DALTON

Kinetic study of stereochemical and other factors governing hydrolytic cleavage of a peptide ligand in binuclear palladium(II) complexes

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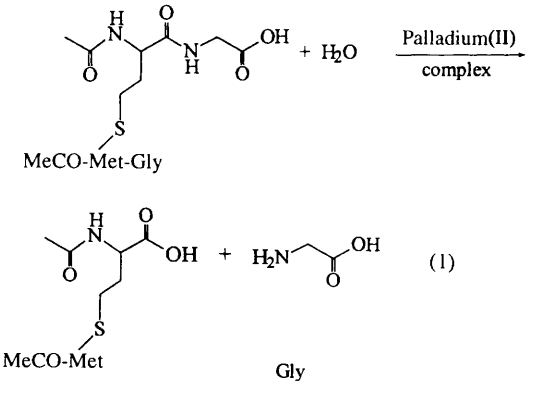
The dipeptide *N*-acetylmethionylglycine (MeCO-Met-Gly) reacted, *via* the thioether group in the methionyl side chain, with five different palladium(II) aqua complexes. The complexes *cis*-$[Pd(en)(H_2O)_2]^{2+}$ (en = $H_2NCH_2CH_2NH_2$) and *cis*-$[Pd(pn)(H_2O)_2]^{2+}$ (pn = $H_2NCH_2CH_2CH_2NH_2$) yield $[Pd_2(\mu\text{-MeCO-Met-Gly})_2(H_2O)_4]^{4+}$ **A**, *cis*-$[Pd(Met\text{-}S,N)(H_2O)_2]^{2+}$ yields *trans*-$[Pd_2(\mu\text{-MeCO-Met-Gly})_2(H_2O)_2(HMet)_2]^{6+}$, **B** and *trans*-$[Pd_2\{Cys(Me)\text{-}S,N\}_2(H_2O)_2]^{4+}$ [Cys(Me) = *S*-methylcysteine] yields *trans*-$[Pd_2\{\mu\text{-}HCys(Me)\}_2(H_2O)_2(MeCO\text{-Met-Gly})_2]^{6+}$ **C**. The complex *cis*-$[Pd(dtco)(H_2O)_2]^{2+}$ (dtco = 1,5-dithiacyclooctane) yields $[Pd_2(\mu\text{-MeCO-Met-Gly})_2(dtco)_2]^{4+}$, **D**. These reactions and hydrolytic cleavage of the methionine–glycine amide bond in the co-ordinated MeCO-Met-Gly are conveniently monitored by ^1H NMR spectroscopy. The rate of cleavage decreases in the order **A** > **B** ≈ **C** > **D**, in which the number of aqua ligands per peptide ligand decreases. Intramolecular attack by aqua ligands is more efficient than external attack by water molecules from the solvent. The peptide ligands occupying terminal and bridging positions in the binuclear palladium(II) complexes undergo hydrolysis at similar rates. This study shows the importance of polynuclear metal complexes in hydrolytic cleavage of peptide bonds.

Hydrolytic cleavage of peptides and proteins is an important metabolic process and a common reaction in analytical biochemistry. Although the half-life for hydrolysis of an unactivated amide bond in neutral aqueous solution is *ca.* 7 years,[1] proteolytic enzymes effect this reaction relatively rapidly and with a considerable degree of selectivity.[2] Unfortunately, only several proteolytic enzymes are efficient enough to be routinely applicable in biochemistry and molecular biology. Moreover, their sequence selectivity, *i.e.* the location of the hydrolytic cuts in the protein, is practically unchangeable.

Chemical reagents are unlikely to surpass enzymes in catalytic turnover, but they hold other potential advantages. Their reactivity and regioselectivity in cleavage can, in principle, be modified by chemical means. Unlike enzymes, chemical reagents can be active under various conditions of pH and temperature. These so-called artificial enzymes have mostly been tested on esters and activated amides, such as *p*-nitroanilides, but seldom on unactivated amides. Of the chemical reagents only cyanogen bromide is being widely used for protein cleavage.[2] Its regioselectivity, cleavage on the carboxylic side of methionine residues, differs usefully from that of common enzymes, but it is highly toxic, requires fairly harsh reaction conditions, and must be applied in considerable excess over the substrate.

