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Reduction of ormaplatin and *cis*-diamminetetrachloroplatinum(IV) by ascorbic acid and dominant thiols in human plasma: kinetic and mechanistic analyses

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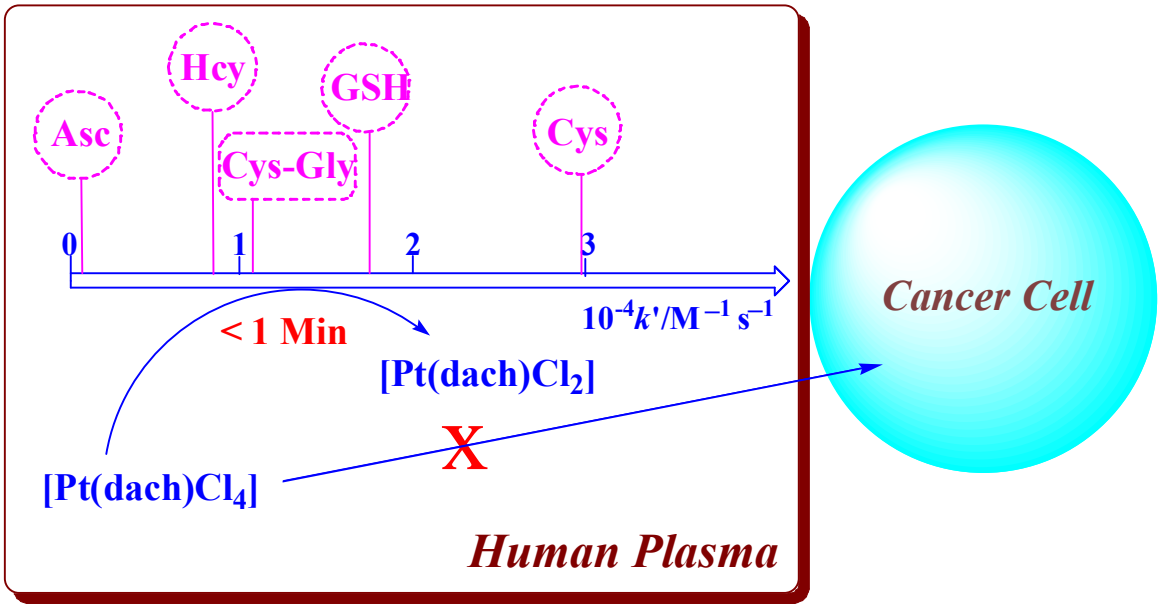
Graphical Abstract

Reductions of Pt(IV) anticancer prodrugs [Pt(dach)Cl_4] (ormaplatin/tetraplatin) and *cis*-[$Pt(NH_3)_2Cl_4$] by several dominant reductants in human plasma follow overall second-order kinetics and the observed second-order rate constants k' at 37.0 °C, 1.0 M ionic strength and pH 7.40 render a similar trend for both prodrugs.



Abstract

Reductions of Pt(IV) anticancer prodrugs [Pt(dach)Cl_4] (ormaplatin/tetraplatin), *cis*-[Pt$(NH_3)_2Cl_4$], and *cis,cis,trans*-[Pt$(NH_3)_2Cl_2Br_2$] by the several dominant reductants in human plasma have been characterized kinetically in this work, including L-ascorbic acid (Asc), L-glutathione (GSH), L-cysteine (Cys), DL-homocysteine (Hcy), and a dipeptide Gly-Cys. All the reductions follow an overall second-order kinetics, being first-order each in [Pt(IV)] and in [reductant]. A general reactivity trend of Asc < Hcy < Cys-Gly < GSH < Cys is clearly revealed for the reductions of [Pt(dach)Cl_4] and [Pt$(NH_3)_2Cl_4$] at 37.0 ^oC and pH 7.40. Analysis of the observed second-order rate constants *k*' implies that these Pt(IV) prodrugs have a very short life time (less than a minute) in human plasma and can hardly enter into cells before reduction and that Asc might not play a dominant role in the reduction process among the reductants. The reductions of [Pt(dach)Cl_4] and [Pt$(NH_3)_2Cl_4$] by Asc have been studied in a wide pH range, and a reaction mechanism has been proposed involving parallel reductions of the Pt(IV) complexes by the Asc protolytic species. Moreover, a halide-bridged (inner-sphere) electron transfer mode for the rate-determining steps is discussed in detail; several lines of evidence strongly bolster this type of electron transfer. Furthermore, the observed activation parameters corresponding to *k*' have been measured around pH 7.40. Analysis of the established *k*' - pH profiles indicates that *k*' is a composite of at least three parameters in the pH range of 5.74 – 7.40 and the measured activation parameters in this range do not correspond to a single rate-determining step. Consequently, the isokinetic relationship reported previously using the measured $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ in the above pH range might be an artifact since the relationship is not justified anymore when our new data are added.

Introduction

In the development of a new generation of platinum-based anticancer drugs, the design, synthesis, screening and physicochemical study of Pt(IV) anticancer active compounds represent an important and big part and have been vigorously pursued.[1-4] In comparison with the Pt(II) drugs such as cisplatin, carboplatin and oxaliplatin, the Pt(IV) compounds have an increased coordination, possessing more structural diversity[4-6] and therefore having more chance to finely tune the pharmacological profile.[5] Pt(IV) compounds are generally substitution inert; this property, together with their more structural diversity, provides more potential possibilities including adjustable lipophilicity, oral administration, reduced side effects and targeted therapy.[5,6] Indeed, several Pt(IV) compounds have entered into clinical trials involving three structural prototypes ormaplatin (tetraplatin, [Pt(dach)Cl_4]), iproplatin, and satraplatin (JM216).[1,7-9] Moreover, these structural prototypes have been followed for a long time.

In contrast to their substitution inert property, Pt(IV) anticancer active compounds are readily reduced to Pt(II) complexes via a reductive elimination process;[2-4] as a consequence, they are generally regarded as prodrugs.[2-4] In fact, this prodrug nature has been explored extensively in the design of drug delivery systems.[10,11] Biologically important reductants, in particular the small molecules with a high abundance in human plasma and cells such as L-ascorbic acid and L-glutathionine (GSH)[12] are commonly believed to be primarily responsible for the reduction of the Pt(IV) prodrugs.[13-35] However, recent work suggests that some less abundant reductants could also play a role in the reduction process, at least under some circumstances.[36-40]

Kinetically, the reduction of Pt(IV) anticancer-model compounds and -prodrugs by Asc (Asc represents ether ascorbic acid or ascorbate or the sum of them) has been studied extensively.[13-28] However, most of these studies were carried out at a single pH or in a very narrow pH region.[13-17,20-28] Only two studies covered a certain pH range,[18,19] enabling to establish the rate expression in which the protolytic equilibria of Asc are involved. Moreover, some discrepancies clearly exist in these studies as shown by the fact that the rate laws, the proposed reaction mechanisms, and the modes of electron transfer differentiate.[13-15,18-21,24] Comparatively, the reduction of Pt(IV) compounds by GSH and other small thiols has been exploited less extensively.[24,29-35]

In this work, we report a kinetic investigation of the reduction of [Pt(dach)Cl_4], *cis*-[$Pt(NH_3)_2Cl_4$], and *cis,cis,trans*-[$Pt(NH_3)_2Cl_2Br_2$] by Asc, and the "observed activation parameters" (*vide infra*) at the physiological pH (7.40); *cis*-[$Pt(NH_3)_2Cl_4$], and *cis,cis,trans*-[$Pt(NH_3)_2Cl_2Br_2$] are the cisplatin prodrugs.[41,42] The structures of these Pt(IV) prodrugs and ascorbic acid are illustrated in Scheme 1. In addition, we studied the reduction of [Pt(dach)Cl_4] and *cis*-[$Pt(NH_3)_2Cl_4$] by Asc in a much wider pH range. We also report the rate constants for the reductions of [Pt(dach)Cl_4] and [$Pt(NH_3)_2Cl_4$] by the four high abundant thiols in human plasma at pH 7.40: GSH, a dipeptide Cys-Gly, L-cysteine (Cys) and DL-homocysteine (Hcy).[12] The aims of the present work were: (1) to establish the rate law in a wide pH range with Asc as reductant; (2) to get a mechanistic insight into the reduction processes of these prodrugs by Asc; (3) to examine the activation parameters collected so far for the Pt(IV)-Asc reactions; (4) to re-examine the isokinetic relationship reported earlier;[18] and (5) to compare the rate constants for the reductions of [Pt(dach)Cl_4] and [$Pt(NH_3)_2Cl_4$] by the four thiols and Asc.

