trans-Dichlorotetracyanoplatinate(IV) as a Reagent for the Rapid and Quantitative Formation of Intramolecular Disulfide Bonds in Peptides

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Oxidation of cysteine thiol groups by *trans*-dichlorotetracyanoplatinate(IV) to form intramolecular peptide disulfide bonds has been studied for a series of dithiol peptides ranging from 4 to 15 amino acid residues in length. The dithiol peptides are rapidly and quantitatively transformed to their intramolecular disulfide forms by a slight excess of $[Pt(CN)_4Cl_2]^{2-}$, as shown by HPLC. Quantitative analyses by HPLC and by spectrophotometric titration confirm a [Pt(IV)]:[dithiol peptide] stoichiometry of 1:1. Under the low pH conditions used, oxidation to form a 38-membered ring in the case of reduced somatostatin is as rapid as that to form much smaller rings, suggesting that ring closure is not the rate-determining step. The oxidation rates increase as the pH is increased. Time-resolved spectra show two isosbestic points, indicating that no peptide−platinum intermediates accumulate to a significant amount. A reaction mechanism similar to that for reduction of $[Pt(CN)_4Cl_2]^{2-}$ by monothiols is proposed. $[Pt(CN)_4Cl_2]^{2-}$ is a mild oxidant and essentially substitution inert; its reduction product, $[Pt(CN)_4]^{2-}$, is stable, has no redox chemistry with peptides, and does not form complexes with peptides. Moreover, $[Pt(CN)_4Cl_2]^{2-}$ and $[Pt(CN)_4]^{2-}$ are nontoxic and readily separable from peptides by HPLC, and the cost of the Pt(IV) complex is negligible compared with that of peptides. The only unwanted side reaction observed with $[Pt(CN)_4Cl_2]^{2-}$ is oxidation of the sulfur of methionine to the sulfoxide form. These characteristics and the results of this study suggest that $[Pt(CN)_4Cl_2]^{2-}$ is an excellent reagent for the formation of intramolecular peptide disulfide bonds.

Introduction

Formation of intramolecular disulfide bonds by oxidation of the corresponding free thiol precursors in solution is usually the last step in the synthesis of disulfide-containing peptides and still remains as a significant challenge.[1−3] Many oxidants, including oxygen, oxidized glutathione (GSSG), dimethyl sulfoxide (DMSO), iodine, ethoxycarbonylsulfenyl chloride (SceCl), and potassium ferricyanide ($K_3Fe(CN)_6$), have been used for this purpose.[1−3] Recently, the use of Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) bound through two sites to a solid support has been used to form disulfide bonds.[4] Although each oxidant has its advantages, most also have drawbacks as well.[1] For instance, advantages of DMSO oxidation include applicability over the wide pH range of 3−8, relatively fast reaction rates, and improved solubility for the materials being oxidized.[1,5,6] However, removing DMSO from the final products can be difficult.[7,8] Moreover, some peptides cannot be oxidized at all, as will be exemplified by peptide **8** in Table 1 of this work (vide infra). Potassium ferricyanide has been found to be useful in the formation of single intramolecular disulfide bonds in small peptides, in particular peptides in the oxytocin and somatostatin families.[1,2] However, methionine and tryptophan residues in the peptides can be oxidized, giving rise to side products.[2] Further, even in the oxidation of relatively small peptides in the oxytocin family, significant amounts of side products, including dimers and polymers, were found.[9] These side reactions can be interpreted in terms of an oxidation mechanism that involves free radicals.[10] Thus, it is not surprising that there are no general rules for selecting an oxidant for the formation of intramolecular peptide disulfide bonds.

Recently, *trans*-dichlorotetracyanoplatinate(IV) ($[Pt(CN)_4Cl_2]^{2-}$) has been used as a model compound for anticancer-active platinum(IV) drugs, and the kinetics and reaction mechanisms for reduction of the model compound by thiols and methionine have been investigated.[11,12] Mechanistically, reduction of chloro−platinum(IV) complexes by thiols shows some features that are similar to thiol−disulfide exchange reactions.[11,13]

