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Discovery of a Highly Selective and Efficient Reagent for Formation of Intramolecular Disulfide Bonds in Peptides

Tiesheng Shi and Dallas L. Rabenstein*

Contribution from the Department of Chemistry, University of California, Riverside, California 92521

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Abstract: We have discovered that *trans*-$[Pt(en)_2Cl_2]^{2+}$ (en = ethylenediamine) is a highly selective and efficient reagent for the quantitative formation of intramolecular disulfide bonds in peptides. A series of 14 dithiol peptides which form disulfide-containing rings ranging in size from 14 to 53 atoms were used to characterize the reagent. The dithiol peptides are cleanly and rapidly converted to their disulfide forms by a slight excess of the platinum complex under mild reaction conditions (slightly acidic and neutral media). For all the dithiol peptides studied, including a penicillamine-derived peptide, the oxidation yields range from 97% to 100%. No side reactions were observed, including no oxidation of the methionine side chain. The reaction kinetics for oxidation of reduced pressinoic acid were found to be second order overall: rate = k'[Pt(IV)][dithiol peptide], where k' is a pH-dependent second-order rate constant. Values of 0.60 ± 0.01, 3.5 ± 0.2, and 22 ± 1 M^{-1} s^{-1} were determined for k' at pH 3.0, 4.0, and 5.0, respectively (25 °C and 0.45 M ionic strength). A reaction mechanism for oxidation of dithiol peptides by $[Pt(en)_2Cl_2]^{2+}$ is proposed. $[Pt(en)_2Cl_2]^{2+}$ and its reduction product $[Pt(en)_2]^{2+}$ are essentially substitution inert under the conditions used for disulfide formation, they are nontoxic, and they are readily separated from peptides by HPLC. The characteristics of $[Pt(en)_2Cl_2]^{2+}$ and its reaction properties with dithiol peptides suggest that $[Pt(en)_2Cl_2]^{2+}$ is a universal reagent for the rapid and quantitative formation of intramolecular disulfide bonds in peptides.

Introduction

Although formation of intrapeptide disulfide bonds can generally be achieved by oxidation of the free thiol or sulfur-protected precursors, a continuing challenge in peptide synthesis has been to develop more efficient and selective methods.[1−6] A variety of oxidants have been explored for formation of disulfide bonds; however, all have drawbacks and some limitations in practical use, including formation of dimers/oligomers and/or side reactions on the side chains of methionine, tyrosine, and tryptophan.[1,6] For instance, thallium(III) trifluroacetate is a mild oxidant that can be employed as an alternative to iodine, and it gives a higher efficiency in some cases.[1,7] The major drawback of this reagent is its high toxicity. Also, thallium can be difficult to remove from sulfur-containing peptides due to its high affinity for sulfur and the oxidation conditions need to be optimized (solvent, reaction temperature, and the concentration of Tl(III)).[1,7]

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Table 1. Dithiol Peptides Used in the Study To Characterize the Disulfide Bond Forming Properties of $[Pt(en)_2Cl_2]^{2+}$

no.	sequence of dithiol peptides	ring size[a]	oxidation yield,[b,c] %
1	Ac-Cys-Gly-Pro-Cys-NH_2	14	100
2	Ac-Cys-Pro-Phe-Cys-NH_2	14	97
3	Ac-Thr-Cys-Pro-Phe-Cys-Arg-NH_2	14	100
4	Ac-Pro-Thr-Cys-Pro-Phe-Cys-Arg-Lys-NH_2	14	100
5	Ac-Lys-Pro-Thr-Cys-Pro-Phe-Cys-Arg-Lys-Thr-NH_2	14	100
6	Ac-Thr-Asp-Ile-Thr-Cys-Gly-Tyr-Cys-His-Lys-Leu-His-NH_2	14	100
7	Ac-Cys-Pro-Phe-Ala-Ala-Cys-NH_2	20	100
8	Cys-Tyr-Phe-Gln-Asn-Cys (reduced pressinoic acid)	20	100
9	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (reduced oxytocin)	20	98
10	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (reduced arginine-vasopressin)	20	100
11	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys (reduced somatostatin)	38	99
12	Arg-Pro-Cys-Pro-Gln-Cys-Phe-Tyr-Pro-Leu-Met-NH_2	14	99
13	Cys-Phe-Gly-Ser-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Met-Gly-Cys-Gly-Arg-Phe	53	100
14	$HSC(Me)_2CH_2CO$-Tyr(Me)-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2[d]	20	97

[a] Ring members for the oxidized forms. [b] Oxidation yield is reported for the single step of conversion of dithiol peptides to their disulfide forms with slight excess of platinum(IV) complex. [c] The experimental errors for the yields are estimated to be ±5%. [d] Reduced form of [1-deaminopenicillamine, 2-(*O*-methyl)tyrosine]arginine-vasopressin.

