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Formation of peptide disulfide bonds through a *trans*-dibromido-Pt(IV) complex oxidation reaction: Kinetic and mechanistic analyses

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ABSTRACT

A *trans*-dibromido-Pt(IV) complex $[PtBr_2(en)_2]Br_2$ was synthesized and evaluated for the synthesis of peptide disulfide bonds in this work. The reactions between the Pt(IV) complex and dicysteine-containing peptides were carried out in various aqueous solutions. As a result, excellent yields with fast reaction rates were achieved. Methionine residue was intact when the Pt(IV) complex reacted with a dicysteine and methionine-containing peptide. 3,6-Dioxa-1,8-octanedithiol (DODT) was selected as a model compound of the dicysteine-containing peptide. Kinetic studies for the reaction between the Pt(IV) complex and DODT were performed in different pH solutions. It is first-order both in [Pt(IV)] and in [DODT]. A reaction mechanism was proposed accordingly. The kinetic and mechanistic results demonstrated that the reaction rate for the formation of peptide disulfide bond *via* the Pt(IV) oxidation is increased with the increase of pH of the reaction medium. The protolytic constants of the two thiol groups in peptide also play a critical role in reaction rate. On the other hand, kinetic studies demonstrated that the Pt(IV) complex *trans*-$[PtBr_2(en)_2]^{2+}$ can be used in an acidic medium for the purpose of synthesis of disulfide bonds.

1. Introduction

Platinum(IV) complexes possess two axial bromide ligands (*trans*-dibromido-Pt(IV)) have attracted much attention due to their promoted antitumor activity [1–5]. The substitution of the axial ligands by a nucleophilic reagent is generally difficult to occur because of the natural kinetics inertness of the Pt(IV) complex. However, a reduction reaction between the Pt(IV) complex and a thiol-containing compound (cysteine, glutathione, homocysteine) is prone to occur through a bromide-bridged electron-transfer process. The thiol-containing compound was oxidized to its corresponding disulfide form [6]. Moreover, *trans*-dibromido-Pt(IV) was easily soluble in aqueous solution. Therefore, this kind of Pt(IV) complexes maybe used as oxidants for synthesis of peptide disulfide bond in aqueous solution through a homogenous oxidation reaction. Although the homogenous oxidation method suffers from the relatively low productivity, resulting from low peptide concentration used in order to minimize intermolecular oligomerization. However, until now, synthesis of peptide disulfide bond in aqueous solution through a homogenous oxidation reaction is generally adopted in industrial peptide production or in Lab peptide research [7–14].

Pt(IV) complexes such as *trans*-$[PtCl_2(CN)_4]^{2-}$, *trans*-$[PtCl_2(en)_2]^{2+}$, *trans*-$[PtCl_2(phen)(en)]^{2+}$, *trans*-$[PtCl_2(bpy)(en)]^{2+}$ and *trans*-$[Pt(OH)_2(phen)(en)]^{2+}$ have been used as very efficient oxidants for synthesizing intramolecular disulfide bonds in peptides [15–20]. The redox potential of Pt(IV) complex has no influence on the rate of formation of peptide disulfide bond by Pt(IV) oxidation. But the thioether group of methionine side chain in peptide can be oxidized to its sulfoxide form by the Pt(IV) complex with higher redox potential. Generally, the rate of formation of peptide disulfide bond by Pt(IV) oxidation was influenced by the equatorial and axial ligands of Pt(IV) complex. Among the five Pt(IV) complexes, *trans*-$[PtCl_2(CN)_4]^{2-}$ can oxidize methionine residue to methionie sulfoxide derivate making it unsuitable used as an oxidant for synthesis of disulfide bond in a methionine-containing peptide [15,16]. The other four Pt(IV) complexes are compatible to methionine residue. Slow reaction rates were achieved when *trans*-$[PtCl_2(en)_2]^{2+}$ and *trans*-$[Pt(OH)_2(phen)(en)]^{2+}$ were used to oxidative synthesis of peptide disulfide in an acidic medium [17–19]. *trans*-$[PtCl_2(phen)(en)]^{2+}$ and *trans*-$[PtCl_2(bpy)(en)]^{2+}$ can rapidly convert dithiol to disulfide bond in peptide in acidic medium [20]. In

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order to expand the toolbox for the synthesis of peptide disulfide, in this work, *trans*-$[PtBr_2(en)_2]^{2+}$ as an oxidant was used to synthesize disulfide bonds, and the kinetics and mechanism of formation of disulfide bond was investigated in detail.