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Table 1. Dithiol Peptides Used in the Study of Intramolecular Peptide Disulfide Bond Formation by Reaction with $[Pt(CN)_4Cl_2]^{2-}$

no.	sequence of dithiol peptides	ring size[a]	% acetonitrile[b]
1	N-Ac-Cys-Pro-Phe-Cys-NH_2	14	17
2	N-Ac-Thr-Cys-Pro-Phe-Cys-Arg-NH_2	14	16
3	N-Ac-Pro-Thr-Cys-Pro-Phe-Cys-Arg-Lys-NH_2	14	14.7
4	N-Ac-Lys-Pro-Thr-Cys-Pro-Phe-Cys-Arg-Lys-Thr-NH_2	14	12.7
5	N-Ac-Cys-Gly-Tyr-Cys-NH_2	14	8
6	N-Ac-Thr-Cys-Gly-Tyr-Cys-His-NH_2	14	8
7	N-Ac-Ile-Thr-Cys-Gly-Tyr-Cys-His-Lys-NH_2	14	12
8	N-Ac-Asp-Ile-Thr-Cys-Gly-Tyr-Cys-His-Lys-Leu-His-Gly-Gln-Met-Lys-NH_2	14	16
9	Cys-Tyr-Phe-Gln-Asn-Cys (reduced pressinoic acid)	20	12
10	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (reduced oxytocin)	20	16
11	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (reduced arginine-vasopressin)	20	12
12	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Try-Lys-Thr-Phe-Thr-Ser-Cys (reduced somatostatin)	38	26

[a] Ring size for the oxidized forms. [b] Percentage of acetonitrile in the mobile phases used for HPLC separations.

$[Pt(CN)_4Cl_2]^{2-}$can be readily prepared,[14] is a stable complex, is substitution inert, and does not hydrolyze over a wide pH range if kept with excess chloride in solution. It is a mild oxidant, reacting with strong reductants such as sulfite, iodide, thioethers, and thiol groups[15] but not with most of the functional groups in peptides, including phenols and disulfide bonds. Moreover, its reduction product, $[Pt(CN)_4]^{2-}$, is substitution inert. Except for extremely strong oxidants such as chlorine gas, most of the common oxidants do not react with $[Pt(CN)_4]^{2-}$.[15] These characteristics suggested to us that $[Pt(CN)_4Cl_2]^{2-}$ might be useful as an oxidant for the formation of intramolecular disulfide bonds in peptides. In this paper, we report the results of a study of the oxidation of a series of dithiol peptides by $[Pt(CN)_4Cl_2]^{2-}$. The results demonstrate $[Pt(CN)_4Cl_2]^{2-}$ to be an efficient reagent for the rapid and quantitative formation of intramolecular disulfide bonds in peptides. This is the first report on the use of a platinum(IV) complex as an oxidant in peptide synthesis.

Results and Discussion

Table 1 lists the dithiol peptides used to study the formation of intramolecular disulfide bonds in peptides by $[Pt(CN)_4Cl_2]^{2-}$. Peptides **1**−**4** are model peptides for the active site of glutathione thioltransferase,[16] peptides **5**−**8** are model peptides for the active site of the disulfide bond-forming protein DsbC,[17] and peptides **9**−**12** are the reduced dithiol forms of four disulfide-containing peptide hormones.

Peptides 1−8. The oxidation was monitored by reversed-phase HPLC. To illustrate, a typical oxidation experiment for decapeptide **4** (Table 1) is shown in Figure 1. In this experiment, peptide **4** (final concentration of 19.4 μM) was dissolved in a mixture of 0.02 M HCl and 20.0 μM *N*-acetyl-L-phenylalanine (which served as an HPLC reference), sparged with helium, and then injected into the HPLC. The chromatogram (Figure 1, top) gives rise to three peaks: the internal standard at 8.3 min, **4** at 12.6 min, and the oxidized form of **4** at 23.5 min. The bottom chromatogram in Figure 1 was obtained for a reaction mixture containing 19.4 μM **4**, 40 μM $[Pt(CN)_4Cl_2]^{2-}$, and 20 μM internal standard in 0.02 M HCl after reaction for 30 min. The peaks for $[Pt(CN)_4]^{2-}$ and $[Pt(CN)_4Cl_2]^{2-}$, peaks B and C respectively, were assigned by use of authentic samples of the two complexes, whereas the peak at 23.5 min was assigned to the oxidized form of **4** by mass and NMR spectroscopies.[18] Chromatograms were also run for the samples in Figure 1 using a higher percentage of acetonitrile in the mobile phase to decrease the retention times; the chromatograms were run for the same length of time as in Figure 1 to determine if there were any late-eluting peaks. No additional peaks were observed, indicating a rapid and clean formation of the intramolecular disulfide bond. Similar results were obtained for the oxidation of **1**−**3** and **5**−**7**. The chromatographic peaks for peptides **1**−**4** and their oxidized forms are broad as a result of the slow *cis-trans* isomerization of the cysteine−proline peptide bonds,[19−23] whereas those for peptides **5**−**7** are sharp.

