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Cisplatin Binding Sites on Human Albumin*

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Reactions of cisplatin (*cis*-[$PtCl_2(NH_3)_2$]) with albumin are thought to play an important role in the metabolism of this anticancer drug. They are investigated here via (i) labeling of cisplatin with ^{15}N and use of two-dimensional $^1H,^{15}N$ NMR spectroscopy, (ii) comparison of natural human serum albumin with recombinant human albumin (higher homogeneity and SH content), (iii) chemical modification of Cys, Met, and His residues, (iv) reactions of bound platinum with thiourea, and (v) gel filtration chromatography. In contrast to previous reports, it is shown that the major sulfur-containing binding site involves Met and not Cys-34, and also a N ligand, in the form of an S,N macrochelate. Additional monofunctional adducts involving other Met residues and Cys-34 are also observed. During the later stages of reactions of cisplatin with albumin, release of NH_3 occurs due to the strong *trans* influence of Met sulfur, which weakens the Pt-NH_3 bonds, and protein cross-linking is observed. The consequences of these findings for the biological activity of cisplatin-albumin complexes are discussed.

Cisplatin, *cis*-[$PtCl_2(NH_3)_2$], is extensively used for the treatment of testicular and ovarian cancers and increasingly against other types of solid tumors (head/neck, lung, cervical, and bladder) (1–4). The cytotoxic effect of cisplatin is thought to be due to attack on DNA bases and induction of apoptosis in cancer cells (5, 6). It is also able to bind to a number of extra- and intracellular proteins (7–11). Inactivation of certain enzymes due to cisplatin binding is likely to be responsible for its appreciable side effects, mainly nephrotoxicity and ototoxicity (12–14).

In body fluids, cisplatin is readily attacked by nucleophiles with exchange of one or both chloride ligands to form high and low molecular mass complexes (15, 16). One day after rapid intravenous infusion of cisplatin, 65–98% of platinum in blood plasma is protein-bound (17–19), while no unbound platinum has been detected at any time in blood plasma of patients after slow 20-h infusions (18). Protein binding results in significantly lower urinary excretion and an increased tissue deposition of platinum (18).

Reaction between cisplatin and serum albumin is thought to be the main route for platinum binding in human blood plasma (16). Cisplatin binding to albumin is essentially irreversible with less than 5% loss of protein-bound platinum after extensive dialysis (15, 20).

Human serum albumin (HSA)[1] is a single-chain 66-kDa protein, which is largely α-helical and consists of three structurally homologous domains, organized into a heart shape (21). HSA contains 17 disulfide bridges and one free thiol at Cys-34. Albumin is known to bind to various endogenous metabolites, metal ions, and drugs (21, 22). Binding of drugs to serum albumin affects their metabolism, efficacy, and body distribution (23).

Although it is widely thought that albumin-bound platinum is therapeutically inactive (19, 24, 25), several clinical and experimental observations are in conflict with this opinion. (i) Hypoalbuminemic patients respond poorly to cisplatin treatment (26–28), (ii) the infusion of preformed cisplatin-albumin complexes significantly increases patient survival times (29, 30), and (iii) HSA-cisplatin complexes are cytotoxic to carcinoma cells (29, 31). Thus, the antitumor activity of infused cisplatin may be determined by both free and albumin-bound drug. Additionally, albumin binding may prevent some of the side effects of cisplatin treatment, especially its nephrotoxicity (30, 32). Therefore, an understanding of the molecular mechanism of albumin-cisplatin interactions may have an impact on the optimization of strategies for cisplatin treatment.

Despite numerous studies of cisplatin-albumin binding during the last few decades (7, 15, 18, 19, 33–37), the precise mechanism of interaction is still poorly understood. Gonias and Pizzo reported that carboxyamidomethylation of the SH group in bovine and human serum albumin resulted in a 4–5-fold decrease in platinum binding and suggested formation of a single monodentate complex of cisplatin with the free sulfhydryl group (Cys-34) (7, 33). Bovine non-mercaptalbumin, obtained via reaction with cystine, also had a low affinity for cisplatin, compared with mercaptalbumin (38). However, the disulfide-type dimers of BSA with no free SH groups still bound substantial amounts of cisplatin (38). In addition, blocking the free SH group has been reported to have no effect on the binding of 2 mol eq of transplatin to HSA (7). These data suggest the existence of additional platinum binding sites on albumin. Momburg *et al*. (34) also suggested the existence of two albumin binding sites for cisplatin, but apart from Cys-34, the nature of the other cisplatin binding site was not elucidated.

The aim of the present work was to study the reaction of cisplatin with intact and chemically modified recombinant hu-

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[1] The abbreviations used are: HSA, human serum albumin; rHA, recombinant human albumin; BSA, bovine serum albumin; HSQC, heteronuclear single-quantum coherence; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); FPLC, fast protein liquid chromatography.

man albumin (rHA), and with HSA using one-dimensional ^1H and two-dimensional $^1H,^{15}N$ HSQC NMR spectroscopy to characterize platination sites under different experimental conditions. Two-dimensional $^1H,^{15}N$ HSQC NMR spectroscopy has proved to be extremely powerful in elucidating the chemistry and biochemistry of ^{15}N-labeled platinum ammine complexes. The ^{15}N chemical shift of the ammine is diagnostic of the ligand coordinated to platinum in the *trans*-position (39). Recent studies have included those on the interaction of platinum drugs with nucleic acids, amino acids, and peptides (39–41).

The experiments reported here reveal for the first time that strong binding sites other than Cys-34 play a major role in albumin-cisplatin interactions in solution.

EXPERIMENTAL PROCEDURES

Materials—*cis*-$[PtCl_2(^{15}NH_3)_2]$ and *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ were prepared according to literature procedures (42). Iodoacetamide, *N*-ethylmaleimide, 5,5′-dithio-bis(2-nitrobenzoic acid), Norit-A (Sigma), iodomethane (Merck), diethylpyrocarbonate (Acros-Organic), DEAE-Sepharose Fast Flow, SP-Sepharose Fast Flow (Amersham Pharmacia Biotech), and polyethylene glycol 4000 (Fluka) were used as received. All other chemicals were of the highest quality available from Sigma and Aldrich.

Albumin Samples—rHA was prepared by Delta Biotechnology Ltd (batches GA950202 and R970103). Samples of rHA were extensively dialyzed against 100 mM ammonium bicarbonate, pH 7.9, and freeze-dried. HSA was obtained from the Scottish National Blood Transfusion Service (batches HA(20)60440 and SPPS (P-3-671)). Crude HSA was precipitated with 25% polyethylene glycol, purified by sequential chromatography on DEAE-Sepharose and SP-Sepharose Fast Flow (43), and defatted by activated charcoal (44). Purified samples were dialyzed against 100 mM ammonium bicarbonate and freeze-dried.

rHA Chemical Modification—Cys-34-blocked rHA was prepared by incubation of albumin solution (40–60 mg ml^{-1}) in 100 mM ammonium bicarbonate, pH 7.9, with 2 mol eq of iodoacetamide (45) or *N*-ethylmaleimide (46) for 12–16 h at ambient temperature in the absence of light. Excess of the blocking agent was removed by dialysis against the same buffer.

Methionine residues were methylated by reaction with iodomethane (47). Iodomethane in 500-fold molar excess was added to an rHA solution (30 mg ml^{-1}) in 0.1 M citric acid-phosphate buffer, pH 4.0. The two-phase solution was stirred at ambient temperature for 20 h in the dark. At the end of this period, iodomethane was removed by dialysis against 0.2% w/v NaN_3 and then water.

Histidine residues were modified by diethylpyrocarbonate (48). This reagent was added to a concentration of 10 mM to rHA solutions (40 mg ml^{-1} or 600 μM) in 0.1 M phosphate, pH 6.0. Solutions were stirred at ambient temperature for 18 h, followed by dialysis against water. The number of modified histidine residues was calculated according to the molar absorption for *N*-carbethoxyimidazole at 240 nm: $\Delta\epsilon_{240} = 3200$ M^{-1} cm^{-1} (48).

