Binding and Aggregation of Human γ-Globulin by cis-Diaminedichloroplatinum (II) through Disulfide Bond

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Received June 6, 1994; accepted August 18, 1994

The incubation of γ-globulin with cis-diaminedichloroplatinum (II) (cis-DDP) resulted in gradual formation of insoluble aggregates. Since the precipitates, composed of polymerized γ-globulin and cis-DDP, were completely solubilized with urea, the reaction mixture containing precipitate was examined in terms of the binding of cis-DDP and the effect on disulfide (S-S) bonds in the γ-globulin. When γ-globulin was incubated with 30 molar excess cis-DDP at pH 7.4 and 37°C, cis-DDP gradually bound to as much as 12 mol per mol of γ-globulin in 14 d. Concurrently, about four disulfide bonds were cleaved without reaching a certain plateau. An sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the aggregated γ-globulin induced by cis-DDP was significantly different from that of the heat-denatured aggregate form or the reduced form by sulfhydryl oxidation. The aquated complex of cis-DDP also produced an insoluble precipitate and affected the S-S bond to a greater extent than the parent drug.

Keywords γ-globulin; cisplatin; cis-diaminedichloroplatinum (II); disulfide bond; drug binding

MATERIALS AND METHODS

Material cis-DDP was purchased from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). Human γ-globulin (No. G4386) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Disodium 2-nitro-5-thiosulfobenzozate (NTSB) was synthesized according to the method of Thannhauser et al. All other chemicals were of reagent grade.

Incubation of γ-Globulin with cis-DDP cis-DDP was dissolved in a phosphate buffer solution (50 mM, pH 7.4) with 0.1 M NaCl. Human γ-globulin was also dissolved in the same medium. Each solution was filtered through a 0.2 μm sterilized filter (Toyo Roshi, Tokyo) and placed in sterilized test tubes with a screw cap after correcting the modified concentration of protein due to adsorption on this nitrocellulose membrane. The concentration of cis-DDP was varied from 0.1 to 0.6 mM, where the protein concentration was always kept at 3 mg/mL. The mixed solutions were protected from light and incubated at 37°C over a 14 d period, during which one of the tubes was used from time to time for various analyses. If γ-globulin was aggregated, it was dissolved in 25% urea solution, after which the analyses were conducted.

Incubation of γ-Globulin with Aquated Form of cis-DDP The reactions of γ-globulin with the aquated form of cis-DDP were conducted as follows. The half-life of the first hydrolysis rate of cis-DDP is reported to be about 9 h at 25°C.11 Thus, the platinum complex solution in 0.25 M NaClO4 without NaCl was allowed to stand for 48 h at 25°C before mixing with γ-globulin. During another 4-d incubation with γ-globulin, however, the monoaquated complex could not avoid the second hydrolysis. Thus Pt (II) species involved in the reaction may include monoaquated ([cis-Pt(NH3)2Cl(OH)]) and [cis-Pt(NH3)2Cl(H2O)]+ and diaquated forms ([cis-Pt(NH3)2(H2O)(OH)])2+ [cis-Pt(NH3)2(OH)2] and a trace amount of [cis-Pt(NH3)2(H2O)2]2+. In the aquated form of cis-DDP, in order to avoid the complex formation with
a buffer component of phosphate ion, 0.25 M sodium perchlorate solution was used and the ionic strength adjusted to 0.25. The other procedure for incubation with γ-globulin was the same as that previously described.

Separation of Unbound cis-DDP At an appropriate time interval, the incubation mixture was centrifuged at 65000 × g for 30 min to obtain the supernatant, and then the unbound cis-DDP fraction was separated by passing it through a column (1.0 cm i.d. × 18 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) equilibrated with a pH 7.4 phosphate buffer containing 0.1 M NaCl.

Analyses of cis-DDP and γ-Globulin cis-DDP was analyzed by the method of Ayres and Meyer with some modification as previously described. The concentration of γ-globulin in the supernatant was determined after centrifugation of the reaction solution at 65000 × g for 30 min. The protein was assayed using the Coomassie Brilliant Blue G kit (Bio-Rad). Determination of S-S Bonds The number of S-S bonds remaining in the γ-globulin was determined using NTSB by a modified method of Kella and Kinsella. γ-Globulin solution (0.5 ml) was mixed with NTSB assay solution (3 ml) containing 0.2 M Tris base, 0.1 M EDTA and 3 M guanidine thiocyanate with a freshly-prepared Na₂SO₄ solution (0.2 M). After 20 min, the absorbance at 412 nm was monitored against an appropriate blank. The concentration of S-S was calculated using a value of 13600 M⁻¹ cm⁻¹ for the extinction coefficient of 2-nitro-5-benzoic acid.