In a previous report, we demonstrated that *trans*-$[Pt(CN)_4Cl_2]^{2-}$ is an efficient reagent for the rapid and quantitative oxidation of dithiol peptides to their disulfide forms.[6] Oxidation takes place by a Cl^+ atom transfer mechanism. Unfortunately, $[Pt(CN)_4Cl_2]^{2-}$ is a sufficiently strong oxidizing agent ($E^{\circ\prime}$ = 0.926 V[8]) that it also oxidizes methionine to methionine sulfoxide.[6,9] However, this was the only side reaction observed for an otherwise excellent reagent for formation of intramolecular peptide disulfide bonds, which encouraged us to investigate other *trans*-dichloro-Pt(IV) complexes; by tuning down the redox potential of the complex, it may be possible to eliminate this side reaction while still maintaining the rapid and quantitative nature of the reaction. A major design consideration was that the equatorial coordination sites should be blocked to avoid reaction of the product Pt(II) complex with other functional groups of the peptide. In this paper, we report that, by pursuing this design approach, we have discovered that *trans*-$[Pt(en)_2Cl_2]^{2+}$ (en = ethylenediamine, $E^{\circ\prime}$ for $[Pt(en)_2Cl_2]^{2+}/[Pt(en)_2]^{2+}$ is 0.58 V[10]) is a highly selective and efficient reagent for formation of intramolecular disulfide bonds in peptides without any observed side reactions. Further, the reaction conditions are mild and the Pt(IV) complex and its Pt(II) reduction product $[Pt(en)_2]^{2+}$ are nontoxic and readily separable from peptides, which are desirable properties for a disulfide bond-forming reagent.[1,2,5] These properties together with the results presented here suggest that the discovery of $[Pt(en)_2Cl_2]^{2+}$ as a disulfide bond-forming reagent represents a particularly significant development in the search for efficient methods for formation of intrapeptide disulfide bonds.

Results and Discussion

The dithiol peptides used in the present study (Table 1) range from 4 to 20 amino acid residues in length and the disulfide-containing rings vary in size from 14 to 53 atoms.

Peptides 1−7. Peptides **1**−**6** are model compounds for the active site of thioredoxin (**1**),[11] glutathione thioltransferase (**2**−**5**),[12] and the disulfide bond-forming protein DsbC (**6**).[13] Peptide **7** is derived from peptide **1**, with two additional alanine residues to increase the ring size in the oxidized form. Reaction of peptides **1**−**7** with $[Pt(en)_2Cl_2]^{2+}$ to form the intramolecular disulfide bond was monitored by isocratic reversed phase HPLC. To illustrate, Figure 1 gives results for the oxidation of **1**. The top chromatogram is for a freshly prepared 80 μM solution of **1**; the bottom chromatogram is for the products obtained after reaction of 80 μM **1** and 150 μM $[Pt(en)_2Cl_2]^{2+}$ in pH 7.3 phosphate buffer for 30 min. The peak assignments given in the legend to Figure 1 for $[Pt(en)_2Cl_2]^{2+}$ and $[Pt(en)_2]^{2+}$ are based on chromatograms for authentic samples. The broad peak at 23.2 min is assigned to the disulfide form of **1** based on the mass spectrometric results (theoretical 416.5, found 417). The broad peak for the disulfide form is ascribed to the slow cis−trans isomerization of the glycine−proline peptide bond and a

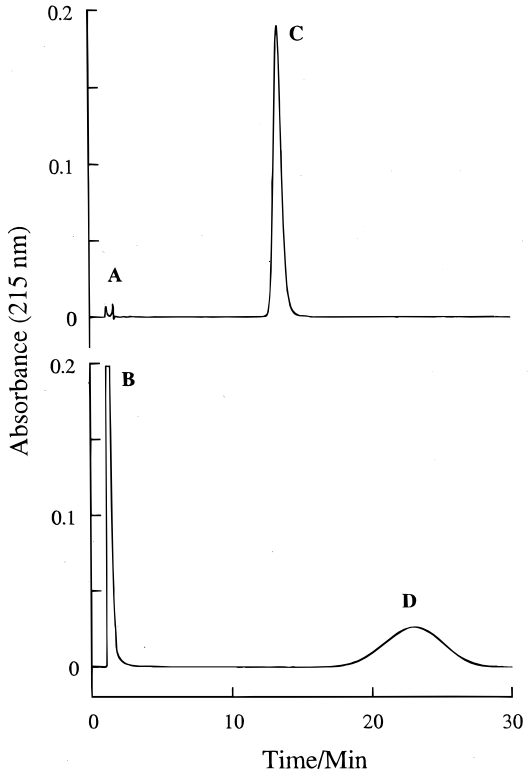

Figure 1. Chromatograms of 80 μM **1** in water (top) and the products from the reaction of 80 μM **1** and 150 μM $[Pt(en)_2Cl_2]^{2+}$ in pH 7.3 phosphate buffer for 30 min (bottom). The mobile phase contained 5% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, solvent + $[Pt(en)_2Cl_2]^{2+}$ + $[Pt(en)_2]^{2+}$; C, **1**; and D, oxidized form of **1**.