2. Experimental section

2.1. Reagents

Dicysteine-containing peptides used in this work were purchased from Nanjing Peptide Company (Nanjing, China) and used as obtained; the purities of all peptides are 90%. The peptide content of iRGD (CRGDKGPDC-NH_2) is reported to be 85%. 3,6-Dioxa-1,8-octanedithiol (DODT) and sodium perchlorate were obtained from Sigma-Aldrich (St. Louis, MO). Standard buffers of pH 4.00, 7.00 and 10.00 were purchased from Fisher Scientific (Fisher Scientific, Pittsburgh, PA). Acetic acid, sodium acetate, sodium dihydrogenphosphate, disodium hydrogenphophate were, all in analytical grade, purchased from Tianjin Chemical Reagent Company (Tianjin, China) and were used for preparation of buffer solutions. Pt(IV) complexes $[PtBr_2(en)_2]Br_2$ and $[PtCl_2(en)_2]Cl_2$ were synthesized according to a method published in the literature [21,22]. The UV–Vis spectrum of $[PtCl_2(en)_2]Cl_2$ is in excellent agreement with that reported earlier for *trans*-$[PtCl_2(en)_2]^{2+}$. $[Pt(en)_2Br_2]Br_2$ was characterized by elemental analysis: Calc. for $C_4H_{16}Br_4N_4Pt\cdot 0.5H_2O$: C, 7.46%; H, 2.66%; N, 8.70%. Found: C, 7.23%; H, 2.64%; N, 8.86%.

2.2. Instruments

Peptides were analyzed on a LC-6 CE high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan). UV–Vis spectra were recorded on a TU-1900 spectrophotometer (Beijing Puxi, Inc., Beijing, China) using 1.00 cm quartz cells. Elemental analysis for C, H, and N was performed on an Elementar instrument (Vario Micro cube, Germany). ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE III 600 MHz digital NMR spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Kinetic measurements were performed on an Applied Photophysics SX-20 stopped-flow spectrometer (Applied Photophysics Ltd., Leatherhead, U.K.) equipped with a thermostat (BG-chiller E10, Beijing Biotech Inc., Beijing). Accumet Basic AB15 Plus pH meter equipped with an Accumet combination pH electrode (Fisher Scientific, Pittsburgh, PA) was used to measure the pH values of buffer solutions.

2.3. Formation of disulfide bonds in peptides

Pt(IV) complex (1–2 equiv.) was reacted with dicysteine-containing peptide in various solvents. The reaction mixtures were analyzed by LC-6 CE HPLC system equipped with a UV–vis detector at 215 nm with a 250 mm × 4.6 mm C_8 column at a flow rate of 1.0 mL/min. Two solvent systems consisting of 0.1% TFA in acetonitrile and 0.1% TFA in water were used for peptide elution with a suitable gradient. It is known that *trans*-$[PtCl_2(en)_2]^{2+}$ can quantitatively convert the dicysteine-containing peptide to its disulfide form [16]. Therefore, the disulfide bridged peptides generated from the *trans*-$[PtCl_2(en)_2]^{2+}$ oxidation were used to characterize and calculate the yields of the disulfide-containing peptides generated from the *trans*-$[PtBr_2(en)_2]^{2+}$ oxidation.

2.4. Kinetic experiments

Kinetic traces were recorded on the Applied Photophysics SX-20 stopped-flow spectrometer. $[PtBr_2(en)_2]Br_2$ was dissolved in a solution containing 0.90 M $NaClO_4$, 0.09 M NaBr, and 0.01 M $HClO_4$ for preparing a 1.0 mM stock solution. This stock solution was used daily in fresh. The solution container was wrapped with aluminum foil and was stored in a refrigerator. For kinetic measurements, solutions of the Pt(IV) complex and DODT were prepared, respectively, by adding an appropriate amount of the Pt(IV) stock solution and of DODT to a specific buffer. All buffer solutions contained 0.10 M NaBr and 2.0 mM EDTA. Sodium perchlorate was used to adjust the ion strength of buffers to 1.0 M. The obtained solutions were flushed for 10 min with nitrogen, and then were loaded on the stopped-flow machine. Equal volumes of the Pt(IV) and DODT solutions were mixed automatically on the machine for recording the kinetic traces under pseudo-first-order conditions with DODT being at least 10-fold excess.