Preparation of rHA-Cisplatin Complexes—In most experiments, *cis*-$[PtCl_2(^{15}NH_3)_2]$ was freshly dissolved in 10 mM phosphate, 100 mM KCl, to a concentration of 1 mM. Freeze-dried albumin was added to an equivalent concentration, followed by the addition of deuterium oxide to 10% to provide an NMR lock signal. The final pH of the solution was adjusted to 6.4 using small aliquots of 2.0 M NaOH. In several experiments, rHA was dissolved to a final concentration of 1 mM in buffer solutions containing 0.5–2 mM cisplatin.

Determination of Free SH Groups—The free SH content of albumin was determined after 24 h of incubation with cisplatin. Free cisplatin was removed by ultrafiltration with a Centricon-10 concentrator (Amicon, Beverly, MA). The thiol contents of rHA and HSA were estimated using Ellman's reagent, 5,5′-dithio-bis(2-nitrobenzoic acid) (49, 50). The amount of generated *p*-nitrothiolate anion was calculated using the extinction coefficient 13,600 M^{-1} cm^{-1} at 412 nm (49). The concentration of albumin was measured using the absorption at 279 nm assuming A_{279} (1 mg ml^{-1}, 1 cm) = 0.556 (51).

FPLC—Gel filtration of cisplatin-albumin complexes was carried out on a Superdex 200 HR column (internal diameter 1 cm; length 30 cm) with an FPLC system (Amersham Pharmacia Biotech) at ambient temperature. Albumin samples were diluted to a concentration of 5 mg ml^{-1} and 200 μl of the protein solution was loaded onto the column. Elution conditions were 50 mM sodium phosphate, 100 mM NaCl, pH 7.0, with flow rate of 0.5 ml min^{-1} and detection at 280 nm.

NMR Spectroscopy—One-dimensional ^1H and two-dimensional $^1H,^{15}N$ HSQC NMR spectra were acquired at 500.13 MHz using a Bruker DMX500 NMR spectrometer equipped with a tunable triple resonance (TBI) probehead [$^1H,^{13}C,X$] incorporating an actively shielded *z*-field gradient coil. One-dimensional ^1H NMR spectra were acquired using a double-pulsed-field-gradient spin-echo pulse scheme (52), based on the WATERGATE sequence, namely $90°_x$-G_z^a-$^{sel}90°_{-x}$-$180°_x$-$^{sel}90°_{-x}$-G_z^a-G_z^b-$^{sel}90°_{-x}$-$180°_x$-$^{sel}90°_{-x}$-G_z^b-ACQ. Typically 32 transients were acquired into 20,000 complex data points using digital quadrature detection over a frequency width of 10 kHz with a recycle delay of 1.4 s. Solvent-selective 90° pulses ($^{sel}90°$) were of a rectangular profile with a pulse duration of 2.3 ms. Sine-shaped *z*-gradient pulses of 2 ms duration were followed by field-gradient-recovery delays of 100 μs. Gradient strengths of 5.5% and 3.0% were used for gradients "a" and "b," respectively. Data were zero-filled to 32,000 data points and apodized using an optimal combination of an exponential line-broadening function of 2 Hz and an unshifted sine-bell function (53) prior to Fourier transformation. All spectra were recorded at 310 K.

Two-dimensional $^1H,^{15}N$ HSQC NMR data were acquired using a gradient-enhanced sequence in which coherence selection is achieved via gradient pulses (54). Typically, 48 transients were acquired over an F2 (^1H) frequency width of 6.6 kHz into 2048 complex data points for each of 128 t_1 increments in TPPI mode with an F1 (^{15}N) frequency width of 3.3 kHz. The sequence was optimized with a delay $1/(4J_{NH})$ of 3.47 ms. Decoupling during the acquisition time was achieved using a GARP decoupling scheme. Data were zero-filled once in F1 followed by apodization using a Gaussian window function in both dimensions prior to Fourier transformation and phase correction. A recycle delay of 1.5 s led to typical experiment times of ~3 h per two-dimensional data set (6 h for thiourea experiments). The reaction times stated in the text refer to the time from mixing to the mid-point of data accumulation. NMR spectra were referenced in the ^1H dimension to internal dioxan at 3.764 ppm and in the ^{15}N indirect dimension to $^{15}NH_4Cl$ in D_2O at 0 ppm (external).

One set of experiments (Fig. 6) was carried out on a Varian Unity 600 spectrometer (^1H, 600 MHz) under conditions similar to those described above.

NMR Peak Integration—All two-dimensional HSQC data acquired at 500 MHz were processed using the software XWINNMR version 1.3 (Bruker Spectrospin Ltd.). Prior to volume integration, flat baseplanes were established for all data sets by applying a baseplane correction. Two types of region were chosen for integration. (i) The overall intensity of each spectrum of protein-bound cisplatin was measured by integrating the entire two-dimensional region where signals were observed, and (ii) the volume integral of the ^1H-^{15}N signal from the starting material was measured excluding the areas in which ^{195}Pt satellites occur.

RESULTS

Reactions of cisplatin with both recombinant human albumin and human albumin isolated from blood serum were studied. Although rHA and HSA are similar in amino acid composition, secondary structure and globular packing, they differ in two respects, namely in free thiol content (approximately 0.9 mol SH mol^{-1} rHA and 0.4–0.5 mol SH mol^{-1} HSA) and in the greater structural heterogeneity of HSA compared with rHA (55). Cisplatin-albumin interactions were studied mainly at pH 6.4, where albumin exists predominantly as one structural isomer, namely the N-form (22). Several experiments were also carried out at pH 7.4 and 5.0. In most experiments, KCl was added to suppress cisplatin hydrolysis (as would be the case under extracellular conditions).

NMR Study of the Reaction of Cisplatin with Intact Human Albumin in Media with Added Chloride

One-dimensional ^1H NMR—One mol eq of cisplatin added to an albumin solution (1 mM rHA in 10 mM phosphate and 100 mM chloride, pH 6.4) induced changes in the protein one-dimensional ^1H NMR spectrum. Specifically, peaks at 2.19 ppm and 2.05 ppm significantly decreased in intensity relative to other peaks in the aliphatic region over a 17 h period at 310 K (data not shown). No other significant changes were observed. There was also a decrease in the overall signal-to-noise ratio, which was significant after 17 h of reaction.

Two-dimensional $^1H,^{15}N$ HSQC NMR—Fig. 1 shows two-

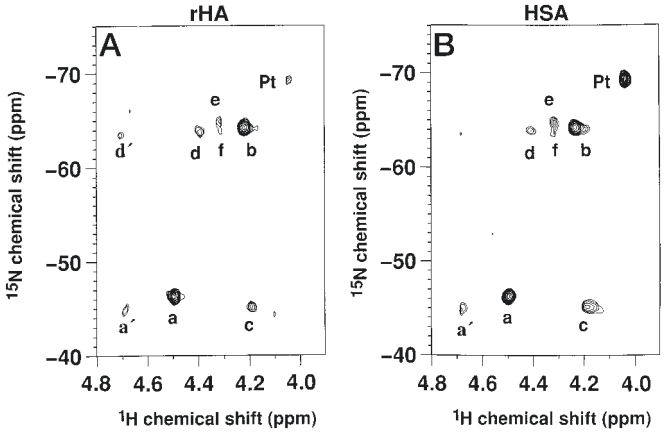


FIG. 1. **Comparison of reactions of cisplatin with recombinant human albumin and human serum albumin.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra for the reaction of rHA (*A*) and defatted HSA (*B*) with 1 mM [^{15}N]cisplatin (1:1 molar ratio); 10 mM phosphate, 100 mM KCl, pH 6.4, 310 K, 13 h after mixing. Labels are referred to in the text. Peaks **b**, **d**, **d′**, and **e/f** are in ^{15}N chemical shift region characteristic of N-Pt *trans* to N or Cl; **a**, **a′**, and **c** are in the region for N-Pt *trans* to S.

dimensional ^{1}H,^{15}N HSQC NMR spectra of solutions of 1 mM [^{15}N]cisplatin with rHA (*A*) and fatty-acid-free HSA (*B*) in a 1:1 molar ratio recorded after a 13-h incubation in 10 mM phosphate buffer, pH 6.4, with 100 mM KCl present at 310 K. The spectrum of [^{15}N]cisplatin-rHA complex contains a cross-peak due to *cis*-[$PtCl_2(^{15}NH_3)_2$] (labeled as **Pt**) and eight cross-peaks attributable to cisplatin-albumin adducts (labeled as **a**, **a′**, **b**, **c**, **d**, **d′**, **e**, and **f**). The spectrum for the [^{15}N]cisplatin-HSA reaction is very similar to that for rHA, and differs only in the higher intensity of the cross-peak from unreacted cisplatin, and in the negligible intensity of cross-peak **d′** (Fig. 1*B*). ^{15}N and ^{1}H chemical shifts of observed cross-peaks are listed in Table I.

