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Disulfide bond cleavage of human serum albumin and alterations of its secondary structure by *cis*-diamminedichloroplatinum(II)

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Summary

cis-Diamminedichloroplatinum(II) (*cis*-DDP) cleaved disulfide (S-S) bonds in human serum albumin (HSA) and brought about alterations of the secondary structure. The α-helix content decreased from 50.5% (native) to approx. 33% (four S-S bonds cleaved). The tendency toward a decrease corresponded only with an increase in the β-sheet. Sulfitolysis of the S-S bonds showed a tendency similar to that of metal binding. Fluorescence and UV difference spectra changed as a function of S-S bond cleavage and led to considerable differences between the two cleaving agents.

Introduction

The antineoplastic activity of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) is now well recognized (Sherman and Lippard, 1987). Because of its strong nucleophilic displacement activity for one or both of the chlorides, *cis*-DDP reacts with naturally occurring small molecules such as cysteine and methionine, and with biomacromolecules such as proteins, enzymes and DNA (Howe-Grant and Lippard, 1980; Pinto and Lippard, 1985).

cis-DDP binds to albumins in an irreversible manner (Repta and Long, 1980). Gonias and Pizzo (1983) reported that human serum albumin (HSA) bound 1 mol of platinum per mol of protein and significantly increased binding was not found at higher concentrations of *cis*-DDP or after longer incubation times. Momburg et al. (1987) observed that the reaction involves the lone sulfhydryl group (Cys-34) in HSA and possibly another site when the drug is incubated with the protein at an equimolar ratio.

We have demonstrated that *cis*-DDP binds to HSA in a multiple manner beyond one or two sites: 1 mol of HSA can bind as much as 20 mol of platinum irreversibly after incubation with an excess amount of the drug over a period of 2 weeks (Yotsuyanagi et al., 1991). In addition, the metal binding resulted in the cleavage of disulfide (S-S) bonds of the protein, which, to our knowledge, has not previously been reported. The metal binding reactions are therefore likely to generate

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significant structural changes of the protein. However, the magnitude of the *cis*-DDP-induced changes was rather qualitative. Therefore, we have estimated the contents of the secondary structure of the metal-bound HSA from CD spectra (Yang et al., 1986). Cleavage of S-S bonds caused by *cis*-DDP was also compared with that by sulfitolysis.

Materials and Methods

Materials

cis-DDP was purchased from Aldrich Chemical Co. and used as received. HSA (fraction V, lot no. 76F-9353, Sigma) was used after defatting according to the method of Chen (1967). A Coomassie Brilliant Blue G kit (Bio-Rad) was used for the protein assay. Disodium 2-nitro-5-thiosulfobenzoate (NTSB) was synthesized according to the method of Thannhauser et al. (1984). Monoiodoacetamide was purchased from Nacalai Tesque, Kyoto. All other chemicals were of reagent grade.

Binding of cis-DDP to HSA

cis-DDP was incubated with HSA in a phosphate buffer (0.05 M, pH 7.4) with NaCl (0.1 M) at 37°C under aseptic conditions. The molar ratio of *cis*-DDP to HSA was varied up to 60 where the protein concentration was always maintained constant at 0.01 mM. At appropriate intervals, one of the tubes was removed separately in order to provide different metal-bound proteins. In every case, the protein fraction was collected by filtration through Sephadex G-25 (Pharmacia) for further studies. Details have been described elsewhere (Yotsuyanagi et al., 1991).

Sulfitolysis of HSA

Sulfitolysis of S-S bonds of HSA was carried out in the absence of a protein denaturant according to the method of Kella and Kinsella (1985) with some modification. An HSA reaction solution (0.1 mM in pH 7.4, 0.05 M phosphate) containing Na_2SO_3 (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. On removal of a 5 ml sample of the reaction solution at appropriate intervals, the sulfitolysis reaction was stopped by adding EDTA (0.2 M, 1.25 ml). Immediately after separating the protein fraction through Sephadex G-25, the resulting sulfhydryl groups were carboxymethylated by the addition of 30 molar equivalents of monoiodoacetamide with respect to S-S bonds (pH 8.0). The carboxymethylated protein was separated by gel filtration through Sephadex G-25, and the protein was assayed by the Coomassie Blue binding method (Bradford, 1976).