Effect of Solubilized Oxygen in the Reaction Medium To determine the effect of solubilized oxygen on the cleavage of the S-S bond of γ-globulin by cis-DDP, the medium was oxygenated by oxygen gas before the cis-DDP and γ-globulin were mixed.

Turbidity of γ-Globulin Solution The turbidity of the reaction solution was measured using a UV-260 (Shimadzu, Kyoto) at a wavelength of 360 nm, where the absorption contribution of the protein was minimal.

Preparation of Heat-Denatured γ-Globulin γ-Globulin solutions (7.5 mg/ml) in a 50 mM phosphate buffer (pH 7.4) were heated in a thermostated water bath at 62°C for 13-60 min. The precipitate of γ-globulin from thermal denaturation dissolved in 25% urea, and the solubilized sample was then used for potassium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of Reduced γ-Globulin Reduced and alkylated γ-globulin was prepared according to a previous report. A γ-globulin solution (3 mg/ml) containing Na₂SO₄ (10 mm) was incubated at 37°C. The reaction was initiated by adding ammoniacal cupric sulfate (0.4 mm) and passing oxygen through a gas dispenser. Upon removal of a 5 ml sample of the reaction solution at appropriate intervals, the sulfhydryl groups were carboxymethylated by the addition of 30 molar equivalents of monoiodoacetamide with respect to the S-S bonds (pH 8.0). The carboxymethylated γ-globulin was obtained by separation again through Sephadex G-25.

Gel Electrophoresis γ-Globulin treated with cis-DDP, heat-denatured and reduced γ-globulin were analyzed by electrophoresis in the presence of 7.5% polyacrylamide gel at pH 8.8. The protein precipitate was completely dissolved in urea before it was applied gel electrophoresis.

RESULTS AND DISCUSSION

Binding of cis-DDP to Human γ-Globulin γ-Globulin (3 mg/ml) was incubated with cis-DDP in pH 7.4 solution (50 mM phosphate buffer containing 0.1 M NaCl) at 37°C. The molar ratio of cis-DDP to γ-globulin was changed to 30. As reported in a preliminary study, a fine precipitate appeared and the solution gradually turned opaque after about 4 d incubation. The solution then changed to one which was white and turbid after continued incubation of 14 d. The turbidity at 360 nm was 1.1 after 14 d for the largest molar ratio of cis-DDP and γ-globulin.

Furthermore, the precipitate was shown to be polymerized γ-globulin probably through cross-linkage between γ-globulin molecules induced by cis-DDP. To investigate the nature of the precipitates, we attempted to redissolve them using a denaturing agent or surfactant. Urea (25%, 4.2 M) dissolved the precipitates, while guanidine hydrochloride, guanidine thiocyanate or SDS did not allow complete dissolution. The urea solution mixed with γ-globulin precipitates showed no light scattering (at 360 nm), suggesting that all of the precipitated material was solubilized by urea. This allowed various assays even in the precipitated reaction mixtures.

Figure 1 shows the cis-DDP bound to γ-globulin, which was estimated from the free cis-DDP eluted from the Sephadex G-25 column. When the reaction solution was turbid, the supernatant was obtained by centrifugation at 65000 × g for 30 min before application on the column. The binding reaction of cis-DDP with γ-globulin was dependent on the cis-DDP concentration and the incubation time. The bound platinum was likely to reach equilibrium after about 10 d. The bound cis-DDP was 12.4 mol per γ-globulin molecule, where the ratio of cis-DDP to γ-globulin was 30. Since aggregated protein does not seem to retain the binding ability for cis-DDP in a similar manner to the native protein in the solution,
the cis-DDP bound to the limited amount of \( \gamma \)-globulin in the solution may have reached a plateau.