Since certain proteolytic enzymes require metal ions for activity, metal complexes hold great promise as cleaving reagents.[3–8] Again, however, many of the previous studies have dealt with esters and activated amides. Those that have dealt with unactivated amides, peptides, and proteins have involved three kinds of metal complexes. First, cobalt(III) complexes, which are inert in substitution reactions, allowed elegant kinetic and mechanistic studies of hydrolysis.[3] These complexes bind only to the N-terminal amino acid and therefore cleave only the first amide bond in the sequence. The cleavage is stoichiometric, not catalytic. Most applications, however, require cleavage of internal amide bonds and turnover. Complexes of copper(II) belong to this first group of reagents.[4] Secondly, iron complexes with ethylenedinitrilotetraacetate (edta) tethered to certain amino acid side chains cleave proximate amide bonds, but the mechanisms of these reactions remain unclear.[9–13] The cleavage can be fast or slow, and the yield can be complete or very low, depending on the substrate, conditions and the tether used. The synthetic chemical work required for this tethering of metal complex to the protein limits the applicability of this clever method.

A third method, developed at Iowa State University,[14–20] involves self-assembly of the reagent–substrate complex upon simple mixing of aqueous solutions. Initial studies with platinum(II) complexes[14] gave way to recent studies with palladium(II) aqua complexes.[15–20] These complexes bind to the side chains of methionine[14–19] and histidine[20] residues and effect selective hydrolytic cleavage of the adjacent amide bond. The anchored palladium(II) atom can act in two ways: by binding the carbonyl oxygen atom of an adjacent amide group, polarizing this group, and activating it for external attack by a water molecule; or by delivering an aqua ligand in an internal attack. When the anchoring residue is methionine the reactions are stoichiometric. When it is histidine there is turnover and the reactions are truly catalytic. This method was applied to various peptides[14,17,19,20] and to a protein.[18] Almost without exception, the cleaved amide bond is the one involving the carboxylic group of the anchoring amino acid, as shown in equation (1). (The leaving glycine is actually an ammonium



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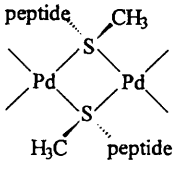

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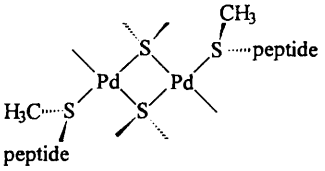


II

cation in the acidic solution used.) Half-lives of the reactions range from less than 1 h to *ca.* 0.5 d. These rates are sufficient for practical applications of the new palladium(II) reagents in biochemistry and molecular biology. Indeed, these reagents cleave the protein cytochrome c regioselectively (at a single site) and with a high yield.[18]

Stringent control experiments showed that peptides are stable for a long time in solutions of pH $\lesssim$ 1.0 and ruled out simple acid catalysis of the hydrolysis in equation (1).[15-17] Acidic solution is needed to suppress deprotonation of aqua ligands and formation of oligomeric palladium(II) complexes with hydroxo bridges.[20]

Although palladium(II) is added to the methionine-containing peptides in the form of mononuclear complexes, the compounds studied so far that actually undergo hydrolysis are binuclear complexes of the type shown in **I**. Both NMR spectra and kinetic experiments support this conclusion.[15,17] In these complexes the thioether group of the methionine side chain acts as a bridging ligand, and the sulfur atoms are tetrahedral. However, this group can act also as a terminal ligand, with pyramidal sulfur atoms, in complexes of the type shown in **II**. Hydrolysis of such a complex is examined for the first time in this study. In complexes of either type the cleaving agent can be a terminal water ligand (not shown) or a water molecule from the solvent.

In this article we compare the kinetics of hydrolysis of the Met-Gly bond in *N*-acetylmethionylglycine (MeCO-Met-Gly) when this dipeptide occupies bridging and terminal positions in binuclear complexes containing one or two terminal aqua ligands per palladium(II) atom. This study contributes to our understanding of the mechanism of hydrolytic cleavage of peptides and brings us a step closer to our ultimate goal, design of palladium(II) complexes as artificial metallopeptidases.

Experimental

Chemicals

Distilled water was further demineralized and purified. The deuterium-containing solvents, $K_2[PdCl_4]$, ethane-1,2-diamine (ethylenediamine, en), propane-1,3-diamine (trimethylenediamine, pn), and 1,5-dithiacyclooctane (dtco) were obtained from Aldrich Chemical Co., methionine (Met), *S*-methylcysteine [Cys(Me)], and the dipeptide methionyl glycine (Met-Gly) from Sigma Chemical Co. All other chemicals were of reagent grade.

The terminal amino group in Met-Gly was acetylated by a published procedure.[21] The ^{1}H NMR spectrum of the product, MeCO-Met-Gly, in D_2O showed the following principal δ values: 2.04 (s, CH_3CO), 2.11 (s, CH_3S) and 3.99 (s, CH_2 of Gly).