Materials and Methods

Materials

Monosodium L-ascorbate, L-ascorbic acid, GSH (γ-Glu-Cys-Gly), Cys, Hcy, and *cis*-diamminetetrachloroplatinum(IV) (*cis*-[$Pt(NH_3)_2Cl_4$]), and *cis*-diamminedichloro-platinum(II) (cisplatin) in their purest forms available were purchased from Sigma-Aldrich (St. Louis, MO). The dipeptide Cys-Gly with a purity > 95% was obtained from Dgpeptides Co., Ltd (Hongzhou, China). Acetic acid, sodium acetate, phosphoric acid, sodium dihydrogenphosphate, disodium hydrogen phosphate, sodium bicarbonate, sodium carbonate, sodium perchlorate, perchloric acid, and sodium chloride, all in analytical grade, were acquired either from Alfa Aesar (in Tianjin, China) or from Fisher Scientific, and were used without further purification. Standard buffers of pH 4.00, 7.00, and 10.00 for the pH electrode calibrations were obtained also from Fisher Scientific. Doubly distilled water was utilized to prepare all the solutions.

[$Pt(dach)Cl_4$] used in this work was from the same batch which was synthesized and characterized recently.[39] *Cis,cis,trans*-[$Pt(NH_3)_2Cl_2Br_2$] was synthesized by oxidation of cisplatin with *N*-bromosuccinimide according to the reported method but with one more recrystallization,[42] yielding [$Pt(NH_3)_2Cl_2Br_2$]•½DMF. Elemental analysis: C, 3.63%; H, 1.92%; N, 7.05%. Found: C, 3.25%; H, 1.96%; N, 6.91%. ^{1}H NMR (600 MHz, DMSO-d_6): δ 5.79 (m, 6H, $^2J_{Pt\text{-}H}$ = 52.4 Hz, $^1J_{N\text{-}H}$ = 52.6 Hz). In addition, δ 2.73 (s, 3H), δ 2.89 (s, 3H), and δ 7.96 (s, 1H) from the associated DMF were also confirmed.[43]

Buffer solutions

Combinations of H_3PO_4/NaH_2PO_4, acetic acid/sodium acetate, NaH_2PO_4/Na_2HPO_4, and $NaHCO_3/Na_2CO_3$ (with concentrations of 0.15 - 0.25 M) were prepared to cover a region of $3.21 \leq pH \leq 8.47$. All the buffers, which encompassed 2 mM EDTA and 0.10 M NaCl, were adjusted to 1.00 M ionic strength (μ) with sodium perchlorate. The role of EDTA was to eliminate any possible catalytic effects of trace of metal ions such as Cu(II) during the autooxidation processes of ascorbate and thiol compounds,[19,44] while the addition of NaCl was to repress the hydrolysis of the tetrachloro-Pt(IV) complexes. The pH values of buffer solutions were measured with an Accumet Basic AB15 Plus pH meter equipped with an Accumet combination pH electrode (Fisher Scientific, Pittsburgh, PA); the electrode was calibrated with the standard buffers of pH 4.00, 7.00 and 10.00 just before the pH measurements. A separate phosphate buffer of pH 7.40 which contained 2 mM EDTA but no sodium chloride or sodium bromide was prepared to study the reduction of $[Pt(NH_3)_2Cl_2Br_2]$ by Asc (*vide infra*).

Kinetic measurements

Stock solutions of 1.0 mM $[Pt(dach)Cl_4]/[Pt(NH_3)_2Cl_4]$ were prepared by dissolving an appropriate amount of $[Pt(dach)Cl_4]/[Pt(NH_3)_2Cl_4]$ in a solution containing 0.90 M $NaClO_4$, 0.09 M NaCl and 0.01 M HCl; these solutions were only used for a few hours. Solutions of the tetrachloro-Pt(IV) complexes and of Asc for kinetic measurements were prepared, respectively, by adding an appropriate amount of the Pt(IV) stock solution and of Asc to a specific buffer. Ascorbic acid was used in the pH range of 3.21 - 4.44 while monosodium ascorbate was employed in the pH range of 5.10 – 8.47. Those solutions were flushed for 10 min with nitrogen before loading onto an Applied Photophysics SX-

20 stopped-flow spectrometer (Applied Photophysics Ltd., Leatherhead, U.K.) and were only used for a couple of hours. Reactions were initiated by mixing equal volumes of Pt(IV) and Asc solutions directly in the stopped-flow machine; the reactions were followed at 320 nm under pseudo first-order conditions with Asc being at least in 10-fold excess. For reduction of the tetrachloro-Pt(IV) complexes by the four thiol compounds, kinetic measurements were similarly performed but only in a pH 7.40 buffer; a wavelength of 280 nm was chosen to follow the kinetic traces.

The reduction of $[Pt(NH_3)_2Cl_2Br_2]$ by Asc was only investigated in the pH 7.40 buffer (*vide supra*) but at 5 temperatures and was monitored at 330 nm. For the reaction, care was taken to minimize some possible side reactions of the Pt(IV) complex induced by light and hydrolysis: (a) the measurements were conducted essentially in dark; (b) the solutions of the oxidant were prepared by directly adding $[Pt(NH_3)_2Cl_2Br_2]$ in the pH 7.40 buffer, flushed by nitrogen gas for 5 min, and were only used up to 30 min.

Stoichiometric assessment

The stoichiometry was investigated for the reaction between $[Pt(NH_3)_2Cl_4]$ and Asc in a pH 7.40 buffer. In one group of experiments, a series of solution mixtures were prepared in which [Pt(IV)] = 0.10 mM was maintained constant while [Asc] was varied from 0 to 0.30 mM, where [Asc] stands for the total concentration of Asc. Absorbance at 265 nm was measured by use of a TU-1900 spectrophotometer (Beijing Puxi, Inc., Beijing, China) and 1.00 cm quartz cells after a reaction time of 4 - 5 min at room temperature for each of the reaction mixtures. In another group of experiments, a series of reaction

mixtures were prepared which contained a constant [Asc] = 0.10 mM and a changing [Pt(IV)] from 0 to 0.30 mM; the absorbances were measured in the same way as above.

Results

Kinetic data and rate law

Reduction of $[Pt(dach)Cl_4]$/$[Pt(NH_3)_2Cl_4]$ by Asc was followed by the stopped-flow machine at 320 nm where the absorbance decreased for the kinetic traces due to the reduction of the Pt(IV) complexes. Pseudo first-order reaction conditions were maintained by use of [Asc] ≥ 10·[Pt(IV)] while a constant pH was controlled by a given buffer. All the kinetic traces could be simulated very well by single exponentials under the above reaction conditions, confirming the first-order in [Pt(IV)]. Pseudo first-order rate constants k_{obsd} were thus derived from the single exponential simulations. Values of k_{obsd} obtained were the averages from 5-7 parallel runs; standard deviations were normally less than 5%.

The effect of varying [Asc] on the reduction rate was investigated in the region 1.00 mM ≤ [Asc] ≤ 10.0 mM in buffer solutions. The use of monosodium ascorbate in the pH region of 5.10 – 8.47 could ascertain that the variation of [Asc] in a buffer solution did not cause any pH changes which were confirmed by the pH measurements. Plots of k_{obsd} versus [Asc] are shown in Figures 1 and 2 for reduction of $[Pt(dach)Cl_4]$ and $[Pt(NH_3)_2Cl_4]$, respectively. These plots clearly pass through the origin without any significant intercepts. Therefore, the redox reactions are first-order in [Asc] and an overall second-order rate law is established:

$$-d[Pt(IV)]/dt = k_{obsd}[Pt(IV)] = k'[Asc][Pt(IV)] \quad (1)$$

and

$$k_{obsd} = k'[Asc] \quad (2)$$

In Equations (1) and (2), k' pertains to the observed second-order rate constants. Values of k' as a function of pH were calculated according to Equation (2) and are summarized in Table 1. The kinetic measurements were carried out in buffers up to pH 8.47 since Asc in buffers of higher pH was not stable, precluding an accurate collection of kinetic data. As a matter of fact, the pH range covered in Table 1 is the widest pH alteration for the Pt(IV) – Asc reaction systems studied so far.[13-28]

"Observed activation parameters"

The reduction of $[Pt(dach)Cl_4]$ and $[Pt(NH_3)_2Cl_4]$ by Asc was studied in a phosphate buffer of pH 7.40 in the temperature range of 15.0 – 37.0 ^oC (cf. Figures 1 and 2). Certainly, the second-order kinetics is strictly held in the temperature range studied; values of k' as a function of temperature are listed in Table 2 and subsequently, the Eyring plots are given in Figure S1 in Supporting Information. Correspondingly, the observed activation enthalpies and entropies were evaluated and are provided in Table 2. The reduction of $[Pt(dach)Cl_4]$ by Asc was also studied in a phosphate buffer at pH 7.12 (*vide infra*) and the results are given in Figure S2 in Supporting Information. Second-order rate constants and the observed activation parameters at pH 7.12 are summarized in Table 2 as well.

Kinetic traces for the reduction of $[Pt(NH_3)_2Cl_2Br_2]$ by Asc in the pH 7.40 buffer were also well simulated by single exponentials; plots of k_{obsd} versus [Asc] are displayed in Figure 3A, conferring the overall second-order kinetics. Values of k' calculated from Figure 3A are listed in Table 2. The Eyring plot is shown in Figure 3B and the observed activation parameters are also given in Table 2.