\( \gamma \)-Globulin used in this study was a polyclonal type and a mixture of four subclasses, IgG1, IgG2, IgG3 and IgG4 with a different number of total S-S bonds. Thus, in order to estimate the bound cis-DDP the average molecular weight of \( \gamma \)-globulin, 150 kDa, was used.

**Precipitation of \( \gamma \)-Globulin** Figure 2 shows the time dependence of the disappearance of \( \gamma \)-globulin from supernatant of the reaction mixture as determined after centrifugation at 65000 \( \times g \) for 30 min. The \( \gamma \)-globulin was precipitated in a biphasic manner. During the first two days, no decrease in the soluble \( \gamma \)-globulin was observed, but then a marked disappearance of the protein from the supernatant in the reaction solution was seen. When 3 mg/ml of \( \gamma \)-globulin was treated with 30-fold cis-DDP, about 83% of the total \( \gamma \)-globulin was aggregated during the 14-d incubation. The untreated control \( \gamma \)-globulin solution was transparent and no precipitation was observed. This result is consistent with the observation that the visible turbidity of the solution was developed only after 3 or 4 d. 6

**Effect on S-S Bond** A previous report demonstrated a decrease in S-S bonds in \( \gamma \)-globulin within the first 3 d of incubation using cis-DDP. 6\footnote{Figure 3 shows time courses for the 14-d incubation. When the reaction mixture was opaque, urea solution was used to dissolve it. A preliminary study confirmed that 25% urea does not affect the determination of the S-S bond. This suggests that carbamoylation by a small amount of cyanic acid derived from urea also does not affect the S-S concentration when added just before the determination.}

The number of S-S bonds continuously decreased in \( \gamma \)-globulin and was dependent on both incubation time and the ratio of cis-DDP to \( \gamma \)-globulin over a 14-d period. During two weeks incubation, the decreasing number of S-S bonds was 1.6, 2.4, 3.1 and 3.8 mol per mol of \( \gamma \)-globulin when the initial ratio of cis-DDP and \( \gamma \)-globulin was 5, 10, 20 and 30, respectively. The \( \gamma \)-globulin used in this study was a polyclonal one and a mixture of four subclasses with different numbers of interchain S-S bonds: 4, 6, 12 or 13, and 4 for IgG1, IgG2, IgG3 and IgG4, respectively, whereas the number of intrachain S-S bonds is always 12 regardless of the subclass. Thus, the average number of S-S bonds was estimated to be 17.1 based on the assumption that the four subclasses were comparable to those in the blood; thus, the number of cysteine residues was calculated to be about 34. These S-S bonds are reported to be slightly reduced to make the free sulfhydryl group (0.2 mol/mol of human IgG), which is known to be a potent binding site for cis-DDP. 2\footnote{On the other hand, in the variable region of IgG, the free sulfhydryl group is reported to be only about 0.01 mol/mol of human IgG. 17}\footnote{We indeed confirmed the almost complete absence of free thiol in \( \gamma \)-globulin, suggesting that the binding of cis-DDP to an intrinsic SH group need not be considered.}

Figure 4 shows the relationship between the bound cis-DDP and the decreased number of S-S bonds in \( \gamma \)-globulin. An approximate linear relationship having a slope of 0.229 (\( r = 0.989 \)) was obtained regardless of the initial cis-DDP to \( \gamma \)-globulin molar ratios. This indicates that the S-S bond was cleaved at the ratio of one S-S bond per 4.4 mol of cis-DDP binding in the range of the total bond cleavage up to at least 4 bonds. In addition to the S-S bond, other residues including Met residue in \( \gamma \)-globulin could conceivably be responsible for the multiple binding of cis-DDP. 21\footnote{\( \gamma \)-Globulin bound cis-DDP to a lesser extent than human serum albumin when...}
one S–S bond was cleaved in the proteins.

**Effect of Oxygenated Medium on the S–S Cleavage** The effect of the oxygen gas dissolved in the reaction medium on the time course of the cleavage of S–S bond was examined. Figure 5 shows representative data obtained using a *cis*-DDP concentration of 0.6 mM. The cleavage in a medium oxygenated by oxygen was slower than that in an untreated medium, as shown in Fig. 2. This result suggests that the thiols, once formed by *cis*-DDP, are then oxidized by the oxygen dissolved in the medium. This explains the apparent reduced cleavage of a S–S bond in the oxygenated medium. Since sulfhydryl groups show a high affinity for platinum, the possibility of free thiol in coordination with another *cis*-DDP molecule cannot be ruled out.

