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# Polymerized precipitation of human γ-globulin induced by *cis*-diamminedichloroplatinum(II)

Danni Chen, Naoko Ohta, Toshihisa Yotsuyanagi *, Ken Ikeda

*Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan*

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**Abstract**

The reaction of human γ-globulin and *cis*-diamminedichloroplatinum(II) (*cis*-DDP) was investigated based on turbidity changes and disulfide bond titration. Precipitate formation of the protein was gradually increased with time, depending on the *cis*-DDP concentration. Disulfide bonds of 2.7 mol of γ-globulin (protein concentration, 3 mg/ml; assumed molecular mass, 150 kDa) were cleaved during the first 3-days of incubation with 0.6 mM *cis*-DDP. SDS-polyacrylamide gel electrophoresis showed that the molecular mass of the precipitated γ-globulin was greater than that of its natural form, suggesting that γ-globulin was polymerized by intermolecular interaction presumably through thiols being exposed after cleavage of the original disulfide bonds.

*Key words: cis*-Diamminedichloroplatinum(II); Human γ-globulin; Disulfide bond cleavage; Polymerization

*cis*-Diamminedichloroplatinum(II) (*cis*-DDP) has been used extensively in the anticancer treatment of solid tumors (Timmer-Bosscha et al., 1992). Due to its strong nucleophilic displacement activity for chloride(s), *cis*-DDP reacts to DNA as well as proteins and enzymes (Geary and Gonias, 1989; Bancroft et al., 1990). Amino acid residues such as cysteine, methionine, and histidine were shown to be preferential sites in the protein binding (Howe-Grant and Lippard, 1980; Pattanaik et al., 1992). Also, it has been demonstrated that the disulfide (S-S) bond can serve as another platinum binding site in serum proteins such as human serum albumin and fibrinogen (Yotsuyanagi et al., 1991; Ohta et al., 1992a; Chen et al., 1994). Furthermore, S-S cleavage by *cis*-DDP resulted in the alteration of their secondary structures (Ohta et al., 1992b, 1993). γ-Globulin, one of the major class of immunoglobulins in the plasma, consists of two sets of light (L) and heavy (H) chains which are held together by interchain S-S bonds (Burton, 1985). These bonds are crucial for establishing a specific conformational integrity and its immunological functions. The present study was carried out to study the effect of *cis*-DDP on the S-S bond in γ-globulin.

*cis*-DDP was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Human γ-globulin (G-4386, lot. 106F-9315) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Disodium 2-nitro-5-thiosulfobenzoate (NTSB)

* Corresponding author. Tel: 052-836-3451; Fax: 052-834-9309.


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was synthesized according to the method of Thannhauser et al. (1984). All other chemicals were of reagent grade. *cis*-DDP was dissolved in a phosphate buffer solution (50 mM, pH 7.4) with 0.1 M NaCl (ionic strength = 0.25). γ-Globulin was also dissolved in the same medium and was filtered through a 0.2 μm sterilized filter (Toyo Roshi, Tokyo). Each reaction solution was again filtered through a 0.2 μm sterilized filter and placed in a sterilized test tube with a screw cap. The medium was prepared under normal conditions and not particularly deoxygenated. 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. At appropriate incubation intervals at 37°C, one of the tubes was separately taken out for measurements of turbidity and the number of S-S bonds.

Turbidity of the reaction solutions was measured using a UV-260 (Shimadzu, Kyoto) at a wavelength of 360 nm, where the absorption contribution of the protein was minimal. The concentration of γ-globulin in the supernatant was determined after centrifugation of the reaction solution at 65 000 × *g* for 30 min. The protein was assayed using the Coomassie brilliant blue G kit (Bio-Rad) (Bradford, 1976). The NTSB assay solution was prepared by diluting a synthesized NTSB solution according to the method of Kella

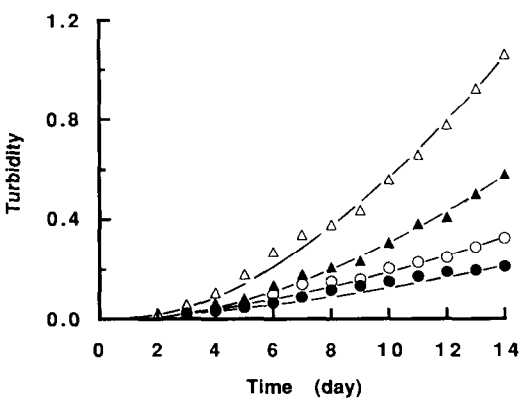


Fig. 1. Turbidity changes of γ-globulin solution incubated with *cis*-DDP at 37°C. Initial γ-globulin concentration: 3 mg/ml. *cis*-DDP concentration: 0.1 mM (●); 0.2 mM (○; 0.4 mM (▲); 0.6 mM (△). Medium: pH 7.4, 50 mM phosphate buffer containing 0.1 M NaCl.