The following dichloro complexes were prepared by published procedures: *cis*-$[Pd(en)Cl_2]$,[22] *cis*-$[Pd(pn)Cl_2]$,[22] *cis*-$[Pd(Met\text{-}S,N)Cl_2]\cdot H_2O$[23-25] and *cis*-$[Pd\{Cys(Me)\text{-}S,N\}Cl_2]$.[23,25,26] The corresponding diaqua complexes were obtained by stirring the precursors with 2.0 equivalents of $AgNO_3$ in a solution of pH* 2 for 4 h at 35 °C and by removal of AgCl by centrifugation, all in the dark.[27] Since the solvent was D_2O the ligands were actually D_2O, but the formulas will be written simply with H_2O. The aqua complexes were always prepared fresh and used as solutions. The ^{1}H NMR spectra of D_2O solutions showed the following principal δ values: *cis*-$[Pd(en)(H_2O)_2]^{2+}$, 2.63 (s, CH_2); *cis*-$[Pd(pn)(H_2O)_2]^{2+}$, 2.39 and 1.75 (both s, both CH_2); *cis*-$[Pd(Met\text{-}S,N)(H_2O)_2]^{2+}$, 2.37 (s, SCH_3); and *trans*-$[Pd_2\{Cys(Me)\text{-}S,N\}_2(H_2O)_2]^{4+}$, 2.55 (s, SCH_3) and 4.32 (q, α-CH).

Measurements

Proton NMR spectra at 500 MHz of D_2O solutions, containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate as an internal reference, were recorded with an AM 500 spectrometer. The sample temperature was kept at 40 ± 0.5 °C. The pH was measured with an Orion 901 instrument and a Phoenix Ag–AgCl reference electrode. The uncorrected values in D_2O solutions are designated pH*. The corrected values are 0.40 ppm greater.[28]

Kinetics of hydrolysis

The palladium(II) complexes were prepared fresh, to avoid or minimize the formation of hydroxo-bridged polynuclear complexes. The stock solutions had concentrations of 30–50 mmol dm^{-3} and pH* 1.50–1.60. Equimolar amounts of the palladium(II) complex and of MeCO-Met-Gly, both as D_2O solutions, were mixed in an NMR tube so that the final concentration of each was 20 mmol dm^{-3}. The pH* value was adjusted with a 1.5 mol dm^{-3} solution of $HClO_4$ in D_2O at the beginning, and was measured again at the end of the reaction. Acquisition of ^{1}H NMR spectra began as soon as possible, and 16 or 32 scans were taken at each time. The temperature was kept at 40.0 ± 0.5 °C. As Fig. 1 shows, reaction (1) is easily followed by monitoring the ^{1}H NMR resonance of the glycyl residue in the dipeptide, which declines, and that of the free glycine, which grows. The concentrations of free glycine (c_t) were determined on the basis of the resonance area and the known initial concentration (c_o) of MeCO-Met-Gly; the estimated error is ±5%. First-order plots of $\ln[(c_o/(c_o - c_t)]$ *versus* time contained 13–20 points and were linear over at least three half-lives, with correlation coefficients 0.995–0.999. The rate constants are the slopes of these plots. The reactions were run for six half-lives.

Results and Discussion

Palladium(II) aqua complexes (promoters of hydrolysis)

The complexes shown are well behaved, and their formation by ligand-substitution reactions is conveniently monitored by ^{1}H NMR spectroscopy.[25,29-32] Especially useful is the resonance of the CH_3S group in the side chains of methionine and of *S*-methylcysteine. Studies of multiple palladium(II) complexes with these and closely homologous amino acids and peptides have shown that the ^{1}H NMR resonances of terminal and doubly bridging thioether ligands fall in two non-overlapping intervals, δ 2.26–2.39 and 2.44–2.55, respectively.[15] The CH_3S resonance of the free thioether occurs at δ 2.11. As expected, bridging co-ordination (in binuclear complexes) causes a greater deshielding than terminal co-ordination (in mononuclear complexes). The net charges shown are for the species in acidic solutions, used in this study.