Stoichiometry

The reaction stoichiometry between $[Pt(NH_3)_2Cl_4]$ and Asc was studied by two approaches at pH 7.40 by use of spectrophotometric titrations. In the first approach, [Pt(IV)] = 0.10 mM was kept constant while [Asc] was varied from 0 to 0.30 mM. Absorbance at 265 nm as a function of [Asc] is shown in Figure S3A in Supporting Information. The intersection point, obtained by extrapolations of the two crossing lines, gives rise to $\Delta[Pt(NH_3)_2Cl_4] : \Delta[Asc] = 0.10 : 0.086 \pm 0.010 = 1 : 0.86 \pm 0.10$. In the second approach, [Asc] = 0.10 mM was maintained constant whereas [Pt(IV)] was changed from 0 to 0.30 mM (Figure S3B in Supporting Information). The intersection point acquired similarly in Figure 3B yields $\Delta[Asc] : \Delta[Pt(NH_3)_2Cl_4] = 0.10 : 0.115 \pm 0.10 = 1 : 1.15 \pm 0.10$. The above two ratios are indicative of a 1:1 stoichiometry within the experimental errors, implying that Asc is oxidized to L-dehydroascorbic acid (DHA, its structure is given in Scheme 1) as described by Equation (3):

$$cis\text{-}[Pt(NH_3)_2Cl_4] + H_2Asc \longrightarrow cis\text{-}[Pt(NH_3)_2Cl_2] + DHA + 2HCl \qquad (3)$$

DHA as the oxidation product of Asc is very common when metal complexes are used as oxidants.[45-49] A reaction stoichiometry similar to Equation (3) is thus assumed for the reductions of [Pt(dach)Cl_4] and [Pt(NH_3)$_2$$Cl_2$$Br_2$] by Asc.

Reduction of Pt(IV) prodrugs by thiol-containing compounds

Reductions of [Pt(dach)Cl_4] and [Pt(NH_3)$_2$$Cl_4$] by the four thiol-containing compounds were studied at pH 7.40 and at two important temperatures 25.0 ^oC and 37.0 ^oC, and were also followed under pseudo first-order conditions with [thiol] $\geq$ 10▪[Pt(IV)]. The reactions were monitored at 280 nm where the thiols have a negligible absorbance at this pH. Plots of k_{obsd} versus [thiol] are displayed in Figures 4 and 5 for reactions of [Pt(dach)Cl_4] and [Pt(NH_3)$_2$$Cl_4$], respectively. Undoubtedly, the linear plots do not exhibit any significant intercepts, designating overall second-order kinetics. The observed second-order rate constants k' were evaluated from these plots, and are summarized in Table 3.

Discussion

Mechanism for the Pt(IV) - Asc redox reactions

In the prepared buffers containing 2 mM EDTA, the second-order rate law is strictly held for reduction of the 3 Pt(IV) prodrugs by Asc. The second-order rate constant k' increases about 5 orders of magnitude when the reaction media are changed from pH 3.21 to 8.47 for both [Pt(dach)Cl_4] and [Pt(NH_3)$_2$$Cl_4$] (Table 1). Since the Pt(IV) complexes do not involve protolytic equilibria, these drastic changes are ascribed certainly to the deprotonations of ascorbic acid. If all the protolytic species of Asc (denoted by H_2Asc,

$HAsc^-$, and Asc^{2-}) are assumed to reduce the Pt(IV) complexes in parallel, Scheme 2 is a modified version of the reaction mechanism proposed earlier by Lemma et al.[18] In the scheme, reactions expressed by $k_1 - k_3$ are all the rate-determining steps. Equation (4) is the rate expression deduced from Scheme 2, where a_H stands for the proton activity which corresponds to the pH measurements.

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

When Equation (4) is compared with Equation 1, Equation (5) is obtained:

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

The protolytic constants of L-ascorbic acid were reported to be $pK_{a1} = 3.96$[50] and $pK_{a2} = 11.24$[51] at 25.0 °C and μ = 1.0 M. Equation (5) was employed to simulate the k' – pH dependence data in Table 1 by use of a weighted non-linear least squares method with the pK_a values as direct inputs and with $k_1 - k_3$ as tunable parameters. The simulations indicated that the k_1 values were indeterminate for both $[Pt(dach)Cl_4]$ and $[Pt(NH_3)_2Cl_4]$. Thus, the contribution of the k_1 term in Equation (5) is so small that it can be neglected, leading to Equation (6):

$$k' = \frac{k_2 K_{a1} a_H + k_3 K_{a1} K_{a2}}{a_H^{\,2} + K_{a1} a_H + K_{a1} K_{a2}} \quad (6)$$

Equation (6) was then utilized to simulate the data again, affording good fittings shown in Figure 6; the values of k_2 and k_3 resulted from the simulations are summarized in Table 4.

Inner-sphere (halide bridged) electron transfer

Reductive eliminations of *trans*-dihalido-Pt(IV) complexes have been interpreted to take place via a bridge formation between one of the *trans*-coordinated halides and the attacking atom of the reductants.[30-33,38-40,52,53] In the present reaction systems, the proposed bridge formation in the transition states is illustrated in Figure S4 in Supporting Information. Concurrent to the bridge formation is the partially breaking of the X-Pt^{IV}-X bonds; by the end, the X-Pt^{IV}-X bonds will be totally broken, resulting in an X^+ transfer to the reductants. This type of electron transfer implies that a better bridging atom will favor the bridge formation with a lower energy barrier, generating a faster reduction rate.[54] $[Pt(NH_3)_2Cl_4]$ has a value of E_p = -0.26 V (E_p stands for a cathodic forward half-wave potential, and a greater E_p value reflects a greater ease of reduction),[41] while $[Pt(NH_3)_2Cl_2Br_2]$ is characterized by a much lower E_p value (E_p = -0.663 V)[42] and is thus expected to be reduced substantially less easy. In contrary, it is observed in the present work that $k'([Pt(NH_3)_2Cl_2Br_2])/k'([Pt(NH_3)_2Cl_4])$ = 52 at pH 7.40 (Table 2). This observation strongly endorses the bridged electron transfer mode since the coordinated-bromide possesses a better bridging effect than the –chloride.[52,54] Moreover, it essentially excludes the possibility of outer sphere electron transfer since in this case the E_p values would play a dominant role (i.e. the reduction of *cis,cis,trans*-$[Pt(NH_3)_2Cl_2Br_2]$ by Asc would proceed with a significantly slower rate). Another clear line of evidence to bolster the bridged electron transfer comes from our recent study where the reduction of *trans*-

$[PtCl_2(CN)_4]^{2-}$ by L-selenomethionine is 3.7 x 10^3 times faster than by L-methionine due to the better bridging effect of selenium than sulfur.[40,55] Quantum mechanical simulations for the reduction of *cis*-$[Pt(NH_3)_2Cl_4]$ by methyl thiolate clearly showed the bride formation and breakage,[56] providing a strongly theoretical support.

The reduction of *cis*-$[Pt(NH_3)_2Cl_4]$ by Asc was studied in a pH 7.30 buffer before,[21] in addition to the second-order kinetic term of Equation (1), a third-order term (k_c[Pt(IV)][Asc][Pt(II)]) was found in the rate law, which suggested that Pt(II) can catalyze the reduction process. However, high Pt(II) concentrations were used (up to 6 mM) in their study to find the third-order term; such high concentrations of Pt(II) can hardly be found *in vivo* as pointed out by Gibson et al.[2] No doubt, the Pt(II) catalyzed reaction path can be neglected *in vivo*.

Analysis of rate constants

From the k' – pH profiles in Figure 6, it is obvious that k' increases almost exponentially with pH in the studied range. Extrapolation of the theoretical curves to higher pH predicts that k' becomes saturated until pH 12 (Figure S5 in Supporting Information). In order to derive the values of k_3 with reliable accuracy it is critical to collect the kinetic data in a pH range as wide as possible, as we pursued in the present work. From the obtained rate constants in Table 4, the ratios of k_3/k_2 are about 10^6 for both $[Pt(dach)Cl_4]$ and $[Pt(NH_3)_2Cl_4]$, revealing a huge reactivity difference between Asc^{2-} and $HAsc^-$.

The Asc species versus pH distribution diagram is shown in Figure 7A. As a comparison, the reactivity versus pH for these Asc species were also calculated and are displayed in Figure 7B for $[Pt(dach)Cl_4]$ and in Figure 7C for $[Pt(NH_3)_2Cl_4]$, respectively.

The distribution diagrams demonstrate that $HAsc^-$ is virtually the only existing form of Asc at pH 7.40 (99.95%), but it has only 1.3% and 2.2% reactivity contributions to the reductions of $[Pt(dach)Cl_4]$ and $[Pt(NH_3)_2Cl_4]$. On contrary, Asc^{2-} contributes about 98% to the total reactivity although it exists in a fraction of only about 0.014% at pH 7.40.

Analysis of the activation parameters

Second-order kinetics is usually accompanied by a negative activation entropy as endorsed by the transition state theory. The activation entropies measured for the Pt(IV)-Asc reactions earlier[18,19] and in the present work in the pH range of 5.74 -7.40 are indeed negative. However, the observed second-order rate constants k' in this region, as expressed by Equation (6), are a composite of k_2, k_3, pK_{a1}, and pK_{a2} which are all temperature-dependent parameters. Even at pH 7.40, k' is at least composed of k_3 and pK_{a2}. Undoubtedly, the activation parameters measured in this region do not correspond to those originating only from k_2 or k_3 and have no clear meaning, and we thus call them "observed activation parameters" as used above.