2.5. Stoichiometry and products analysis

The stoichiometry was determined by reaction of 1.0 mM Pt(IV) complex with various concentrations of iRGD (0.1 ≤ [iRGD] ≤ 2.0 mM) in H_2O. The peak area of disulfide bridged iRGD peptide generated

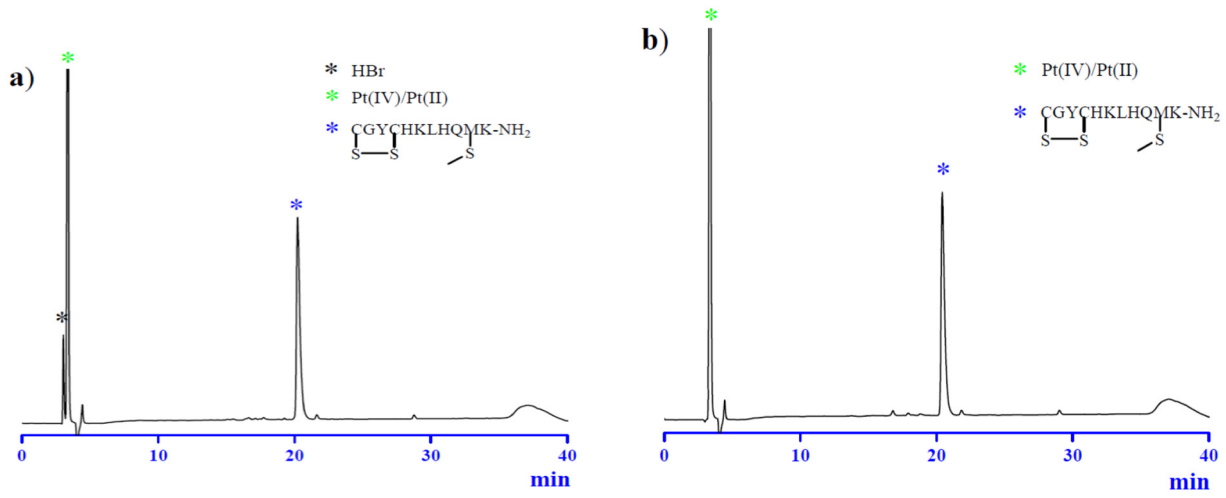


Fig. 1. HPLC chromatograms of a) the reaction between *trans*-$[PtBr_2(en)_2]^{2+}$ (1.0 mM) and peptide 1 (0.8 mM) in H_2O; b) the reaction between *trans*-$[PtCl_2(en)_2]^{2+}$ (1.0 mM) and peptide 1 (0.8 mM) in H_2O.

Table 1
Synthesis of disulfide bonds in peptides by *trans*-$[PtBr_2(en)_2]^{2+}$ oxidation in H_2O at room temperature [a].

No.	Peptide	Sequence of peptide	Reaction time (min)	Yield [b] (%)	Purity [c] (%)
1	peptide 1	CGYCHKLHQMK-NH_2	<1	99	99
2	peptide 2	CGYCHKLHQGK-NH_2	<1	99	99
3	oxytocin	CYINQCPLG-NH_2	<1	95	85
4	arginine vasopressin	CYFQNCPRG-NH_2	<1	97	95
5	somatostatin	AGCKNFFWKTFTSC-OH	<1	99	96
6	brain binding peptide	CLSSRLDAC-NH_2	<1	96	91
7	iRGD	CRGDKGPDC-NH_2	<1	99	98
8	crustacean cardioactive peptide	PFCNAFTGC-NH_2	<1	98	92
9	phenypressin	CFFQNCPRG-NH_2	<1	99	90
10	vasotocin	CYIQNCPKG-OH	<1	99	98
11	atriopeptin II	SSCFGGRIDRIGAQSGLGCNSFR-OH	<1	99	98