Fig. 2*A* shows two-dimensional ^{1}H,^{15}N HSQC NMR spectra at various times during the reaction of rHA with [^{15}N]cisplatin (1 mM, 1:1), whereas Fig. 2 (*B* and *C*) shows the time dependence over a 17 h period of volume integrals of the main cross-peaks in spectra of [^{15}N]cisplatin-rHA complexes. Cross-peaks **a**, **b**, **c**, **d**, and **e/f** appeared in the first few hours of reaction, grew in intensity up to 6–9 h, and either reached a plateau (peak **b**) or slightly decreased in intensity (**a**, **c**, **d**, and **e/f**) at later times (Fig. 2, *A* and *B*). In contrast, cross-peaks **a′** and **d′**, which are close to the H_2O peak, were intense in the early stages, but had negligible intensities after 17 h of reaction (Fig. 2*A*). The increase in the intensities of the cross-peaks for bound cisplatin was accompanied by a large decrease in the signal intensity of unbound drug, which had all reacted after 17 h (Fig. 2*C*). However, the loss of intensity of signal for free cisplatin was not accompanied by a similar increase in total intensity of cross-peaks of protein-bound cisplatin, and there was an approximate 9-fold reduction in the general area intensity in the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum of [^{15}N]cisplatin-rHA complexes between 2 and 17 h of reaction (data not shown).

The most intense cross-peaks for albumin-bound cisplatin are in the ammine *trans* to sulfur region at −46.2/4.5 ppm (peak **a**) and in the ammine *trans* to nitrogen/chloride region at −64.2/4.2 ppm (peak **b**) of the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum (Fig. 1). These peaks (**a** and **b**) seem to be a pair and therefore are likely to represent two NH_3 ligands bound to the same platinum in one type of cisplatin-albumin adduct. They have similar relative intensities throughout the reaction (Fig. 2*B*) and exhibit the same behavior under the different experimental conditions described below.

TABLE I
^{1}H and ^{15}N NMR chemical shifts of cross-peaks observed during reaction of cisplatin with human albumin

See Fig. 1 for peak labels.

Peak label	$\delta(^{15}N)/\delta(^{1}H)$ for Pt-NH_3	Pt-NH_3 *trans* to
a	−46.2/4.50	S
b	−64.2/4.24	N/Cl
c	−45.3/4.21	S
d	−64.0/4.40	N/Cl
e	−64.8/4.33	N/Cl
f	−63.4/4.33	N/Cl
a′	−44.5/4.68	S
d′	−63.5/4.71	N/Cl
Pt	−69.1/4.06	Cl

The pair of cross-peaks **a′** at −44.5/4.68 ppm and **d′** at −63.5/4.71 ppm, which quickly decreased in intensity after 9 h, probably represent $\{Pt(^{15}NH_3)_2\}^{2+}$ bound to another site on albumin.

In contrast, it is difficult to pair unambiguously other signals observed in the ammine *trans* to nitrogen/chloride region at −64.2/4.3 ppm (poorly resolved peaks **e** and **f**) and −64.0/4.4 ppm (**d**), and in the ammine *trans* to sulfur region at −45.3/4.2 ppm (**c**) (Figs. 1 and 2). During the first two hours of reaction of rHA with [^{15}N]cisplatin (1 mM, 1:1), cross-peaks **d** and **c** were more intense than peaks **e/f**, but, with time, peaks **c** and **e/f** became more intense than **d** (Fig. 2). After long term reaction (25–30 h) of 1 mM rHA with 0.5 or 1 mM [^{15}N]cisplatin, only intense cross-peaks **a**, **b**, **c**, and **e/f** were observed in two-dimensional ^{1}H,^{15}N HSQC NMR spectra, while the intensity of signal **d** was negligible (Fig. 3*A*). At these reaction times, cross-peak **d** was as intense as signals **c** and **e/f** only when reactions involved a molar excess of [^{15}N]cisplatin:rHA, *e.g.* 1.5:1 (data not shown) and 2:1 (Fig. 3*B*). No signal from free cisplatin was observed in the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum after 28 h of reaction of rHA (1 mM) with 2 mol eq of [^{15}N]cisplatin (Fig. 3*B*).

To aid pairing and assignment of the above cross-peaks, we studied the reaction of albumin-bound cisplatin with thiourea, at pH 7.4. Interaction of [^{15}N]cisplatin with rHA (1 mM, 1:1) for 17 h at pH 7.4 resulted in the same signals in the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum as were described for pH 6.4 (Fig. 4*A*). Addition of thiourea (1 mM, 1 mol eq) to the above cisplatin-rHA solution significantly changed the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum recorded after a further 10 h incubation at 310 K (Fig. 4*B*). Thiourea induced the disappearance of cross-peaks **c**, **d**, and **e/f** and led to the appearance of three new signals in the ammine *trans* to nitrogen/chloride region at −66.3/4.33 ppm (labeled as $\mathbf{th}_1$), −66.3/4.42 ppm ($\mathbf{th}_2$) and −66.0/4.52 ppm ($\mathbf{th}_3$). Incubation of thiourea with [^{15}N]cisplatin alone for 5 h in 10 mM phosphate buffer, 100 mM KCl, pH 7.4, gave rise to cross-peaks with ^{195}Pt satellites in the ammine *trans* to nitrogen/chloride region ($\mathbf{th}_4$ at −67.6/4.18 ppm) and in the ammine *trans* to sulfur region ($\mathbf{th}_5$ at −45.0/4.10 ppm, $\mathbf{th}_6$ at −43.5/4.01 ppm, and $\mathbf{th}_7$ at −44.5/3.95 ppm) (Fig. 4*C*).

NMR Study of the Reaction of Cisplatin with Intact rHA in Media without Added Chloride

Two-dimensional ^{1}H,^{15}N HSQC NMR—The reaction of rHA with [^{15}N]cisplatin (1 mM, 1:1) was also studied in 10 mM phosphate buffer pH 6.4 without added chloride. In the early stages of this reaction, the two-dimensional ^{1}H,^{15}N HSQC NMR spectra contained the same cross-peaks as described for rHA-[^{15}N]cisplatin in the presence of 100 mM chloride (Fig. 5, *A* and *B*). However, cross-peaks **d** and **e/f** had completely disappeared from the spectrum after 17 h of reaction (Fig. 5*C*).