**SDS-PAGE of Heat-Denatured γ-Globulin and Reduced One** γ-Globulin or IgG is known to form insoluble aggregates due to heat-denaturation. Upon reduction, H chains of immunoglobulins are also insoluble at neutral pH. These precipitates were prepared in order to compare them with *cis*-DDP-treated γ-globulin. Protein solution in pH 7.4 phosphate buffer was heated at 62°C for different time intervals of 13–60 min as previously described, then cooled. The extent of precipitation was dependent on heating time. The precipitated protein was completely solubilized with 25% urea. No turbidity was detected at 360 nm in the solubilized solution. Figure 6 shows SDS-PAGE of urea-solubilized heat-denatured γ-globulin (1–4), and urea-solubilized γ-globulin that was aggregated during incubation with different concentrations of *cis*-DDP at 37°C for 14 d (5–8). Native γ-globulin shows a broad band due to the heterogeneous feature of the polyclonal one. The pattern obtained for heat-denatured γ-globulin differs significantly from that of the *cis*-DDP-treated protein. The apparent molecular weight of heat-denatured γ-globulin was not altered to the native form, which is in agreement with a report by Williams et al., whereas *cis*-DDP induced high molecular weight adducts in a dose-dependent manner. Since the denaturant would disrupt only the aggregates, which are held together by noncovalent forces, both of the precipitated γ-globulins due to heat and *cis*-DDP are likely to be attached noncovalently. However, *cis*-DDP-treated γ-globulin, even after being solubilized by urea, still has polymeric characteristics as is evident from the

![Fig. 5. Disulfide Bond Cleavage in an Oxygenated Medium γ-Globulin, 3 mg/ml; cis-DDP, 0.6 mM.](image)

<table>
<thead>
<tr>
<th>S</th>
<th>N</th>
<th>denatured globulin</th>
<th><em>cis</em>-DDP treated globulin</th>
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<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>106.0 kDa</td>
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Fig. 6. SDS-PAGE of Heat-Denatured γ-Globulin and *cis*-DDP-Treated γ-Globulin

Heat-denatured γ-globulin was prepared by heating at 62°C for 1, 13, 2, 28, 3, 45, 4; 60 min. *cis*-DDP-treated γ-globulin was prepared by incubation at 37°C for 14 d with *cis*-DDP, 5, 0.1; 6, 0.2; 7, 0.4; 8, 0.6 mM. S is standard SDS-PAGE molecular weight marker. N is native γ-globulin.

![Fig. 7. SDS-PAGE of Reduced Form of γ-Globulin and *cis*-DDP-Treated γ-Globulin](image)

<table>
<thead>
<tr>
<th>number of S–S bond cleaved</th>
<th>NaSO₃</th>
<th><em>cis</em>-DDP</th>
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<tbody>
<tr>
<td>1.4</td>
<td>1.5</td>
<td>1.6</td>
</tr>
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γ-Globulin was reduced by NaSO₃ and then alkylated as described in Materials and Methods. The incubation time with 10 mM NaSO₃ or 0.1–0.6 mM *cis*-DDP was controlled to adjust the approximate number of cleaved S–S bonds. STD is standard SDS-PAGE molecular weight marker. N is native γ-globulin.
band in Fig. 6, which might involve platinum covalently bound to γ-globulin in an undefined manner.

Figure 7 shows SDS-PAGE for two types of γ-globulin being prepared so as to adjust the cleaved S-S bonds to similar extents by incubation with 10 mM Na₂SO₄ or cis-DDP (0.1—0.6 mM) at adequate intervals. The IgG molecule consists of two sets of L and H chains (LHHL). An L chain is linked to an H chain by a single S-S bond. The two H chains are linked together by different numbers of S-S bonds dependent on subclasses. The fragments obtained by reducing γ-globulin are derived from IgG and are L, H, and some combinations of these chains, with a molecular weight range from about 25 kDa to about 150 kDa. No bands were detected in the upper region other than the native protein. In the course of reducing γ-globulin, no appreciable precipitate was observed.

cis-DDP-treated γ-globulin, on the other hand, had a distinct pattern from the reduced one, irrespective of the similar extent of cleaved S-S bonds. No bands were detected in the lower region other than the native protein. Instead, γ-globulin was gradually changed to a polymer form with increasing cleavage of S-S bonds which is not clearly shown in Fig. 6.