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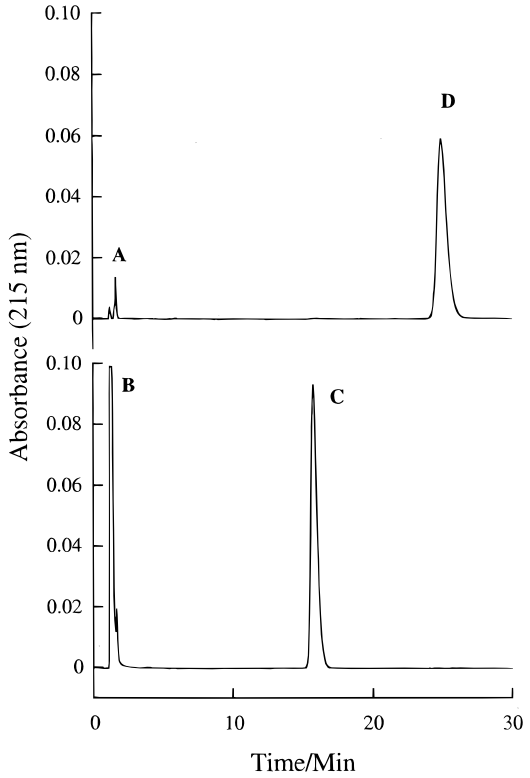

Figure 2. Chromatograms of 100 μM **8** (top) and a reaction mixture of 100 μM **8** and 150 μM $[Pt(en)_2Cl_2]^{2+}$ in a pH 7.3 phosphate buffer after reaction for 30 min (bottom). The mobile phase contained 12% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, solvent + $[Pt(en)_2Cl_2]^{2+}$ + $[Pt(en)_2]^{2+}$; C, oxidized form of **8**; and D, **8**.

relatively high population of the cis isomer.[14,15] The bottom chromatogram was also run for a longer time than shown in Figure 1; no late-eluting peaks due to dimers or higher oligomers were observed. The chromatograms in Figure 1 indicate that a slight excess of $[Pt(en)_2Cl_2]^{2+}$ cleanly and rapidly transforms **1** to its intramolecular disulfide form. It was determined by using the peak area and the HPLC response factor for the disulfide form of the peptide, together with an HPLC detection limit of 3%, that the transformation is quantitative within experimental error, cf. Table 1. Chromatographic patterns for the products of the oxidation of peptides **2**−**7** are similar to that for **1** except that peaks for the disulfide forms are sharp, cf. Supporting Information.

Reduced Peptide Hormones 8−11. Peptides **8**−**11** are the reduced forms of the disulfide-containing hormones pressinoic acid, oxytocin, arginine-vasopressin, and somatostatin, respectively. Figure 2 presents results for oxidation of peptide **8** by $[Pt(en)_2Cl_2]^{2+}$. The top chromatogram is for a freshly prepared 100 μM solution of **8**. The bottom chromatogram is for a reaction mixture of 100 μM **8** and 150 μM $[Pt(en)_2Cl_2]^{2+}$ in pH 7.3 phosphate buffer after 30 min of reaction. The peak at 15.7 min in the bottom chromatogram was assigned to the oxidized form of **8** by comparison to the chromatogram of an authentic sample of pressinoic acid. Peak area measurements indicate that conversion of **8** to its disulfide form is quantitative. The reaction of $[Pt(en)_2Cl_2]^{2+}$ with peptides **9**−**11** was also found to be rapid and quantitative for these reaction conditions, cf. Supporting Information.

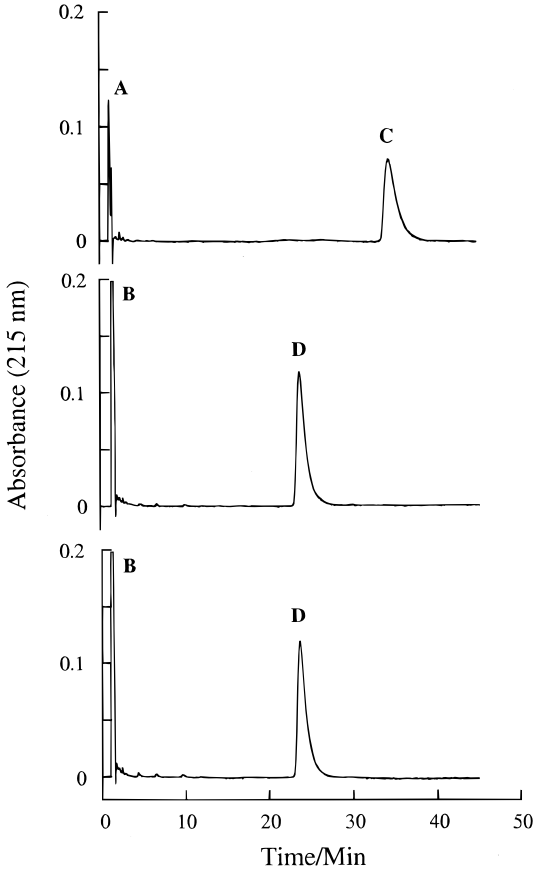

Figure 3. Chromatograms of 40 μM **13** (top) and the products from the reaction of 40 μM **13** and 150 μM $[Pt(en)_2Cl_2]^{2+}$ in pH 6.0 phosphate buffer for 30 min (middle) and 10 h (bottom). The mobile phase contained 20% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, solvent + $[Pt(en)_2Cl_2]^{2+}$ + $[Pt(en)_2]^{2+}$; C, **13**; and D, oxidized form of **13**.

In previous syntheses of oxytocin and arginine-vasopressin and their derivatives,[16−19] it was reported that oxidation of the dithiol groups by ferricyanide results predominantly, but not exclusively, in formation of intramolecular disulfide bonds. This, however, was not the case for the related hexapeptides, tocinamide and pressinamide.[19] Oxidation of the dithiol forms of tocinamide and pressinamide by ferricyanide results mainly in the formation of dimers and higher polymers.[19] In contrast, reaction with $[Pt(en)_2Cl_2]^{2+}$ results in the quantitative formation of intramolecular disulfide bonds, regardless of the peptide sequence.