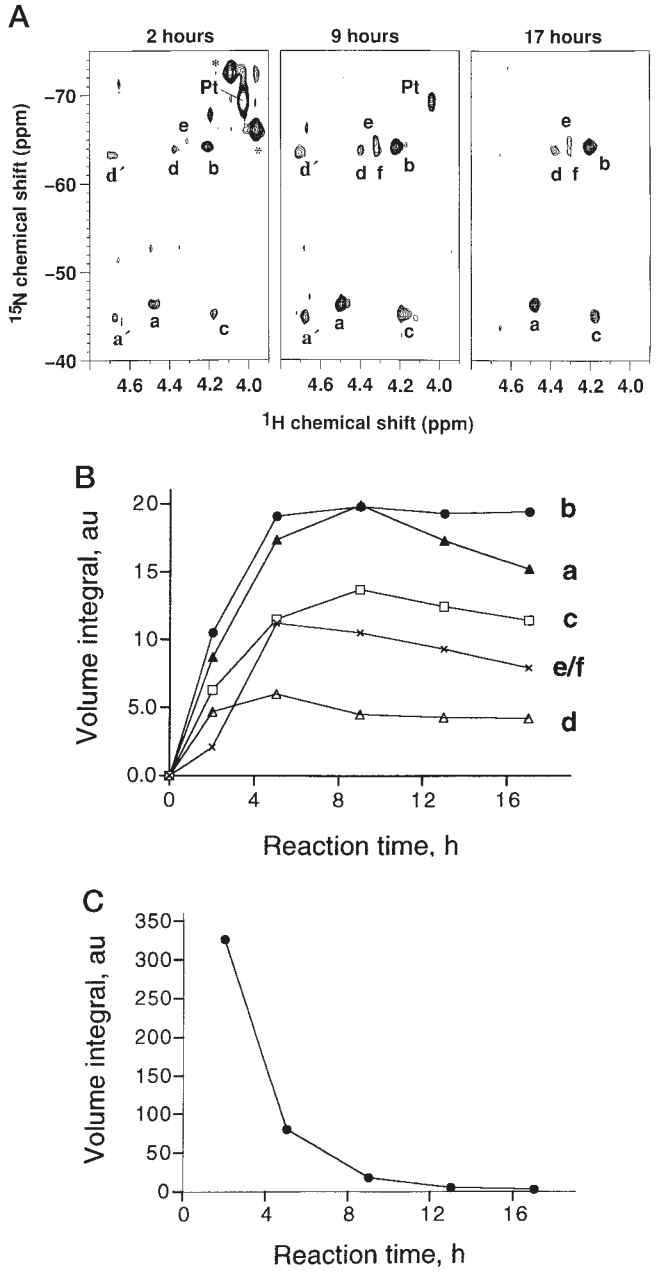


FIG. 2. **Time dependence of the reaction of cisplatin with recombinant human albumin in the presence of added chloride.** *A*, two-dimensional ^{1}H,^{15}N HSQC NMR spectra at various times during the reaction of rHA with 1 mM [^{15}N]cisplatin (1:1 molar ratio); 10 mM phosphate, 100 mM KCl, pH 6.4. *B*, time dependence of cross-peak volume integrals of cisplatin-albumin adducts. *C*, time dependence of volume integral of the free cisplatin cross-peak. *Asterisks* represent ^{195}Pt satellites.

When rHA reacted with *cis*-[Pt($^{15}NH_3$)$_2$(H_2O)$_2$]$^{2+}$ (*i.e.* in the absence of chloride ligands on platinum), only the intense cross-peaks **a** and **b** for albumin-bound Pt-NH_3 groups were observed in the spectrum after 13 h of reaction (Fig. 6). The most intense signals were present in the ammine *trans* to oxygen region of the spectrum at −82.4/3.97, −82.4/3.95, and −78.8/3.88 ppm and are assignable to unbound platinum aqua/hydroxo complexes.

Effect of Cisplatin on the Free Thiol Content and Heterogeneity of rHA

SH Group Determination—The free SH content of two batches of rHA and two of HSA before and after reaction with

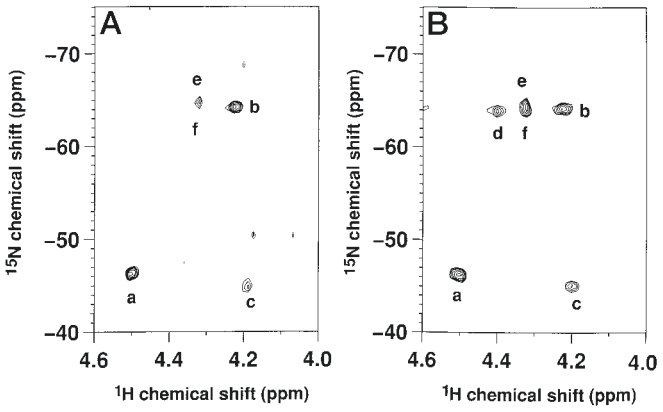


FIG. 3. **Comparison of reactions of cisplatin with recombinant human albumin at different cisplatin-albumin molar ratios.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra for the reaction of 1 mM rHA with 0.5 mM [^{15}N]cisplatin (*A*) or 2 mM [^{15}N]cisplatin (*B*), plus 10 mM phosphate, 100 mM KCl, pH 6.4, at 28 h after mixing.

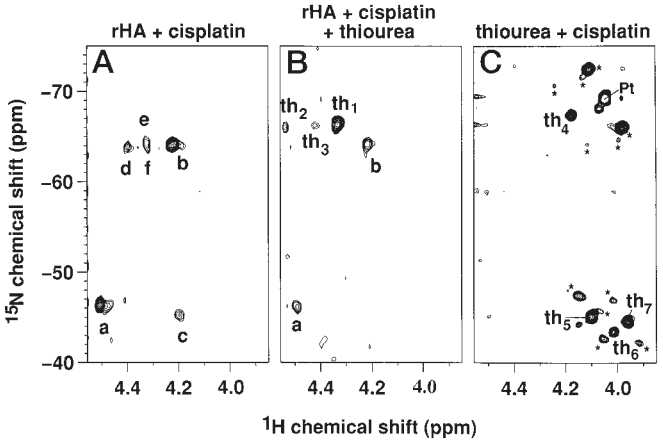


FIG. 4. **Reactions of thiourea with cisplatin-albumin complexes and free cisplatin.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra as follows. *A*, [^{15}N]cisplatin-rHA reaction (1 mM, 1:1) after 17 h, 10 mM phosphate, 100 mM KCl, pH 7.4; *B*, after an additional 10-h incubation of the sample used for *A* with 1 mol eq of thiourea; *C*, reaction of [^{15}N]cisplatin (1 mM) with an equimolar concentration of thiourea after 5 h in 10 mM phosphate, 100 mM KCl, pH 7.4.

cisplatin was determined by the DTNB-method. rHA samples contained 0.78 and 0.75 mol of free thiol/mol of protein, while for HSA samples the amount of free SH was significantly lower, 0.49 and 0.29 mol mol^{-1} (Table II). After 24 h of reaction with an equimolar concentration of cisplatin, the SH content of the two rHA samples decreased similarly, from 0.78 to 0.69 and from 0.75 to 0.67 mol mol^{-1}. Incubation of rHA (1 mM) with a 2-fold molar excess of cisplatin resulted in a more pronounced decrease of free SH, from 0.75 to 0.54 mol mol^{-1}. Reaction of HSA (1 mM) either with an equimolar concentration or with a 2-fold molar excess of cisplatin did not change the SH content of either batch of HSA. Additionally, albumin-bound cisplatin had no influence on the reaction of DTNB with SH-blocked rHA (Table II).

FPLC Data—Fig. 7 shows typical gel-filtration chromatograms of control rHA and its complexes with cisplatin. The chromatogram of control rHA consisted of one strong peak with a retention time of 28.6 min (91% calculated by absorbance at 280 nm) due to rHA monomer and a small peak at 25.1 min due to albumin dimers. The chromatogram of the 1:1 cisplatin-albumin reaction (1 mM, reacted 24 h in 10 mM phosphate, 100 mM KCl, pH 6.4) was significantly different from the control; the intensity of the monomer peak decreased to 58% of the total, that of the dimer peak increased, and a new peak with a retention time of 23.3 min appeared (Fig. 7*B*). After reaction of

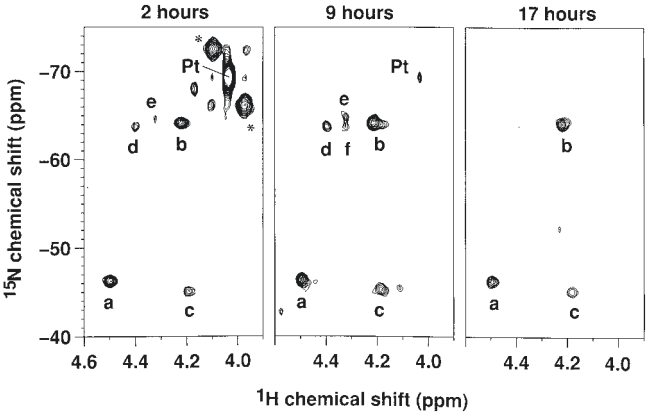


FIG. 5. **Reaction of cisplatin with recombinant human albumin in the absence of chloride.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra at various times during the reaction of rHA with [^{15}N]cisplatin (1 mM, 1:1); 10 mM phosphate pH 6.4 without added chloride. In comparison with Fig. 2*A*, it can be seen that peaks **d** and **e/f** disappeared from the spectrum after long reaction times, suggesting that they belong to species with chloride ligands that undergo hydrolysis. *Asterisks* represent ^{195}Pt satellites.