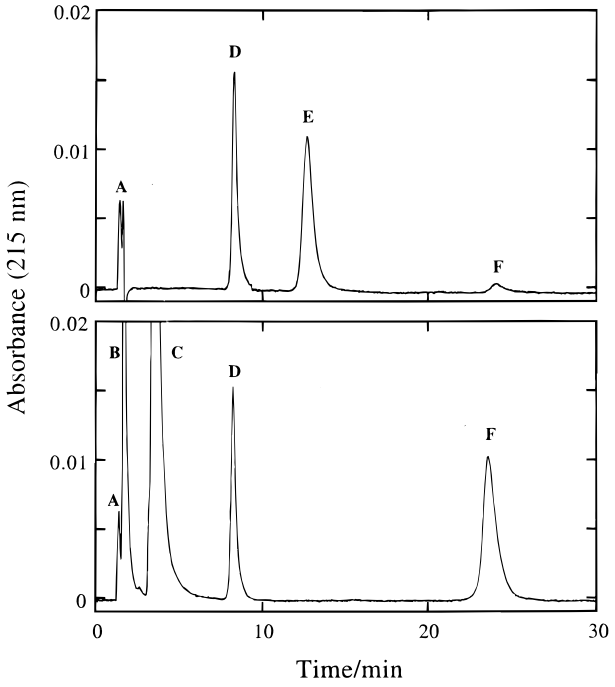

Figure 1. Chromatograms of 19.4 μM decapeptide **4** in 0.02 M HCl (top) and a reaction mixture of 19.4 μM **4** and 40 μM $[Pt(CN)_4Cl_2]^{2-}$ in 0.02 M HCl (bottom). The mobile phase contained 14.7% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, $[Pt(CN)_4]^{2-}$ + solvent; C, $[Pt(CN)_4Cl_2]^{2-}$; D, internal standard acetyl-L-phenylalanine; E, decapeptide **4**; F, oxidized form of **4**.

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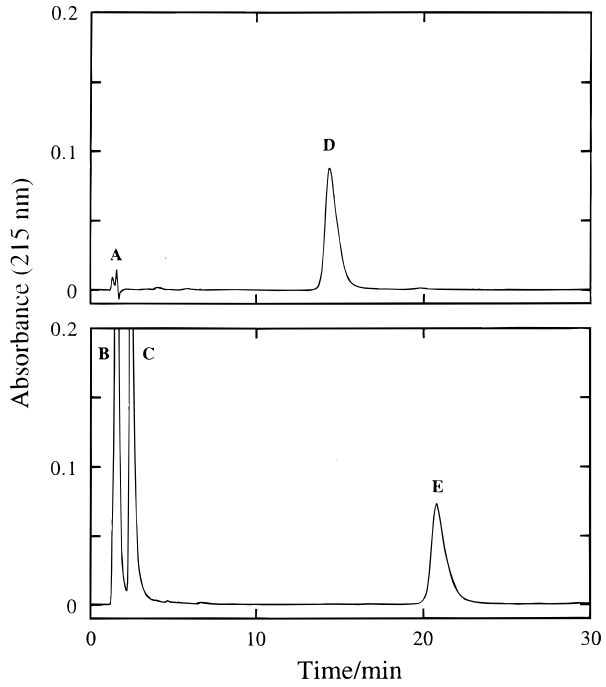

Figure 2. Chromatograms of 80.5 μM tetrapeptide **1** in 0.02 M HCl (top) and a reaction mixture of 402.5 μM **1** and 500 μM $[Pt(CN)_4Cl_2]^{2-}$ in 0.02 M HCl, which was diluted five times (bottom). The mobile phase contained 17% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, $[Pt(CN)_4]^{2-}$ + solvent; C, $[Pt(CN)_4Cl_2]^{2-}$; D, tetrapeptide **1**; E, oxidized form of **1**.

