Surprising Reactions of Iodo Pt(IV) and Pt(II) Complexes with Human Albumin: Detection of Cys34 Sulfenic Acid

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Abstract: *cis*-$[PtCl_2(NH_3)_2]$ is the anticancer drug cisplatin, but the iodide analogue *cis*-$[PtI_2(NH_3)_2]$ is inactive. This inactivity is usually attributed to the greater stability and lower reactivity of Pt−I bonds compared to Pt−Cl in aqueous solution. Interest in reactions of Pt(IV) complexes with thiols arises from the reductive activation of Pt(IV) anticancer drugs in blood plasma. Recently, we found (*J. Am. Chem. Soc.* **1998**, *120*, 8253−8254) that low M_r thiols react with Pt(IV)−I bonds of *trans,cis*-$[Pt(en)(OH)_2I_2]$ via attack on the coordinated iodo ligand giving rise to reactive chelate-ring-opened Pt(II) ethylenediamine species. Here we report reactions of the Pt(II) and Pt(IV) complexes $[Pt(en)I_2]$ and *trans,cis*-$[Pt(en)(OH)_2I_2]$ with the major thiol in blood plasma, Cys34 of the protein albumin (66 kDa). Unexpectedly, $[Pt(en)I_2]$ reacted more rapidly with albumin than the cisplatin analogue, $[Pt(en)Cl_2]$, and did not give products with Pt bound to Cys34. The Pt(IV) chloro analogue, *trans,cis*-$[Pt(en)(OH)_2Cl_2]$, did not react at all with albumin. Reactions of *trans,cis*-$[Pt(en)(OH)_2I_2]$ with the protein, via direct attack of an iodo ligand on Cys34, gave rise to a relatively stable sulfenic acid derivative, in contrast to reactions with low M_r thiols. Reactions of Pt complexes with thiols in proteins can therefore take a different course: albumin can exert control over the reactivity of Cys34 and stabilize activated derivatives such as the sulfenyl iodide and sulfenic acid. The reactivity of iodide ligands in Pt complexes is much higher than has been previously recognized, and it may be possible to incorporate them into drug design strategies.

Introduction

Current clinical platinum anticancer complexes always contain chloride and not bromide or iodide as halide ligands. Examples are cisplatin,[1] *cis*-$[PtCl_2(NH_3)_2]$, sterically hindered complex *cis*-$[PtCl_2(NH_3)(2\text{-picoline})]$ (ZD0473),[2] the trinuclear Pt(II) complex BBR 3464,[3] and *cis,trans,cis*-$[PtCl_2(OAc)_2NH_3(c\text{-}C_6H_{11}\text{-}NH_2)]$ (JM216).[1] The latter, an orally active octahedral Pt(IV) complex, is believed to be a prodrug for an active chloro Pt(II) complex.[4] The lack of exploration of the activity of Br and I complexes appears to stem from early findings[5] that the activity of *cis*-$[PtX_2(NH_3)_2]$ and $[Pt(en)X_2]$ in several animal tumor models follows the order: $X = Cl > Br \gg I$. It seems reasonable to suppose that Pt−I bonds (soft metal ion, soft ligand) are too stable to allow activation via hydrolysis, the usual mechanism for attack of Pt(II) chloro complexes on DNA. Iodo Pt(II) complexes are therefore mainly used as intermediates in the synthesis of chloro Pt(II) complexes,[6] for example in the synthesis of mixed-amine Pt(II) complexes,[7] and as heavy-atom compounds for X-ray structure determination of large biomolecules.[8] There are relatively few reports of the chemistry[9] of iodo Pt(II) complexes and their reactivity toward biomolecules.[10]

We are interested in the coordination chemistry of iodo Pt(IV) complexes, designed as oxygen-independent photoactivatable metallodrugs for phototherapy. The iodo ligands shift the ligand-to-metal charge-transfer band (LMCT) into the visible region of the spectrum. These photoactive analogues of the anticancer drug cisplatin, *cis*-$[PtCl_2(NH_3)_2]$, are promising in this respect, because their cell cytotoxicity is potentiated by visible light, and stereospecific reactions between the diiodo Pt(IV) complexes and nucleotides can be photoinduced.[11]

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Success with the design of photoactivated metallodrugs is, however, highly dependent on their stability under physiological conditions. A better and more detailed understanding of redox interactions of Pt(IV) complexes with biomolecules is therefore crucial. Due to their inertness to substitution, antitumor-active chloro Pt(IV) complexes are thought to be prodrugs activated in vivo through reduction to their Pt(II) analogues by biological reductants with the loss of two axial ligands.[7b,12]

There is relatively little information in the literature concerning the mechanisms of reduction of antitumor Pt(IV) diamines by biologically important thiols. Shi et al.[13] proposed a mechanism consisting of nucleophilic attack by the thiol of glutathione on one of the axial chloride ligands of *trans*-$[PtCl_2(CN)_4]^{2-}$ during the redox reaction to form the corresponding Pt(II) complex $[Pt(CN)_4]^{2-}$. Recently we have shown that diiodo Pt(IV) complexes, *trans*,*cis*-$[Pt(en)(OR)_2I_2]$ (where R = H, $COCH_3$), are highly reactive toward low molecular weight thiols, such as glutathione and *N*-acetyl-L-cysteine, and undergo electron-transfer-driven chelate ring-opening.[14] The thiol appears to attack one of the equatorial iodide ligands of the Pt complex forming a surprisingly long-lived chelate-ring-opened Pt(II) complex and the disulfide.

Since the major thiol in blood plasma is the protein albumin (M_r 66 kDa, concentration 0.53−0.75 mM),[15] we have now made a detailed study of its reactions with Pt(IV) and Pt(II) iodo complexes under physiologically relevant conditions. Reactions of diiodo Pt(II) and Pt(IV) complexes were followed by 2D [^{1}H,^{15}N] HSQC NMR techniques, and by ICP-MS for determination of free and bound Pt and I. Unexpectedly, the diiodo Pt(II) complex, $[Pt(en)I_2]$, reacted very rapidly with rHA, and the products from reaction with the Pt(IV) complex *trans*,*cis*-$[Pt(en)(OH)_2I_2]$ were different from those observed with low M_r thiols. In particular, the protein is able to stabilize a sulfenic acid intermediate.

Materials and Methods

Materials. General chemicals and meta-arsenite (*m*-$NaAsO_2$, S-7400), glutathione (G-6529), L-methionine, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, C-5261), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, D-8130), and iodine were purchased from Sigma/Aldrich, and H_2O_2 (30%) from Prolab.

$[Pt(en)I_2]$, $[Pt(en)Cl_2]$, *trans*,*cis*-$[Pt(en)(OH)_2I_2]$ and *trans*,*cis*-$[Pt(en)(OH)_2Cl_2]$ and the (^{15}N-en)-labeled Pt complexes, $[Pt(^{15}N\text{-}en)I_2]$ and *trans*,*cis*-$[Pt(^{15}N\text{-}en)(OH)_2I_2]$, were synthesized according to previously described procedures[11a] and were fully characterized by elemental analysis, NMR, and FAB-MS.

Table 1. SH Content of Recombinant Human Albumin (rHA) before and after 24 h Incubation with Pt Complexes at a Molar Ratio of 1:1 (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K

Pt complex		SH content (mol mol^{-1})	decrease (%)
none		0.86 ± 0.01[a]	0
trans,*cis*-$[Pt^{IV}(en)(OH)_2I_2]$	**1**	0.02 ± 0.01	98
trans,*cis*-$[Pt^{IV}(en)(OH)_2Cl_2]$	**2**	0.78 ± 0.04	9
$[Pt^{II}(en)I_2]$	**3**	0.79 ± 0.05	8
$[Pt^{II}(en)Cl_2]$	**5**	0.79 ± 0.03	8

[a] Mean ± SD, $N = 3$.

Recombinant human albumin (rHA) (batch R970103), supplied by Delta Biotechnology, was dialyzed against 100 mM NH_4HCO_3, pH 7.9, and freeze-dried. Cys34-blocked rHA (*N*-ethylmaleimide treated rHA, NEM-rHA) was prepared by incubation of rHA (40−60 mg mL^{-1}) in 100 mM NH_4HCO_3, pH 7.9, with 2 mol equiv of *N*-ethylmaleimide for 12−16 h at ambient temperature in the dark. Excess blocking agent was removed by dialysis against the same buffer.

Calibration materials for ICP-MS analysis were: KI (99.5%) and single element standards for ICP at 1000 mg/L of Pt (Pt in HCl 20%), and Te (Te in HCl), all "Aristar" grade, from Merck Ltd., Leics, U.K. KOH was analytical grade from Fisher Scientific, Loughborough, U.K. The reference materials NIST SRM 2670 "Toxic metals in urine" (U.S. National Institute of Standards and Technology, Gaithersburg, Maryland) and BCR CRM 063 R "Skim milk powder" (EU Institute for Reference Materials and Measurements, Geel, Belgium) were used to assess the accuracy of Pt and I determinations, respectively.