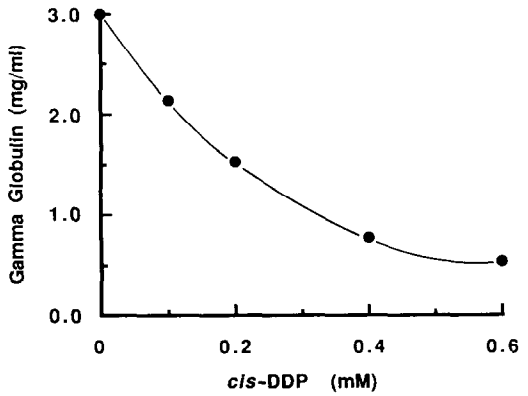


Fig. 2. The concentration of the γ-globulin in the supernatant of the reaction solution incubated with *cis*-DDP for 14 days. Initial γ-globulin concentration: 3 mg/ml.

(1988) with some modifications as described previously (Ohta et al., 1992b). The concentration of S-S bonds was calculated based on the extinction coefficient of 2-nitro-5-thiobenzoic acid, 13 600 $M^{-1}$ $cm^{-1}$ (Ellman, 1959). Reduced and alkylated γ-globulin was prepared based on the method of Davies et al. (1989). γ-Globulin was reduced by incubation with dithiothreitol (DTT) (0.25–4.0 mM) for 30 min. The reaction was stopped by the addition of iodoacetamide at a level of 2–5-times the concentration of DTT. γ-Globulin treated with *cis*-DDP or DTT was analyzed by electrophoresis in the presence of sodium dodecyl sulfate (SDS) on 7.5% polyacrylamide gel at pH 8.8. Protein precipitate was dissolved in 25% urea before being applied on gel electrophoresis.

Incubation of γ-globulin with *cis*-DDP in pH 7.4 buffer at 37°C resulted in the appearance of fine precipitation and the solution gradually turned opaque. As shown in Fig. 1, the turbidity developed with time in a dose-dependent nonlinear manner. It appeared not to reach a plateau over an incubation period of 14 days. In order to confirm the constituents of the precipitate, the protein concentration in the supernatant was assayed after 14 days (Fig. 2). As the protein content significantly decreased during incubation, the precipitate was ensured to be predominantly made of γ-globulin. Although the precipitate was not observed over an initial period after incuba-

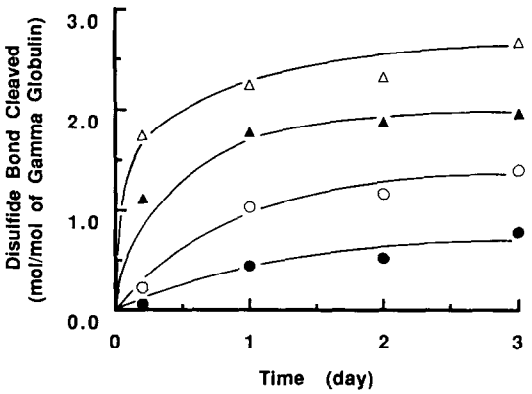


Fig. 3. The number of cleaved disulfide bonds of γ-globulin incubated with *cis*-DDP for the initial 3 days. Temperature: 37°C. Initial γ-globulin concentration: 3 mg/ml. *cis*-DDP concentration: 0.1 mM (●); 0.2 mM (○); 0.4 mM (▲); 0.6 mM (△). Medium: pH 7.4, 50 mM phosphate buffer containing 0.1 M NaCl.

tion, the number of S-S bonds was examined for the initial 3 days during which the solution remained transparent. Fig. 3 shows that the number of S-S bonds cleaved increased with time and concurrent *cis*-DDP. However, the time course of S-S cleavage did not correlate with the turbidity change. This discrepancy suggests that the initial precipitation develops only when cleavage occurs to a certain degree. The 3 days incubation at a 30-fold excess of *cis*-DDP (0.6 mM) to the protein resulted in the cleavage of 2.7 mol S-S per mol of protein, where the average molecular mass of gamma globulin was assumed to be 150 kDa, as L and H chains are of approx. 25 and 50 kDa, respectively. As γ-globulin used in this study was a polyclonal one and a mixture of four subclasses, $IgG_1$, $IgG_2$, $IgG_3$ and $IgG_4$ with different numbers of total S-S bonds, the average number of S-S bonds was estimated to be 17.1 on the assumption that the fractions of the four subclasses were comparable to those in serum (Nisonoff, 1984). As indicated in Fig. 1 and 3, the S-S scission of at least one to two S-S bonds might play a key role in inducing precipitation which was able to be detected by turbidity at 360 nm. In preliminary experiments, S-S cleavage in a medium oxygenated by oxygen gas showed a slightly slower tendency than that deoxygenated by $N_2$ gas. This suggests that thiols once formed by *cis*-DDP might be oxidized by the oxygen dissolved in the reaction medium.