Aquation of the starting dichloro complexes simply gives the first four aqua complexes shown. The simple ^{1}H NMR patterns show that the ethylenediamine, propylenediamine, and dithiacyclooctane ligands are symmetrical, therefore

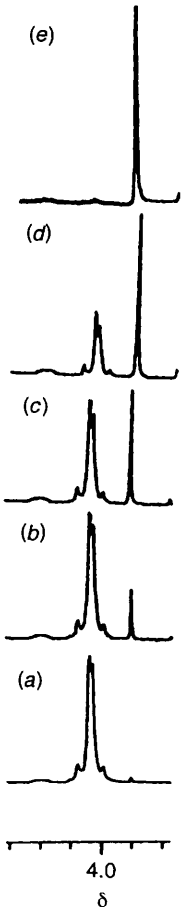
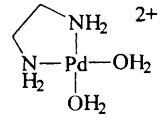
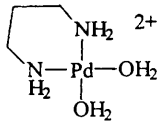


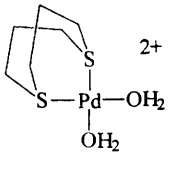
Fig. 1 Proton NMR spectra of a mixture containing 1 equivalent of *trans*-$[Pd_2\{Cys(Me)\text{-}S,N\}_2(H_2O)_2]^{4+}$ and 2 equivalents of the dipeptide MeCO-Met-Gly in D_2O, at pH* 0.93 and 40 ± 0.5 °C, at the following times after mixing: (*a*) 5 min; (*b*) 0.50 h; (*c*) 1.4 h; (*d*) 3.6 h and (*e*) 12 h. The CH_2 resonances of the glycyl residue in the dipeptide and of free glycine appear at δ 4.03 and 3.90, respectively. The interval on the scale of chemical shifts is 0.10 ppm



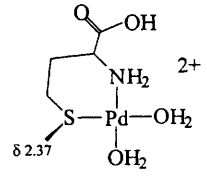
cis-$[Pd(en)(H_2O)_2]^{2+}$



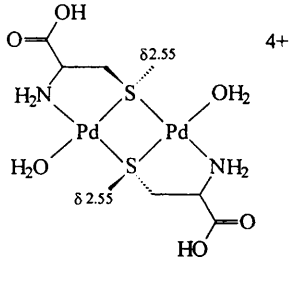
cis-$[Pd(pn)(H_2O)_2]^{2+}$



cis-$[Pd(dtco)(H_2O)_2]^{2+}$



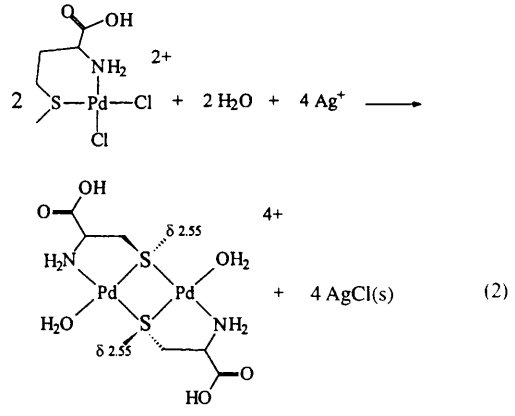
cis-$[Pd(Met\text{-}S,N)(H_2O)_2]^{2+}$



trans-$[Pd_2\{Cys(Me)\text{-}S,N\}_2(H_2O)_2]^{4+}$

bidentate. The resonance at δ 2.37 shows that the thioether group of methionine is a terminal ligand, *i.e.*, that this is a mononuclear complex. In the absence of potential bridging ligands such as chloride, these four aqua complexes remain mononuclear.

Aquation of *cis*-$[Pd\{Cys(Me)\text{-}S,N\}Cl_2]$, however, yields the binuclear complex shown above and in equation (2). In this



(2)

complex the free amino groups favourably close two five-membered chelate rings. A related binuclear complex is formed according to Scheme 1, also at pH* 2. In this case, however, the amino groups are acetylated and do not close chelate rings. Chelation *via* the carboxylate group would yield six-membered rings, which are less favourable. Protonation and displacement of ethylenediamine in the second step in Scheme 1 is facilitated by the *trans* effect of the thioether ligands, as shown in one of our previous studies.[17] The binuclear complexes shown earlier in equation (2) and in Scheme 1 have a common feature, bridging thioether ligands. (The sulfur atoms are tetrahedral, a feature not shown explicitly in the graphics.) Indeed, the ^1H NMR resonances of CH_3S fall in the correct range: δ 2.55 for the former and 2.50 for the latter. The resonance at δ 2.50 appears several minutes after mixing the two reactants in Scheme 1 and then grows because both the first and the second binuclear complexes show it. The resonance of the free H_2en^{2+}, at δ 3.37, grows over the next hour, as the second step follows the first. Neither the reaction in equation (2) nor that in Scheme 1 forms a product with a terminal thioether ligand, as is evident from the ^1H NMR spectrum of the mixture at the end which shows no resonances in the interval δ 2.26–2.39. Evidently, the conversions shown in equation (2) and Scheme 1 are complete, as judged from the ^1H NMR spectra.