Methionine-Containing Peptides 12 and 13. Peptides **12** and **13** are the dithiol forms of the methionine-containing peptides [$Cys^{3,6}$, Tyr^8, Pro^9]-Substance P (selective NK-1 agonist) and Atrial Natriuretic Factor (ANF, 11-30, frog), respectively.[20,21] Figure 3 shows chromatograms for the oxidation of **13** in a phosphate buffer at pH 6.0; peak assignments

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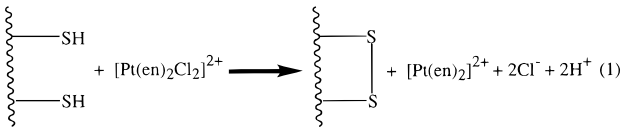
and reaction conditions are given in the figure legend. The top chromatogram is for a fresh solution of 40 μM **13**, and the middle and bottom chromatograms are for a mixture of 40 μM **13** and 150 μM $[Pt(en)_2Cl_2]^{2+}$ in pH 6.0 phosphate buffer after 30 min and 10 h of reaction, respectively. The peak at 23.7 min is assigned to the oxidized form based on its retention time being identical with that for an authentic sample and the molecular weight of the isolated peptide (theoretical 2116.6; found 2116). As determined by HPLC analysis, 100% of the dithiol peptide was converted to the disulfide form after reaction for 30 min. Also, the excess $[Pt(en)_2Cl_2]^{2+}$ and the Pt(II) product in the reaction mixture did not interact with the oxidized peptide including the methionine side chain, as evidenced by the fact that the chromatogram obtained after 10 h of reaction was identical with that obtained after 30 min of reaction. Similar results were obtained for peptide **12**, which also contains a methionine residue. Formation of the 53-membered disulfide-containing ring in the oxidized form of peptide **13**, and also the 38-membered ring in **11**, was found to be as rapid and as efficient as formation of the much smaller rings in peptides **1**−**7**.

Oxidation yields, which range from 97% to 100%, are given in Table 1. It is noteworthy that the peptides studied include all the amino acids with readily oxidizable side chains (methionine, tyrosine, and tryptophan). Thus, the oxidation of dithiol peptides to their disulfide forms by $[Pt(en)_2Cl_2]^{2+}$ is quantitative within experimental error, with no known side reactions. The oxidation reaction can be described by eq 1.



Peptide 14. The oxidation of dithiol peptides by $[Pt(en)_2Cl_2]^{2+}$ was extended to penicillamine-derived peptide **14**,[22] the purpose being to examine the oxidation efficacy when steric hindrance near the thiol groups is increased. Figure 4 shows results for oxidation of **14**; the reaction conditions are given in the figure caption. The bottom chromatogram was also run for a longer time; no late-eluting peaks due to dimers or higher oligomers were observed. The peak at 12.3 min was assigned to the disulfide form of **14** by comparison to the retention time of an authentic sample. It is of interest to note that, although steric hindrance due to the introduction of a deamino-penicillamine increases the difficulty for ring closure, formation of the disulfide form of **14** (yield 97%) is still the predominant reaction. However, some minor peaks (ca. 3%) are observed in Figure 4, which are probably due to side products as discussed below.

Oxidation by $[Pt(CN)_4Cl_2]^{2-}$. Oxidation of peptides **2**−**5** and **8**−**11** by $[Pt(CN)_4Cl_2]^{2-}$ to form the corresponding disulfides was found previously to also be essentially quantitative, with no undesirable side reactions.[6] Oxidation of the peptide Ac-Asp-Ile-Thr-Cys-Gly-Tyr-Cys-His-Lys-Leu-His-Gly-Gln-Met-Lys-NH_2 by $[Pt(CN)_4Cl_2]^{2-}$ also resulted in quantitative formation of the intrapeptide disulfide bond; however, the methionine side chain was oxidized to the sulfoxide form.[6] For a direct comparison of $[Pt(CN)_4Cl_2]^{2-}$ and $[Pt(en)_2Cl_2]^{2+}$ as reagents for disulfide bond formation in methionine-containing peptides, peptide **12** was also oxidized with $[Pt(CN)_4Cl_2]^{2-}$ in the present study using the reaction conditions reported previously.[6] The predominant reaction product was found to be the disulfide peptide, but with the methionine side chain converted to the sulfoxide form (M^+: theoretical 1366.8; found 1366.4).

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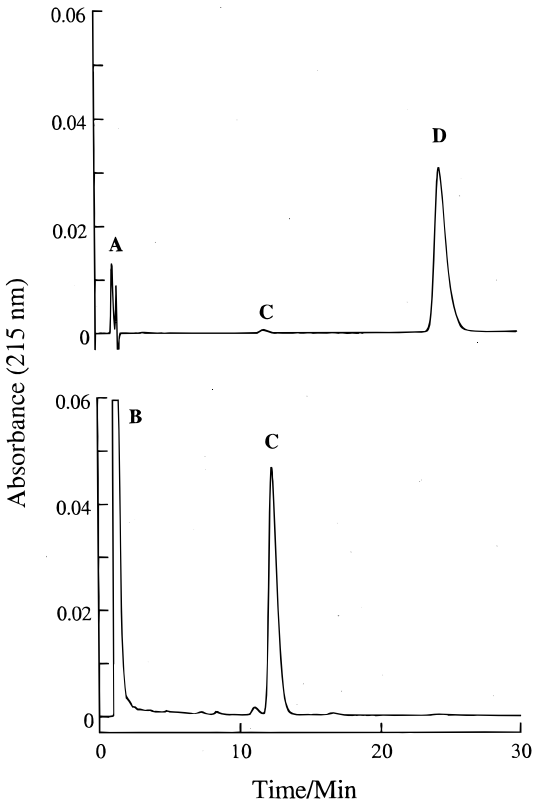