Ultrapure water (resistivity 18 MΩ, <5 ppb) was obtained from a combined ion-exchange/reverse osmosis system (Elga Ltd., Bucks, U.K.) and used in all experiments.

Measurements of pH. Values of pH were measured with a Corning 145 pH-meter equipped with a microcombination electrode calibrated with Aldrich standard buffers (pH 4, 7, and 10) and were adjusted with dilute solutions of HCl and NaOH.

UV−Vis Spectroscopy. Electronic absorption spectra were recorded at 298 or 310 K on a Shimadzu spectrophotometer with temperature control using 1-cm path-length cells.

UV−vis kinetic studies of the reaction of *trans*,*cis*-$[Pt(en)(OH)_2I_2]$ with either rHA or NEM-rHA in a molar ratio of 1:1 (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K were monitored at different pH values over 20 min by the decrease of the LMCT band at 386 nm, a characteristic feature of all diiodo Pt(IV) complexes.

Sample Preparation for Thiol and Sulfenyl Iodide/Sulfenic Acid Determinations. Pt complexes (as listed in Table 1) were incubated with rHA at a molar ratio of 1:1 (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K for 24 h. Additionally, the kinetic course of the reaction between *trans*,*cis*-$[Pt(en)(OH)_2I_2]$ and rHA was followed by taking samples at the following times: 0, 0.3, 0.8, 1.5, 5, and 24 h. Unbound Pt complex was then removed by three cycles of ultrafiltration (Amicon, Beverly, MA, cutoff 30 000 Da, 10 min at 277 K and 2516 g). The thiol content and the amount of sulfenyl iodide/sulfenic acid were then determined.

For control studies, H_2O_2, an oxidizing agent known to produce the metastable sulfenic acid derivative of albumin, was used.[16,17] In addition, the reaction with iodine was studied. This is known to give rise to a sulfenyl iodide derivative of albumin.[18] Both oxidizing agents were incubated with rHA at 310 K for 20 min at different molar ratios in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4. Excess reagent was then removed by three cycles of ultrafiltration, as described above, and then thiol and sulfenyl iodide/sulfenic acid determinations were carried out.

Determination of rHA Thiol Content. Ellman's reagent, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), was used and the absorption

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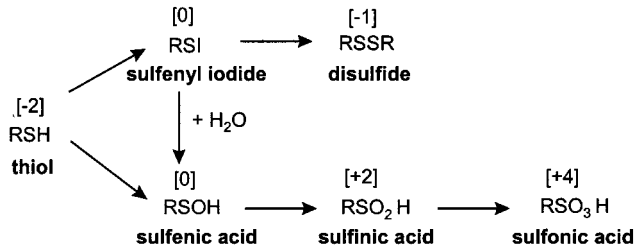
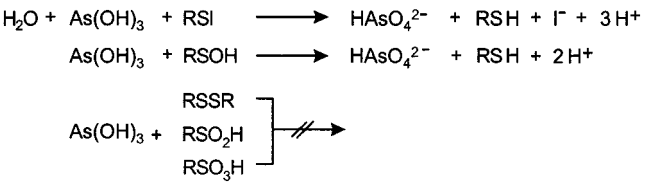
Scheme 1. Major Pathways for Thiol Oxidation: Oxidation State of Sulfur is Given in Square Brackets



Scheme 2. Reduction of Oxidized Thiol Species by Arsenite[a]



[a] Arsenite probably exists as $As(OH)_3$ at pH 7.4 and is oxidized largely to $HAsO_4^{2-}$.[44]

at 412 nm (ϵ_{412} = 13 600 M^{-1} cm^{-1}) was measured.[19] Samples containing 36 nmol of rHA were diluted 1:4 in 10 mM phosphate, 2 mM EDTA, pH 7.4, and incubated with 505 nmol of DTNB (50.5 mM stock solution, in ethanol) for 30 min at ambient temperature before measuring the absorption at 412 nm.

Protein concentrations were measured using the Bradford assay: 0.8 nmol of protein was added to 1.5 mL of Bradford reagent and the absorption measured at 595 nm. A calibration curve was constructed by assuming that the absorption of a 1 mg mL^{-1} rHA solution at 279 nm is 0.558.[15b,19] The thiol content of the rHA was then calculated by dividing the thiol concentration by the protein concentration of each sample.

Determination of Sulfenyl Iodide/Sulfenic Acid. For this, *m*-$NaAsO_2$ was used. Arsenite reduces sulfenyl iodides and sulfenic acids to thiols, but does not reduce other oxidation states of sulfur, such as disulfides, sulfinic and sulfonic acids (see Schemes 1 and 2).[17,20] Recovery of the oxidized thiol was determined by using Ellman's reagent (DTNB).

Samples of rHA (54 nmol) were incubated with *m*-$NaAsO_2$ (5 μmol) for 45 min at 310 K, and the thiol content was determined as described above. For the controls, the same procedure was used but without addition of m-$NaAsO_2$.

Gel Filtration Chromatography. *Trans,cis*-[Pt(en)$(OH)_2I_2$] was incubated with rHA at a molar ratio of 1:1 (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K for 24 h. Unbound Pt complex was then removed by three cycles of ultrafiltration (Amicon, Beverly, MA, cutoff 30 000 Da, 10 min at 277 K and 2516 g). Gel filtration of the Pt−albumin complex was carried out on a Superdex 200 HR column (internal diameter 1 cm, length 30 cm) with an FPLC system (Amersham Pharmacia Biotech, Bucks, U.K.) at ambient temperature. Albumin samples were diluted to a concentration of 5 mg mL^{-1} and 0.2 mL was loaded onto the column. Elution conditions were 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.0, with a flow rate of 0.5 mL min^{-1} and detection at 280 nm.

ESI-Mass Spectrometry. Positive ion electrospray mass spectrometry was performed on a Platform II mass spectrometer (Micromass, Manchester, U.K.). The samples were infused at 10 μL min^{-1} and the ions produced in an atmospheric pressure ionization (API)/ESI ion source. The source temperature was 338 K and the drying gas flow rate 300 L h^{-1}. A potential of 3.5 kV was applied to the probe tip, and a cone voltage gradient of 50−90 V over 1000−2000 Da was used. The quadrupole was scanned at 100 amu s^{-1}. The acquisition and deconvolution of data were performed on a Mass Lynx (V. 2.3) Windows NT PC data system using the MaxEnt Electrospray software algorithm. The mass accuracy of all measurements was within 0.1 *m/z* unit. ESI-MS was used to detect the sulfenic acid derivative of rHA after reaction with the diiodo Pt(IV) complex by using the trapping agent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl).[21]

Recombinant albumin and *trans,cis*-[Pt(en)$(OH)_2I_2$] (1:1, 100 μM) were incubated in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, for 0 and 20 min at 310 K. For control studies, H_2O_2 was added to produce the metastable sulfenic acid derivative of albumin.[16,17] H_2O_2 (3 mol equiv) was incubated with rHA for 20 min under the same conditions. Excess of reagent was removed by three cycles of concentration and redilution with phosphate−saline buffer in a Centricon-30 concentrator at 2516 g, for 10 min at 277 K. Samples containing 60 nmol of rHA, NEM-rHA, and reacted rHA (with either H_2O_2 or *trans,cis*-[Pt(en)$(OH)_2I_2$]) were incubated with 2 mol equiv of NBD-Cl (10 mM in dimethyl sulfoxide) for 30 min at ambient temperature and then concentrated and rediluted in phosphate−saline buffer three times in a Centricon-30 concentrator, as above, to remove excess of NBD-Cl. Prior to injection, aliquots of the rHA sample were diluted 1:1000 in 50/50% CH_3CN/H_2O, 0.2% HCOOH.

UV−vis spectra (see UV−Vis Spectroscopy) of NBD-modified rHA samples were recorded at 298 K prior to ESI-MS analysis.

ICP-Mass Spectrometry. A Plasma Quad 3 instrument (VG Elemental, Winsford, U.K.), equipped with the S-option for increased sensitivity, was used for all measurements. Details of the procedure used for the simultaneous determination of Pt and I on the high and low M_r fractions by ICP-MS are reported elsewhere.[22] Briefly, Pt complexes were incubated with either rHA or NEM-rHA at a molar ratio of 1:1 (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K for 24 h. The binding of I^- to albumin was assessed independently by incubating KI (200 μM) with rHA for 24 h in a 1:2 molar ratio.

Additionally, the kinetic courses of the reactions between either rHA and *trans,cis*-[Pt(en)$(OH)_2I_2$] or [Pt(en)I_2] were followed. Aliquots were taken from reaction mixtures at either 0, 0.5, 1, 1.5, 2.5, 6, 20.5, or 24 h to determine the Pt and I distribution among high M_r and low M_r fractions at different stages of the reaction with rHA. The two fractions were separated by ultrafiltration, and weakly bound iodide was removed by three cycles of concentration and redilution with phosphate−saline buffer in a Centricon-30 concentrator (Amicon, Beverly, MA) with a 30 kDa cutoff for 10 min at 277 K, 2516 g. The last centrifugation was carried out for 15 min, and all low M_r fractions were pooled. The final volumes of the high and low M_r fractions were recorded. Samples and standards for calibration were diluted in 10 mM KOH containing 20 μg L^{-1} Te and analyzed by ICP-MS. The limits of detection of the method were 0.18 nmol L^{-1} for I and 11 pmol L^{-1} for Pt. The analysis of certified reference materials (CRM 063R "Skimmed milk powder" and SRM 2670 "Toxic metals in urine") was satisfactory, and the accuracy of the method for the determination of Pt:I ratios ranging from 0.240 to 1.035 was 101 ± 2.7%.