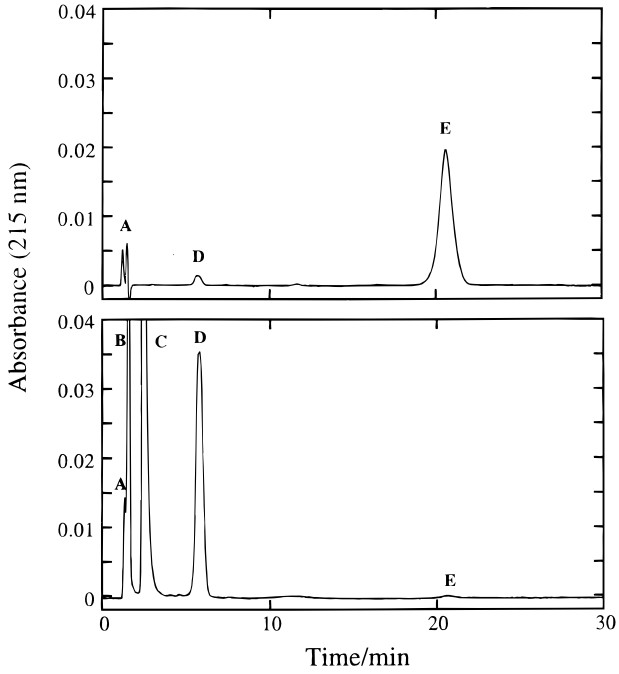

Figure 3. Chromatograms of ca. 35 μM peptide **8** in 0.02 M HCl (top) and a reaction mixture of ca. 35 μM peptide **8** and 50 μM $[Pt(CN)_4Cl_2]^{2-}$ in 0.02 M HCl after reaction for 1 h (bottom). The mobile phase contained 16% acetonitrile, 0.10 M NaH_2PO_4, and sufficient H_3PO_4 to adjust the pH to 2.5. Peak assignments: A, solvent; B, $[Pt(CN)_4]^{2-}$ + solvent; C, $[Pt(CN)_4Cl_2]^{2-}$; D, oxidized form of **8** (methionine side chain oxidized to methionine sulfoxide); E, **8**.

Previous experience with peptide synthesis[1,2] suggests that oxidation reactions to form intramolecular peptide disulfide bonds should be carried out at high dilution, typically at 10−100 μM, to avoid aggregation and intermolecular disulfide bond formation. With $[Pt(CN)_4Cl_2]^{2-}$ as an oxidant, however, oxidation to form intramolecular disulfide bonds was found to be quantitative even at millimolar concentrations, as evidenced by the reaction between the Pt(IV) complex and **1** (Figure 2).

It was observed that peptide **8** was not oxidized to its disulfide form by some commonly used oxidants, including oxygen, GSSG, DMSO, and iodine. When $[Pt(CN)_4Cl_2]^{2-}$ was used, the oxidation was clean and complete within 1 h in a 0.02 M HCl medium. Chromatograms for this oxidation are shown in Figure 3. From the mass spectrum of the oxidized form of peptide **8**, $[\text{peptide} + 3H]^{3+} = 621.3$ (base peak) or molecular weight = 1860.9, indicating that not only was the 14-membered disulfide bond-containing ring formed but the methionine residue was oxidized to methionine sulfoxide as well. The extent of methionine oxidation depends on the [dithiol peptide]:[Pt(IV)] ratio; at a ratio ≥ 1:2, the oxidation is quantitative, as indicated by a single peak in the chromatogram. The oxidation of the methionine side chain by the Pt(IV) complex is an expected result.[12] The methionine sulfoxide group can be conveniently reduced with ammonium iodide while preserving the disulfide bond.[24,25]

Oxidation of Reduced Hormones. The reduced dithiol forms of the peptide hormones pressinoic acid, oxytocin, arginine-vasopressin, and somatostatin (**9**−**12** in Table 1) are also rapidly and cleanly oxidized by $[Pt(CN)_4Cl_2]^{2-}$, as illustrated for reduced somatostatin **12** by the chromatograms in Figure 4. The peak assignments and reaction conditions are given in the figure legend. Chromatogram a is for a solution containing only **12**, chromatogram b is for a solution containing **12** plus less than a stoichiometric amount of $[Pt(CN)_4Cl_2]^{2-}$, chromatogram c is for **12** plus an excess of $[Pt(CN)_4Cl_2]^{2-}$, and chromatogram d is for the same solution as in c but after a longer reaction time. The chromatograms indicate that, when less than a stoichiometric amount of $[Pt(CN)_4Cl_2]^{2-}$ is used, only a fraction of the reduced somatostatin is converted to the oxidized form. In the presence of excess $[Pt(CN)_4Cl_2]^{2-}$, all of the reduced somatostatin is converted to the disulfide form, and the disulfide bond is stable toward the excess $[Pt(CN)_4Cl_2]^{2-}$. Formation of the disulfide bond of somatostatin was found to be as rapid as formation of the disulfide bonds of the other peptides in Table 1, which suggests that, under the conditions used, ring closure to form the disulfide bond is probably not the rate-controlling step in the oxidation reaction for this series of peptides. It also is interesting to note that the disulfide forms of the peptide hormones elute from the column before the