The reduction of $[Pt(dach)Cl_4]$ by Asc was studied by Choi et al. at a single pH (7.1 ± 0.1) by use of ^1H NMR spectroscopy; and $\Delta H^{\ddagger}$ = 99.4 ± 13.5 kJ·mol^{-1} and $\Delta S^{\ddagger}$ = 114 ± 32 J·K^{-1}·mol^{-1} were reported at this particular pH.[16] The large positive activation entropy seems abnormal and unreasonable for second-order kinetics. We thus re-measured the reaction in phosphate buffer of pH 7.12 (cf. Figure S2 in Supporting Information and Table 2) and we obtained the values of $\Delta H^{\ddagger}$ = 56.2 ± 1.9 kJ·mol^{-1} and $\Delta S^{\ddagger}$ = -9 ± 3 J·K^{-1}·mol^{-1}. Our values are reasonable and very different from those obtained by Choi et al., since they did not control pH by a buffer for the reaction.[16] For

the reduction of $[Pt(NH_3)_2Cl_4]$ by Asc, $\Delta H^{\ddagger}$ = 60 ± 2 kJ·mol^{-1} and $\Delta S^{\ddagger}$ = -14 ± 7 J·K^{-1}·mol^{-1} measured in a buffer of pH 5.74 were reported.[19] In the preset work, we measured the activation parameters for this reaction again but in a buffer of pH 7.40 (Table 2). Clearly, the two sets of activation parameters are significantly different. All these large discrepancies are easy to understand when the property of the observed activation parameters is considered.

Re-examination of the isokinetic relationship

Previously, Lemma et al. reported an isokinetic relationship by use of the observed activation parameters measured between pH 5.74 and 7.12 for the Pt(IV)-Asc redox systems (re-plotted in Figure 8A).[18,19] When our new data in Table 2 are added to this plot, Figure 8B is obtained. After the addition, the scattered data points in Figure 8B do not justify the isokinetic relationship anymore. In fact, the reported isokinetic relationship could be an artifact since the observed activation parameters used to make this relationship have no meaning as discussed above. *It follows that whether this kind of relationship does exist for the reduction of Pt(IV) prodrugs by a single reductant will need to be verified by a large set of clean activation parameters*. In fact, some clean activation parameters have been obtained for reduction of Pt(IV) compounds by other reductants such as L-methionine,[55] L-selenomethionine,[40] and thiosulfate.[57] However, when Asc and the thiol-containing compounds are used as reductants,[13-35] clean activation parameters are difficult to get because their k' – pH profiles are all similar to those shown in Figure 6.

Rate comparisons

Intravenous administration is a common approach for clinical use of platinum anticancer drugs.[1,58] Thus, the interaction of these drugs with human plasma takes place first after administration. In the present work, the reductions of $[Pt(dach)Cl_4]$ and $[Pt(NH_3)_2Cl_4]$ by several abundant small reductants involving ascorbic acid and the four thiol-containing compounds in human plasma[12] all followed second-order kinetics. Moreover, the observed rate constants at pH 7.40 and at two important temperatures were obtained (Table 3). A general reactivity trend of Asc < Hcy < Cys-Gly < GSH < Cys is clearly revealed. However, Hcy, Cys-Gly and GSH have a very close reactivity; consequently, the roles played by Hcy and Cys-Gly in the reduction process are anticipated to be small due to their less abundance than GSH.[12] Cys has a reactivity about 2 times higher than GSH, and certainly, its role in the reduction process should be taken into account.

Roughly, the above observed reactivity trend is inversely correlated to the pK_a values of the thiol groups and pK_{a2} of Asc, cf. the pK_a order: Asc (pK_{a2} =11.24) >> Hcy (pK_{SH} = 8.9)[59] > GSH (pK_{SH} = 8.64)[60] > Cys (pK_{SH} = 8.10)[59]. It does not ascertain if Cys-Gly (pK_{SH} = 7.9)[61] is an exceptional one since the reported pK_a value was measured under different conditions. The rough inverse correlation can be explained as follows: a thiol compound with a higher pK_a value possesses a lower existing fraction of the thiolate species at pH 7.40. The lower existing fraction will clearly generate a lower reactivity due to the predominant roles played by the thiolate species in the reduction of the Pt(IV) complexes at pH 7.40.[39,62] The reactivity of Asc apparently follows this order since it has the highest pK_a value and displays the lowest reactivity.

On the other hand, big ratios of k'(GSH)/k'(Asc) = 13 - 20 and k'(Cys)/k'(Asc) = 22 - 45 at pH 7.40 were found. This finding suggests that Asc may not be more important than GSH and Cys in the reduction of the Pt(IV) prodrugs in human plasma even if their abundances are considered (the total concentration of the small reduced thiols is 12 - 20 μM while the concentration of reduced Asc is 30-150 μM).[12] Calculations show that the longest half-life of ormaplatin is 17 seconds when Asc is assumed to be the only reductant and 19-32 seconds when only the small thiols are assumed to be the reductants. Thus, ormaplatin has life time of less than one minute in human plasma; and the life times for prodrugs *cis*-[$Pt(NH_3)_2Cl_4$] and *cis,cis,trans*-[$Pt(NH_3)_2Cl_2Br_2$] are even shorter. Clearly, with such a short life, these prodrugs can hardly enter into cells before reduction and the reduction takes place extracellularly. By analogy, the Pt(IV) anticancer active compounds developed recently bearing a similar structure to ormaplatin are also anticipated to be reduced extracellularly.[63-65]

Also recently, the oxidation of DNA related molecules by ormaplatin was carried out experimentally or theoretically,[66-68] highlighting the importance of ormaplatin and its structural type; the oxidation was found to proceed via several pathways different from those described in Scheme 2 but the redox rates were sluggish or much slower than those between ormaplatin and the reductants observed in this work. Therefore, the slow redox kinetics and mechanisms are very difficult to validate *in vivo,*[66-68] because the surviving time of ormaplatin is too short, according to the kinetics studied here.

Conclusion

Several conclusions can be drawn from the present study: (1) The k' – pH profiles have been established for the reductions of Pt(IV) anticancer prodrugs [Pt(dach)Cl_4] and *cis*-[$Pt(NH_3)_2Cl_4$] by Asc, enabling us to examine the reactivity - pH distribution of Asc protolytic species. The observed second-order rate constants k' are a composite of at least three parameters (k_2, k_3 and K_{a2}) in the pH range of 5.74 – 7.40. Thus, the activation parameters determined for k' in this pH range are only the observed ones, not corresponding to any specific rate constant of the rate-determining steps. As a consequence, the isokinetic relationship reported earlier might be an artifact due to the non-meaningful activation parameters used to make this relation. (2) Results obtained in the present work strongly support the bridged electron transfer (inner-sphere), virtually excluding the outer-sphere electron transfer mode. (3) At 37.0 ^{o}C and pH 7.40, the reactivity follows a trend of Asc < Hcy < Cys-Gly < GSH < Cys for both [Pt(dach)Cl_4] and *cis*-[$Pt(NH_3)_2Cl_4$]. A reactivity comparison indicates that Asc may not be a dominant reductant in the reduction processes of the Pt(IV) prodrugs in human plasma and the small thiols play an essentially equivalent role as Asc. (4) Ormaplatin is reduced quickly in human plasma, making its life time very short; its reduction certainly takes place extracellularly. Other Pt(IV) anticancer prodrugs bearing a similar configuration as ormaplatin are also anticipated to be reduced rapidly in human plasma. The kinetic and mechanistic analysis presented in this work not only has clarified several important issues and but also provides a deeper understanding of the reduction process for some Pt(IV) prodrugs.

Acknowledgements

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Table 1. Observed second-order rate constants k' for reduction of the Pt(IV) prodrugs by Asc as a function of pH at 25.0 ^{o}C and μ = 1.0 M.

Pt(IV) complex	pH	$k'/M^{-1}s^{-1}$
[Pt(dach)Cl_4]	3.21	0.14 ± 0.02
	3.59	0.26 ± 0.02
	4.03	0.58 ± 0.02
	4.44	1.41 ± 0.05
	5.10	4.7 ± 0.2
	5.57	18.0 ± 0.5
	6.10	55.6 ± 0.9
	6.70	150 ± 5
	7.12	290 ± 9
	7.40	555 ± 15
	7.72	(1.04 ± 0.04) x 10^3
	7.89	(1.23 ± 0.05) x 10^3
	8.47	(1.09 ± 0.04) x 10^4
cis-[Pt(NH_3)$_2$$Cl_4$]	3.21	0.32 ± 0.03
	3.59	0.51 ± 0.06
	4.03	1.16 ± 0.03
	4.44	2.41 ± 0.07
	5.10	8.2 ± 0.2
	5.64	29.3 ± 1.5
	6.26	84.3 ± 2.0
	6.70	206 ± 7
	7.40	726 ± 36
	7.72	(1.33 ± 0.06) x 10^3
	7.89	(1.84 ± 0.08) x 10^3
	8.47	(1.18 ± 0.05) x 10^4

Table 2. Observed second-order rate constants k' for reduction of Pt(IV) prodrugs by Asc and the "observed activation parameters" at pH 7.12 - 7.40 and μ = 1.0 M.