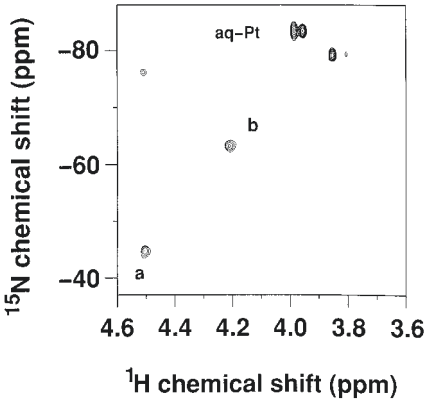


FIG. 6. **Reaction of recombinant human albumin with diaqua cisplatin.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra for the reaction of rHA (1 mM) with *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ in 10 mM phosphate pH 6.4, 13 h after mixing.

rHA with a 2-fold molar excess of cisplatin under similar conditions, only 43% of the monomer form remained, the peak at 23.3 min was more intense, and a new broad peak with a retention time of ~20 min was present (Fig. 7*C*). Incubation of cisplatin with 1 mol eq of SH-blocked (via reaction with iodoacetamide) rHA under similar conditions, led to a smaller decrease in the amount of monomeric albumin: from 91% to 70%.

NMR Study of the Reaction of Cisplatin with Chemically Modified rHA in Media with Chloride

SH-blocked rHA—On reaction of Cys-34 blocked rHA (iodoacetamide) with *cis*-$[PtCl_2(^{15}NH_3)_2]$, the ^{1}H,^{15}N cross-peaks **a**, **b**, **c**, **d**, and **e/f** were still observed in the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum at various incubation times up to 17 h (Fig. 8). The main difference between spectra from the reactions of blocked and unblocked albumin was the absence of cross-peaks **a′** and **d′** in spectra of SH-blocked rHA. Additionally, in the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum of SH-blocked rHA, the cross-peak for unbound cisplatin (peak **Pt**, Fig. 8) was still visible after a reaction time of 17 h, while this signal disappeared from the spectrum of unblocked albumin after 13 h incubation with cisplatin under similar conditions. Exactly the same changes in the two-dimensional ^{1}H,^{15}N HSQC NMR spectra were obtained for the reaction of cisplatin with *N*-ethylmaleimide-modified albumin (data not shown).

Methionine-modified rHA—The methionine residues of rHA were methylated by a 500-fold molar excess of iodomethane at pH 4.0. The reaction with iodomethane resulted in suppression of the singlet resonances assignable to methionine S-CH_3 groups in the region 1.9–2.3 ppm of the ^{1}H NMR spectrum (data not shown). Fig. 9*A* shows the two-dimensional ^{1}H,^{15}N HSQC NMR spectrum obtained after 13 h of the reaction of [^{15}N]cisplatin with methionine-modified rHA (1:1, 1 mM, in the presence of chloride, pH 6.4). The intense signals in this spectrum can be assigned to free cisplatin and its partially hydrolyzed products. Only a very weak cross-peak **e/f** was observed in the ammine *trans* to nitrogen/chloride region together with a weak signal at −46.2/4.13 ppm in the ammine *trans* to sulfur region, probably attributable to cross-peak **c** with a low frequency ^{1}H shift. The above cross-peaks were clearly visible in the spectrum after only 9 h and retained weak intensities up to 17 h of reaction (data not shown).

Histidine-modified rHA—Histidine residues of rHA were modified by reaction with diethylpyrocarbonate in 0.1 M phosphate buffer pH 6.0. This procedure resulted in modification of 6 His imidazole rings, as calculated from the absorption at 240 nm of the *N*-carbethoxyimidazole formed. Histidine modification produced substantial changes in the ^{1}H NMR spectrum of rHA, in particular, suppression of the six most intense (sharp) resonances in the aromatic region of the spectrum (data not shown). The two-dimensional ^{1}H,^{15}N HSQC NMR spectrum of histidine-modified rHA after 13 h of reaction with [^{15}N]cisplatin contained the same cross-peaks **a**, **b**, **c**, **d**, and **e/f** as the spectrum of unmodified albumin (Fig. 9*B*). The differences concerned a slight decrease in the relative intensities of cross-peaks **d**, **e/f**, and **c**, and a high frequency ^{1}H shift of peak **e/f** in the spectrum of histidine-modified rHA.

DISCUSSION

The biotransformation of cisplatin occurs directly in biological fluids via the formation of complexes with plasma proteins, especially serum albumin (15, 16, 18–20). Cisplatin-albumin adducts may play an important role in determining the body distribution of platinum, and in reducing platinum nephrotoxicity and ototoxicity (32, 56). However, there were only a few studies of the fate of albumin-bound platinum, and its role, if any, in the antitumor activity is controversial.

Takahashi *et al*. (57) and Hoshino *et al*. (31) have reported that cytotoxic effects occur only at very high concentrations of cisplatin-albumin and concluded that the complex was unlikely to contribute to the antitumor activity of cisplatin. A short report by DeSimone *et al*. (29), however, claimed that a complex of cisplatin and albumin possessed an equivalent antitumor activity to free cisplatin in seven transplantable animal tumor models. They also obtained several responses from patients with various types of tumor using a cisplatin-albumin complex. Holding *et al*. (30) noted the effectiveness of cisplatin-albumin complexes in the treatment of patients with squamous cell carcinoma of the head and neck. An interesting observation in this study concerned the tumor concentration of platinum, which was considerably higher after cisplatin-albumin administration than after conventional cisplatin therapy.

The reported contradictions concerning the antitumor activity of cisplatin-albumin complexes may arise from the use of different methods for the preparation of cisplatin-albumin complexes. Understanding the exact mechanism of cisplatin-albumin binding may enable improved cytotoxic formulations to be developed.

Two-dimensional ^{1}H,^{15}N HSQC NMR spectroscopy has proved to be an extremely useful tool in elucidating the chemistry of ^{15}N-labeled Pt-NH_3 complexes (39–41). The ^{15}N chemical shift of the ammine is diagnostic of the ligand coordinated to platinum in the *trans*-position (39). If the ligand *trans* to

TABLE II

Free SH content (mol/mol albumin) of rHA and HSA (1 mM) samples before and after 24 h of reaction with cisplatin in 10 mM phosphate, 100 mM KCl, pH 6.4

Data presented as mean ± S.D. values of three measurements; I and II refer to different albumin batches.

Albumin sample	Cisplatin:albumin molar ratio	Free SH without cisplatin	Free SH with cisplatin	Change
				%
rHA-I	1:1	0.78 ± 0.01	0.69 ± 0.01	−12
rHA-II	1:1	0.75 ± 0.02	0.67 ± 0.01	−11
rHA-II	2:1	0.75 ± 0.02	0.54 ± 0.02	−28
rHA-SH blocked	1:1	0.03 ± 0.01	0.02 ± 0.01	0
HSA-I	2:1	0.49 ± 0.01	0.47 ± 0.01	0
HSA-II	1:1	0.29 ± 0.02	0.27 ± 0.02	0
HSA-II	2:1	0.29 ± 0.02	0.30 ± 0.02	0

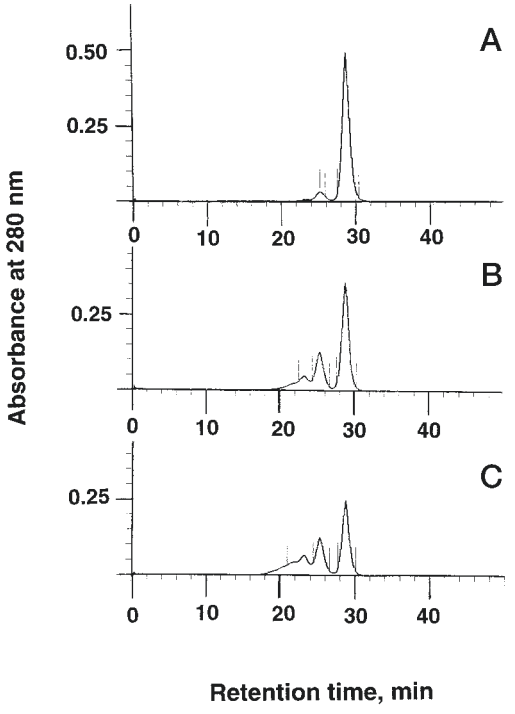


FIG. 7. **Gel filtration chromatography of recombinant human albumin complexes with cisplatin.** FPLC traces for rHA control (*A*); rHA after a 24-h reaction with 1 mM cisplatin (1:1) in 10 mM phosphate, 100 mM KCl, pH 6.4 (*B*); and rHA after reaction with 2 mM cisplatin (1:2) (*C*). An increase in the amount of dimers and formation of higher polymer forms of rHA due to reaction with cisplatin can be seen (see text).