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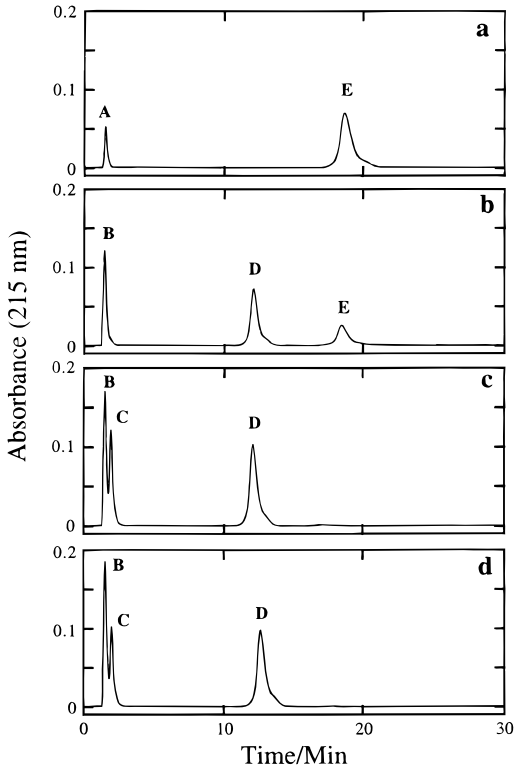


Figure 4. Chromatograms for the oxidation of reduced somatostatin **12**: (a) 27.04 μM **12**; (b) a reaction mixture of 27.04 μM **12** and 20 μM $[Pt(CN)_4Cl_2]^{2-}$ after reaction for 1 h at pH $\approx$ 2.3; (c) a reaction mixture of 27.04 μM **12** and 50 μM $[Pt(CN)_4Cl_2]^{2-}$ after reaction for 1 h at pH $\approx$ 2.3; and (d) the same reaction mixture as in (c) after reaction for 12 h. Peak assignments: A, solvent; B, $[Pt(CN)_4]^{2-}$ + solvent; C, $[Pt(CN)_4Cl_2]^{2-}$; D, somatostatin; E, reduced somatostatin **12**.

reduced forms, in contrast to the chromatographic behavior of peptides **1**−**7** (Figures 1 and 2).

Quantitation of the Oxidation Reactions. The reaction of $[Pt(CN)_4Cl_2]^{2-}$ with dithiol peptides was quantitatively characterized by two experiments. In the first experiment, a somatostatin sample obtained from Bachem (peptide content reported to be (89.2 ± 3)%, which was confirmed previously by NMR[23]) was used as a standard to prepare an HPLC peak area vs somatostatin concentration calibration curve. Reduced somatostatin **12** was then prepared by reduction of somatostatin with a 10-fold excess of dithiothreitol in pH 7.0 buffer, the pH was reduced to 2.5, and the reduced somatostatin was isolated from the reaction mixture by semipreparative scale HPLC. The chromatogram of the sample isolated (Figure 4a) indicates the absence of native somatostatin. The concentration of reduced somatostatin was determined spectrophotometrically by reaction with Ellman's reagent to be 33.8 μM. Aliquots of the reduced somatostatin solution were then reacted with $[Pt(CN)_4Cl_2]^{2-}$ at pH $\approx$ 2.3 using four reaction conditions: 27.04 μM **12** + 50 μM Pt(IV) reacted for 1 h; 27.04 μM **12** + 200 μM Pt(IV) reacted for 1 h; 27.04 μM **12** + 100 μM Pt(IV) reacted for 1.5 h; and 27.04 μM **12** + 50 μM Pt(IV) aged for 12 h. The reaction solutions were analyzed by HPLC. Using the peak area/concentration calibration curve, reduced somatostatin was found to be converted to native somatostatin with a yield of (93 ± 8)%. The large error is estimated from four sources: the somatostatin content of the sample used to prepare the calibration curve

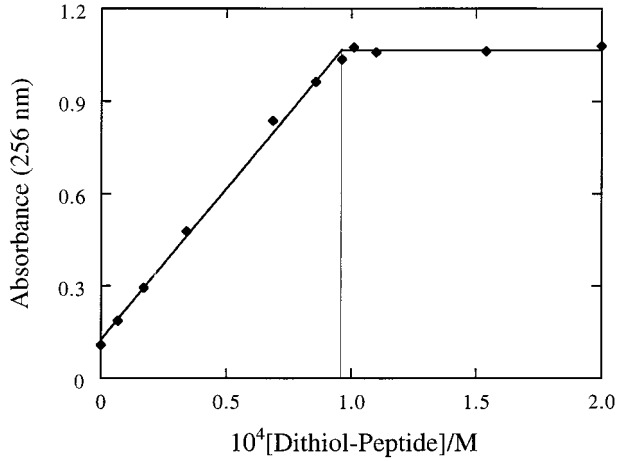


Figure 5. Absorbance at 256 nm for solutions with constant [Pt(IV)] and increasing [**1**]. Conditions: $[Pt(CN)_4Cl_2^{2-}] = 0.10$ mM, [HCl] = 0.02 M, and room temperature.