Co-ordination of MeCO-Met-Gly to the promoters

Results of treating the complexes with the dipeptide MeCO-Met-Gly are shown in Table 1 and structures **A**–**D**. Since the terminal amino group is protected by acetylation the dipeptide co-ordinates only *via* the methionine side chain. The reaction mixtures were readily analysed by ^1H NMR spectroscopy. The thioether group acts as a bridging ligand (in complexes **A**, **B** and **D**) or as a terminal ligand (in **C**).

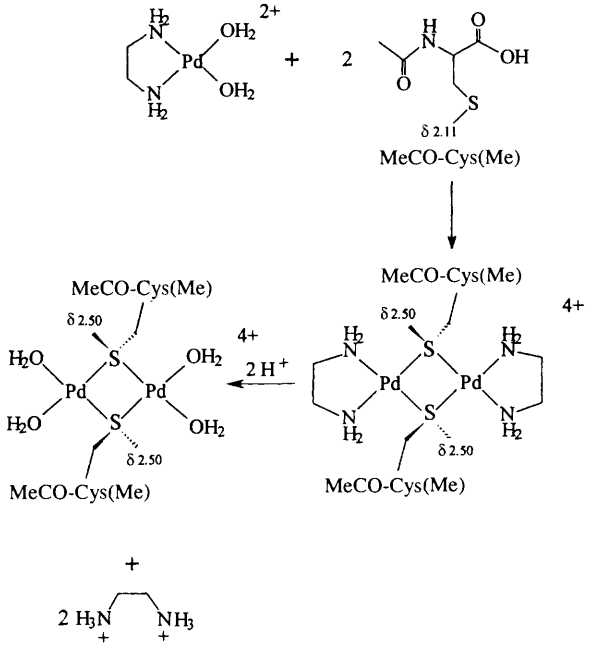
The ethylenediamine (en) and propylenediamine (pn) ligands are displaced as diammonium cations within minutes of mixing the corresponding mononuclear complexes with MeCO-Met-Gly. The ^1H NMR resonance of H_2en^{2+} is a singlet at δ 3.37, whereas the resonances of H_2pn^{2+} are a quintet at δ 2.07 and a triplet at 3.11. The ^1H NMR spectra show the absence of terminal thioether ligands; only the complexes of type **A** are formed.

The mononuclear palladium(II) complexes with the methionine and dithiacyclooctane ligands react with an equimolar amount of MeCO-Met-Gly to give products containing both

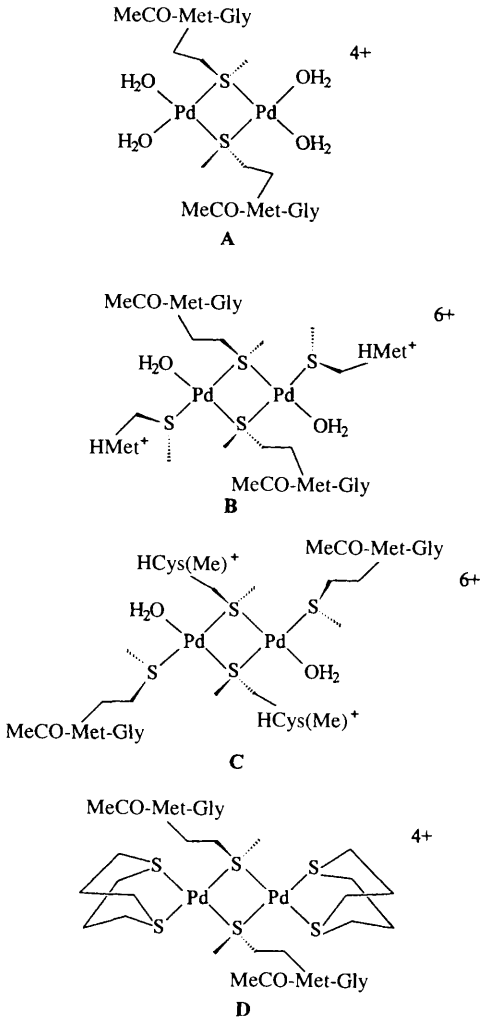
Table 1 Diagnostic chemical shifts of the CH_3S group in complexes that the dipeptide MeCO-Met-Gly forms with various mononuclear palladium(II) complexes at 40 °C and pH* 0.83–0.99