[a] Reaction conditions: $[PtBr_2(en)_2^{2+}] = 1.0$ mM, [dicysteine-containing peptide] = 0.5–1.0 mM. [b] Yields of disulfide-containing peptides were calculated according to the peak areas of disulfide bridged peptides generated from *trans*-$[PtCl_2(en)_2]^{2+}$ oxidation reactions. [c] Purities of disulfide-containing peptides were calculated by the areas of the chromatographic products peaks recorded at 215 nm; Peak areas of Pt(IV)/Pt(II) are not included in the calculation.

form the reaction was determined by HPLC. Excess of *trans*-$[PtBr_2(en)_2]^{2+}$ (0.6 mM) was reacted with iRGD peptide (0.5 mM) in D_2O, the reaction mixture was analyzed by NMR spectra for characterization of the product of *trans*-$[PtBr_2(en)_2]^{2+}$.

3. Results and discussion

The reaction between peptide 1 and *trans*-$[PtBr_2(en)_2]^{2+}$ was investigated in H_2O, the reaction mixture was maintained for 1 min, and then analyzed by HPLC (Fig. 1a). A yield of 99% was obtained for the disulfide bridged peptide 1, while no methionine sulfoxide derivate was observed. It is known that *trans*-$[PtCl_2(en)_2]^{2+}$ is an excellent oxidant for the formation of disulfide bond in the methionine-containing peptide [16]. The reaction of *trans*-$[PtCl_2(en)_2]^{2+}$ with peptide 1 was also investigated in this work (Fig. 1b). The yield of disulfide bridged peptide 1 is 98%, and the methionine residue is intact. However, it needs about 4 h to convert the two thiol groups in cysteine residues to disulfide bond.

In order to demonstrate the efficiency of *trans*-$[PtBr_2(en)_2]^{2+}$ for disulfide bonds formation, other ten peptides with variable lengths were selected and reacted with *trans*-$[PtBr_2(en)_2]^{2+}$. The results are summarized in Table 1. As can be seen, all the reactions are very fast, and excellent yields were achieved for these peptides, illustrating that this Pt(IV) complex is a very efficient oxidant for synthesizing peptide disulfide bond. HPLC chromatograms are displayed in the supplementary material. On the other hand, formation of disulfide bond in the antitumor drug somatostatin was also investigated in aqueous mediums with different hydrogen ion concentration; the results are summarized in Table 2. These reactions with good yields are very fast. So, the Pt(IV) complex tolerates a range of hydrogen ion concentration upon the formation of peptide disulfide bonds.

The reaction stoichiometry was determined by reaction of iRGD peptide with the Pt(IV) complex in H_2O. All the reactions were aged for 1 min, and then were analyzed by HPLC. Plot of the peak areas of disulfide bridged iRGD *versus* [iRGD] is displayed in Fig. 2.

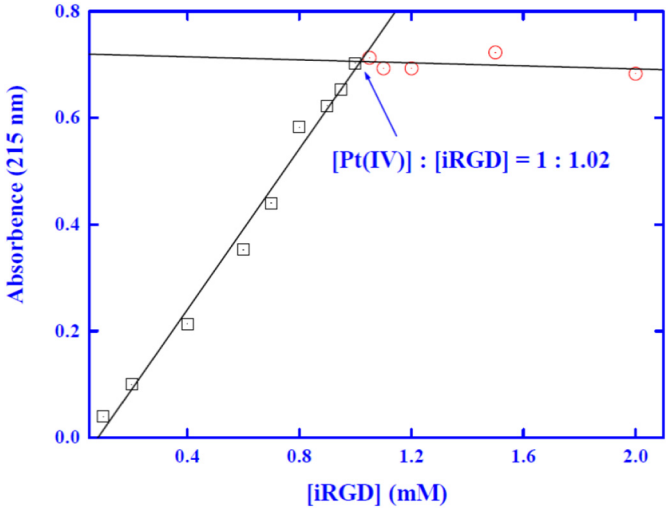


Fig. 2. HPLC peak areas of disulfide bridged iRGD at 215 nm for a series of reaction mixtures in which the concentrations of the iRGD were varied in the region 0.1 ≤ [iRGD] ≤ 2.0 mM and $[PtBr_2(en)_2^{2+}] = 1.0$ mM was kept constant. Reaction medium: H_2O; room temperature.