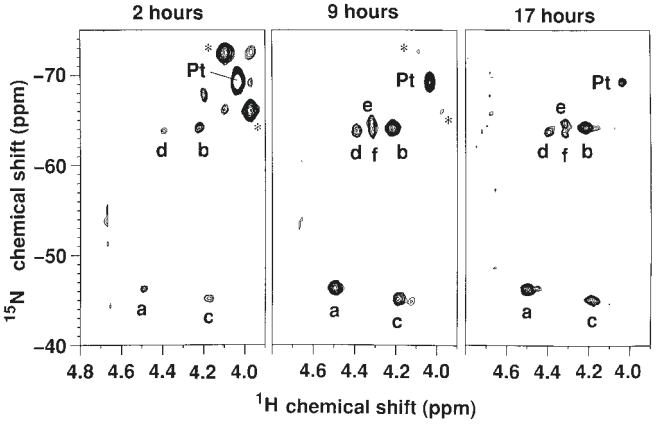


FIG. 8. **Reaction of cisplatin with SH-blocked rHA in the presence of added chloride.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra at various times during the reaction of carboxyamidomethylated rHA with [^{15}N]cisplatin (1 mM, 1:1); 10 mM phosphate with 100 mM KCl, pH 6.4. In comparison with Fig. 2*A*, it can be seen that peaks **a′** and **d′** are absent at all reaction times, suggesting that they belong to an adduct with Cys-34. *Asterisks* represent ^{195}Pt satellites.

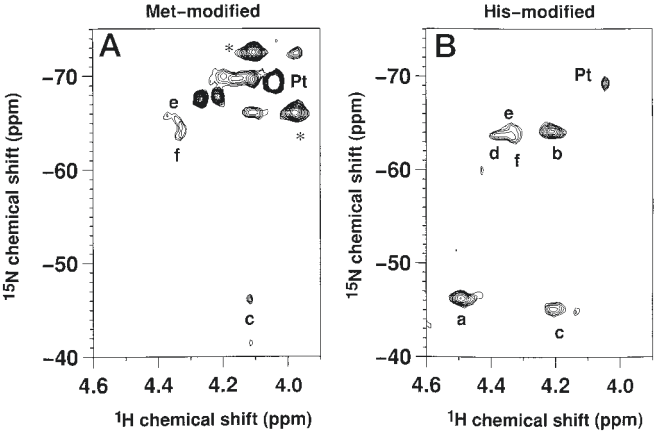


FIG. 9. **Effect of Met and His modification on reaction of cisplatin with recombinant human albumin.** Two-dimensional ^{1}H,^{15}N HSQC NMR spectra for the reaction of [^{15}N]cisplatin with methionine-modified rHA (*A*) and histidine-modified rHA (*B*) (1 mM, 1:1, 10 mM phosphate, 100 mM KCl, pH 6.4, 13-h incubation).

ammine is an oxygen-donor ligand, then the ammine chemical shift falls between −75 and −95 ppm, if *trans* to nitrogen/chloride −55 to −70 ppm, and *trans* to sulfur between −40 and −50 ppm. Since about 34% of platinum is ^{195}Pt, which has a spin quantum number I = ½, ^{1}H,^{15}N Pt-NH_3 peaks are 1:4:1 triplets due to ^{195}Pt spin-spin coupling. The ^{195}Pt satellites are usually observed only for low M_r platinum complexes. For larger complexes, such as platinum-albumin adducts, they are broadened beyond detection due to relaxation via chemical shift anisotropy (58).

In the current work, reactions of cisplatin with intact and chemically modified rHA and HSA have been studied using one-dimensional ^{1}H and two-dimensional ^{1}H,^{15}N HSQC NMR spectroscopy, together with liquid chromatography, to characterize platination sites under different experimental conditions.

Reaction of Intact Human Albumin with Cisplatin in Media with Chloride

^{1}H NMR—Reaction of cisplatin with 1 mol eq of rHA significantly reduced the intensity of singlets at 2.05 and 2.19 ppm assignable to ϵCH_3 groups of methionine residues (59, 60) in the spectrum of the protein, but formation of cisplatin-albumin adducts at pH 6.4 did not result in significant suppression of any signals in the aromatic region of the ^{1}H NMR spectrum. This region contained six major singlet resonances assignable to imidazole ring protons of histidines (61). Previous ^{1}H NMR titration experiments have shown that most of these histidines have pK_a values between 6.5 and 7.5 (61), close to that of free histidine, and are therefore likely to be exposed to the solvent.

Two-dimensional ^{1}H,^{15}N HSQC NMR—Reaction of cisplatin

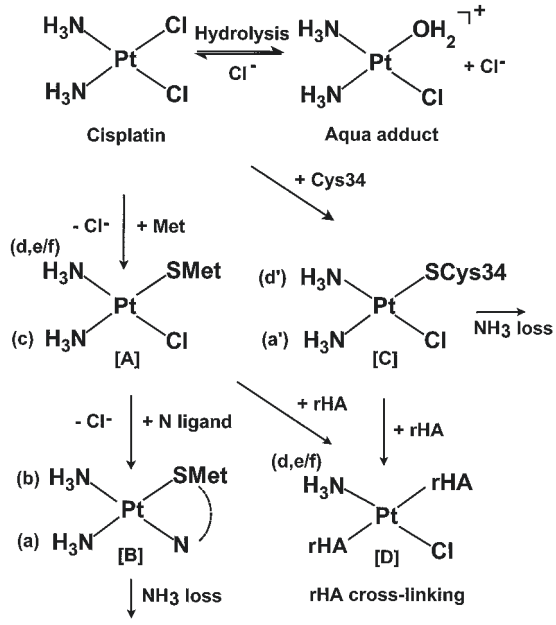
with human albumin was readily monitored by two-dimensional $^{1}H,^{15}N$ HSQC NMR spectroscopy, both by the appearance of new cross-peaks from drug-albumin adducts and by the disappearance of the cross-peak for free [^{15}N]cisplatin (Figs. 1 and 2).

Eight new cross-peaks attributable to protein-bound drug were observed in reactions of 1 mM rHA or defatted HSA with an equimolar amount of [^{15}N]cisplatin (100 mM KCl, pH 6.4, 310 K) (Fig. 1). Two of these, labeled **a′** and **d′**, grew in intensity up to 6 h of reaction, but then significantly decreased and had negligible intensities 17 h after mixing (Fig. 2*A*). Other cross-peaks, labeled **a**, **b**, **c**, **d**, and **e/f**, were clearly visible in spectra acquired over the whole course of the reaction (Fig. 2, *A* and *B*). There were no differences in the number and the chemical shifts of cross-peaks attributable to protein-bound drug in spectra of rHA and HSA, which implies that these two proteins have identical binding sites for cisplatin (Fig. 1). Additionally, after a 17-h reaction of rHA with an equimolar concentration of cisplatin at pH 7.4, the same cross-peaks were observed as at pH 6.4 (Fig. 3*A*). It therefore seems likely that the two main structural isomers of albumin (namely the N- and B-forms; Ref. 22), which coexist at physiological pH, form similar adducts with cisplatin.

The $^{1}H,^{15}N$ cross-peak for unbound [^{15}N]cisplatin decreased sharply in intensity with time, and was not detectable in the spectrum after 17 h of reaction (Fig. 2*C*). Simultaneously the general intensity of the spectrum greatly decreased, being 9-fold less after 17 h than after 2 h of reaction. These results imply that some of the ^{15}N-containing products are not detectable. Different processes may reduce the signal intensities of cisplatin-albumin adducts. First, the loss of $^{15}NH_3$ ligands of cisplatin may occur during interaction with the protein; second, ^{1}H exchange on $^{15}NH_4^+$ may be too rapid at this pH to allow detection. Additionally, reductions in relaxation times for bound Pt-NH_3 may lead to line-broadening and loss of signal intensities (62). The initial growth of cross-peaks **a′**, **d′**, **a**, **c** and **e/f** followed by decay after 6–9 h of reaction (Fig. 2*B*) suggests the loss of NH_3 from bound platinum. Displacement of NH_3 has been observed previously in reactions of cisplatin with the sulfur donor of free methionine and cysteine-containing peptides in model solutions (60, 63, 64) and in human blood plasma (65).