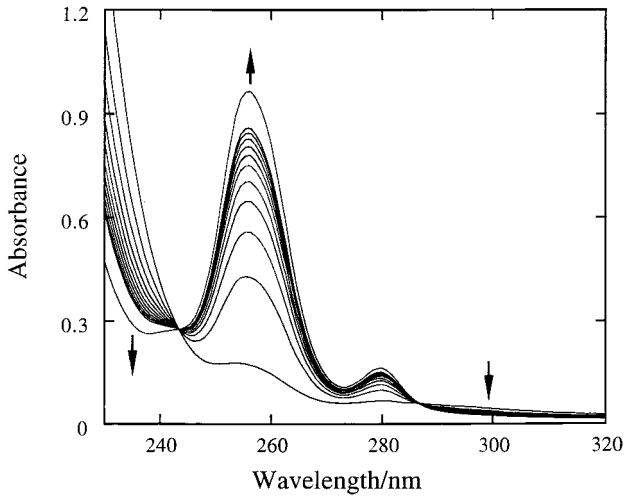
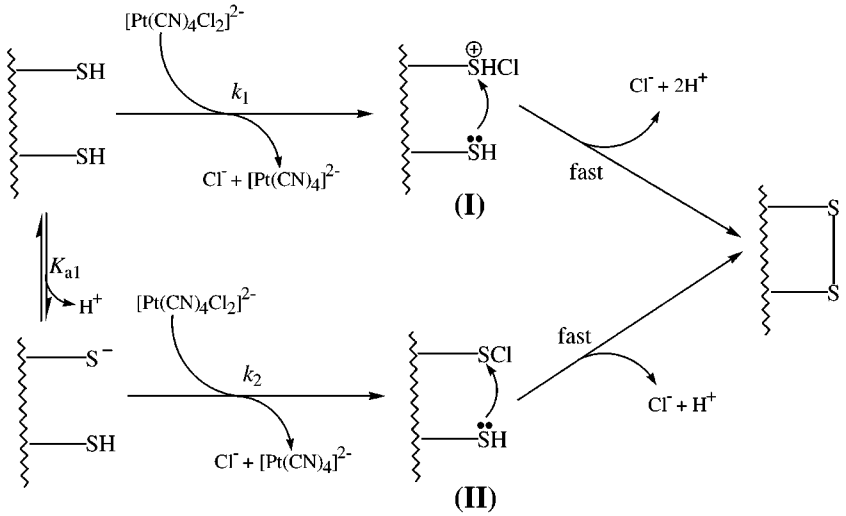


Figure 6. Time-resolved spectra for reduction of $[Pt(CN)_4Cl_2]^{2-}$ by tetrapeptide **1**. Conditions: $[Pt(CN)_4Cl_2^{2-}] = 0.10$ mM, [**1**] = 0.0858 mM, [HCl] = 0.02 M, and room temperature. A solution of 0.02 M HCl was used as reference. The first spectrum was obtained ca. 10 s after mixing $[Pt(CN)_4Cl_2]^{2-}$ and **1**. The time interval between scans was 2 min. The last scan was obtained 1 h after mixing; no spectral changes were observed thereafter.

(±3%); errors in the determination of the concentration of reduced somatostatin using Ellman's reagent (±3%); weighing errors (±2%); and sample injection errors (±2%).

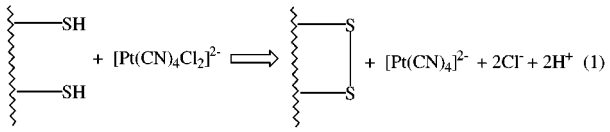
In the second quantitation experiment, a spectrophotometric titration was performed with peptide **1**. The absorption peak for $[Pt(CN)_4]^{2-}$ at 256 nm (cf. Figure 6) was used. Absorption spectra were measured for a series of solutions in which the Pt(IV) concentration was held constant at 0.100 mM and the concentration of **1** was increased from 0 to 0.20 mM. After oxidation was complete (checked by scanning the UV−vis spectrum), the absorbance was measured at 256 nm for each solution. The titration curve shown in Figure 5 gives rise to a stoichiometry of [Pt(IV)]:[**1**] = 1:(0.96 ± 0.06). If errors from the concentration assay of **1** (±3%) and from locating the end point in the titration curve (±3%) are taken into account, this stoichiometry is in good agreement with the HPLC results. Thus, these two experi-

Scheme 1



ments indicate quantitative formation of the disulfide bond, with no significant formation of intermolecular disulfides to give higher polymers.