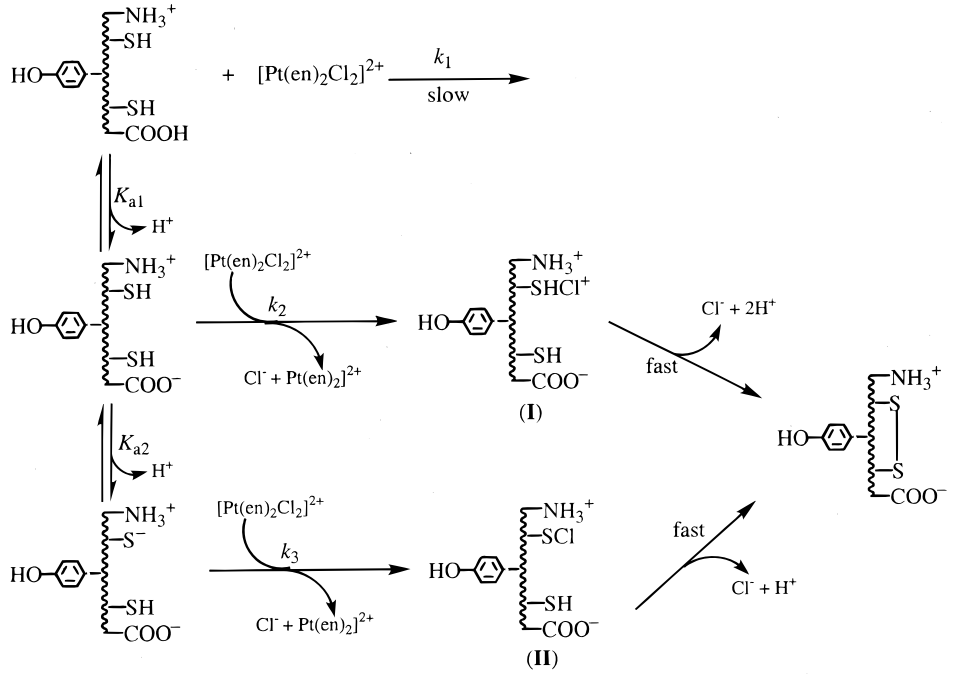
Figure 4. Chromatograms of 100 μM **14** (top) and the products from the reaction of 100 μM **14** and 200 μM $[Pt(en)_2Cl_2]^{2+}$ in pH 4.0 phosphate buffer for 1.5 h (bottom). The mobile phase contained 24% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, solvent + $[Pt(en)_2Cl_2]^{2+}$ + $[Pt(en)_2]^{2+}$; C, oxidized form of **14**; and D, **14**. Minor peaks at 11.0 and 16.7 min are probably from side reaction products, cf. text.

Reaction Conditions. The oxidation reaction was studied under various conditions. To determine if it is necessary to isolate the dithiol peptide in pure form before formation of the disulfide bond, $[Pt(en)_2Cl_2]^{2+}$ was reacted directly with the crude product obtained by cleavage of peptide **6** from solid-phase peptide synthesis resin and deprotection of side chain functional groups (vide infra). Chromatograms obtained by semipreparative HPLC for a reaction mixture containing ca. 2 mM **6** and 3 mM $[Pt(en)_2Cl_2]^{2+}$ (phosphate buffer at pH 6.0, reaction time 30 min) indicate that **6** is rapidly oxidized to its disulfide form, as identified by mass spectrometry, cf. Supporting Information. No additional peaks due to dimer or polymers of **6** were observed, which indicates that oxidation is still clean and quantitative even at millimolar peptide concentrations.

The rate of formation of the intramolecular disulfide bond by reaction with $[Pt(en)_2Cl_2]^{2+}$ is pH dependent. Formation of the disulfide bond by reaction of 100 μM **8** with 250 μM $[Pt(en)_2Cl_2]^{2+}$ was found to be complete in 3 h, 20 min, 3 min, and 20 s at pH 4.0, 5.0, 6.0, and 7.0, respectively, at room temperature. No peaks were detected from side products, even for the pH 7.0 conditions where the reaction took place very rapidly.