A stoichiometry was estimated as $[PtBr_2(en)_2^{2+}]$: [iRGD] = 1: 1.02. The reduction product of *trans*-$[PtBr_2(en)_2]^{2+}$ was studied by NMR (Fig. S1). As shown, *trans*-$[PtBr_2(en)_2]^{2+}$ was reduced to $[Pt(en)_2]^{2+}$

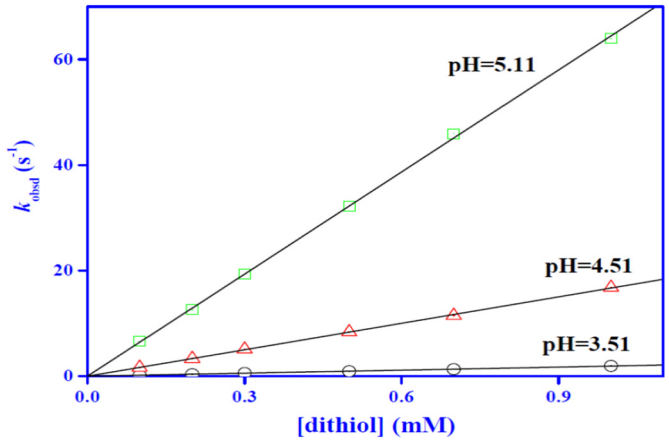


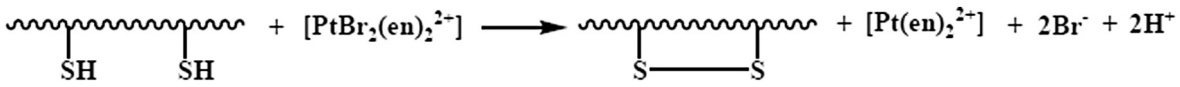
Fig. 3. Plots of k_{obsd} *versus* [DODT] at 25.0 °C.

Table 2
Formation of disulfide bridged somatostatin by *trans*-$[PtBr_2(en)_2]^{2+}$ oxidation in various solvents at room temperature.[a]

No.	Peptide	Solvent	$[H^+]$/(mol/L)	yield [a] (%)	Reaction time (min)
1	somatostatin	HAc/NaAc	$10^{-3.65}$	97	<1
2		TFA	$10^{-4.50}$	95	<1
3		TFA	$10^{-6.27}$	96	<1
4		TFA	0.01	96	<1
5		TFA	0.05	95	<1

[a] Reaction conditions: $[PtBr_2(en)_2^{2+}] = 1.0$ mM, [dicysteine-containing somatostatin] = 0.5–1.0 mM.

with loss of two bromide ligands. Therefore, the reaction between *trans*-$[PtBr_2(en)_2]^{2+}$ and dicysteine-containing peptide can be described by Eq. (1):



The reaction between *trans*-$[PtBr_2(en)_2]^{2+}$ and dicysteine-containing peptide is very fast as shown in Table 1. So, we want to study the reaction kinetics using stopped-flow spectrometer. Unfortunately, all the peptides used in this work have a predominant absorbance at 235 nm, which is the maximum absorption wavelength of *trans*-$[PtBr_2(en)_2]^{2+}$. In our previous work, 3,6-dioxa-1,8-octanedithiol (DODT) was oxidized to an intramolecule disulfide loop by Pt(IV) complex, and the size of the loop is close to those of the oxidized active site Cys-Xaa-Yaa-Cys of thioredoxins. Moreover, the p*K*a values ($pK_{a1} = 8.7$ and $pK_{a2} = 9.6$) for the DODT are about the same to the active site peptide [23]. Most importantly, DODT has no absorption at 235 nm. Therefore, DODT was chosen as a model compound for the dicysteine-containing peptide used in this work.