Pt(IV)	t/°C	k'/$M^{-1}s^{-1}$	$\Delta H^{\ddagger}$/kJ·mol^{-1}	$\Delta S^{\ddagger}$/J·K^{-1}·mol^{-1}
$[Pt(dach)Cl_4]$	15.0	279 ± 15	51.5 ± 2.5	-19 ± 5
(pH 7.40)	20.0	401 ± 20		
	25.0	602 ± 30		
	30.0	846 ± 43		
	37.0	(1.37 ± 0.07) x 10^3		
$[Pt(dach)Cl_4]$	15.0	131 ± 4	56.2 ± 1.9	-9 ± 3
(pH 7.12)	20.0	200 ± 5		
	25.0	293 ± 9		
	30.0	444 ± 12		
	35.0	643 ± 15		
$[Pt(NH_3)_2Cl_4]$	15.0	358 ± 18	46.7 ± 1.5	-34 ± 9
(pH 7.40)	20.0	520 ± 26		
	25.0	726 ± 36		
	30.0	910 ± 40		
	37.0	(1.61 ± 0.08) x 10^3		
$[Pt(NH_3)_2Cl_2Br_2]$	15.0	(1.97 ± 0.06) x 10^4	43.4 ± 1.5	-12 ± 6
(pH 7.40)	20.0	(2.63 ± 0.08) x 10^4		
	25.0	(3.77 ± 0.12) x 10^4		
	30.0	(5.14 ± 0.15) x 10^4		
	35.0	(6.66 ± 0.19) x 10^4		

Table 3. Summary of the observed second-order rate constants for reduction of $[Pt(dach)Cl_4]$ and *cis*-$[Pt(NH_3)_2Cl_4]$ by the dominant reductants in human plasma at pH 7.40 and μ = 1.0 M.

Pt(IV) complex	Reductant	$k'/M^{-1}s^{-1}$ (25.0 °C)	$k'/M^{-1}s^{-1}$ (37.0 °C)
$[Pt(dach)Cl_4]$	Asc	602 ± 30	(1.37 ± 0.07) x 10^3
	GSH	(6.4 ± 0.2) x 10^3	(1.79 ± 0.06) x 10^4
	Cys-Gly	(4.9 ± 0.2) x 10^3	(1.07 ± 0.05) x 10^4
	Cys	(1.16 ± 0.05) x 10^4	(2.98 ± 0.09) x 10^4
	Hcy	(3.08 ± 0.09) x 10^3	(9.0 ± 0.3) x 10^3
$[Pt(NH_3)_2Cl_4]$	Asc	726 ± 36	(1.61 ± 0.08) x 10^3
	GSH	(1.23 ± 0.05) x 10^4	(3.26 ± 0.09) x 10^4
	Cys-Gly	(1.17 ± 0.05) x 10^4	(2.14 ± 0.08) x 10^4
	Cys	(2.8 ± 0.1) x 10^4	(7.3 ± 0.3) x 10^4
	Hcy	(7.1 ± 0.3) x 10^3	(1.84 ± 0.06) x 10^4

Table 4. Values of rate constants of the rate-determining steps for the reduction of Pt(IV) complexes by Asc derived from curve-fittings at 25.0 ^{o}C and μ = 1.0 M

Pt(IV) Complex	k_m	Value/$M^{-1}s^{-1}$
[Pt(dach)Cl_4]	k_1	0 [a]
	k_2	0.90 ± 0.08
	k_3	(4.5 ± 0.2) x 10^6
cis-[Pt(NH_3)$_2$$Cl_4$]	k_1	0 [a]
	k_2	2.0 ± 0.2
	k_3	(6.1 ± 0.3) x 10^6

[a] too small to be determined with accuracy and assumed to be 0.

Figure Captions

Figure 1. Observed first-order rate constants k_{obsd} as a function of [Asc] at 25.0 ^{o}C and different pHs (**A**), and at pH 7.40 and several temperatures (**B**) for the reduction of [Pt(dach)Cl_4] by Asc.

Figure 2. Observed first-order rate constants k_{obsd} as a function of [Asc] at 25.0 ^{o}C and different pHs (**A**), and at pH 7.40 and several temperatures (**B**) for the reduction of *cis*-[$Pt(NH_3)_2Cl_4$] by Asc.

Figure 3. (**A**) Plots of k_{obsd} versus [Asc] at pH 7.40 and several temperatures for the reduction of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2Br_2$] by Asc. (**B**) The Eyring plot for the observed second-order rate constant at pH 7.40.

Figure 4. Observed first-order rate constants k_{obsd} at 25.0 ^{o}C and 37.0 ^{o}C in pH 7.40 phosphate buffer for reduction of [Pt(dach)Cl_4] by GSH, Cys-Gly, Cys and Hcy.

Figure 5. Observed first-order rate constants k_{obsd} at 25.0 ^{o}C and 37.0 ^{o}C in pH 7.40 phosphate buffer for reduction of *cis*-[$Pt(NH_3)_2Cl_4$] by GSH, Cys-Gly, Cys and Hcy.

Figure 6. Observed second-order rate constants, k', as a function of pH at 25.0 ^{o}C (data points) for the reductions of [Pt(dach)Cl_4] and *cis*-[$Pt(NH_3)_2Cl_4$] by Asc. The solid curves were obtained from the best fits of Equation (6) to the experimental data by a weighted nonlinear least-squares routine.

Figure 7. (**A**) Asc species versus pH distribution diagram by use of pK_{a1} = 3.96 and pK_{a2} = 11.24 at 25.0 ^{o}C and μ = 1.0 M.[50,51] (**B**) Reactivity of Asc species versus pH distribution diagram for the reduction of [Pt(dach)Cl_4] by Asc. (**C**) Reactivity of Asc species versus pH distribution diagram for the reduction of *cis*-[$Pt(NH_3)_2Cl_4$] by Asc. The pK_a values and the rate constants in Table 4 were employed to generate the diagrams in (B) and (C).

Figure 8. (**A**) The isokinetic relationship between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ reported and discussed earlier in refs 18 and 19 for the Pt(IV)-Asc reactions; $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were obtained between pH 5.74 and 7.12. **1,** *cis*-$[Pt(NH_3)_2Cl_4]$; **2**, *trans*-$[Pt(NH_3)_2Cl_4]$; **3**, *trans*-$[PtCl_2(en)_2]^{2+}$; **4**. $[PtCl_6]^{2-}$; **5**, $[PtBr_6]^{2-}$. (**B**) The relation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ after addition of the data points obtained in the present work: **1′**, *cis*-$[Pt(NH_3)_2Cl_4]$ (obtained at pH 7.40); **6,** ormaplatin (obtained at pH 7.40); **6'**, ormaplatin (obtained at pH 7.12); **7**, *cis,cis,trans*-$[Pt(NH_3)_2Cl_2Br_2]$ (obtained at pH 7.40).

Scheme 1. Structures of $[Pt(dach)Cl_4]$, *cis*-$[Pt(NH_3)_2Cl_4]$, *cis,cis,trans*-$[Pt(NH_3)_2Cl_2Br_2]$, L-ascorbic acid, and L-dehydroascorbic acid.

Scheme 2. The proposed reaction mechanism for reduction of Pt(IV) anticancer prodrugs by Asc.