NMR Spectroscopy. Two-dimensional [^{1}H,^{15}N] HSQC NMR spectra were recorded on a Bruker DMX 500 (^{1}H, 500 MHz, ^{15}N, 50.7 MHz) NMR spectrometer equipped with a tunable triple resonance (TBI) probehead [^{1}H, ^{13}C, X] incorporating an actively shielded *z*-field gradient coil. 2D [^{1}H,^{15}N] HSQC NMR data were acquired using a pulse sequence in which coherence selection is achieved via gradients, and procedures and conditions similar to those described previously.[19]

Solutions containing either [Pt(^{15}N-en)I_2], [Pt(^{15}N-en)Cl_2], or *trans,cis*-[Pt(^{15}N-en)$(OH)_2I_2$] and either rHA or NEM-rHA at a 1:1 molar ratio (150 μM) were prepared, and D_2O (final concentration 10% v/v) was added. The final pH was either 6.1 or 7.4. Dioxane was used as an internal ^{1}H standard (3.764 ppm relative to TSP); ^{15}N chemical shifts were referenced externally to $^{15}NH_4Cl$ at 0 ppm.

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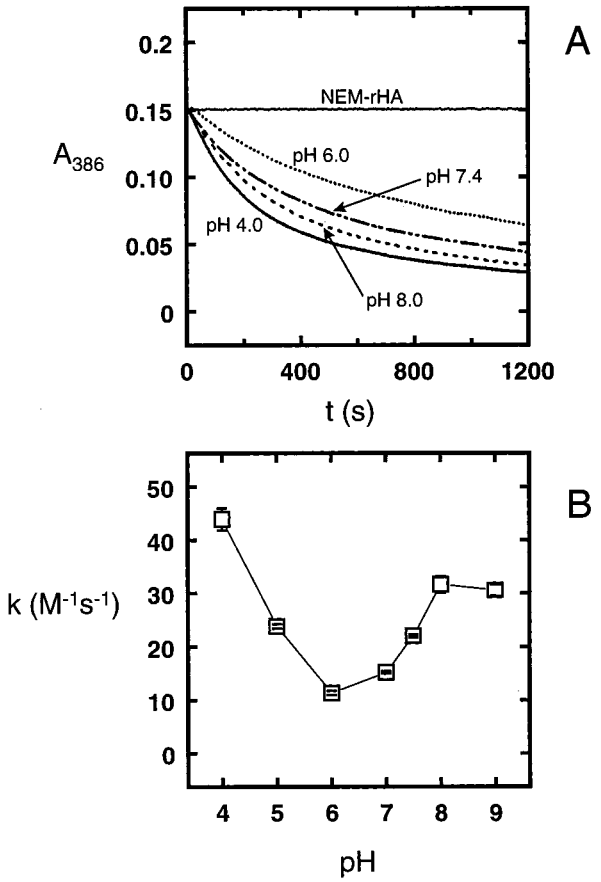


Figure 1. (A) Decrease in the intensity of the LMCT band at 386 nm during the reaction of *trans,cis*-[Pt(en)$(OH)_2I_2$] (**1**) with rHA at different pH values and NEM-rHA at pH 7.4 and (B) pH-dependent reactivity profile. The decrease of the LMCT of **1** was pH dependent and exhibited a minimum at pH 6. The diiodo Pt(IV) complex, however, did not react with the thiol blocked albumin, NEM-rHA, at any pH values.

Results

Reactions between diiodo Pt(IV) and Pt(II) complexes and recombinant human albumin were studied by UV−vis spectroscopy, gel filtration, NMR, and mass spectrometry. Particular emphasis was placed on the determination of the nature of the modification to the only free thiol group in albumin, Cys34. We used recombinant human albumin (rHA), which is more homogeneous than albumin isolated from blood plasma or serum[23] and, in particular, has an SH content (ca. 0.85 SH mol^{-1})[19] similar to that of albumin in vivo.

Reactions of rHA with *trans,cis*-[Pt(en)$(OH)_2I_2$] (1). Oxidation of Cys34. Reaction of the diiodo Pt(IV) complex **1** with rHA at a molar ratio of 1:1 (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4 buffer, pH 7.4, produced a rapid decrease in the intensity of the characteristic Pt(IV)−I LMCT band at 386 nm (Figure 1A). The rate of decline of the LMCT band over the pH range 4−9 exhibited a minimum at pH 6 (Figure 1B). In contrast, no change in this band was detected on reaction of complex **1** with SH-blocked rHA (NEM-rHA) at any pH value.

Samples were taken during the incubation of complex **1** with rHA to assess the thiol content of rHA. First, the reaction between the Pt complex and rHA was stopped after 0, 0.3, 0.8, 1.5, 5, and 24 h by removing free Pt complex by three cycles of ultrafiltration, and then the thiol content was determined by using Ellmans' reagent (DTNB). Figure 2 (open bars) shows that the thiol group of Cys34 was almost completely blocked within the first hour of the reaction.

(23) Differences between isolated albumin and the albumin in blood plasma are discussed in: Christodoulou, J.; Sadler, P. J.; Tucker, A. *FEBS Lett.* **1995**, 376, 1−5.

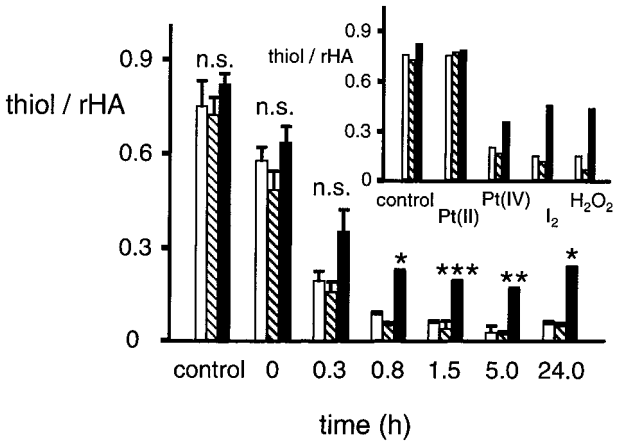
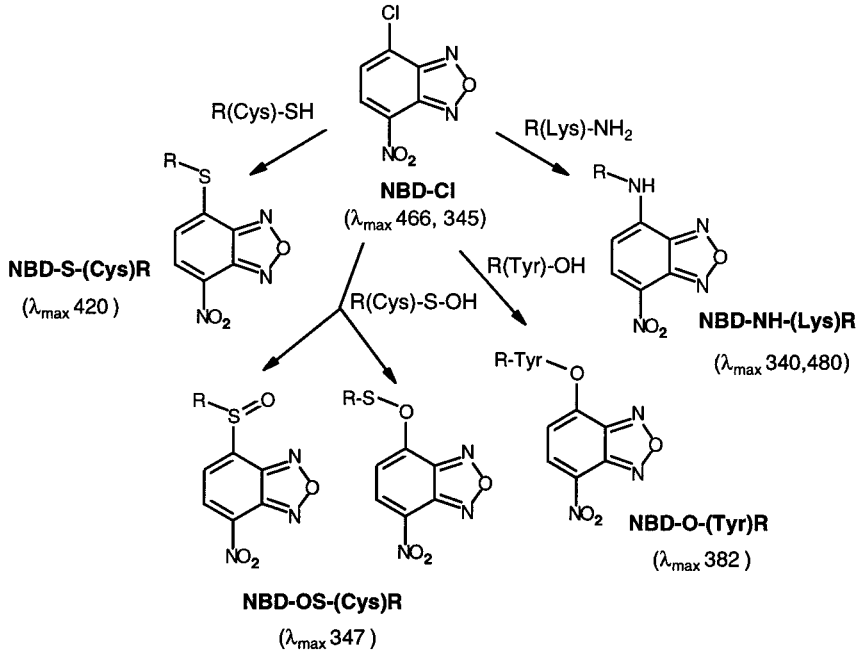


Figure 2. Changes of the thiol/protein ratios during the reaction of *trans,cis*-[Pt(en)$(OH)_2I_2$] with rHA with time (mol ratio 1:1, 100 μM, in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K). Samples were taken at the times indicated, and the reaction was stopped by removing unbound Pt complex by three cycles of ultrafiltration The thiol content of rHA was then determined by using Ellman's reagent. Open bars show that the thiol of rHA was blocked almost completely within 48 min. The amounts of the sulfenyl iodide/sulfenic acid derivative of rHA formed during the reaction with **1** were determined by incubating the rHA samples for a further 45 min at 310 K with (closed bars) or without (hatched bars) arsenite after stopping the reaction by ultrafiltration, as described above. The recovery of the thiol after addition of arsenite shows that a significant amount of sulfenyl iodide/sulfenic acid derivative of rHA is formed during the reaction with **1**. Control values represent the thiol/protein ratios of rHA. Inset: Comparison of the thiol/protein ratios with and without arsenite after 20 min incubation of rHA (100 μM, in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K) with either [Pt(en)I_2] (**3**) (mol ratio 1:1), *trans,cis*-[Pt(en)$(OH)_2I_2$] (**1**) (mol ratio 1:1), I_2 (mol ratio 1:1), or H_2O_2 (mol ratio 1:3). The recovery of the thiol of rHA after addition of arsenite was similar in the reactions with the diiodo Pt(IV) complex **1**, H_2O_2, and I_2. No change of the thiol content was observed during the reaction of [Pt(en)I_2] (**3**) with rHA, showing that the thiol content/arsenite assay is not influenced by Pt(II), and the observed effect during the reaction of **1** with rHA is only due to the formation of sulfenic acid/sulfenyl iodide. Data points are the means (±SD) of three independent experiments. Significant differences between closed and hatched bars are: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.002$ (two-sided, paired Student's *t*-test). n.s., not significant.