If cleavage occurred between the inter H chains or the H and L chains to release H or L chain, it could be identified through gel electrophoresis. Fig. 4 demonstrates SDS-polyacrylamide gel electrophoresis of γ-globulin which was treated with *cis*-DDP for 3 or 14 days, in comparison with

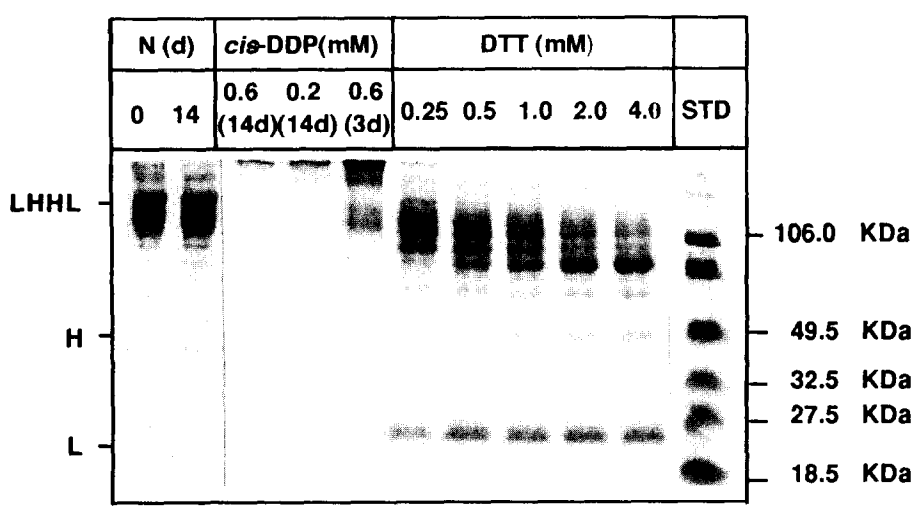


Fig. 4. SDS-polyacrylamide gel electrophoresis of γ-globulin incubated with *cis*-DDP for 3 or 14 days. N, native γ-globulin incubated without *cis*-DDP at 37°C for 0 and 14 days. γ-Globulin was partially reduced by DTT (0.25–4 mM).

γ-globulin reduced by DTT and alkylated with iodoacetamide. When DTT was used, several fragments, including L and H chains, were detected, indicating that S-S bonds, both inter H chains and H-L chains, were cleaved by DTT. The heterogeneous feature of the polyclonal γ-globulin was reflected in the broad bands. The pattern obtained for *cis*-DDP-treated globulin was substantially different from that of the fragmentation by DTT. When γ-globulin was incubated with *cis*-DDP for 3 days, half of the protein remained at the origin, while the other half stayed at the LHHL zone. Furthermore, γ-globulin incubated for 14 days remained at the origin with no appreciable amount of the intact molecules. The results suggest that the γ-globulin changed to a high-molecular-mass form, such as a polymerized species. This polymerization might be attributed to S-S reformation caused in part by the interaction between the intermolecular thiols being exposed due to S-S scission by *cis*-DDP. The polymerization process is likely to be accompanied by an alteration in the structure of γ-globulin, leading to lower solubility. The number of intrachain S-S bonds is always fixed at 12 and the interchain bonds depend on the subclasses. Under mild conditions for reduction, such as with 10 mM DTT or 0.2 M 2-mercaptoethanol, interchain bonds are known to be more readily reduced than intrachain bonds (Nisonoff, 1975). However, from these limited results, it could not be determined which type of S-S bonds was mainly involved in the *cis*-DDP-treated γ-globulin.

In conclusion, *cis*-DDP is likely to be involved in the cleavage of S-S bonds in human γ-globulin, which subsequently allows formation of precipitates composed of polymerized γ-globulin. Although the initial concentration of γ-globulin is about one-fifth of the physiological concentration, the present results suggest that insoluble *cis*-DDP-globulin complexes may have an important implication for the nephrotoxicity often observed in *cis*-DDP medication. The in vivo biochemical reaction mechanism of nephrotoxicity is unknown, but soluble or insoluble immune complexes are known to occur in the normal immune defense mechanism and may be trapped by renal glomeruli under certain conditions like impaired phagocytic functions (Boers et al., 1992). Similar entrapment may be involved in the treatment by *cis*-DDP.

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