To determine if $[Pt(en)_2Cl_2]^{2+}$ can be used to form disulfide bonds in peptides which are only slightly water-soluble but are soluble in aqueous−organic mixtures, the oxidation of **13** was studied in aqueous/acetonitrile solution. Peptide **13** was found to be cleanly and quantitatively oxidized in aqueous phosphate buffer containing up to 50% acetonitrile. $[Pt(en)_2Cl_2]Cl_2$ is

Scheme 1



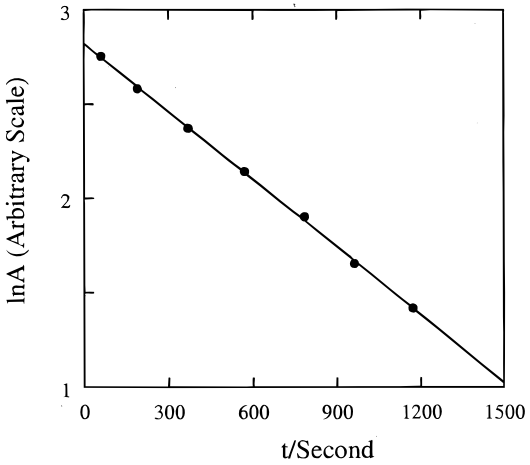


Figure 5. Plot of ln *A* versus reaction time according to eq 2 for oxidation of peptide **8** by $[Pt(en)_2Cl_2]^{2+}$ in pH 3.0 phosphate buffer. Reaction conditions: [**8**] = 80 μM, [Pt(IV)] = 2.00 mM, ionic strength of 0.45 M, and 25 °C.

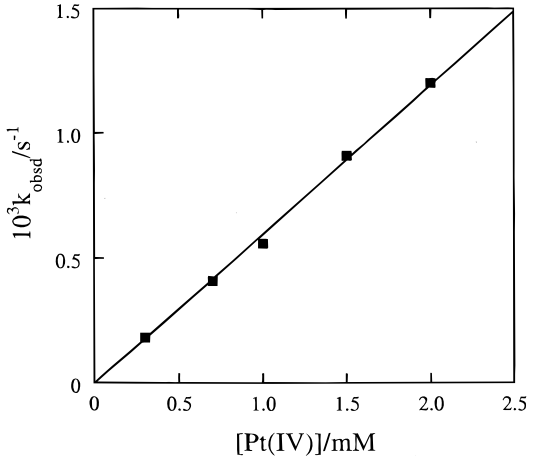


Figure 6. Plot of k_{obsd} versus [Pt(IV)] according to eq 3 for reaction between peptide **8** and $[Pt(en)_2Cl_2]^{2+}$ at pH 3.0, 25 °C, and ionic strength of 0.45 M.

soluble at the millimolar level in mixed aqueous/organic solvent mixtures with methanol, ethanol, acetonitrile, and acetone.

Kinetics and Reaction Mechanism. The kinetics of the reaction of peptide **8** with $[Pt(en)_2Cl_2]^{2+}$ were studied. The oxidation reaction was followed by isocratic HPLC by monitoring the disappearance of **8** under pseudo-first-order conditions with $[Pt(en)_2Cl_2]^{2+}$ present in a 10-fold or larger excess. Because **8** can be totally converted to its disulfide form by reaction with $[Pt(en)_2Cl_2]^{2+}$ (Figure 2), the decrease in the area of the peak for **8** vs time can be described by eq 2 if the reaction is first order in **8**, where A_0 and A are the peak areas of **8** at the start of the reaction and at time t, respectively. Plots of ln A versus

$$\ln A = \ln A_0 - k_{obsd}t \qquad (2)$$

time are linear, as shown in Figure 5 for pH 3 data, which indicates that the reaction is indeed first order in **8**; a pseudo-first-order rate constant of $k_{obsd} = 1.20 \times 10^{-3}$ s^{-1} was obtained from the linear plot in Figure 5.

To determine the order of the reaction with respect to $[Pt(en)_2Cl_2]^{2+}$, the concentration of $[Pt(en)_2Cl_2]^{2+}$ in a pH 3.0 reaction mixture containing 30−80 μM **8** was varied from 0.30 to 2.50 mM. The dependence of k_{obsd} on $[Pt(en)_2Cl_2]^{2+}$ is linear with a zero intercept, as shown in Figure 6, which demonstrates that the oxidation reaction is also first order with respect to the $[Pt(en)_2Cl_2]^{2+}$. Thus, the oxidation reaction follows an overall second-order rate law as described by eq 3, where k' denotes the pH-dependent second-order rate constant. Values of 0.60 ± 0.01, 3.5 ± 0.2, and 22 ± 1 M^{-1} s^{-1} were determined for k'

$$-\mathrm{d}[\mathbf{8}]/\mathrm{d}t = k_{obsd}[\mathbf{8}] = k'[\mathrm{Pt(IV)}][\mathbf{8}] \qquad (3)$$

at 25 °C and ionic strength 0.45 M at pH 3.0, 4.0, and 5.0, respectively. The reaction is too fast to be followed by HPLC at pH ≥6. The oxidation reaction was also studied in 1.00 M HCl; the reaction of 60 μM **8** with 1.00 mM $[Pt(en)_2Cl_2]^{2+}$ yielded ca. 4% and 10% oxidized peptide after 4 and 10 h, respectively, at room temperature. The yields are comparable to those obtained by air oxidation, which suggests that the reaction of $[Pt(en)_2Cl_2]^{2+}$ in 1.00 M HCl is negligibly slow.