Reversal of Oxidation at Cys34. To investigate the nature of the blocking group, we studied the reversibility of Cys34 sulfur modification. Arsenite, *m*-$NaAsO_2$, was employed to determine the amount of reversibly oxidized sulfur species, such as sulfenyl iodide and sulfenic acid (see Schemes 1 and 2). The Pt(IV) complex **1** was incubated with rHA under the same conditions as above, and samples were taken at 0, 0.3, 0.8, 1.5, 5, and 24 h. The reaction was stopped by three cycles of ultrafiltration, and aliquots of the rHA samples were then further incubated for 45 min at 310 K, with or without addition of excess arsenite (16.7 mM). After the incubation, the thiol content was determined by using Ellmans' reagent (DTNB). The closed bars in Figure 2 show the thiol content of rHA after addition of excess arsenite during the reaction with the Pt(IV) complex **1** and of the corresponding rHA controls (without addition of arsenite, hatched bars). Statistical analysis of the data showed that the differences between the closed and hatched bars are significant at later time points (0.8−24 h of the reaction of rHA with Pt(IV)), and 27% of Cys34 thiol was recoverable by

Scheme 3. Structures, Absorption Maxima (λ_{max}, nm) and Pathways for the Reactions of 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) with Functional Groups of Amino Acid Residues in Proteins[21,24]



addition of arsenite, suggesting that a significant amount of sulfenyl iodide/sulfenic acid is formed.

Gel filtration chromatography showed that even after 24 h only monomeric albumin was present after incubation of **1** with rHA (i.e., no disulfide-bridged dimers were detected).

Trapping Cys34 Adducts with NBD-Cl. To confirm the formation of Cys34-sulfenyl iodide/sulfenic acid of rHA, rHA and Cys34-blocked rHA (NEM-rHA as control) were treated with a trapping agent for cysteine sulfenic acid, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl,[21] Scheme 3),[24] with and without prior exposure to the Pt(IV) complex **1**. Recombinant albumin and complex **1** were incubated in a molar ratio of 1:1 at 310 K (100 μM) in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, and the reaction was stopped immediately after mixing or after 20 min by three cycles of ultrafiltration. Protein samples were then incubated with 2 mol equiv of NBD-Cl for 30 min at ambient temperature, and excess of NBD-Cl was removed by ultrafiltration. As controls, rHA and NEM-rHA were treated in the same way but without addition of complex **1**. In the UV−vis spectrum (see Figure 3), NBD-treated rHA showed two absorption maxima at 344 and 408 nm, the latter absorption being due to the thiol−NBD adduct (NBD-S−Cys34). As expected, NBD-treated NEM-rHA gave rise to only one band at 344 nm, and the band for the thiol−NBD adduct was absent. The presence of two bands for the rHA reaction suggests that there are two types of binding sites on rHA. After incubation of rHA with complex **1**, reactions with NBD-Cl did not give rise to the absorption band at 408 nm (Figure 3), indicating the blockage of the thiol during the reaction, whereas the band at 344 nm was shifted only slightly to 347 nm. The wavelength of the latter band is characteristic of a trapped protein sulfenic acid adduct NBD-OS−(Cys)R, but the presence of the 344 nm band for NBD-treated rHA prevented the identification of the sulfenic acid based on the UV−vis data only. Electrospray mass spectrometry (ESI-MS) was used to clarify the identity of this product.

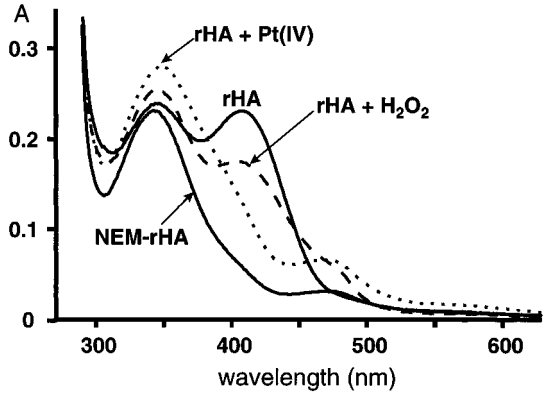


Figure 3. UV−vis spectra of the products of the reaction of NBD-Cl with rHA, Cys34-blocked rHA (NEM-rHA), rHA preincubated for 1 h with *trans,cis*-[Pt(en)$(OH)_2I_2$], and rHA preincubated for 20 min with H_2O_2 in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K. The decrease in intensity of the band at 408 nm, characteristic for NBD-S−(Cys34)rHA, shows that the sulfur of Cys34 is blocked during the reaction with either the diiodo Pt(IV) complex **1** or H_2O_2, preventing the reaction with the trapping agent NBD-Cl (Scheme 3).

Analysis of rHA alone by ESI-MS gave a molecular mass of 66 440.5 Da (theoretical value 66 438.41 Da) (Figure 4A). In the ESI mass spectrum of NBD-treated rHA, there were peaks at 66 607.6 and 66 766.9 amu, which can be assigned to rHA with either one (theoretical mass: 66 604.2 Da) or two (theoretical mass: 66 768.3 Da) molecules of bound NBD. When rHA was reacted with complex **1** for 20 min and then treated with NBD-Cl, a major peak of mass 66 621.8 amu was observed, consistent with the expected value for the sulfenic acid adduct NBD-OS−(Cys)R (theoretical mass: 66 621.2 amu, Figure 4B). Therefore, the ESI-MS results confirm the thiol determination data showing that a sulfenic acid derivative of Cys34 is formed during the reaction of *trans,cis*-[Pt(en)$(OH)_2I_2$] (**1**) with rHA.

Release of I^- from Complex 1 during Reaction with rHA. To investigate the release of iodide from complex **1** during reactions with rHA, Pt and I were determined simultaneously in the low and high M_r fractions by inductively coupled plasma mass spectrometry (ICP-MS). First the binding of I^- to rHA

(24) (a) Miki, M. *J. Biochem.* **1985**, *97*, 1067−1072. (b) Aboderin, A. A.; Boedefeld, E.; Luisi, P. L. *Biochim. Biophys. Acta* **1973**, *328*, 20−30.

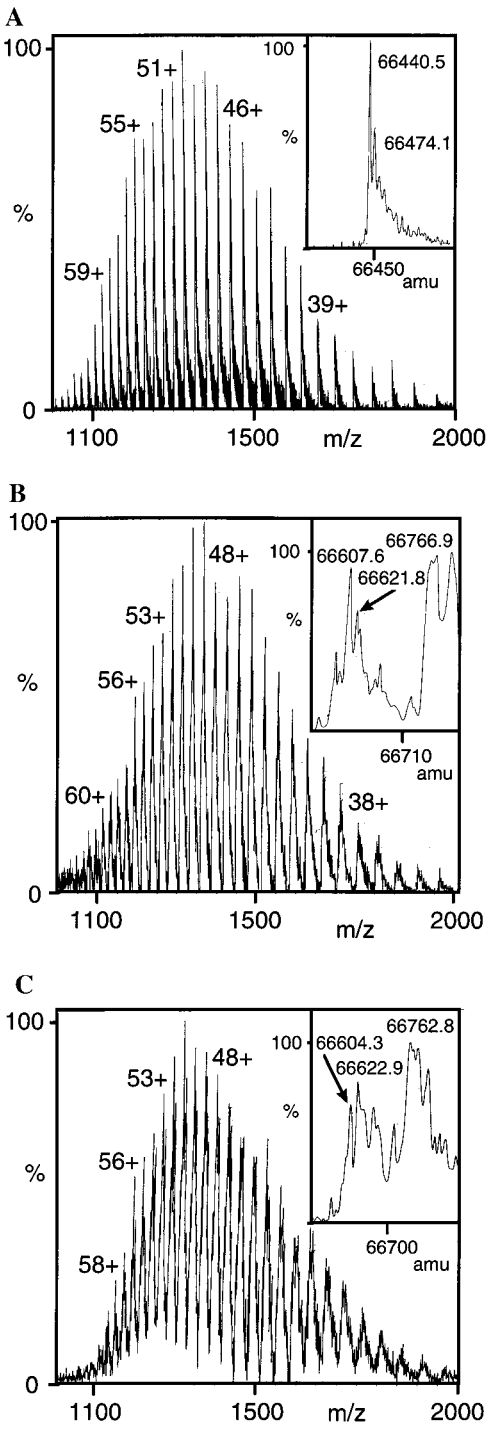


Figure 4. Electrospray mass spectra for (A) rHA and NBD-modified products of rHA with either (B) *trans,cis*-[Pt(en)$(OH)_2I_2$] or (C) H_2O_2. The MaxEnt Electrospray software algorithm was used to deconvolute the charged ion series. (A) ESI-MS analysis of rHA gave a molecular mass of 66 440.5 Da (theoretical value 66 438.41 Da). The ESI mass spectra B and C show peaks due to rHA with either one (theoretical mass: 66 604.2 Da) or two (theoretical mass: 66 768.3 Da) molecules of bound NBD, and a peak at mass 66 622, which might be due to a sulfenic acid adduct, NBD-OS−(Cys)rHA (theoretical mass of 66 621.2 amu).