Precursor	Mole ratio substrate : precursor	Product	δ(CH_3S) bridging	δ(CH_3S) terminal
cis-$[Pd(en)(H_2O)_2]^{2+}$	1 : 1	**A**	2.50	
cis-$[Pd(pn)(H_2O)_2]^{2+}$	1 : 1	**A**	2.48	
cis-$[Pd(Met\text{-}S,N)(H_2O)_2]^{2+}$	1 : 1	**B**	2.50	2.39
	1 : 2		2.46	2.36
	2 : 1		2.50	2.31
trans-$[Pd_2\{Cys(Me)\text{-}S,N\}_2(H_2O)_2]^{4+}$	1 : 1	**C**	2.49	2.28
	2 : 1		2.47	2.31
cis-$[Pd(dtco)(H_2O)_2]^{2+}$	1 : 1	**D***	2.50	

* Ref. 15.



Scheme 1 Formation of the complex A in solution of pH* 1

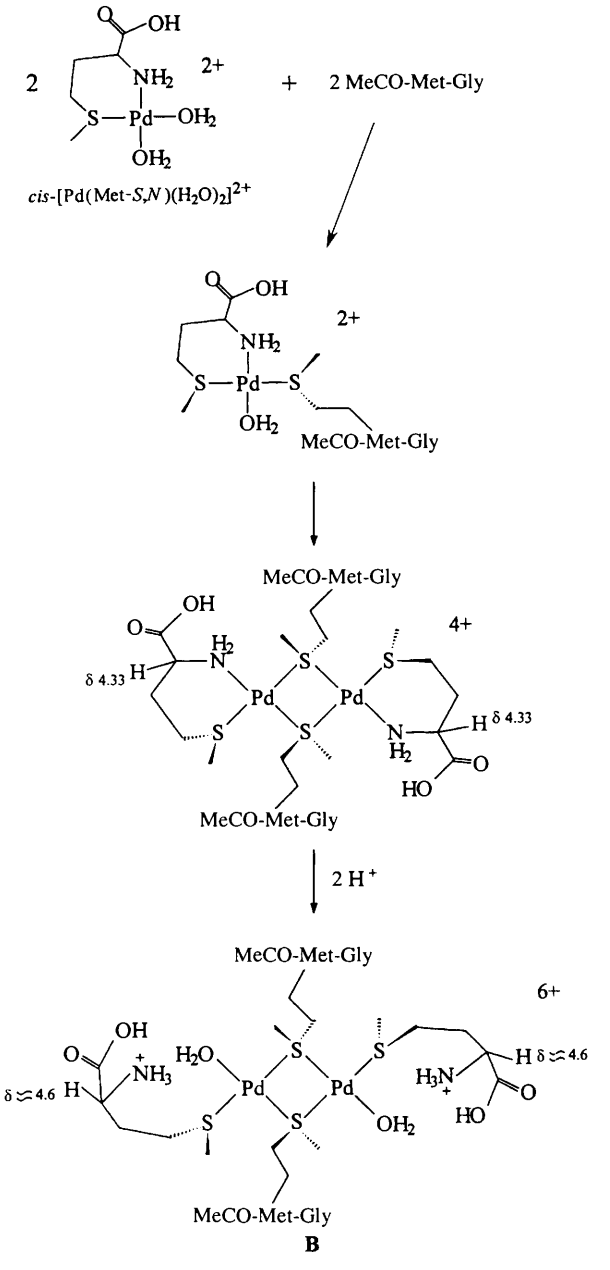


doubly bridging and terminal thioether ligands. These reactions were readily followed by monitoring both the CH_3S and α-CH resonances. As Scheme 2 shows, the thioether group of MeCO-Met-Gly displaces the aqua ligand labilized by the thioether group of the bidentate methionine. Dimerization yields the binuclear complex (presumably of *trans* structure) in which the amino group of methionine finds itself *trans* to the (bridging) thioether ligand and is therefore labilized for displacement assisted by protonation. Owing to this protonation, the α-CH resonance moves downfield and becomes buried under the unavoidable HDO resonance. This effect of single protonation (*ca.* 0.3 ppm) is lesser than the effect of double protonation (*ca.* 1.0 ppm), as the co-ordinated ethylenediamine and propylenediamine are converted into H_2en^{2+} and H_2pn^{2+} in the formation of complex **A** (see above). In Scheme 2, binding of MeCO-Met-Gly precedes and facilitates opening of the chelate rings.