The reaction between *trans*-$[PtBr_2(en)_2]^{2+}$ and DODT was followed at 235 nm [6] for recording the kinetic trace. As shown in Fig. S2, the decrease in absorbance was simulated by Eq. (2), where A_t, A_0 and A_∞ stand for absorbance at time *t*, zero and infinity, respectively.

$$A_t = (A_0 - A_\infty)\exp.(-k_{obsd}t) + A_\infty \quad (2)$$

The good simulation demonstrated that the reaction is first order in [Pt(IV)]. Pseudo first-order rate constant k_{obsd} was thus derived from the simulation; the average value of k_{obsd} was from five to seven duplicate runs; standard deviation was usually less than 5%. The influences of varying [DODT] on the reaction rates were investigated in various buffers. Values of k_{obsd} are summarized in Tables S1. Plots of k_{obsd} *versus* [DODT] are straight lines with no significant intercepts (Fig. 3), indicating that the reaction is also first-order in [DODT].

An overall second-order rate law is established as Eq. (3), where *k*' denotes the observed second-order rate constant. Values of *k*' were calculated from the plots of k_{obsd} *versus* [DODT], and are listed in Table 3. The values of *k*' listed in Table 3 increase several orders of magnitude when the reaction medium is changed from acidic solution to slightly acidic ones. The large increase of *k*' reflects that the deprotonated forms thiolates are much more reactive than the protonated thiols.

$$-d[Pt(IV)]/dt = k\prime[Pt(IV)][DODT] \quad (3)$$

The oxidation product of DODT was characterized by mass spectrum (Fig. S3); the value of *m*/*z* was determined as 181.03506 corresponding to the disulfide form of DODT. According to the kinetic results, a mechanism including different protolytic species of DODT reduction of *trans*-$[PtBr_2(en)_2]^{2+}$ was proposed, and is displayed in Scheme 1.

Table 3
Values of *k*' at 25.0 °C and 1.0 M ionic strength.

$[H^+]$/M	pH	$k'/M^{-1}\ s^{-1}$
0.2		1.27 ± 0.03
0.1		4.03 ± 0.11
0.05		7.93 ± 0.14
0.03		14.1 ± 0.3
0.02		20.9 ± 0.4
0.01		46.8 ± 1.0
0.005		94.5 ± 2.7
	3.18	743 ± 12
	3.51	(1.95 ± 0.04) × 10^3
	3.85	(4.24 ± 0.03) × 10^3
	4.12	(7.46 ± 0.05) × 10^3
	4.51	(1.67 ± 0.02) × 10^4
	4.74	(2.95 ± 0.02) × 10^4
	5.11	(6.44 ± 0.06) × 10^4
	5.55	(1.93 ± 0.02) × 10^5

A rate law as Eq. (4) was deduced according to the proposed mechanism, where a_H stands for the proton activity which corresponds exactly to the pH measurements or is calculated by equation: pH = − log[H^+] + 0.20 for acidic solution [24]. Thus, the second order rate constant *k*' is depended on a_H and the protolytic constants of DODT. The Eq. (5) was used to fit the *k*' - pH data listed in Table 3.

