogen bonds with the phenolic hydroxyl group and an imidazole NH group. In this case the hydroxyl group does not engage in any specific interactions; it simply contributes to the volume of the leaving amino acid.

Catalytic Turnover. There is no tunrover in cleavage when palladium(II) complexes are attached to methionine and cysteine side chains.[9−14] When a peptide AcHis-Aa and *cis*-[Pd(en)$(H_2O)_2]^{2+}$ are mixed in equimolar amounts, their binding is incomplete in the acidic solutions used; see Figures 1b and 2a. Although some of the peptide always remains free, all of it becomes cleaved; see Figure 1a. Even when AcHis-Gly and the catalyst were mixed in the molar ratio 4:1, the cleavage was complete, owing to the lability (i.e., facile ligand displacement) of palladium(II) complexes. Although modest, this turnover is significant for it proves the principle of catalysis and validates this laboratory's approach to artificial metallopeptidases. In our future work we will try to change the reaction conditions and improve the turnover.

Fate of the Catalyst. Figure 1b shows the free dipeptide and at least seven other imidazole-containing species simultaneously present in the middle stage of the hydrolysis reaction. Since palladium(II) complexes are labile with respect to ligand substitution, the substrate, the catalyst, the hydrolytic products, and various complexes that the catalyst forms with the substrate and with the hydrolytic products exist in an extended equilibrium, only some features of which can be shown in Scheme 1.[47] The hydrogen ions in the acidic solution compete with palladium(II) for the nitrogen and oxygen donor atoms in the dipeptide and in the amino acids Aa, formed by its cleavage. This competition labilizes all the palladium(II) complexes shown in Scheme 1 and makes possible shifts in the equilibria.

The five complexes that *cis*-[Pd(en)$(H_2O)_2]^{2+}$ forms initially with AcHis-Aa, the hydrolysis substrate, were discussed above. In a separate experiment, reaction between the equimolar amounts of *cis*-[Pd(en)$(H_2O)_2]^{2+}$ and AcHis, one of the hydrolysis products, also yielded five complexes. Their mixture and the reaction mixture at the end of hydrolysis of AcHis-Aa gave identical ^{1}H NMR spectra in the imidazole region. Three of the complexes containing AcHis are indistinguishable by ^{1}H NMR spectroscopy from their counterparts designated A, B, and D containing AcHis-Gly, but the complexes of the types C and E show slight differences when the ligand is AcHis or AcHis-Gly. These findings are borne out by the spectra in Figure 1b. As the H-5 resonances of the AcHis-Gly complexes designated C (at 6.58 ppm) and E (at 6.77 ppm) decline, the resonances of the corresponding AcHis complexes at 6.56 and 6.76 ppm grow. It is very likely that the AcHis-Aa complexes designated A, B, and D disappear and are replaced by the AcHis complexes of the same types. Although this change is undetectable by ^{1}H NMR spectroscopy, there is no reason why the complexes of these three types should behave differently from the complexes of the type C and E, the conversion of which is evident in Figure 1b.

The experimental evidence is still insufficient for conclusive identification of the substrate−catalyst complexes in Scheme 1 that are and are not active in hydrolysis. The evidence, however, allows some inferences to be drawn. The complex designated D, in which the palladium(II) atom is chelated on the far side of the scissile bond, almost certainly is catalytically inactive, and yet it is consumed in the reaction; Figure 1b shows its disappearance. Other catalytically-inactive complexes probably are consumed as well. Since the reaction must be "funneled" through the catalytically-active complex or complexes, the consumption of the catalytically-inactive complexes means that they are converted into the active one(s), so that the dipeptide is fully hydrolyzed. Indeed, the extended equilibria partially shown in Scheme 1 make this interconversion possible.[47]

So far we discussed the imidazole-containing species—the substrate AcHis-Aa, the cleavage product AcHis, and their complexes with the palladium(II) catalyst. But the other product of cleavage, the leaving amino acid, also binds to palladium(II). Initial unidentate coordination via the carboxylate group gives rise to the final bidentate coordination via the carboxylate and amino groups. This sequence is well documented.[48] In separate experiments under the hydrolytic conditions, we mixed equimolar amounts of *cis*-[Pd(en)$(H_2O)_2]^{2+}$ and each of the amino acids listed in Table 1. Their ^{1}H NMR chemical shifts matched those observed at the end of the hydrolysis of the corresponding dipeptides; these chemical shifts are given in Table 1. Removal of palladium by precipitation with sodium diethyldithiocarbamate at the end of hydrolysis reactions restored the free leaving amino acids. Evidently, hydrolysis of the His−Aa bond in the dipeptides AcHis-Aa is selective and complete.

Conclusions and Prospects

Many previous studies have dealt with complexes of transition metals with histidine and histidine-containing peptides. To our knowledge, this is the first study to show that unidentate coordination of the imidazole group is followed by hydrolytic cleavage of the coordinated peptides. This cleavage is regioselective—only the amide bond on the carboxylic side of histidine is cleaved. The interesting dependence of the hydrolysis rate on the steric bulk of the leaving fragment and on hydrogen bonding may be exploited in future attempts at sequence-selectivity. This study, in which cleavage occurs next to the histidine residue, validates the inorganic-chemical principle of cleavage selectivity. Whereas selectivity of natural peptidases is based on a fit of the substantial segment of the substrate into the enzymatic active site, selectivity of so-called artificial metallopeptidases seems to be based on the "narrower" binding affinity between the anchoring side chain—in this case the imidazole group—and the palladium(II) atom in the catalyst. The facility with which palladium(II) complexes undergo ligand-substitution reactions makes possible catalytic turnover. We will continue to investigate the mechanism of this new reaction and will apply it to proteins.

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(47) For practical reasons of layout, many equilibrium relationships could not be shown in Scheme 1. For example, the complexes designated **A** through **E** may interconvert without the dissociation into AcHis-Aa and the free catalyst. The corresponding five complexes containing AcHis may also interconvert, and they can do so with or without the dissociation into AcHis and the free catalyst.

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