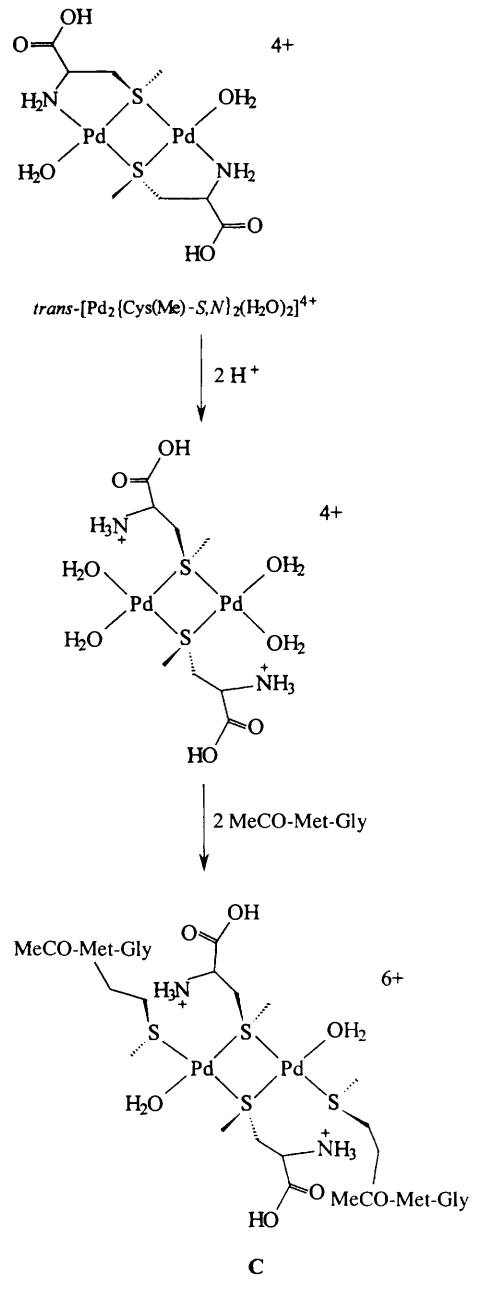
In Scheme 3, however, the amino group of *S*-methylcysteine in the starting complex is already labilized by the *trans* effect of the thioether group. Protonation in acidic solution opens the chelate rings even before MeCO-Met-Gly is added; now this dipeptide co-ordinates, *via* the methionine side chain, as a terminal ligand.

As Table 1 shows, complexes **B** and **C** have virtually identical ^1H chemical shifts of the bridging thioether group, but different shifts of the terminal thioether group. Indeed, as illustrated, the terminal methionine ligand in **B** is a cation, whereas the terminal methionyl group in **C** is a part of the neutral dipeptide. Hence the greater deshielding in **B**.

Complex **D** was reported in one of our earlier studies.[15] Proton NMR spectra show that dithiacyclooctane (dtco) remains asymmetrical, therefore a bidentate ligand. Since it is not a Brønsted–Lowry base, the chelate rings are not readily opened in acidic solution. The spectra of aged solutions show a small amount of free MeCO-Met-Gly, evidence that the central four-membered ring in **D** is opened to a small extent. The departed bridging MeCO-Met-Gly ligand is replaced by water.



Scheme 2 Formation of complex **B** in solution of pH* 1

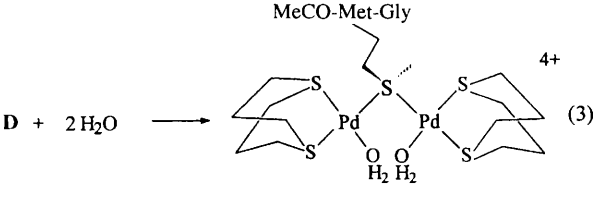


Scheme 3 Formation of complex **C** in solutions of pH* 1

Kinetics of hydrolysis of the Met–Gly bond

The complexes **A**–**D** form an interesting series. In **A**, **B** and **D** the dipeptide MeCO-Met-Gly acts as a bridging ligand, whereas in **C** it is terminal. The number of aqua ligands per molecule of MeCO-Met-Gly is two in **A**, one in **B** and **C**, and none in **D**.