In the reaction of rHA with [^{15}N]cisplatin, the most intense peaks **a** and **b** are likely to represent the major cisplatin-albumin adduct (Fig. 2, *A* and *B*). The ^{15}N chemical shift of **a** is indicative of an ammine ligand *trans* to a sulfur donor ligand, and that of **b**, of an ammine *trans* to nitrogen/chloride, *i.e.* to species A or B in Scheme I. The other pair of cross-peaks in the ammine *trans* to sulfur (**a′**) and ammine *trans* to nitrogen/chloride regions (**d′**), is likely to represent a second type of cisplatin-albumin complex with a high rate of ammonia loss. Additional cisplatin-albumin adducts give rise to intense cross-peaks in the ammine *trans* to sulfur (**c**) and in the ammine *trans* to nitrogen/chloride regions (**d** and **e/f**). It is likely that cross-peak **c** is paired with both peaks **d** and **e/f**, representing two cisplatin-albumin adducts (**c**-**d** and **c**-**e/f**) with similar sulfur donors and different types/environments of nitrogen/chloride donors. These adducts have different rates of formation and subsequent loss of NH_3, which results in variations in their peak intensities at different times of reaction (Figs. 2 and 3).

The nature of the above cisplatin-albumin adducts was clarified by studies of their reactivity toward thiourea, which is expected to react rapidly only with monofunctional adducts of cisplatin and not with bifunctional ones, as has been demonstrated for DNA (66). When an equimolar amount of thiourea was added to a preincubated 1:1 reaction mixture of cisplatin and rHA at pH 7.4, cross-peaks **c**, **d**, and **e/f** disappeared and gave rise to three new peaks $\mathbf{th}_1$, $\mathbf{th}_2$, $\mathbf{th}_3$ in the two-dimensional $^{1}H,^{15}N$ HSQC NMR spectrum (Fig. 4*B*). Thiourea adducts with [^{15}N]cisplatin alone gave rise to cross-peaks in the ammine *trans* to sulfur ($\mathbf{th}_5$, $\mathbf{th}_6$, and $\mathbf{th}_7$) and in the ammine *trans* to nitrogen/chloride regions ($\mathbf{th}_4$) of the spectrum (Fig. 4*C*), all with detectable platinum satellites. Hence, cross-peaks $\mathbf{th}_1$, $\mathbf{th}_2$, and $\mathbf{th}_3$ are not due to low molecular mass platinum-thiourea complexes but to protein-bound Pt-NH_3. A new $^{1}H,^{15}N$ cross-peak at −44.5/4.42 ppm for a cisplatin-albumin complex with *trans* sulfur donor of thiourea was observed only during the first 3 h after addition of thiourea (data not shown), suggesting that there is a subsequent rapid loss of NH_3 from this disulfur adduct. Our data are consistent with previous observations that the sulfur ligands thiosulfate and *N,N′*-diethyldithiocarbamate interact with cisplatin-albumin adducts but do not release substantial amounts of protein-bound platinum (67, 68). The experiments with thiourea described here suggest strongly that cross-peaks **c**, **d**, and **e/f** arise from monofunctional cisplatin-albumin adducts containing a sulfur donor ligand from albumin (signal **c**) and a chloride ligand (cross-peaks **d** and **e/f**) (species A in Scheme I).



SCHEME I. **Reactions of cisplatin with human albumin.** Labels in *brackets* refer to $^{1}H,^{15}N$ cross-peaks in two-dimensional spectra.

Reaction of Intact Human Albumin with Cisplatin without Added Chloride

The assignment of cross-peaks **d** and **e/f** to Pt-NH_3 *trans* to chloride ligands in cisplatin-albumin adducts was supported by the investigation of cisplatin-rHA reactions without added chloride (Fig. 5). All the $^{1}H,^{15}N$ cross-peaks for protein-bound cisplatin observed during the first 9 h of reaction were similar to those observed in the presence of chloride (Fig. 5, *A* and *B*). However, more prolonged incubation resulted in the elimination of cross-peaks **d** and **e/f**. In this case, these monofunctional cisplatin-albumin adducts would be further modified by hydrolysis with displacement of chloride ligands by aqua/hydroxo ligands.

In addition, rHA was incubated with *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ at pH 5.0 to minimize the formation of hydroxy-bridged dimeric and polymeric platinum species that readily occur above the first pK_a value of coordinated H_2O (5.37) (69). It is noteworthy that, at this pH, albumin still exists in the native, completely folded N-form

(22). Reaction of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ with equimolar rHA resulted in only the pair of intense cross-peaks **a** and **b** for protein-bound drug in the two-dimensional $^1H,^{15}N$ HSQC NMR spectrum (Fig. 6). Other strong peaks were observed in the ammine *trans* to oxygen region of the spectrum, and probably represent hydroxy-bridged dimeric and polymeric platinum species.

These investigations of the interaction of [^{15}N]cisplatin with rHA under different experimental conditions allow the following types of cisplatin binding sites on albumin to be proposed. (i) The major adduct is a single bifunctional complex of $\{Pt(NH_3)_2\}^{2+}$ involving sulfur and nitrogen donor ligands giving rise to the pair of cross-peaks **a** and **b** (*e.g.* species B in Scheme I); (ii) the second type of site accounts for the two monofunctional adducts giving pairs of signals **c-d** and **c-e/f**, involves a sulfur donor of albumin and also contains a chloride ligand, which is apparently rather unreactive perhaps because it is shielded by the protein (species A in Scheme I); (iii) the third type of site is probably also a monofunctional adduct with a sulfur donor from albumin, and appears to undergo rapid loss of platinum-bound NH_3 (species C in Scheme I).

Our data show that cisplatin binds to two sites even when albumin is present in a 2-fold molar excess, giving a major bifunctional adduct and a monofunctional one (Fig. 3*A*). With a 2-fold molar excess of [^{15}N]cisplatin, the proportion of monofunctional adducts significantly increases (Fig. 3*B*).

The two-dimensional $^1H,^{15}N$ HSQC NMR data suggest that only sulfur and nitrogen/chloride donor ligands of albumin are involved in platinum binding. There are three types of sulfur atom in albumin, namely Cys-34 SH, Met thioether, and cystine disulfide. Although previous work on cisplatin-albumin interactions has suggested that platinum can induce cleavage of disulfide bonds, implying binding of platinum to cystine sulfur (35, 36), a high excess of platinum and very long incubation times were required to achieve this. Therefore, Cys-34 and Met are the likely sulfur ligands for platinum in the present work. From consideration of published x-ray structures of several proteins, it is likely that Pt(II) binds more readily to histidine and the N-terminal amino group (70) than other N donors. However, in our study cisplatin binding did not perturb the resonances for Asp-1 and Ala-2, which were clearly visible in the aliphatic region of the one-dimensional ^{1}H NMR spectrum of rHA. Therefore, the most likely source of a nitrogen donor in the formation of the bifunctional cisplatin-albumin adduct is the imidazole ring of histidine. Pt(II) chelation to S and deprotonated amide N is known for Met peptides and is favored at high pH (71).

To further elucidate the nature of the amino acid residues of albumin that are involved in platinum binding, we studied the free thiol content, aggregation status of rHA, and influence of selective modification of Cys-34, methionines, and histidines on cisplatin-rHA interactions.

Influence of Cisplatin Binding on Free Thiol Content and Heterogeneity of rHA

SH Group Determination—The reaction of cisplatin with rHA (1:1 molar ratio in the presence of chloride) resulted in a 11–12% decrease in the free SH content for the two batches of rHA investigated over 24 h, while 2:1 reaction of cisplatin:rHA decreased the free thiol content of rHA by an average of 28% (Table II). Reaction of cisplatin with the two human serum albumin samples had no effect on the free thiol content. It is unlikely that the presence of protein-bound platinum influences the DTNB method, since the cisplatin adducts of SH-blocked rHA did not induce cleavage of DTNB.

This provides the first direct evidence that only a small fraction of cisplatin binds to Cys-34 in rHA and does not bind significantly to the SH group of HSA. In the abstract of their article, Momburg *et al*. (34) suggested the involvement of the SH group of HSA in cisplatin binding according to DTNB titration, but surprisingly, did not describe these results. Tosetti *at al*. (72) observed a 10–40% decrease of free SH content in the blood plasma of patients treated with cisplatin. In their work, the initial concentration of free thiol in blood plasma was about 300 μM, while the highest concentration of platinum in the samples did not exceed 4 μM. Therefore, the observed effects cannot be explained by direct interaction of cisplatin with protein thiols in blood plasma.