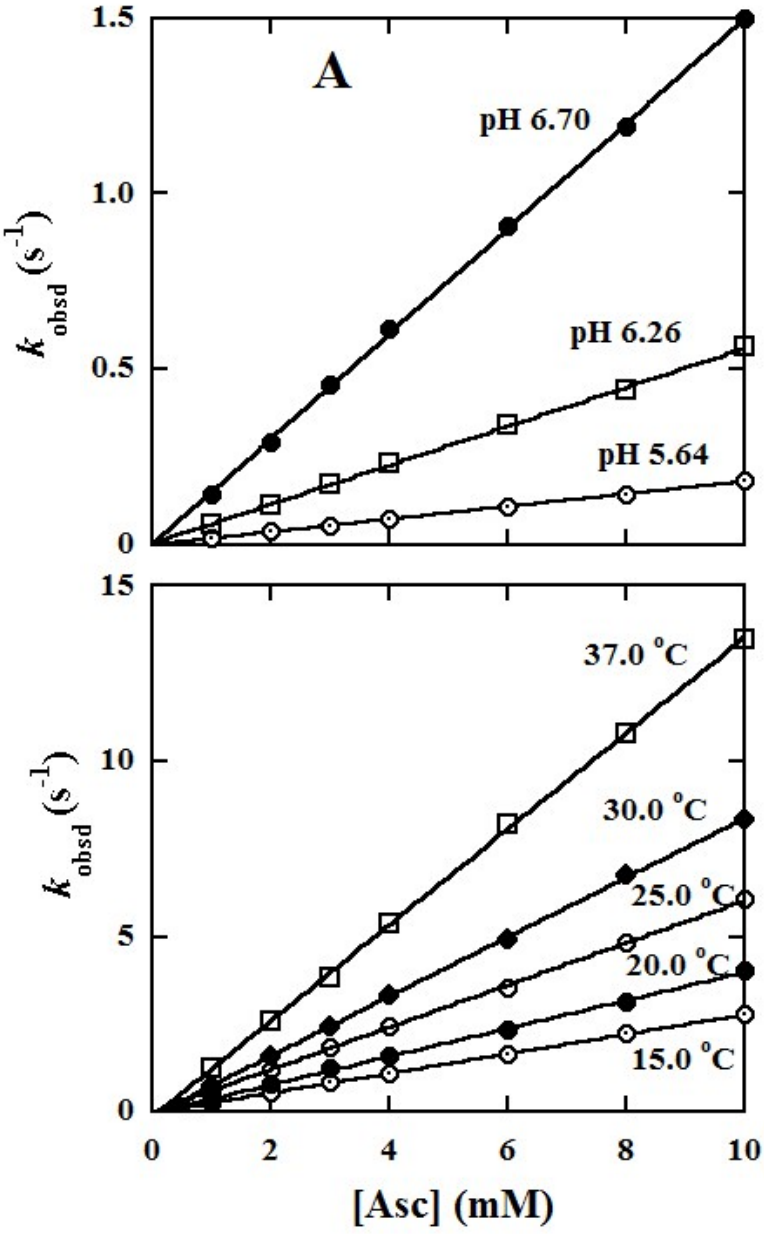


Figure 1

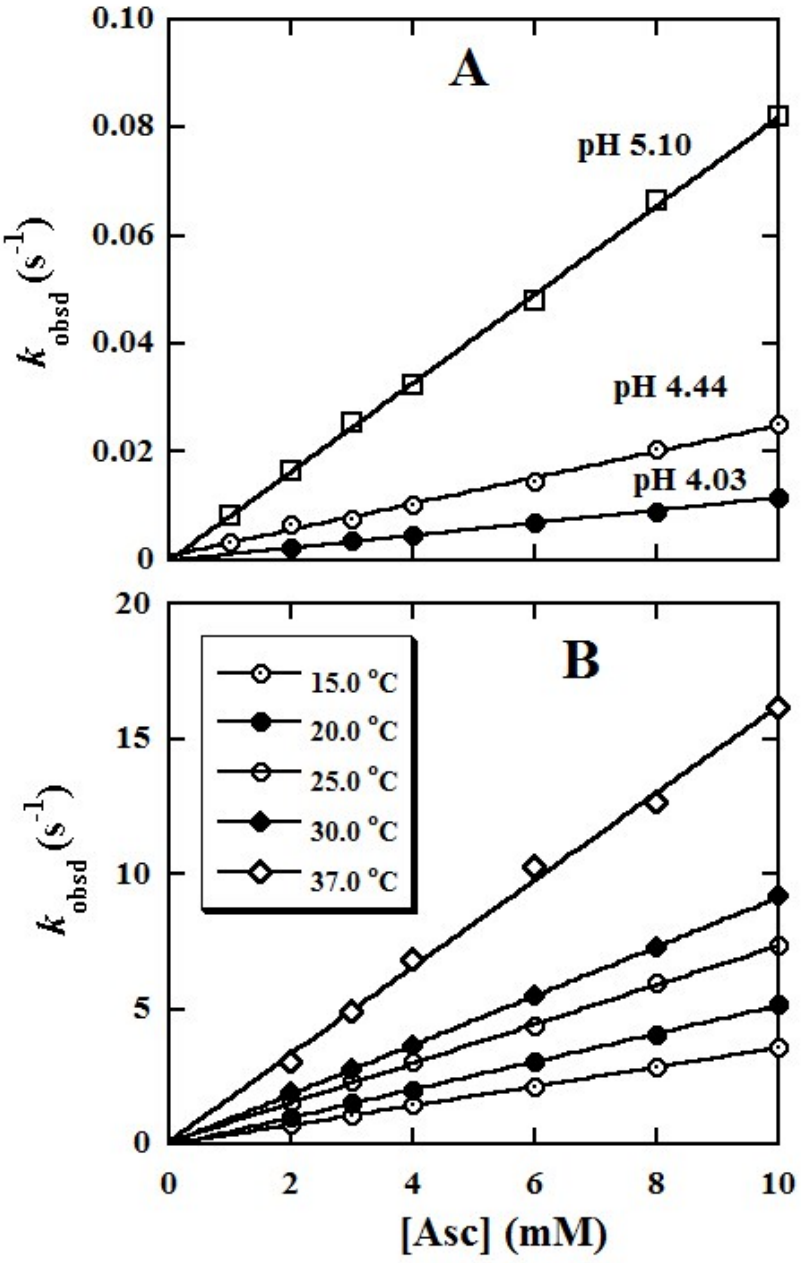


Figure 2

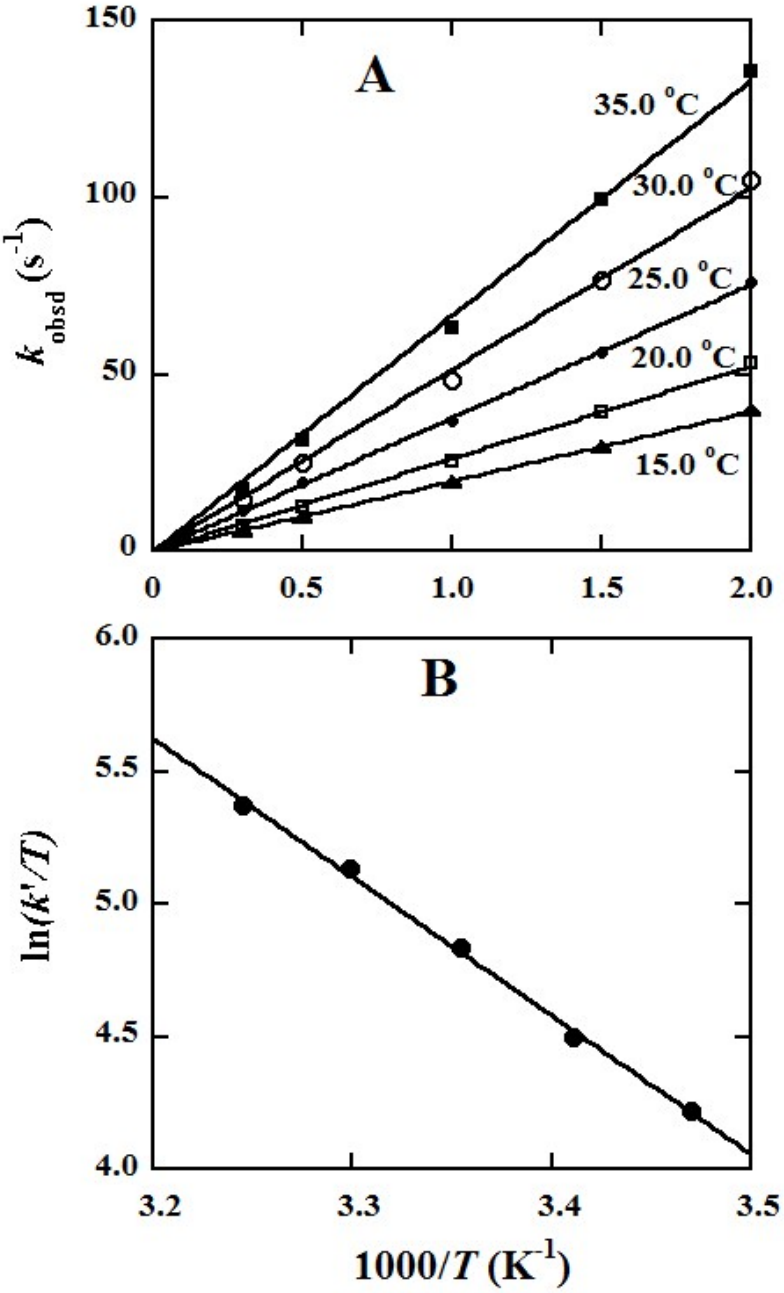


Figure 3

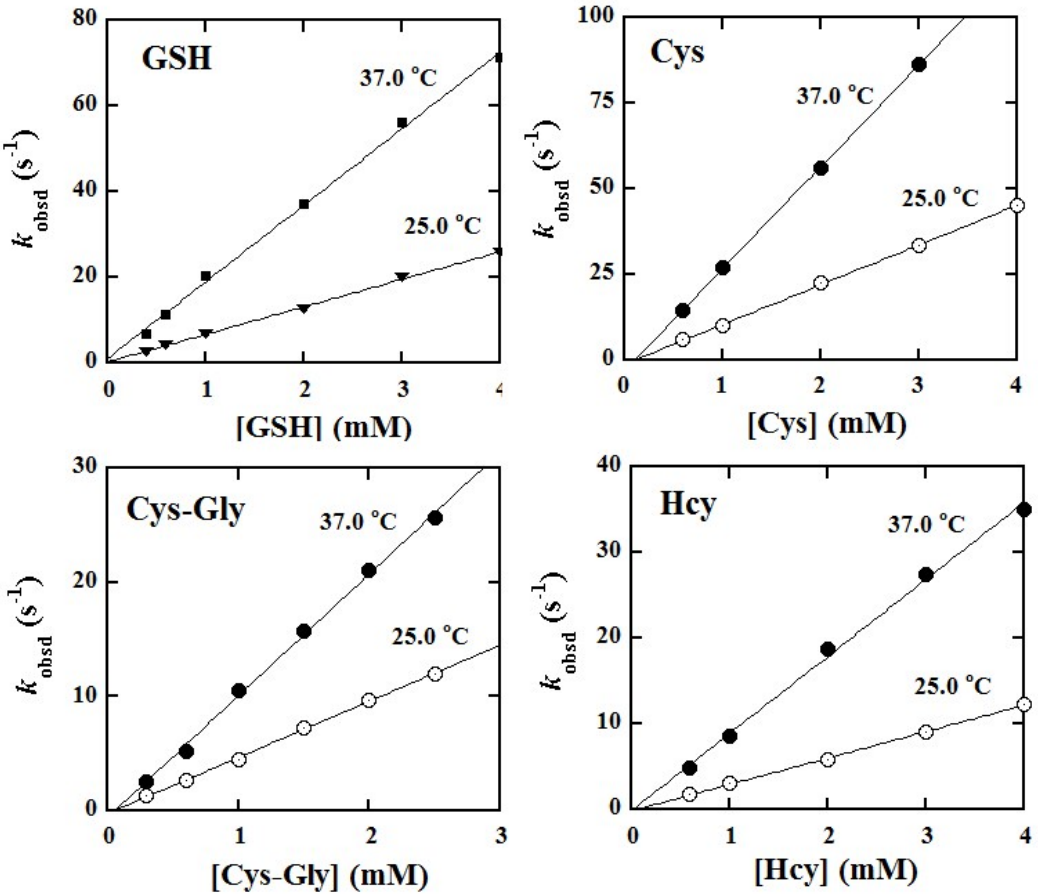


Figure 4

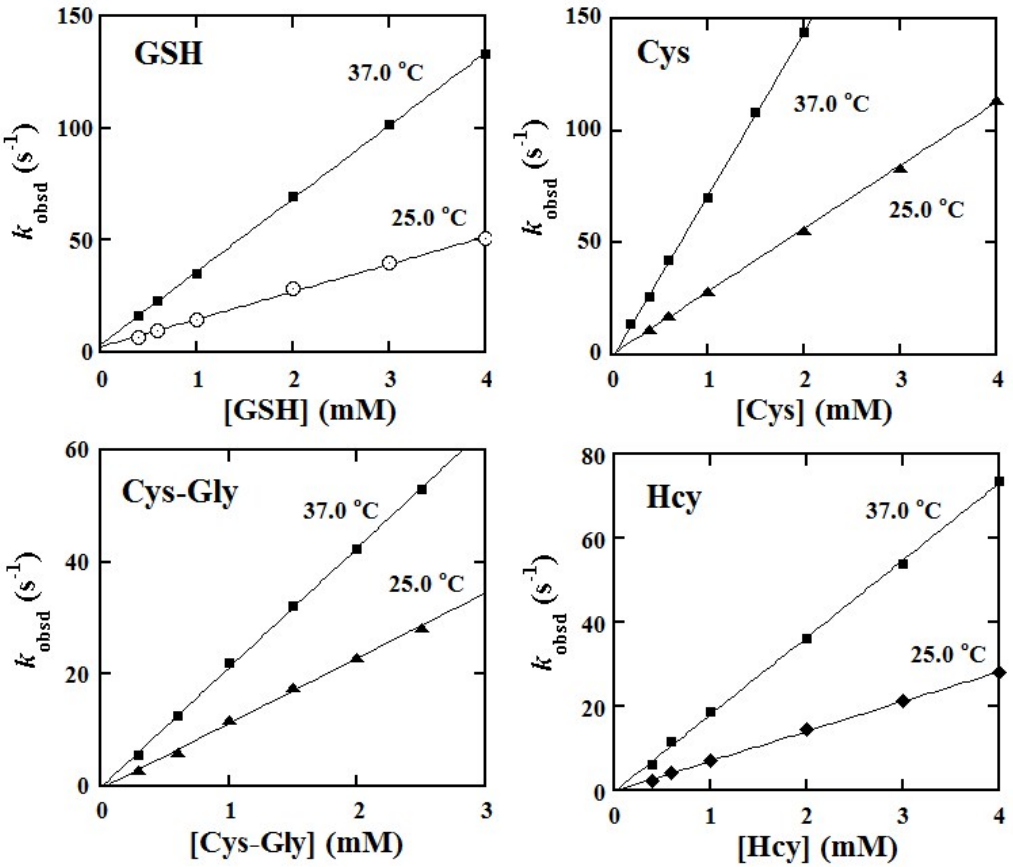


Figure 5

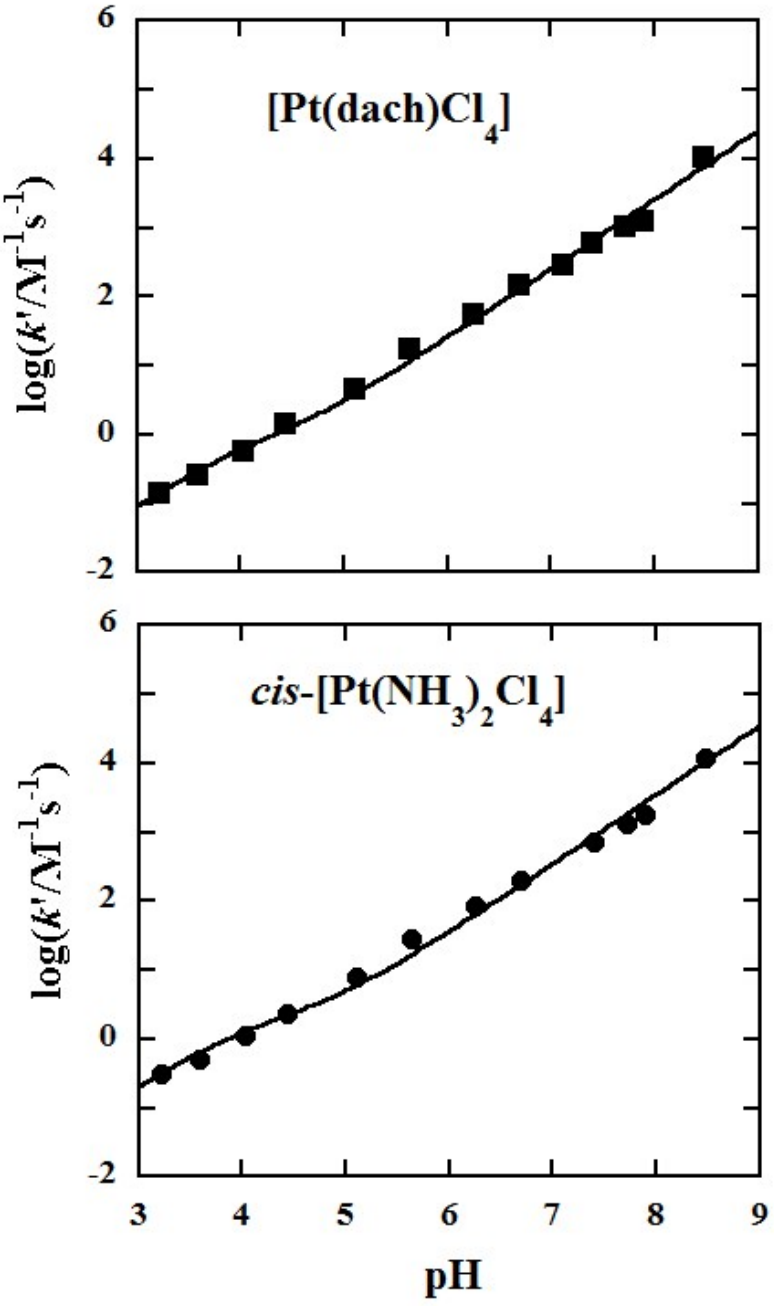


Figure 6