Effect of Aquated Complexes of cis-DDP on γ-Globulin cis-DDP readily undergoes hydrolysis to give the monoauqua or diaqua complex at physiological pH, and temperature and in a very low level of Cl⁻ (4 mM) like intracellular space. As reported by others, the first step in cis-DDP binding to the intracellular target DNA is controlled by the rate of hydrolysis.²⁰ The hydrolysis products formed via nucleophilic substitution by solvent water are known to be the active anti-tumor species. The reactive form, the aquated complexes of cis-DDP, may also give rise to favorable binding to proteins or sulfur-containing compounds which may have some relevance to the severe side effects of cisplatin administration.²⁰ LeRoy and Thompson reported that prior aquation was also required for the binding of cis-DDP to human plasma protein.²¹ In high chloride environments, such as the extracellular space or plasma (103 mM), hydrolysis is thought to be significantly inhibited. However, Cl⁻ would not completely prevent the formation of any aquated complexes in an acceptable concentrations, since the loss of the chloro ligands from cis-DDP follows equilibrium reactions.²² Administration of cis-DDP in hypertonic saline brought about decreased nephrotoxicity, which is presumably due to Cl⁻ inhibition of hydrolysis and/or the reaction with DNA in nontarget organs, tissue, and proteins.²³

In this study, the effect of the aquated complexes on γ-globulin was examined. γ-Globulin incubated with the 0.4 mM aquated complexes of cis-DDP exhibited an extremely rapid aggregation relative to cis-DDP: the turbidity at 360 nm reached about 2 following just a 4-d incubation as shown in Fig. 8. Although the turbidity is dependent on the particle size and the number of the particles, it is not always directly proportional to the concentration of the material composing the particle. If the diameter of a particle is significantly smaller than the wavelength, Rayleigh scattering gives a linear relationship between turbidity and concentration. In this case of particles which consisted of polymerized γ-globulin and cis-DDP, we should regard the observed turbidity as reflecting only the approximate size or concentration of the particle since the size was not measured.

Figure 9 shows the corresponding increase in the number of cleaved S-S bonds. The effects of the aquated form on the precipitation and the S-S decrease were larger than those by cis-DDP. For example, the turbidity development of 1.9 provoked by 4-d incubation with the aquated complexes was 3-fold greater than that (0.6) by 14-d incubation with cis-DDP.²⁵ Also, the number of cleaved S-S bonds of γ-globulin induced by 4-d incubation with the aquated form, 5.6, was about 2-fold greater than that by the 14-d incubation with cis-DDP of 3.1 (Fig. 3). γ-Globulin, the highest plasma protein next to albumin, interacted with cis-DDP in a peculiar way. The cis-DDP induced aggregate was clearly different from heat-denatured aggregation or the reduced form. The aggregation process may be derived from a noncovalent association of polymerized γ-globulin that is induced by the covalently bound platinum complex. The aquated form of cis-DDP causes γ-globulin aggregation and its S-S bond cleavage to a greater extent than does cis-DDP. This is attributed to potent electrophilic characteristics that will
react with any nucleophile including sulfur-containing residues on protein and nucleophilic groups on nucleic acids.

Associated or denatured γ-globulin often shows antigenicity, and may be responsible for rheumatoid arthritis or other connective tissue diseases.\(^7\) For example, human rheumatoid factors are IgM, IgG, IgA, or IgE antibodies binding to the Fc portion of IgG, which are autoantibodies.\(^8\) It is well known that the insoluble antibody complex, which is formed as the result of the immune defense reactions is likely to deposit at the kidney, leading to nephrotoxicity. There is a possibility that the γ-globulin precipitation induced by cis-DDP and its aquated forms may contribute to the platinum nephrotoxicity.

Acknowledgements We thank Professor Ken Ikeda of Nagoya City University for helpful advice and continuous encouragement and Mr. T. Matsuo and Mr. H. Muta for their assistance in the experimental work.

REFERENCES