Gel-filtration Chromatography—It is known that platinum compounds can induce intermolecular cross-linking of proteins (73, 74). For example, albumin aggregation has been reported to arise from reaction of HSA with K_2PtCl_4 (75). FPLC data demonstrated that the reaction of cisplatin with rHA (1:1, 1 mM) was accompanied by a decrease in the amount of monomeric albumin to an average of 58%, by an increase of dimer content, and appearance of higher molecular mass polymers (Fig. 7*B*). These changes were also observed for 0.5:1 cisplatin:rHA reactions (data not shown) and were enhanced after reaction with a 2-fold molar excess of cisplatin (Fig. 7*C*). SH blocking of rHA reduced albumin aggregation, but did not prevent formation of significant amounts of dimers and polymers. These data suggest that cisplatin-induced albumin aggregation occurs mainly via direct intermolecular cross-linking, probably via monofunctional adducts or via NH_3 release (species D in Scheme I).

It is noteworthy that we observed cisplatin-induced aggregation of albumin even at low cisplatin/albumin molar ratios. This observation may have important implications for the biological activity and therapeutic effects of cisplatin-albumin complexes such as those prepared at 1:1 cisplatin:albumin molar ratios by DeSimone *et al*. (29) and Holding *at al*. (30), or with a high molar excess of drug (7:1) by Hoshimo *et al*. (31). Cross-linked forms of albumin may not be effective delivery agents for cisplatin, being quickly eliminated from the circulation by hepatocytes and liver macrophages (76, 77). Cisplatin-induced protein cross-linking may also explain reported differences in the body distribution of platinum after infusion of free cisplatin or its complex with plasma proteins; in the former case, a major amount of platinum was detected in the kidney (78) and, in the latter, in liver tissue (20).

Reaction of Cisplatin with Chemically Modified rHA in Media with Chloride

SH-blocked Albumin—Carboxyamidomethylation of the free SH group with iodoacetamide (Fig. 8) or its modification with *N*-ethylmaleimide confirmed the assignment of cross-peaks **a′** and **d′** in the two-dimensional $^1H,^{15}N$ HSQC NMR spectrum for cisplatin bound to the SH group of rHA. SH blocking eliminated cross-peaks **a′** and **d′** but did not influence the chemical shifts and intensities of the other cross-peaks of protein-bound cisplatin (Fig. 8*A*). Additionally, modification of the free thiol decreased the rate of reaction of rHA with cisplatin, as was observed previously (33, 38). The decrease in the rate of this reaction may explain the decrease in the amount of dimer and polymer formed from reaction of SH-blocked rHA with cisplatin.

Methionine-modified rHA—The involvement of methionine in cisplatin binding was demonstrated via methylation of methionines with iodomethane (forming -SMe_2^+ groups), which dramatically reduced the extent of cisplatin binding to albumin (Fig. 9*A*). Following modification, (i) only one pair of weak cross-peaks (**e/f** and **c**) was observed in the two-dimensional $^1H,^{15}N$ HSQC NMR spectrum after 13 h and (ii) most of the

cisplatin was unbound over 17 h of reaction.

$^{1}H,^{15}N$ NMR data for iodomethane-modified rHA strongly suggest that methionine is the sole source of sulfur in the major bifunctional (cross-peaks **a** and **b**) and two monofunctional (cross-peaks **c**, **d**, and **e/f**) cisplatin-albumin adducts. Weak signals **e/f** and **c** are likely to represent monofunctional cisplatin adducts formed by reaction with the small fraction of unmodified methionines or with disulfide groups.

Histidine-modified Albumin—Modification of six histidines of rHA via reaction with diethylpyrocarbonate only slightly decreased the intensities of $^{1}H,^{15}N$ cross-peaks **c** and **d** and changed the ^{1}H shift of cross-peak **e/f** (Fig. 9*B*), assigned to a monofunctional adduct. It seems likely that the observed changes are due to indirect effects of histidine modification on cisplatin binding sites via small changes in protein conformation. Since histidine modification induced a dramatic decrease of the intensities of six relatively sharp signals in the aromatic region of the ^{1}H NMR spectrum of albumin (ϵCH), it is probable that the modification affects only solvent-exposed His residues. Additionally, we did not observe any significant changes to these peaks upon cisplatin binding; therefore, imidazole rings of exposed histidines do not serve as nitrogen donors in cisplatin adducts. X-ray crystallographic studies of several proteins have suggested that only Met and His side chains exposed to the solvent are able to interact with $[PtCl_4]^{2-}$, while buried ones do not bind to platinum (70). His residues of rHA which do not react with diethylpyrocarbonate are probably buried inside the protein, and it seems unlikely that these are involved in cisplatin binding, although this cannot be ruled out. Backbone amide nitrogens or Lys or Arg side chains with abnormally low pK_a values may also serve as possible N donors. Recently, it has been shown that a lysine amino group in a high mobility group protein is involved in cisplatin binding (79).

Possible Albumin Binding Sites for Cisplatin

The NMR results suggest that platinum reacts mainly with methionine residues of rHA creating bifunctional (cross-peaks **a** and **b**) and two monofunctional (peaks **c** and **d** or **c** and **e/f**) complexes as well as an adduct with Cys-34. Our data can be contrasted with previous reports that a free thiol group in albumin is the major site for cisplatin binding, based on experiments with blocking of Cys-34 of HSA and BSA (7, 33, 38). However, albumin samples in the previously reported experiments probably had a low initial level of free thiol (can be as low as 0.3 mol mol^{-1} protein; Table II). It is also possible that bovine and human serum albumin have different binding sites for cisplatin due to small differences in amino acid sequence. In particular, HSA has six methionine residues, whereas BSA has only four (substitutions M123L, L185M, M298I, M329S; Ref 21).

The crystal structure of human albumin has been published (21, 80); however, the atomic coordinates have not yet been made available. Attempts to locate possible platinum binding sites can therefore be made only on the basis of published ribbon diagrams of the structure of albumin, domain II and subdomains IIA and IIIB. These views were used to assess the positions and the relative solvent accessibilities of the six methionine side chains on human serum albumin, residues 87, 123, 298, 329, 446, and 548.

We can speculate that the major cisplatin binding site which appears to be an S,N macrochelate (peaks **a** and **b**) may utilize Met-298 as a ligand. Met-298 appears to be the most surface-accessible Met residue, and most likely accounts for the sharp singlet in the ^{1}H NMR spectrum of albumin, which was suppressed upon cisplatin binding. The ^{1}H chemical shift of the ammine *trans* to sulfur cross-peak for this macrochelate complex (4.50 ppm) is shifted significantly to higher frequency (~0.3 ppm) than has been observed for ammine *trans* to a methionine sulfur ligand (81). A possible reason for this is the presence of a H bond or salt-bridge interaction between the ammine hydrogen and nearby acceptor. Met-298 is in a region of high negative charge, with Asp-296 and Asp-301 nearby, together with glutamate residues 292, 294, and 297. Furthermore, this may be important for site preference if the reactant is positively charged, as is *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{2+}$ and monoaqua cisplatin.

Apart from Met-298, Met-87 and Met-446 may also be involved in the formation of monofunctional adducts with cisplatin since these appear to be exposed residues.

Conclusions

Contrary to previous reports, we find that the free thiolate group of Cys-34 of albumin is not the locus of the major cisplatin binding site. Our $^{1}H,^{15}N$ NMR data, obtained via the use of *cis*-$[PtCl_2(^{15}NH_3)_2]$, combined with comparison of human serum albumin and recombinant human albumin, SH blocking, and His and Met modification reactions, suggest that the major binding site involves a Met S,N macrochelate, together with minor monofunctional sites involving Met S and Cys-34. The high *trans* influence of Cys S and Met S leads to the eventual displacement of platinum-bound NH_3 ligands and protein cross-linking detectable by gel filtration chromatography. Platinum-induced formation of polymers may be of significance to the *in vivo* activity of cisplatin. The complexity of the reactions of cisplatin may explain why previous reports of the biological properties of cisplatin-albumin complexes have sometimes appeared to be contradictory.

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