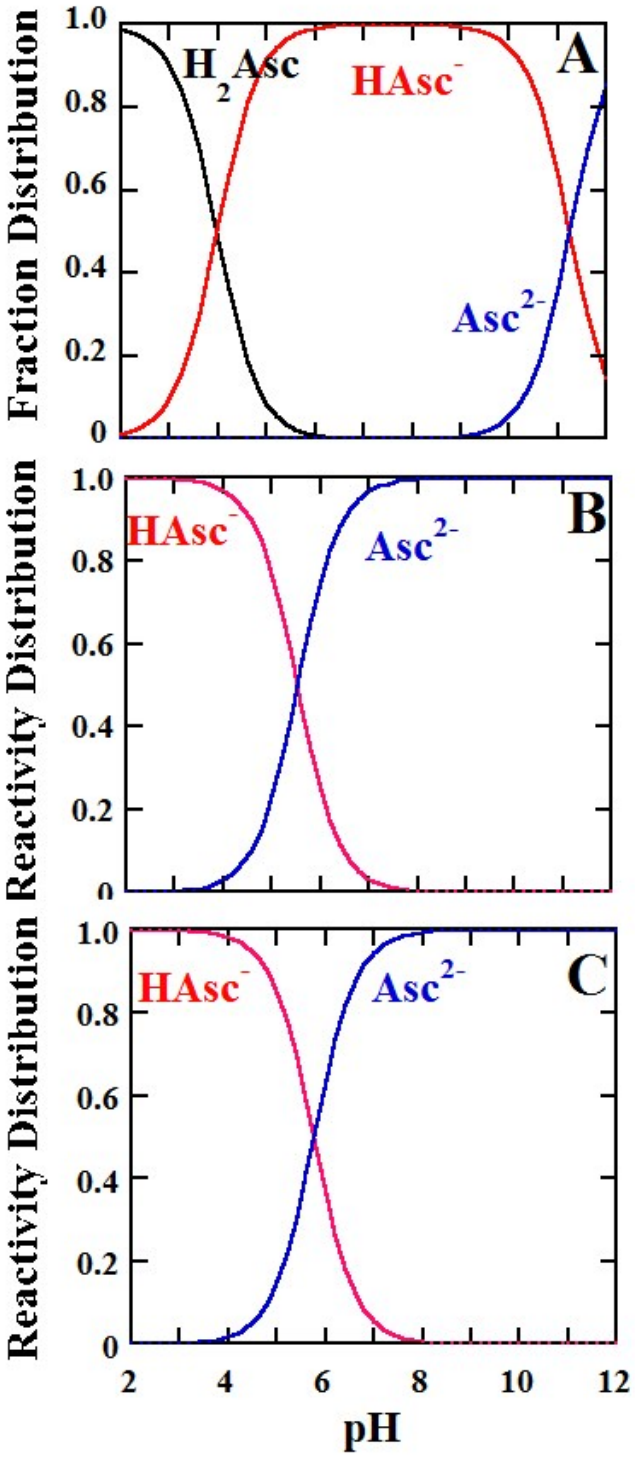


Figure 7

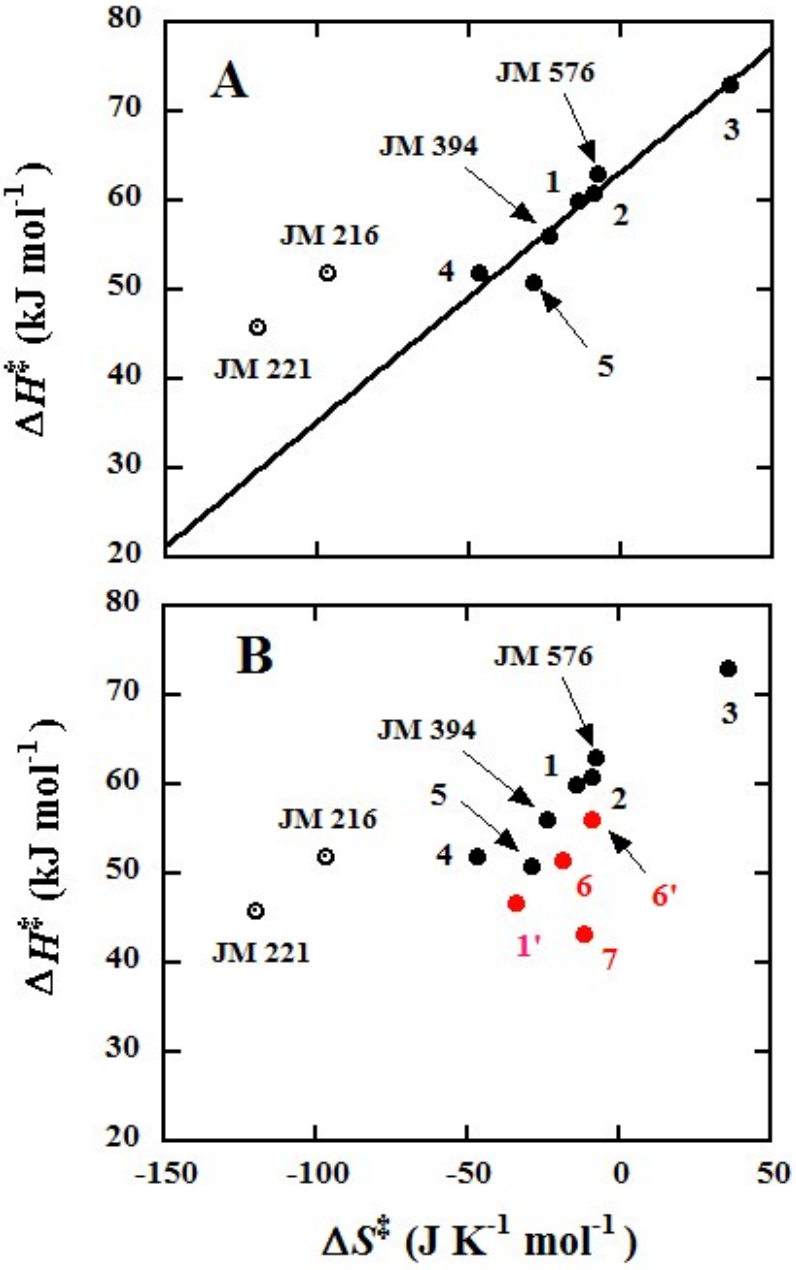
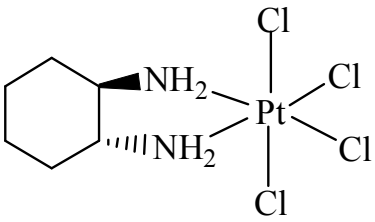
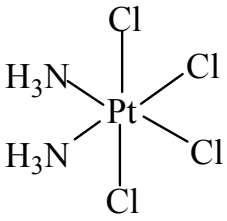


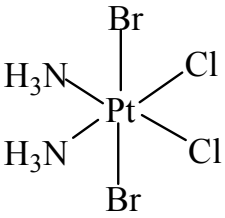
Figure 8



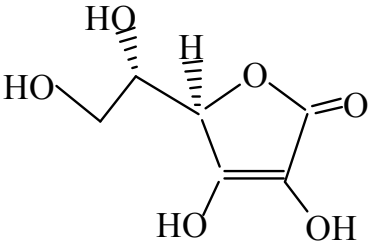
[Pt(dach)Cl_4] (ormaplatin)