The stoichiometry for oxidation of dithiol peptides (without a methionine residue) by $[Pt(CN)_4Cl_2]^{2-}$ can be described by eq 1:



Reaction Mechanism. Time-resolved spectra were measured for the oxidation of tetrapeptide **1**; the results are shown in Figure 6. The growing peak at 256 nm is ascribed to formation of $[Pt(CN)_4]^{2-}$. Two clear-cut isosbestic points are observed at 243.4 and 286.6 nm, indicating that no platinum-containing intermediates accumulate to any significant concentration. Thus, $[Pt(CN)_4Cl_2]^{2-}$ is directly reduced to $[Pt(CN)_4]^{2-}$, further confirming the quantitative nature of the oxidation reaction.

Reduction of $[Pt(CN)_4Cl_2]^{2-}$ by monothiols (RSH) was shown previously to proceed according to eq 2 in the

$$[Pt(CN)_4Cl_2]^{2-} + 2RSH \rightarrow [Pt(CN)_4]^{2-} + RSSR + 2Cl^- + 2H^+ \quad (2)$$

presence of a large excess of RSH.[11] Reduction of platinum(IV) halide complexes is rationalized in terms of halide-bridged electron transfer,[11−15] and reduction by thiols is assumed to take place by attack of the sulfur on a coordinated chloride. Accompanying the attack is a Cl^+ transfer from the Pt(IV) center to the incoming sulfur atom, resulting in RSCl and $RSHCl^+$ as initial short-lived products.[11,15] RSCl and $RSHCl^+$ can hydrolyze quickly, giving rise to RSOH, which subsequently is trapped by RSH and RS^- according to reactions 3 and 4.[27] When

$$RSOH + RSH \rightarrow RSSH + H_2O \quad (3)$$

$$RSOH + RS^- \rightarrow RSSH + OH^- \quad (4)$$

monothiols are oxidized under conditions of excess $[Pt(CN)_4Cl_2]^{2-}$, as in the present work, the [Pt(IV)]:[RSH] stoichiometry deviates significantly from 1:2.[26] Under these conditions, RSOH can accumulate to a significant amount and be further oxidized by the excess Pt(IV) to RSO_2H and RSO_3H.[26]

A reaction mechanism similar to that proposed previously for reduction of $[Pt(CN)_4Cl_2]^{2-}$ by monothiols[11,15] is presented in Scheme 1.

In this reaction mechanism, dithiol peptide and its deprotonated species reduce $[Pt(CN)_4Cl_2]^{2-}$ in parallel reactions, giving two short-lived intermediates (**I** and **II**), which then undergo intramolecular attack to form the disulfide bond. The Pt(IV) complex is substitution inert and no Pt(IV)−dithiol peptide reaction intermediates are observed, as evidenced by the time-resolved spectra (Figure 6). The rate of oxidation of dithiol peptides increases when the pH of the reaction medium is increased. A similar pH dependence was observed previously for reduction of $[Pt(CN)_4Cl_2]^{2-}$ by monothiols and is due to the much faster reaction of the thiolate form with the Pt(IV) complex.[11] Although there is very little of the thiolate species present at the low pH used in this work, there is nevertheless some reaction by the lower pathway in Scheme 1 because the rate of reaction of $[Pt(CN)_4Cl_2]^{2-}$ with the thiolate group is orders of magnitude faster than with the thiol group.[11] A low pH was used to minimize potential side reactions in which the free thiol group in intermediates **I** and **II** would react with the excess $[Pt(CN)_4Cl_2]^{2-}$ before it could undergo intramolecular attack at the SCl or $SHCl^+$ group to form the disulfide bond. Working in acidic media is also convenient because thiol−disulfide exchange and air oxidation side reactions are essentially eliminated. Theoretically, an intermolecular attack on the intermediates by another peptide could produce a dimer as in the case of monothiol oxidations.[11] However, no chromatographic peaks that could be assigned to intermolecular disulfides were observed, suggesting that the rate for intermolecular attack is much less than that for intramolecular attack under the conditions used. In other words, rapid conformational change of intermediates **I** and **II** to make ring closure possible is a highly favorable step. In this regard, the possibility for formation of the hydrolyzed intermediate similar to RSOH as discussed above is also less likely.