The rate law and k' vs pH dependence for the reaction of $[Pt(en)_2Cl_2]^{2+}$ with **8** are similar to those found for reactions between *trans*-dichloro-platinum(IV) complexes and monothiols such as glutathione.[23,24] By analogy with the reaction mechanisms proposed previously for the oxidation of monothiols by Pt(IV) complexes,[23,24] a reaction mechanism for oxidation of peptide **8** by $[Pt(en)_2Cl_2]^{2+}$ is described in Scheme 1. The reaction proceeds through a transition state in which Cl^+ is transferred from the Pt(IV) center to an incoming thiol or thiolate nucleophile.[6,9,23−26] The mechanism in Scheme 1 shows all possible protonation states of **8** under the reaction conditions used in the kinetic study.[27] However, as described above, there is little or no oxidation of peptide **8** by $[Pt(en)_2Cl_2]^{2+}$ in 1.00 M HCl, which indicates that oxidation of the fully protonated form of the peptide is negligibly slow. The reactions described by k_2 and k_3 are the rate-determining steps and are assumed to take place via parallel attack by thiol and thiolate on the coordinated chloride, resulting in a Cl^+ transfer from $[Pt(en)_2Cl_2]^{2+}$ to the attacking group.[6,9,23−26] The intermediates formed, denoted by **I** and **II** in Scheme 1, then undergo an intramolecular nucleophilic attack to form the disulfide bond. The rates of formation of the disulfide bond from intermediates **I** and **II** are expected to be largely controlled by conformational changes, which are expected to be fast relative to the atom transfer step.[28] This is similar to what has been found in detailed studies of the kinetics of formation of intramolecular disulfide bonds in peptides such as **9**−**11** by thiol−disulfide exchange,[29,30] where the nucleophilic displacement reaction in the second step of the overall intramolecular disulfide bond forming reaction is formally similar to the nucleophilic displacement of Cl^- from intermediates **I** and **II**. The relatively fast rates of ring closure by formation of intramolecular disulfide bonds by thiol−disulfide exchange reactions, even in the formation of the disulfide bond to give the 38-membered ring of somatostatin, are attributed to high effective concentrations of the attacking thiolate. [29,30]

The reaction rate increases almost exponentially as the solution pH is increased, which indicates that the thiolate anion is much more reactive than the protonated thiol forms.[23,24] Thus, at the pHs used in the kinetic studies, the reaction described by k_3 in Scheme 1 is the predominant oxidation pathway, while the reaction path described by k_2 contributes only slightly to the overall reaction at pH 3−5.

Reaction mechanisms similar to that described in Scheme 1 can be derived for the other peptides in Table 1. However, for peptide **14**, reaction intermediates similar to **I** and **II** in Scheme 1 apparently undergo ring closure reactions more slowly due to steric hindrance from the two methyl groups on the deaminopenicillamine residue. Under such circumstances, the reaction intermediates are sufficiently long-lived that hydrolysis to form RSOH competes with ring closure by nucleophilic displacement of Cl^-. The RSOH formed can then be further oxidized to give RSO_2H and RSO_3H, or it can undergo intermolecular reactions with the thiol or thiolate groups of other molecules, resulting in formation of dimers and higher polymers.[6] This may explain the presence of up to ∼3% side reaction products in the oxidation of peptide **14**, as indicated by the extra peaks in the chromatograms shown in Figure 4.

It also is of interest to compare the properties of $[Pt(en)_2Cl_2]^{2+}$ with those of $[Pt(CN)_4Cl_2]^{2-}$ as an oxidant for the formation of intramolecular disulfide bonds in peptides. $[Pt(CN)_4Cl_2]^{2-}$ is a somewhat stronger oxidizing agent, as indicated by its redox potential, and as a result it can rapidly convert dithiol peptides to their disulfide forms at lower pH (1−3) than $[Pt(en)_2Cl_2]^{2+}$. The disadvantage, however, is that $[Pt(CN)_4Cl_2]^{2-}$ is a sufficiently strong oxidizing agent that it can oxidize the sulfur of the methionine side chain. Similar reaction rates can be achieved with $[Pt(en)_2Cl_2]^{2+}$ by carrying out the reaction at higher pH (4−7) where a larger fraction is in the thiolate form; however, oxidation of the methionine sulfur, which is a pH-independent oxidation, does not occur.

The advantages and limitations of other reagents for forming intrapeptide disulfide bonds, including the metal complexes $[Fe(CN)_6]^{3-}$ and $Tl(tfa)_3$ and nonmetallic oxidants such as oxygen, disulfides, dimethyl sulfoxide, iodine, and a mixture of methyltrichlorosilane−diphenylsulfoxide, have been described in previous publications, including the comprehensive review by Annis et al.[1c] In general, these reagents are nonselective toward formation of intrapeptide disulfide bonds; dimers and higher oligomers can also form and the oxidation conditions need to be optimized with respect to pH, solvent, and concentrations of peptide and oxidant. Also, depending on the oxidant, side products can be formed by reaction with the side chains of Met, Trp, and Tyr. In contrast, the results presented here show that $[Pt(en)_2Cl_2]^{2+}$ is a highly selective oxidant for the formation of intramolecular disulfide bonds over a range of solution conditions.