was assessed by incubating rHA and KI (200 μM) in a molar ratio of 1:2 in the same buffer as that above (100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) for 24 h at 310 K. It was found that 33% of I (66 μM) was bound to the protein after the first ultrafiltration step. This was reduced to <0.45% (0.9 μM) after four ultrafiltration steps using the same buffer. Washing with

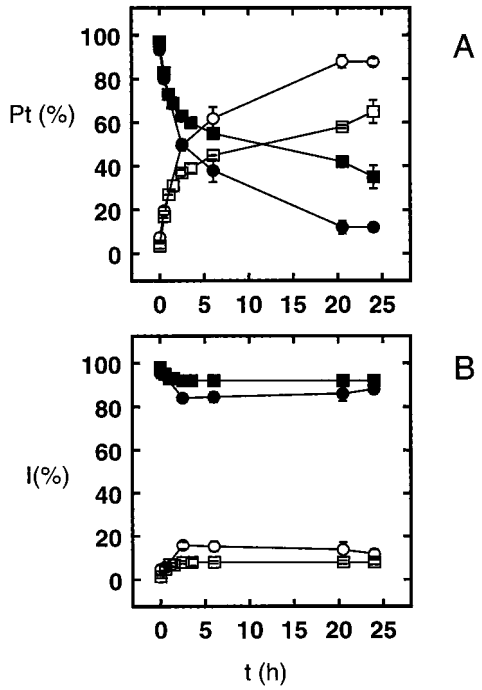


Figure 5. Reactions of *trans,cis*-[Pt(en)$(OH)_2I_2$] (□, ■) or [Pt(en)I_2] (●, ○) with rHA (mol ratio of 1:1, 100 μM, 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K): percent distribution of bound (□, ○) and unbound (■, ●) Pt (A) or I (B) determined by ICP-MS. The reaction course of Pt shows that the Pt(II) complex reacts slightly faster with rHA compared to the Pt(IV) complex, and only a small percentage of the iodide is bound to the protein at any time.

10 mM bis-tris-propane, pH 9, gave similar results. After establishing the washing procedure, the Pt(IV) complex **1** was incubated with rHA or NEM-rHA for 24 h at 310 K at a molar ratio of 1:1 (100 μM) in the same buffer. The high and low M_r fractions were separated by four cycles of ultrafiltration and analyzed by ICP-MS. The distribution of Pt and I species in these fractions is reported in Table 2. The recovery of Pt and I from the complex, *trans,cis*-[Pt(en)$(OH)_2I_2$], and from KI alone after the separation procedure was 100%. Blocking the thiol group of Cys34 led to a dramatic decrease in the amount of bound Pt, from 65.2 ± 5.3% (8.1%, RSD) for rHA to 15.0 ± 2.9% (19.3%, RSD) for NEM-rHA after 24 h. In addition, the kinetics of binding of both Pt and I to rHA after reaction with **1** at 310 K at a molar ratio of 1:1 were studied over a period of 24 h using the same procedure as described above. At 2.5 h, 92.0 ± 0.7% (0.8%, RSD) of I and 63.4 ± 1.4% (2.2%, RSD) of Pt were found in the low M_r fraction, Figure 5 (■), and only 36.6 ± 1.4 (3.7%, RSD) (□) of platinum was bound to the protein. The Pt/I ratio in the low M_r fraction decreased from 0.48 ± 0.004 (0.89% RSD) corresponding to 1 Pt:2 I in the Pt(IV) complex **1** just after mixing, to 0.18 ± 0.03 (15.5% RSD) after 24 h, indicating that iodide is released from the Pt(IV) complex. The increase in the Pt/I ratio for bound complex from 1 to 4 over 24 h suggests that the initial binding involves loss of one I^- ligand followed by the gradual loss of the second.

Two-dimensional [^{1}H,^{15}N] HSQC NMR spectroscopy provided insight into the low M_r products formed during the reaction of complex **1** with rHA. The kinetic course of the reaction of *trans,cis*-[Pt(^{15}N-en)$(OH)_2I_2$], **^{15}N-1**, with rHA and NEM-rHA at a molar ratio of 1:1 (150 μM) was studied at 310 K. After 2 h, two new cross-peaks assignable to [Pt(^{15}N-en)ClI] (50%) and [Pt(^{15}N-en)Cl_2] (10%) were observed, accounting for 60% of Pt not bound to the protein (^{15}N/^{1}H chemical shifts listed in Table 3). After 6.5 h, only a weak cross-peak for [Pt(^{15}N-en)-

Table 2. ICP-MS Determination of Percentage Distribution of Pt and I in Low and High M_r Fractions Separated after a 24 h Incubation of Pt(IV) Complexes and KI with either rHA or NEM-rHA (1:1 mol ratio, 100 μM, in 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, at 310 K)

compd		albumin	time (h)	bound Pt (%)	unbound Pt (%)	bound I (%)	unbound I (%)
cis,trans-[Pt(en)I_2(OH)$_2$]	**1**	−	0	0.5	99.5	0.5	99.5
		rHA	0	3.5 ± 1.2[a]	96.5 ± 1.2	1.4 ± 0.6	98.6 ± 0.6
			24	65.2 ± 5.3	34.8 ± 5.3	7.8 ± 1.1	92.2 ± 1.1
		NEM-rHA	0	1.2 ± 0.2	98.9 ± 0.2	0.8 ± 0.2	99.3 ± 0.2
			24	16.9 ± 2.0	83.1 ± 2.0	2.0 ± 0.1	98.0 ± 0.1
cis,trans-[Pt(en)Cl_2(OH)$_2$]	**2**	−	0	0	100		
		rHA	0	0.3 ± 0.5	99.7 ± 0.5		
			24	2.3 ± 0.5	97.7 ± 0.5		
		NEM-rHA	0	0.7 ± 0.3	99.3 ± 0.3		
			24	1.6 ± 0.1	99.0 ± 0.6		
KI		rHA	0			1.0	99.0
			24			1.5	98.5

[a] Mean ± SD, $N = 3$.

Table 3. $^{15}N/^{1}H$ NMR Chemical Shifts of Pt(II) and Pt(IV) Complexes

complex		δ ($^{15}N/^{1}H$)	^{15}N *trans* to
trans,cis-[Pt(^{15}N-en)(OH)$_2$$I_2$]	**1**	13.92/6.52	I
[Pt(^{15}N-en)I_2]	**3**	−14.69/5.12	I
[Pt(^{15}N-en)ClI]	**4**	−35.70/5.14	Cl
		−11.43/5.28	I
[Pt(^{15}N-en)Cl_2]	**5**	−31.35/5.23	Cl
[Pt(^{15}N-enH)I(OH)$_2$]$^-$ [a]		−26.92/5.56	I

[a] This long-lived ring-opened Pt(II) species was detected after reaction of *trans,cis*-[Pt(^{15}N-en)(OH)$_2$$I_2$] with glutathione.[14]

Cl_2] was visible, and the cross-peaks for [Pt(^{15}N-en)ClI] had disappeared. Cross-peaks for protein-bound ^{15}N−Pt species were not observed and were presumably too broad to be detected since there was a reduction in the total peak intensity in the 2D [^{1}H,^{15}N] HSQC NMR spectrum of about 8-fold.

Incubation of **^{15}N-1** with NEM-rHA for 24 h at 310 K gave rise only to the $^{15}N/^{1}H$ cross-peak for the starting material, indicating that *trans,cis*-[Pt(^{15}N-en)(OH)$_2$$I_2$] is stable when Cys34 is blocked.