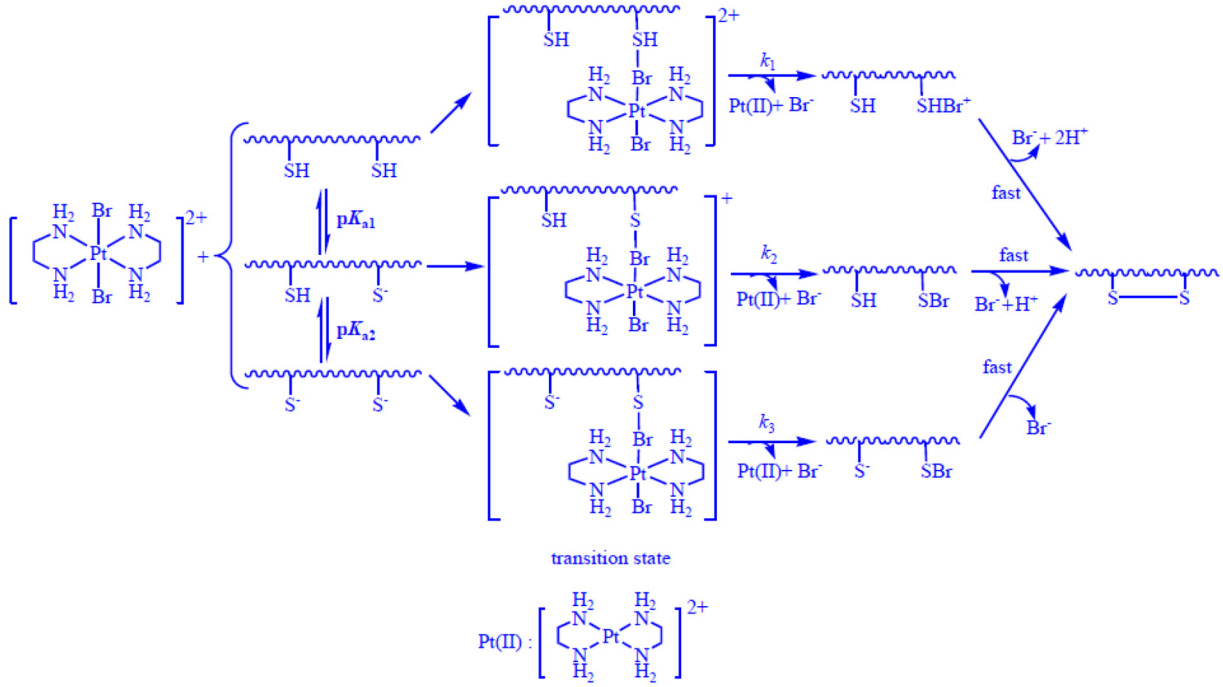
$$-d[Pt(IV)]/dt = \frac{k_1 a_H^2 + k_2 K_{a1} a_H + k_3 K_{a1} K_{a2}}{a_H^2 + K_{a1} a_H + K_{a1} K_{a2}} \quad (4)$$

$$k' = \frac{k_1 a_H^2 + k_2 K_{a1} a_H + k_3 K_{a1} K_{a2}}{a_H^2 + K_{a1} a_H + K_{a1} K_{a2}} \quad (5)$$

A good fit was achieved as displayed in Fig. 4; the rate constants of rate determining steps listed in Table 4 were calculated from the fit. Unfortunately, k_1 was undetermined. By comparison, the rate of oxidation of DODT by *trans*-$[PtBr_2(en)_2]^{2+}$ is faster than that of oxidation of DODT by *trans*-$[PtCl_2(CN)_4]^{2-}$, while the redox potential of *trans*-$[PtBr_2(en)_2]^{2+}$ ($E^{o\prime} = 0.54$ V) [25] is lower than that of *trans*-$[PtCl_2(CN)_4]^{2-}$ ($E^{o\prime} = 0.926$ V) [26]. *trans*-$[PtCl_2(CN)_4]^{2-}$ possesses higher redox potential making it can oxidize the methionine residue in peptide 1 to methionine sulfoxide derivate [27], while *trans*-$[PtBr_2(en)_2]^{2+}$ unable to oxidize the methionine residue.

The kinetic results demonstrated that thiolate (deprotonated form of thiol) are much more reactive than the protonated thiol. As a result, the reaction rate for the formation of peptide disulfide bond *via* Pt(IV) oxidation is increased with the increase of pH of the reaction medium. Therefore, the protolytic constants of the two thiol groups in peptide play a critical role in the reaction rate. On the other hand, kinetic studies demonstrated that the Pt(IV) complex *trans*-$[PtBr_2(en)_2]^{2+}$ can be used in acidic medium for the purpose of synthesis of disulfide bond.