Conclusions. We have discovered a highly selective and efficient reagent, *trans*-$[Pt(en)_2Cl_2]^{2+}$, for formation of intramolecular disulfide bonds in peptides. It rapidly and quantitatively converts the dicysteine peptide precursor, even at millimolar concentrations, to its disulfide form in slightly acidic and neutral media. As compared to $[Pt(CN)_4Cl_2]^{2-}$, $[Pt(en)_2Cl_2]^{2+}$ is a somewhat weaker oxidizing agent. No side reactions were observed with $[Pt(en)_2Cl_2]^{2+}$, including no oxidation of the methionine side chain. $[Pt(CN)_4Cl_2]^{2-}$ is a sufficiently strong oxidizing agent that it can rapidly oxidize dicysteine peptides at pH 1−3. Similar rates can be achieved with $[Pt(en)_2Cl_2]^{2+}$ by running the reaction at pH 4−7, where a larger fraction of the thiol groups are in the thiolate form. The Pt(IV) complex is also highly efficient for the oxidation of penicillamine-derived dithiol peptides. Moreover, $[Pt(en)_2Cl_2]^{2+}$ and its reduction product $[Pt(en)_2]^{2+}$ are essentially substitution inert under the conditions used for disulfide bond formation, nontoxic, and readily separable from peptides by HPLC. Thus, $[Pt(en)_2Cl_2]^{2+}$ should be widely useful for the rapid and quantitative formation of intramolecular disulfide bonds in synthetic peptides.

Experimental Section

Materials. $[Pt(en)_2]Cl_2$ and dithiothreitol (DTT) were obtained from Aldrich. Phosphoric acid, sodium dihydrogen phosphate, sodium monohydrogen phosphate, trifluoroacetic acid (TFA), and HPLC-grade acetonitrile were purchased from Fisher Scientific Co. $[Pt(en)_2Cl_2]Cl_2$ was prepared from $[Pt(en)_2]Cl_2$ according to a literature method;[31,32] the UV−visible spectrum was in good agreement with that reported.

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The peptide hormones pressinoic acid, oxytocin, arginine-vasopressin, and somatostatin, [Cys[3,6], Tyr[8], Pro[9]]-Substance P,[20] and [1-deaminopenicillamine, 2-(*O*-methyl)tyrosine]arginine-vasopressin[22] were obtained from Bachem Inc. (Torrance, CA). The peptide ANF (11−30, frog)[21] was supplied by PepSyn Co. (Dublin, CA). Water was purified with a Millipore water purification system.

Peptide Synthesis and Purification. Peptides **1**−**7** in Table 1 were synthesized on a Millipore Model 9050 Plus peptide synthesizer using FMOC solid-phase peptide synthesis methods. A reagent of 88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane was used to cleave the peptide chain from the resin and remove side chain protecting groups; crude peptide product was obtained after lyophilization. Peptides **1**−**7** were isolated from the crude mixture by reversed-phase gradient HPLC on a 100 mm × 250 mm C18 column with an acetonitrile−water mobile phase containing 0.1% TFA. The identities of the peptides were confirmed by molecular weight. Peptides **8**−**14** were prepared by reduction of their disulfide forms with a large excess of DTT at pH 7.0 and were isolated from the reaction mixture using the same HPLC system.

Analysis of the Oxidation Reactions by HPLC. Oxidation reaction mixtures were analyzed by isocratic HPLC on a 3.2 mm × 100 mm C18 reversed-phase column (particle size 3 μm). The UV-detector was set at 215 nm. Mobile phases were prepared by addition of NaH_2PO_4 (0.10 M final concentration) and acetonitrile to water, and the pH was then adjusted to 2.5 with phosphoric acid. Chromatographic conditions were optimized for separation of the reduced dithiol and oxidized forms of the peptides by varying the percentage of acetonitrile in the mobile phase.[33] HPLC mobile phases were filtered through a 0.45 μm cellulose nitrate filter, sparged with helium for ca. 15 min just before the experiment, and were used for less than a week.

Conditions for Oxidation of Dicysteine Peptides with $[Pt(en)_2Cl_2]^{2+}$. The optimum conditions for quantitative formation of intramolecular peptide disulfide bonds are slightly acidic to neutral solution with a slight excess of $[Pt(en)_2Cl_2]^{2+}$. We have found it convenient to use a 2−10 mM stock solution of $[Pt(en)_2Cl_2]^{2+}$ in 1.00 M NaCl and phosphate buffer (pH 4−7) containing 1 mM EDTA. A solution of ca. 2 mM or less dithiol peptide (or crude peptide product cleaved from synthesis resin) is mixed with the $[Pt(en)_2Cl_2]^{2+}$ reagent in phosphate buffer, with $[Pt(en)_2Cl_2]^{2+}$ in slight excess. The mixture is allowed to react from 2 h (pH 4) to 30 min (pH 7), after which oxidation of the dithiol peptide to its disulfide form is complete.

Kinetic Measurements. Phosphate buffer solutions (0.10 M) at pH 3.0, 4.0, 5.0, and 6.0 that contained 1 mM EDTA were prepared. Stock solutions of ca. 1 mM peptide **8** were prepared by dissolving **8** in water and flushed with helium for 10 min. Each solution was only used for 1 h. Kinetic experiments were conducted by combining stock solutions of the reactants and buffer which were equilibrated at 25 °C before mixing. Ionic strength was adjusted to 0.45 M with stock solutions of 1.00 M NaCl. After initiation, the reaction mixture was kept at 25 °C throughout the experiment. Aliquots were removed as a function of time and quenched by adding an equal volume of 2.0 M HCl. The quenched solutions were then analyzed by isocratic HPLC within 4 h. Control experiments showed that the oxidation of **8** in 1.0 M HCl solution is negligibly slow, confirming that addition of the reaction mixture to 2.0 M HCl quenches the reaction.

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Supporting Information Available: Chromatograms for the oxidation of **2**, **6**, **7**, **10**, and **11** by *trans*-$[Pt(en)_2Cl_2]^{2+}$ in phosphate buffer (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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