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Conclusions. This work has demonstrated that $[Pt(CN)_4Cl_2]^{2-}$ is an efficient oxidant for the rapid and quantitative formation of intramolecular peptide disulfide bonds. The Pt(IV) complex has several advantages: (1) it is a mild oxidant and essentially substitution inert; (2) its reduced product $[Pt(CN)_4]^{2-}$ is also substitution inert; (3) both $[Pt(CN)_4Cl_2]^{2-}$ and $[Pt(CN)_4]^{2-}$ are nontoxic and readily separable from peptides by HPLC; and (4) the cost of the Pt(IV) complex is negligible compared to that of peptides. The only drawback observed is that $[Pt(CN)_4Cl_2]^{2-}$ can oxidize the methionine side chain to methionine sulfoxide.

Experimental Section

Chemicals. $K_2[Pt(CN)_4]\cdot 3H_2O$ (Johnson Matthey), dithiothreitol (DTT, Aldrich), *N*-acetyl-L-phenylalanine (Sigma), and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Sigma) were used as received. $K_2[Pt(CN)_4Cl_2]$ was synthesized by the procedure described previously.[14] Stock solutions of 1.00 mM and 5.00 mM $K_2[Pt(CN)_4Cl_2]$ in 0.02 M HCl were prepared. Sodium dihydrogen phosphate, phosphoric acid, hydrochloric acid, trifluroacetic acid (TFA), and acetonitrile (Optima) were obtained from Fisher Scientific Co. The peptide hormones arginine-vasopressin, oxytocin, pressinoic acid, and somatostatin were obtained from Bachem Inc. (Torrance, CA). Peptides **1**−**8** were synthesized by solid-phase peptide synthesis using FMOC methodology and characterized by LC−MS and NMR spectroscopy in this laboratory.[18] Water was purified with a Millipore water purification system. Mobile phases were filtered through a 0.45 μm cellulose nitrate filter, sparged with helium for ca. 15 min just before the experiment and used for less than a week.

Purification of the Peptides. The reduced dithiol forms of the peptide hormones were prepared by reduction of the oxidized forms with a 10-fold excess of DTT at pH 7.0 and were isolated immediately by reversed-phase HPLC on a 100 mm × 250 mm C18 column. The reduced peptides were eluted with an acetonitrile−water gradient containing 0.1% TFA. The pH of the mobile phases was low (ca. 2.5) to minimize oxidation of the reduced peptides by air. The other peptides used in this work were also purified by HPLC. The reduced peptide content was determined spectrophotometrically by use of Ellman's reagent at pH 7.4 in a phosphate buffer containing 1 mM EDTA.[28,29]

Oxidation Protocol. Oxidation reactions were carried out in 20 mM HCl solution at room temperature. A stock solution of 5.00 mM $K_2[Pt(CN)_4Cl_2]$ in 20 mM HCl was prepared; $[Pt(CN)_4Cl_2]^{2-}$ is stable indefinitely under these conditions. A 100 μM to 1 mM solution of the dithiol peptide (or crude product cleaved from the synthesis resin) was also prepared in 20 mM HCl. The dithiol peptide and $[Pt(CN)_4Cl_2]^{2-}$ solutions were mixed at [dithiol peptide]:[Pt(IV)] ratios ranging from 1:1.2 to 1:2 and then flushed for several minutes with nitrogen or argon. The solutions were then allowed to react for ∼1 h, after which conversion of the dithiol peptide to its disulfide form was complete.

Monitoring the Oxidation Reactions by HPLC. Oxidation of dithiol peptides by $[Pt(CN)_4Cl_2]^{2-}$ was monitored by isocratic HPLC using a 100 mm × 3.2 mm ODS (C18) column (particle size 3 μm). The 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 then the pH was adjusted to 2.5 with 1 M H_3PO_4. 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;[30] the percentage of acetonitrile used for each peptide is given in Table 1.

Time-Resolved Spectra and Spectrophotometric Titration. Time-resolved spectra for the reaction of $[Pt(CN)_4Cl_2]^{2-}$ with peptide **1** in 20 mM HCl were recorded with a diode array spectrophotometer. The reaction solution was contained in 1.00 cm quartz cells. Solutions of 2.0×10^{-4} M $[Pt(CN)_4Cl_2]^{2-}$ and of 1.716×10^{-4} M **1** were prepared fresh in 20 mM HCl and were sparged with helium for ca. 10 min before mixing and recording the spectra. For the spectrophotometric titration, a series of solutions with [Pt(IV)] = 0.100 mM, [HCl] = 20 mM, and $0 \le$ [**1**] $\le$ 0.200 mM were freshly prepared and flushed with helium, and their absorbances were measured at 256 nm (the absorption peak of $[Pt(CN)_4]^{2-}$) after the solutions had reacted for ca. 3 h.

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