Cisplatin Binding Sites on Human Albumin*

Andrei I. Ivanov‡§, John Christodoulou‡, John A. Parkinson‡, Kevin J. Barnham‡, Alan Tucker‡, John Woodrow‡, and Peter J. Sadler‡**

From the ‡Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, the *Department of Chemistry, Birkenhead College, University of London, WC1H OPP London, and ‡Delta Biotechnology Ltd, Nottingham NG7 1FD, United Kingdom

Reactions of cisplatin (cis-[PtCl2(NH3)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 15N and use of two-dimensional 1H,15N 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 NH3 occurs due to the strong trans influence of Met sulfur, which weakens the Pt-NH3 bonds, and protein cross-linking is observed. The consequences of these findings for the biological activity of cisplatin-albumin complexes are discussed.

Cisplatin, cis-[PtCl2(NH3)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 is detected 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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Man albumin (rHA), and with HSA using one-dimensional \(^1\)H and two-dimensional \(^1\)H,\(^{15}\)N HSQC NMR spectroscopy to characterize platination sites under different experimental conditions. Two-dimensional \(^1\)H,\(^{15}\)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\(_2\)(\(^{15}\)NH\(_3\))\(_2\)\((H_2O)_2\)]\(^2+\) were characterized according to literature procedures (42). Iodoacetamide, N-ethylmaleimide, 5,5′-dithio-bis(2-nitrobenzoic acid), Norit-A (Sigma), iodoethane (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 SPSS (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, and freeze-dried. HSA was obtained from the Scottish National Blood Transfusion Service (batches HA(20)60440 and SPSS (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.

Histidine residues were modified by reaction with iodoethane (47). Iodoethane 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. 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).

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 \(^1\)H NMR—One mol eq of cisplatin added to an albumin solution (1 mM rHA in 10 mM phosphate, pH 6.4) induced changes in the protein-one-dimensional \(^1\)H NMR spectra. 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.

Two-dimensional \(^1\)H,\(^{15}\)N HSQC NMR—Fig. 1 shows two-
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. 5C).
When rHA reacted with cis-[Pt(15NH3)2(H2O)2]2+ (i.e., in the absence of chloride ligands on platinum), only the intense cross-peaks a and b for albumin-bound Pt-NH3 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 282.4/3.97, 282.4/3.95, and 278.8/3.88 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 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. 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. 7B). After reaction of 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).

**FIG. 2.** Time dependence of the reaction of cisplatin with recombinant human albumin in the presence of added chloride. A, two-dimensional 1H,15N HSQC NMR spectra at various times during the reaction of rHA with 1 mM [15N]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 195Pt satellites.

When rHA reacted with cis-[Pt(15NH3)2(H2O)2]2+ (i.e., in the absence of chloride ligands on platinum), only the intense cross-peaks a and b for albumin-bound Pt-NH3 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.

**Fig. 3.** Comparison of reactions of cisplatin with recombinant human albumin at different cisplatin-albumin molar ratios. Two-dimensional 1H,15N HSQC NMR spectra for the reaction of 1 mM rHA with 0.5 mM [15N]cisplatin (A) or 2 mM [15N]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 1H,15N HSQC NMR spectra as follows. A, [15N]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 [15N]cisplatin (1 mM) with an equimolar concentration of thiourea after 5 h in 10 mM phosphate, 100 mM KCl, pH 7.4.
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**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 $^{[15N]}$cisplatin (1 mM, 1:1); 10 mM phosphate pH 6.4 without added chloride. In comparison with Fig. 2A, it can be seen that peaks d and ef 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-$^{[15N]}$PtCl$_2$(NH$_3$)$_2$ 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. 7C). 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%.

**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. (37) and Hoshino et al. (31) have reported that cytotoxic effects occur only at very high concentrations of cisplatin–albumin and concluded that the complex is 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
ammine is an oxygen-donor ligand, then the ammine chemical shift falls between 275 and 295 ppm, if trans to nitrogen/chloride 255 to 270 ppm, and trans to sulfur between 240 and 250 ppm. Since about 34% of platinum is 195Pt, which has a spin quantum number I = 5/2, 1H,15N Pt-NH3 peaks are 1:4:1 triplets due to 195Pt spin-spin coupling. The 195Pt satellites are usually observed only for low Mr 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 1H and two-dimensional 1H,15N 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**

1H 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 eCH3 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 1H NMR spectrum. This region contained six major singlet resonances assignable to imidazole ring protons of histidines (61). Previous 1H NMR titration experiments have shown that most of these histidines have pKa 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 1H,15N HSQC NMR—Reaction of cisplatin

**TABLE II**

| 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                           | 0.77 ± 0.02              | 0.54 ± 0.02            | −28    |   |
| rHA-SH blocked | 1:1                           | 0.63 ± 0.01              | 0.22 ± 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      |   |
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. 2A). 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. 3A). 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. 2C). 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 \( ^{15}NH_3 \) 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. 2B) 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 cisplatin-albumin adducts. First, the loss of \( ^{15}NH_3 \) ligands of trans and that of \( trans \) to nitrogen/chloride regions \( (a', d') \) 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 \( e, d, \) and \( e/f \) disappeared and gave rise to three new peaks \( th_1, th_2, th_3 \) in the two-dimensional \( ^{1}H,^{15}N \) HSQC NMR spectrum (Fig. 4B). Thiourea adducts with \([^{15}N] \) cisplatin alone gave rise to cross-peaks in the ammine \( trans \) to sulfur \( (th_1, th_2, th_3) \) and in the ammine \( trans \) to nitrogen/chloride regions \( (th_4) \) of the spectrum (Fig. 4C), all with detectable platinum satellites. Hence, cross-peaks \( th_1, th_2, \) and \( th_3 \) 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 \( e \)) and a chloride ligand (cross-peaks \( d \) and \( e/f \) (species A in Scheme I).

**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 hydrolisis with displacement of chloride ligands by aqua/hydroxo ligands.

In addition, rHA was incubated with cis-[Pt(\( ^{15}NH_3 \))_2(H_2O)]^{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.
Reaction of cis-[Pt(NH₃)₂(H₂O)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,¹⁵N HSQC NMR spectrum (Fig. 6). Other strong peaks were observed in the ammine trans to oxygen region of the spectrum, and probably represent hydroxyl-bridged dimeric and polymeric platinum species.

These investigations of the interaction of [¹⁵N]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(NH₃)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 e-f, involving 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 NH₃ (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. 3A). With a 2-fold molar excess of [¹⁵N]cisplatin, the proportion of monofunctional adducts significantly increases (Fig. 3B).

The two-dimensional ¹H,¹⁵N 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 ¹H 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 et 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 K₂PtCl₄ (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. 7B). 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. 7C). 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 NH₃ 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 et 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,¹⁵N 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. 8A). 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 -SMe₂ groups), which dramatically reduced the extent of cisplatin binding to albumin (Fig. 9A). Following modification, (i) only one pair of weak cross-peaks (e'f and c') was observed in the two-dimensional ¹H,¹⁵N 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 diethyллоркарбонат 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. 5B), 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 (eCH), 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 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 diethylлоркарбонат 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 79). 

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$_2$(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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