Oxidation of Cys34 with H_2O_2 and I_2. H_2O_2 is known to oxidize Cys34 of albumin to sulfenic acid,[16,17] and reaction with iodine gives rise to the sulfenyl iodide derivative.[18] The thiol content of rHA (100 μM) was determined after incubation with either H_2O_2 or I_2 at different molar ratios for 20 min at 310 K. Excess of reagent was removed by three cycles of ultrafiltration. Figure 2 (inset, open bars) shows that iodine (1:1 mol ratio) and H_2O_2 (1:3 mol ratio) reduced the thiol content of rHA from 0.79 to 0.14 (82%) within 20 min. The amount of sulfenic acid and sulfenyl iodide (see Schemes 1 and 2) formed during the reaction of rHA with H_2O_2 and iodine, respectively, was determined using arsenite. The reagents were incubated with rHA (100 μM) at different ratios in 100 mM NaCl, 10 mM NaH_2PO_4 buffer, pH 7.4, for 20 min, followed by ultrafiltration three times and further incubation for 45 min at 310 K with or without addition of excess arsenite (16.7 mM). Figure 2 (inset) shows that the recovery of reduced thiol after incubation of rHA for 20 min with either H_2O_2 (molar ratio 1:3) or iodine (molar ratio 1:1) and further incubation with arsenite (black bars) was ca. 52% for both oxidizing agents compared to 37% after a 20 min incubation with **1**, *trans,cis*-[Pt(en)(OH)$_2$$I_2$] (1:1 mol ratio).

The reaction of H_2O_2 with rHA was used to verify that NBD-Cl[21] traps the sulfenic acid derivative of Cys34 (Scheme 3). rHA and H_2O_2 were incubated in a molar ratio of 1:3 (100 μM, 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) and the reaction was stopped after 20 min at 310 K by three cycles of ultrafiltration. The sample was then incubated with 2 mol equiv of NBD-Cl for 30 min at ambient temperature, and excess NBD-Cl was removed by ultrafiltration. The UV−vis spectrum (see Figure 3) showed two absorption bands with maxima at 347 and 408 nm. The decrease in absorption at 408 nm (NBD-S−(Cys)R) compared to NBD-treated rHA indicated that Cys34 was partly oxidized after a 20 min incubation of rHA with H_2O_2. The absorption band at 347 nm is assigned to the sulfenic acid adduct NBD-OS−(Cys)R, but because NBD-treated rHA gave rise to a band at 344 nm, further confirmation of this assignment was needed. Analysis of rHA samples incubated with H_2O_2 and then treated with NBD-Cl by ESI-MS (Figure 4C) gave rise to peaks at masses of 66 604.3 and 66 762.8 amu. These can be assigned to rHA species with either one (theoretical mass: 66 604.2 Da) or two (theoretical mass: 66 768.3 Da) bound molecules of NBD. A major peak at 66 622.9 amu was detectable in the ESI-MS spectrum, which is consistent with the expected value for the sulfenic acid adduct NBD-OS−(Cys)R (theoretical mass: 66 621.2 amu).

Reactions of rHA with the Dichloro Complex *trans,cis*-[Pt(en)(OH)$_2$$Cl_2$] (2). The thiol content of rHA was determined 24 h after incubation with complex **2** (100 μM, molar ratio of 1:1, 100 mM NaCl, 10 mM NaH_2PO_4 buffer, pH 7.4). Three cycles of ultrafiltration were used to remove unbound Pt complex. Table 1 shows the thiol content of rHA 24 h after reaction with the Pt(IV) complexes **1** and **2**. In contrast to the diiodo Pt(IV) complex, the dichloro complex **2** did not affect the thiol content of rHA compared to the control. To determine if complex **2** was binding at sites other than Cys34, Pt determinations were carried out by ICP-MS. Complex **2** was incubated with rHA or NEM-rHA for 24 h at 310 K (molar ratio of 1:1, 100 μM, 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4), and the high M_r and low M_r fractions were then separated by four cycles of ultrafiltration and analyzed by ICP-MS. As can be seen from Table 2, complex **2** did not react with either rHA or NEM-rHA, showing that the presence of iodide ligands in *trans,cis*-[Pt(en)(OH)$_2$$I_2$] is critical for its reaction with Cys34 of rHA.

Reactions of rHA with the Pt(II) Complex [Pt(en)I_2] (3). Effect on Cys34. Complex **3** was incubated with rHA in 100 mM NaCl, 10 mM NaH_2PO_4 buffer, pH 7.4, at a molar ratio of 1:1 (100 μM), and samples were taken at 0, 0.6, 1.1, 4.5, and 24 h. Low M_r Pt and I species were removed by three cycles of ultrafiltration before determining the thiol content of rHA. The diiodo Pt(II) complex did not affect the thiol content of rHA during the 24 h period (Table 1). In view of this, the Pt(II) complex was chosen as a control to assess if arsenite, *m*-$NaAsO_2$, which was employed to determine the amount of reversibly oxidized sulfur species (see Schemes 1 and 2) formed during the reaction of rHA with **1**, was affected by the presence of Pt(II). Complex **3** was incubated with rHA at a molar ratio

of 1:1 (100 μM) in the same buffer and samples were taken at 0, 0.57, 1.08, 4.5, and 24 h. The reaction was stopped by three cycles of ultrafiltration, and aliquots of the protein fraction were then further incubated for 45 min at 310 K with or without addition of excess arsenite (16.7 mM). The thiol content after the incubation was determined using Ellman's reagent (DTNB). It was apparent that Pt(II) did not affect the determination of the thiol content in the arsenite assay, since the same results were obtained for the controls (Figure 2, inset).

The possibility that [Pt(en)I_2] binds at sites other than Cys34 of rHA was then investigated. Samples from a 1:1 mixture (100 μM, 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, 310 K) were taken over a period of 24 h (Figure 5, circles), and the distribution of Pt and I in the high and low M_r fractions separated by four cycles of ultrafiltration were determined by ICP-MS. The Pt(II) complex (Figure 5, circles) reacted slightly faster with rHA than the diiodo Pt(IV) complex **1** (Figure 5, squares). After 2.5 h, 49.6% of the Pt was bound to the protein and 84.2% after 24 h, whereas only 63.4% of the Pt was bound to rHA after reaction with the diiodo Pt(IV) complex **1**. The reaction of [Pt(^{15}N-en)I_2], **^{15}N-3**, with rHA in a 1:1 molar ratio (150 μM, 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, 310 K) was also followed by 2D [^{1}H,^{15}N] HSQC NMR spectroscopy. Only [^{1}H,^{15}N] peaks for [Pt(^{15}N-en)Cl_2] and [Pt(^{15}N-en)ClI] were observed, and no cross-peaks for protein-bound platinum were detected (^{15}N/^{1}H chemical shifts, see Table 3). After 2.5 h, 50% of Pt, which was not bound to the protein, could be assigned to [Pt(^{15}N-en)I_2] (33%), [Pt(^{15}N-en)ClI] (15%), and [Pt(^{15}N-en)Cl_2] (2%), by 2D [^{1}H,^{15}N] HSQC NMR spectroscopy.

Discussion

Anticancer Pt(IV) complexes, such as orally active JM216, *cis,trans,cis*-[$PtCl_2(OAc)_2NH_3$(*c*-$C_6H_{11}NH_2$)], are thought to be prodrugs reduced to their active chloro Pt(II) complexes by biomolecules.[4,7b,12] It is known that the redox reactivity of Pt(IV) complexes is strongly dependent on the coordinated ligands. Replacement of axial chloro ligands by acetato or hydroxo ligands, for example, stabilizes the Pt(IV) complex against attack by reducing agents.[25] Replacement of chelating amine ligands, however, by mono-coordinated am(m)ine ligands, like ammonia, pyridine, and 3-picoline, increases the redox potentials of Pt(IV) complexes and their reactivity.[26] Studies of redox reactions of Pt(IV) complexes with inorganic reductants, such as thiocyanate, iodide, and thiosulfate, have shown that Pt(IV) complexes are reduced more easily as the π-acceptor character of the ligands increases and the σ-donor character decreases.[27] It has also been noted by Peloso that sterically hindered ligands with low σ-donor character increase the reactivity of Pt(IV) complexes toward inner and outer sphere redox reactions.[28]

Key reactions in a biological context involve thiolates.[7b,12,13] Since the major thiol in blood plasma is albumin, we have studied reactions of the photoactive complex *trans,cis*-[Pt(en)-$(OH)_2I_2$] (**1**) with this protein and compared them with those of the dichloro analogue of **1**, *trans,cis*-[Pt(en)$(OH)_2Cl_2$] (**2**), and with those of the diiodo Pt(II) complex [Pt(en)I_2] (**3**). Little attention has previously been paid to the biological chemistry of iodo Pt(II) am(m)ine complexes,[10] probably because of the early findings that *cis*-[$PtI_2(NH_3)_2$] is inactive as an anticancer agent.[5] It is usually assumed that this is due to the high stability and low reactivity of Pt(II)−I bonds in aqueous solution.[5,9a] Our new data are remarkable in that they demonstrate entirely the opposite trend: Pt(II)−I bonds can be very reactive.