By comparison, the reaction of *trans*-$[PtCl_2(en)_2]^{2+}$ with dicysteine-containing somatostatin was also studied in 10.0 mM HCl solution. It needs about 15 h to complete the reaction, while only 1 min is need for *trans*-$[PtBr_2(en)_2]^{2+}$ oxidation (See Table 2). Therefore, the rate for formation of peptide disulfide by *trans*-$[PtBr_2(en)_2]^{2+}$ oxidation is dramatically faster than that of formation of peptide disulfide by *trans*-$[PtCl_2(en)_2]^{2+}$ oxidation in acidic solution. However, the redox potential ($E^{o\prime} = 0.54$ V [25]) of $[PtBr_2(en)_2^{2+}]/[Pt(en)_2^{2+}]$ is almost equal to that of $[PtCl_2(en)_2^{2+}]/[Pt(en)_2^{2+}]$ ($E^{o\prime} = 0.58$ V [25]). Therefore, the redox potential of Pt(IV) complex has no influence on the rate of disulfide bond formation. This was also observed in the reduction of Pt(IV) complexes with axially coordinated-chloride/bromide ligands by cysteine or selenomethionine in our previous works [28,29]. In these reactions, a parallel reaction mechanism was proposed, and a chloride/bromide bridge mediated inner sphere electron transfer was occurred in these reactions. Generally, redox potential plays a dominant role in the outer sphere electron transfer process and not in the inner sphere electron transfer. Thus, the formation of peptide disulfide bond by *trans*-$[PtBr_2(en)_2]^{2+}$ oxidation was also occurred through the inner sphere electron transfer (As shown in Scheme 1). Moreover, this observation strongly emphasizes that the axially coordinated-bromide is a better bridging ligand.



Scheme 1. The proposed mechanism for the formation of disulfide bond *via trans*-$[PtBr_2(en)_2]^{2+}$ oxidation.

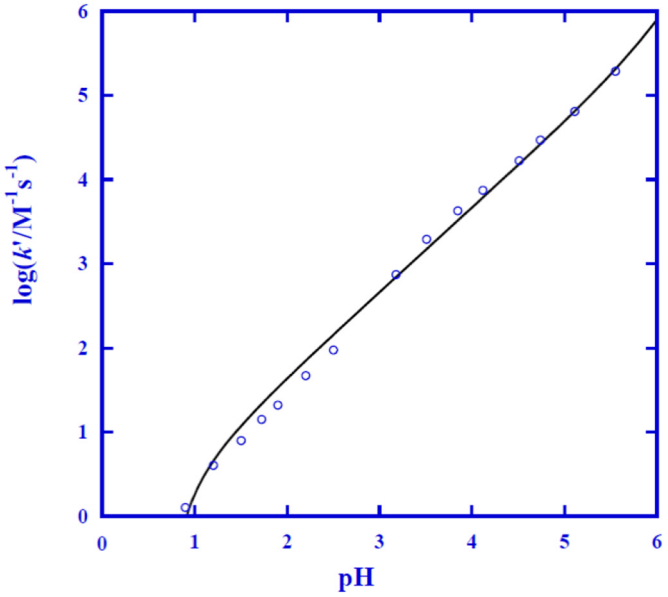


Fig. 4. Observed second-order rate constants, *k'*, as a function of pH at 25.0 °C (data points). The solid curve represents the best fit of Eq. (5) to the experimental data by a weighed nonlinear least-squares routine.

Table 4
Values of the rate-determining steps derived from curve-fitting at 25.0 °C and 1.0 M ionic strength.

Pt(IV) Complex	k_m	Value/M^{-1} s^{-1}	Ref
trans-$[PtCl_2(CN)_4]^{2-}$	k_1	38 ± 2	[23]
	k_2	(2.32 ± 0.01) × 10^7	
	k_3	_ a	
trans-$[PtBr_2(en)_2]^{2+}$	k_1	_ b	this work
	k_2	(5.37 ± 0.02) × 10^8	
	k_3	(6.71 ± 0.34) × 10^{11}	

[a,b]Could not be derived from the kinetic data collected.

4. Conclusion

Pt(IV) complex *trans*-$[PtBr_2(en)_2]^{2+}$ was synthesized and was successfully used to prepare disulfide bonds in peptides. Excellent yields with fast reaction rates were achieved in acidic or neutral reaction medium. Methionine residue is compatible to the Pt(IV) complex oxidation method. Therefore, *trans*-$[PtBr_2(en)_2]^{2+}$ is a very efficient oxidant for the purpose of formation of disulfide bonds. The kinetics and mechanism for the reaction between *trans*-$[PtBr_2(en)_2]^{2+}$ and the model compound DODT was investigated in detail. The results demonstrated that the rate of formation disulfide is very fast, and is depended on both pH of reaction medium and p*K*a of thiol group in peptide. Moreover, the rate of formation of peptide disulfide is influenced by the axial ligands of Pt(IV) complex but not its redox potential.

Declaration of Competing Interest

Authors report no conflict of interest in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2020.115195.

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