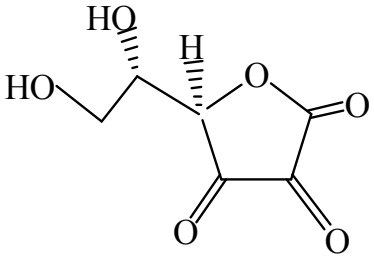
cis-[Pt(NH_3)$_2Cl_4$]



cis,cis,trans-[Pt(NH_3)$_2Cl_2Br_2$]

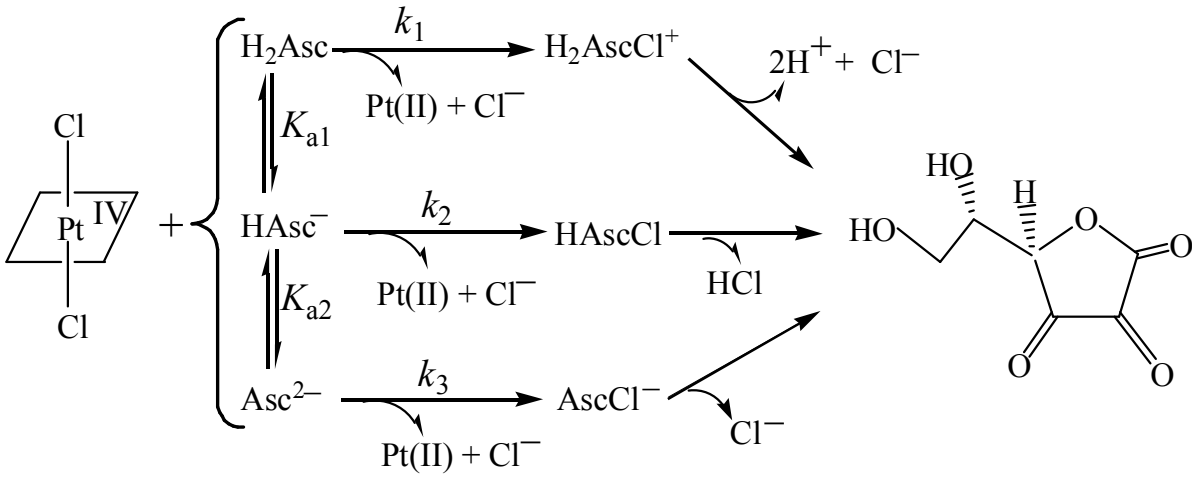


L-Ascorbic Acid



L-Dehydroascorbic Acid (DHA)

Scheme 1



Pt(IV) = $[Pt(dach)Cl_4]$/*cis*-$[Pt(NH_3)_2Cl_4]$;

Pt(II) = $[Pt(dach)Cl_2]$/*cis*-$[Pt(NH_3)_2Cl_2]$

Scheme 2