In the past, chemical studies on albumin have often been hampered by its heterogeneity.[15,19] Typically, commercial human albumin has a free thiol content of only ca. 0.1 SH mol^{-1}.[23] In this work, we have been able to use recombinant human albumin (rHA) in which Cys34 is largely unblocked, the thiol content being ca. 0.85 SH mol^{-1}.[19] The courses of reactions have been followed by UV−vis (Pt(IV)−I LMCT band), ICP-MS and NMR spectroscopy (with ^{15}N-labeling of en), gel filtration chromatography, and determination of the extent of thiol blocking using DTNB assays. The nature of the modifications at Cys34 have been identified via mass spectrometry trapping of adducts with NBD-Cl and arsenite assays.

Reactions of Pt Complexes with the Thiol of rHA, Cys34. The study of the LMCT band of *trans,cis*-[Pt(en)$(OH)_2I_2$] (**1**) showed that it reacted only with recombinant human albumin (rHA), not with Cys34-blocked rHA (NEM-rHA), indicating that the thiol of Cys34 plays a dominant role in this reaction. The diiodo Pt(IV) complex induced a complete blockage of the thiol group of Cys34 within 1 h. The pH profile of the reaction resembles that reported previously for reactions of human albumin with the oxidant dithiopyridine.[29] The minimum rate of the reaction is close to the pK_a of Cys34 (between 5 and 7),[15,30] a pK_a which is low compared to that of Cys in smaller peptides, for example, glutathione (pK_a 8.7).[31] The increase in reactivity at higher pH is probably attributable to the increased proportion of $CysS^-$ present, and at low pH to the increased accessibility due to a conformational change in the protein (N−F transition).[15] The importance of the iodo ligands of complex **1** was apparent from studies of the analogous chloro complex *trans,cis*-[Pt(en)$(OH)_2Cl_2$] (**2**) which had no effect on the thiol content of rHA. Also, the Pt(II) diamines, [Pt(en)I_2] and [Pt(en)Cl_2], had little effect on the SH content of rHA, and therefore platination of Cys34 does not appear to play a major role in reactions of albumin with Pt(II) diamines. We have reached a similar conclusion for reactions of cisplatin with rHA.[19]

Nature of the Cys34 Modification Induced by the Pt(IV) Complex *trans,cis*-[Pt(en)$(OH)_2I_2$]. Reaction of complex **1** with glutathione (GSH) gives rise to GSSG,[14] but the formation of a disulfide-bridged albumin dimer was ruled out since only monomeric albumin was detected by gel filtration. It is known that Cys34 sulfenic acid is produced on reaction of albumin with H_2O_2[16,17] and that iodine oxidizes the thiol to the sulfenyl iodide derivative.[18] Sulfur in the oxidation state zero, as in sulfenic acid and sulfenyl iodide, can be reduced to the thiol by arsenite, and recovery of the thiol can be determined. Studies with arsenite showed that the thiol at Cys34 of rHA could be partly recovered (37%) after reaction with the diiodo Pt(IV) complex **1**, suggesting that Cys34 sulfenic acid/sulfenyl iodide[32] was formed. The remaining irreversibly oxidized Cys34 (63%)

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may be either sulfinic or sulfonic acid arising from further oxidation of sulfenic acid by oxygen. Similar thiol Cys34 recoveries (ca. 52%) were achieved with arsenite after incubation of rHA with H_2O_2 and I_2, reagents which are known to give sulfenic acid and sulfenyl iodide species. It was clear that the increase of the thiol content after reaction with arsenite in the case of complex **1**, iodine, and H_2O_2 is due only to the formation of sulfenic acid/sulfenyl iodide, because no change of thiol content was observed after addition of arsenite during the reaction of [Pt(en)I_2], **3**, with rHA. Radi et al.[17] have reported that 52% of the thiol of bovine albumin is oxidized to the sulfenic acid by H_2O_2. Reaction of glutathione and L-cysteine with albumin−sulfenic acid formed by reaction with NO, H_2O_2, and peroxynitrite are thought to be important pathways for the formation of mixed disulfides in plasma.[16]

The electrophilic trapping agent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was used to detect the formation of sulfenyl iodide and sulfenic acid during reaction of the diiodo Pt(IV) complex **1** with rHA. The absorption bands of the NBD adducts allow identification of the thiol derivative (Scheme 3).[21,24] UV−vis experiments showed that rHA reacts with two molecules of NBD-Cl, giving rise to the thiolate trapped species Cys34-S−NBD (λ_{max} 408 nm) and an unknown NBD adduct (λ_{max} 344 nm). The latter was still present after treatment of rHA with the Cys34 blocking agent (NEM) and must therefore be due to a nonthiol adduct. NBD-Cl is also known to form adducts with phenols (Tyr) and amino groups,[24] which might be responsible for the second NBD-rHA product. The Cys34-S(O)−NBD adduct (λ_{max} 347 nm) could not clearly be identified by UV−vis spectroscopy because of the presence of the overlapping band at 344 nm. In contrast to rHA, no interfering band at 344 nm has been reported for protein tyrosine phosphatases[21a] and alkyl hydroperoxide reductase.[21b] In the latter cases, the absorption maximum of the Cys-S−NBD product occurred at 420 nm, compared to 408 nm in our work.

Analysis of rHA samples treated with NBD-Cl by ESI-MS after incubation with either H_2O_2 or *trans*,*cis*-[Pt(en)$(OH)_2I_2$] (**1**), however, gave rise to a major peak at 66 622 amu in both spectra, which is consistent with the formation of the sulfenic acid adduct NBD-OS−(Cys)R (66 621 amu). This confirms that a sulfenic acid intermediate is formed during the reaction of **1** with rHA.

Fate of the Iodide Ligands. The simultaneous determination of Pt and I by ICP-MS showed clearly that iodide was released from *trans*,*cis*-[Pt(en)$(OH)_2I_2$] during reaction with rHA, since the Pt/I ratio in the low M_r fractions decreased from 0.5 (the Pt/I ratio of complex **1**) to 0.2 during the reaction. The detection of 92% of I in the low M_r fraction indicates that sulfenyl iodide, formed during the attack of *trans*,*cis*-[Pt(en)$(OH)_2I_2$] on Cys34, is rapidly hydrolyzed to sulfenic acid. Our results are consistent with earlier studies, which reported that iodide is not incorporated into proteins after oxidation of cysteine by iodine/iodine monochloride, suggesting that sulfenyl iodide is hydrolyzed to sulfenic acid.[20,33]

The increase in the Pt/I ratio for the high M_r fraction from 1.0 (monoiodo platinum−protein adduct) to 2.0 within the first 2 h suggests either that there is substitution of the second iodo ligand by chloride or that chelated Pt adducts without iodo ligands are formed. Little reaction of *trans*,*cis*-[Pt(en)$(OH)_2I_2$] with the Cys34-blocked rHA was observed, indicating that Cys34 is responsible for the reduction process.

In contrast, the dichloro analogue of **1**, *trans*,*cis*-[Pt(en)$(OH)_2Cl_2$] (**2**), did not react with either rHA or Cys34-blocked rHA (NEM-rHA). Similarly, other Pt(IV) complexes with axial hydroxo/acetato ligands, such as anticancer drugs JM216 (*cis*,*trans*,*cis*-[$PtCl_2(OAc)_2NH_3$(*c*-$C_6H_{11}NH_2$)]), JM383 (*cis*,*trans*,*cis*-[$Pt(OH)_2(OAc)_2NH_3$(*c*-$C_6H_{11}NH_2$)]), and CHIP (*cis*,*trans*,*cis*-[$PtCl_2(OH)_2$(*i*-$C_3H_7NH_2)_2$]), have been reported not to react with human albumin after 24 h.[34] Pt(II) analogues of JM216 and CHIP, which have lost the axial OAc or OH ligands, have been detected in human plasma, but the endogenous reducing agents have yet to be identified.[4,35]

Unexpectedly, the Pt(II) complex [Pt(en)I_2] (**3**) reacted faster with rHA than *trans*,*cis*-[Pt(en)$(OH)_2I_2$]. Intriguingly, [Pt(en)I_2] reacted rapidly with rHA via reactions which do not appear to give products in which Pt is bound to Cys34 (no thiol blockage), although most of the Pt (but not iodide) is bound at other sites. Since complex **3** reacts rapidly with L-methionine forming the chelate [Pt(en)(Met-*S*,*N*)]$^+$,[36] it is possible that the Pt(II) binding sites are the sulfur atoms of methionine residues in albumin (Met 87, 123, 298, 329, 446, and 548),[19] especially Met 298, which is positioned on the surface of the protein.