As Table 2 shows, hydrolysis according to equation (1) is fastest in complex **A**. In this complex there are two aqua ligands per molecule of the dipeptide. Both of these ligands are well suited for intramolecular attack on the scissile Met–Gly bond. The aqua ligands are labilized by the thioether in the *trans* position, and they are *cis* to the substrate. Although MeCO-Met-Gly is conformationally flexible, there is a good probability of nucleophilic attack by a proximate aqua ligand. The number of aqua ligands is the same, one, in complexes **B** and **C**. The rate constants are similar for the bridging (in **B**) and terminal (in **C**) positions of the dipeptide. Since in **C** the aqua ligand is located *cis* to the dipeptide it is reasonable to conclude that also in **B** the aqua ligand attacks the dipeptide molecule *cis* to itself, not that *trans*. Complex **D**, which lacks aqua ligands, must be heated above 40 °C for the hydrolysis to set in, slowly. Since under these conditions there is a slight release of MeCO-Met-Gly from the complex, a bridging MeCO-Met-Gly molecule probably is replaced by terminal aqua ligands [equation (3)], which then attacks the second molecule which



remains in the other bridging position. Undetectability of hydrolysis in the intact complex **D** indicates that external attack by the solvent water on the co-ordinated dipeptide is relatively ineffective. Although co-ordination to palladium(II) renders an aqua ligand less nucleophilic than a free water molecule, this decrease in nucleophilicity is outweighed by the benefit of

Table 2 Hydrolysis of the methionine–glycine bond in MeCO-Met-Gly when this dipeptide is co-ordinated in different binuclear palladium(II) complexes

Complex	pH*	$10^3 k_{obs}$/min^{-1} at 40 °C
A[a]	0.99	38.1
B	0.94	6.8
C	0.93	4.3
D[b]	1.00	3.1

[a] Formed from *cis*-$[Pd(pn)(H_2O)_2]^{2+}$. [b] At 50 °C, ref. 15.

proximity when both the aqua ligand and the dipeptide exist in the same complex.

Complexes obtained when the mononuclear complexes were treated with non-equimolar amounts of MeCO-Met-Gly gave kinetic results consistent with those just discussed. The complex *cis*-$[Pd(Met\text{-}S,N)(H_2O)_2]^{2+}$ reacts with an equimolar amount of the dipeptide to form **B**, as explained above. A similar reaction with 2 equivalents of the dipeptide, so that the mole ratio Pd_2:MeCO-Met-Gly is 1:4, yields a complex devoid of aqua ligands. This is derived from **B** by displacement of both aqua ligands by the thioether groups of MeCO-Met-Gly. A reaction with 5 equivalents (an excess) of the dipeptide MeCO-Met-Gly produced the same binuclear complex. In these two cases hydrolysis was not detected. These results confirm our conclusion that at least one aqua ligand is required for palladium(II) to promote peptide hydrolysis.

Final evidence for this conclusion came from inhibition experiments. When complex **B** was prepared in the presence of an equimolar amount of thiourea this nucleophile displaced the aqua ligands, and hydrolysis of the dipeptide was not detected.

Effects of reaction conditions on the rate of hydrolysis

The reaction of complex **B** was studied also under conditions different from those in Table 2. The quantity $10^3 k_{obs}$ was 6.8, 5.5, 4.2 and 1.3 min^{-1} in solutions having pH* values of 0.94, 1.16, 1.38 and 2.06, respectively; the temperature was always 40 ± 0.5 °C. This decrease is indicative of the decay of the palladium(II) complexes, probably owing to oligomerization. Since control experiments showed no appreciable hydrolysis in the absence of the metal complexes the decrease cannot be due to hydrolysis catalysed simply by the acid.[14-20]

When D_2O was replaced as a solvent by CD_3OD, $10^3 k_{obs}$ increased from 19.9 to 41 min^{-1}. This promising result, and other solvent effects on the kinetics and mechanism of hydrolysis, will be the subject of a future study.

Conclusion

This study explains why the rate of MeCO-Met-Gly hydrolysis promoted by palladium(II) decreases as the bidentate ligand in the precursor complexes *cis*-[Pd(bidentate ligand)Cl_2] is changed from the *N,N*-donors ethylenediamine and propylenediamine to the *S,N*-donors methionine and *S*-methylcysteine, to the *S,S*-donor dithiacyclooctane. The explanation lies in the lability in acidic solution of amine ligands *trans* to thioether ligands and in the inertness of thioether ligands. The *N,N*-donors are completely displaced, by two aqua ligands, the *S,N*-donors are partially displaced, by one aqua ligand, and remain co-ordinated *via* the thioether group and the S,S-donor is not displaced and remains bidentate. The promoters and the peptide form the binuclear complexes **A**–**D**, which were readily identified on the basis of their ^1H NMR spectra. This study shows the importance of polynuclear metal complexes as promoters of peptide hydrolysis and points the way to our ultimate goal, simple transition-metal complexes as artificial peptidases.

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