Formation of Chloro Complexes during the Reactions of Pt Complexes with rHA followed by NMR. For physiological relevance, reactions of *trans*,*cis*-[Pt(^{15}N-en)$(OH)_2I_2$] and [Pt(^{15}N-en)I_2] with rHA were carried out in a chloride-containing buffer. The formation of 50% [Pt(^{15}N-en)ClI] and 10% [Pt(^{15}N-en)Cl_2] was detected as low M_r products (60% unbound Pt) by NMR spectroscopy after 3 h. These chloro complexes can then react with the protein via substitution reactions. The detection of [Pt(^{15}N-en)ClI] after the redox reaction of *trans*,*cis*-[Pt(^{15}N-en)$(OH)_2I_2$] with rHA is in agreement with the detection of free iodide by ICP-MS. Cross-peaks for protein-bound ^{15}N−Pt species were not observed, although there was an 8-fold reduction in the total peak intensity in the 2D [^{1}H,^{15}N] HSQC NMR spectrum, presumably because of broadening due to slow tumbling and low mobility. Importantly, the reaction of [Pt(^{15}N-en)I_2] with albumin was faster than that of the analogous chloro complex, [Pt(^{15}N-en)Cl_2].[37] After 2 h, only 33% [Pt(^{15}N-en)I_2], 15% [Pt(^{15}N-en)ClI], and 2% [Pt(^{15}N-en)Cl_2] were found as low M_r products (50% of unbound Pt). Such enhanced reactivity of iodo complexes toward biomolecules does

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not appear to have been recognized previously, although it has been noted that [Pt(en)I_2] undergoes more rapid solvolysis in DMSO than the chloro complex.[9d] Also, the iodo ligand of [Pt(dienMe)I]$^+$ is replaced much faster by halides, SCN^-, and thiourea than the chloro ligand in [Pt(dienMe)Cl]$^+$ due to greater nucleophilic discrimination, whereas the hydrolysis of the iodo complex is slower.[9c]

Significance of Sulfenic Acid and Sulfenyl Iodide Derivatives of Proteins. Recently, X-ray crystal structures of low M_r organic sulfenic acid[32d,e] and sulfenyl iodide[38] derivatives have been reported. These provide clues as to how such reactive intermediates might be stabilized in proteins. The reversible oxidation of cysteines in catalytic sites of proteins to sulfenic acids[39] by second messengers, such as H_2O_2 and NO, is important for the intracellular redox regulation of the activity of DNA binding domains of proteins, such as nuclear factor I (NFI),[39g] bovine papillomavirus,[39a] Fos, Jun,[39h] and OxyR transcription factors[39i] and enzymes, such as NADH peroxidase,[39c] peroxiredoxins (Prx),[39f] papain and glyceraldehyde-3-phosphate dehydrogenase,[39j] alkyl hydroperoxide reductase,[21b] creatine kinase,[33a] and tyrosine phosphatases.[21a] Criteria for the stabilization of Cys-SOH in proteins are[39c] (1) the absence of other vicinal protein thiols, (2) limited solvent accessibility and association with apolar elements of the protein structure, (3) ionization to the conjugate sulfenate base, and (4) intramolecular hydrogen bonding. In rHA, Cys34 is the only cysteine in the protein and is situated in a hydrophobic crevice, which would fulfil the first two criteria. Remarkably, Tyr84 lies near to Cys34 (see Figure 6, Cys34-S to Tyr84-O distance: 2.7 Å) and may provide an apolar environment and limit solvent accessibility. His39 is within 5 Å of Cys34 and may play a role in lowering the pK_a of the thiol and in the stabilization of the sulfenate base. A similar interaction has been observed in the X-ray structure of enterococcal NADH peroxidase[39c] and human peroxidase.[39f]

Conclusion

These studies show that both the Pt(IV) and the Pt(II) diiodo complexes *trans,cis*-[Pt(en)$(OH)_2I_2$] and [Pt(en)I_2] are highly reactive toward human albumin, the major protein in blood plasma. The single free thiol group at Cys34 might be expected to reduce the Pt(IV) complex to Pt(II). However, the reaction pathway does not simply involve loss of hydroxo ligands and formation of [Pt(en)I_2] together with albumin disulfide. The mechanism appears to involve attack of the thiolate of Cys34 on an **iodo ligand** of *trans,cis*-[Pt(en)$(OH)_2I_2$] (Scheme 4), forming Cys34 sulfenyl iodide, which is then hydrolyzed to sulfenic acid. The sulfenic acid may then be further oxidized to sulfinic or sulfonic acid. Interactions within the crevice of albumin containing Cys34 probably control the kinetic and thermodynamic stability of reactive sulfenyl iodide and sulfenic acid derivatives, and this may be crucial to the natural biological activity of albumin.

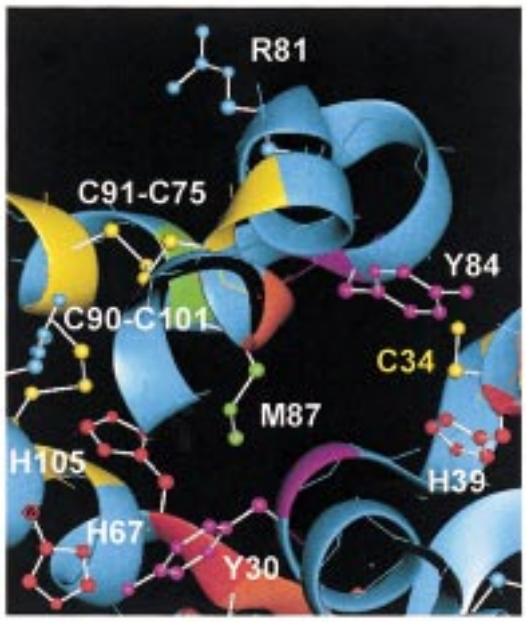
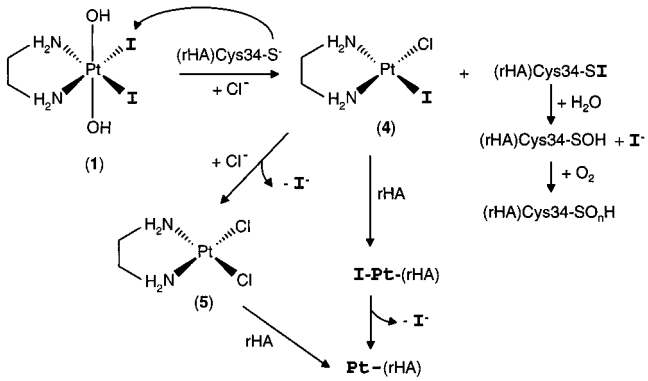


Figure 6. View of the region around Cys34 in the X-ray crystal structure of human albumin, based on S. Sugio, S. Mochizuki, M. Noda, A. Kashima **1997**, PDBid 1A06. Cys34 lies in a hydrophobic pocket and is close to Tyr84 and His39. Both interactions may play a role in lowering the pK_a of Cys34 and stabilization of the sulfenic acid.

Scheme 4. Proposed Mechanism for the Reduction of *trans,cis*-[Pt(en)$(OH)_2I_2$] (**1**) by rHA[a]



[a] Initially, Cys34 attacks a coordinated iodo ligand of **1**, giving rise to the sulfenyl iodide which then hydrolyzes to the sulfenic acid, together with the monoiodo monchloro Pt(II) complex **4** (100 mM NaCl present in the medium). This Pt(II) complex can undergo further substitution reactions, giving rise to [Pt(en)Cl_2] (**5**), and adducts with the protein at sites other than Cys34. Further oxidation of the sulfenic acid is irreversible.

The reduction of *trans,cis*-[Pt(en)$(OH)_2I_2$] by glutathione appears to occur via attack of the thiol on an iodo ligand of the Pt(IV) complex, forming a reactive chelate-ring-opened Pt(II) ethylenediamine complex.[14,40] Attack on the highly reactive sulfenyl iodide by another glutathione molecule gives rise to the disulfide. The ring-opened Pt(II) species undergoes further ring-closure reactions and reacts with the released I^-, forming

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$[Pt(en)I_2]$ and with RSH or RSSR[41] giving $[\{Pt(en)(\mu\text{-}SR)\}_2]^{2+}$.[42] A similar electron-transfer-driven *trans*-ligand labilization reaction probably occurs during reactions of *trans*,*cis*-$[Pt(en)(OH)_2I_2]$ with rHA.[43] The ring-opened Pt(II) species undergoes rapid ring closure and reacts with the large excess of chloride ions forming [Pt(en)ClI], which is the only product observed by NMR spectroscopy. The Pt(II) complex, [Pt(en)ClI], can then undergo substitution reactions with chloride ions and rHA forming, $[Pt(en)Cl_2]$ and Pt−protein adducts. Surprisingly, in these Pt−protein adducts, Pt is not bound to Cys34. Our ICP-MS data show that iodide is released from the Pt(IV) complex during the redox reaction. In contrast to reactions of complex **1** with the low M_r thiol glutathione, for which glutathione disulfide is the major product, rHA can stabilize the reactive intermediate sulfenic acid of Cys34.

These new findings of the unexpectedly enhanced reactivity of iodo platinum complexes toward the abundant blood plasma protein albumin, which is also an essential component of most cell culture media, will be of value when iodide ligands are considered in Pt drug design. Interestingly, mono(iodo)mono(chloro) Pt(II) diam(m)ines, such as *cis*-$[Pt(NH_3)_2ClI]$, are antitumor-active[5] like the dichloro complexes, and therefore, the high cytotoxicity of the diiodo Pt(IV) complexes against tumor cells in vitro in the absence of light can be explained.[11a] Improved understanding of how thermodynamic and kinetic control of the redox state of albumin can be achieved may lead to the design of novel drugs and to a better understanding of the natural functions of this protein.

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