Determination of S-S bonds

The NTSB assay solution was prepared by diluting a synthesized NTSB solution 50 times with a solution containing 0.4 M Tris base, 120 mM EDTA and 4.3 M guanidine thiocyanate (Kella and Kinsella, 1985), and then adjusting the pH to 9.5 with NaOH. The HSA solution (0.5 ml) was mixed with an NTSB assay solution (3 ml) containing a freshly prepared Na_2SO_3 solution (0.4 M). After 20 min the absorbance at 412 nm was monitored against an appropriate blank. The concentration of S-S bonds was calculated by a value for the extinction coefficient of 2-nitro-5-benzoic acid of 13 600 M^{-1} cm^{-1} (Ellman, 1959).

Fluorescence and UV difference spectral measurements

A spectrofluorophotometer (RF-520, Shimadzu, Kyoto) was used at a wavelength of the excitation beam of 300 nm (25°C). The concentration of HSA was maintained fixed at 2 μM throughout. The relative fluorescence intensity at the emission maximum of 350 nm was followed as a function of S-S bond cleavage. The UV difference spectral measurement was carried out at 5 μM protein.

CD spectral measurements and determination of secondary structure

CD spectral measurements were made on a Jasco J-600 circular dichroism instrument. Calibration was achieved using *d*-camphorsulfonic acid. CD spectra were recorded at a protein concentration of 1 μM using a quartz cuvette

with a path length of 0.1 cm. The cuvette chamber atmosphere was purged using nitrogen gas at a flow rate of 5 l/min in order to prevent the formation of ozone. The spectra between 196 and 240 nm were analyzed according to the method of Yang et al. (1986) in which the spectra of 15 water-soluble reference proteins of known three-dimensional structure were integrated. Least-squares curve fitting was carried out at 1 nm intervals in the range of wavelength recorded. The molecular weight of the mean residue was assumed to be 113.8. The quality of the fit was evaluated by calculating a normalized standard deviation (NRMSD) (Brahms and Brahms, 1980). An NRMSD value of less than 0.1 was assumed to provide a good fit of a CD spectrum obtained.

Results and Discussion

Relationship between cis-DDP binding and cleavage of S-S bonds of HSA

Fig. 1 shows the relationship between platinum binding and cleavage of S-S bonds (Yotsuyanagi et al., 1991). The plot was represented by a linear relationship having a slope of about 0.188 (r = 0.991). This indicates that the S-S bond was cleaved at a ratio of 1 mol S-S bond per 5.3 mol *cis*-DDP binding in the range of total bond cleavage up to about 4, where one HSA molecule contains 17 S-S bonds. This relationship, however, could not always be explained in terms of the number of metal atoms bound and of S-S bonds cleaved, if the metal binding occurred only at S-S bonds in addition to Cys-34 (Gonias and Pizzo, 1983). While amino acids such as methionine (six residues in HSA) and histidine (16 residues) are coordinated with platinum(II) complexes (Howe-Grant and Lippard, 1980), these residues may be involved in such multiple binding.

Fluorescence and UV difference studies

Tryptophan fluorescence is most frequently examined among three intrinsic aromatic fluors in HSA. On excitation of the protein at 300 nm, the resulting fluorescence efficiency reflects changes of the neighboring microenvironment of the lone

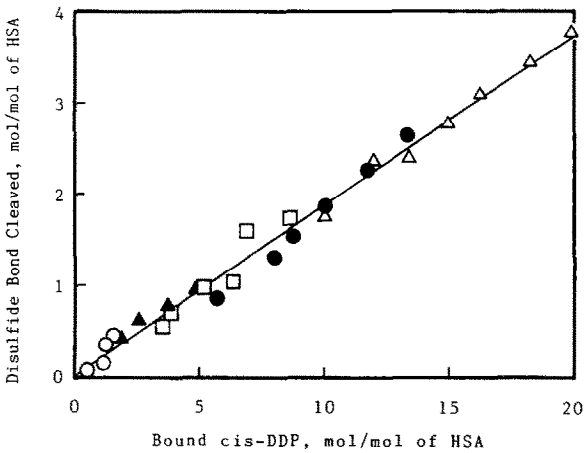

Fig. 1. Relationship between *cis*-DDP binding and cleavage of S-S bonds. *cis*-DDP and HSA were incubated at pH 7.4 (phosphate buffer with 0.1 M NaCl) and 37°C for 2 weeks. Molar ratio (*cis*-DDP/HSA): (○) 5, (▲) 10, (□) 20, (●) 40, (△) 60. Concentration of HSA: 10 μM. A linear regression analysis gave $y = 0.188x - 0.001$ ($r = 0.991$).

tryptophan residue (Trp-214) (Steinhardt et al., 1971). Fig. 2 shows changes of the relative fluorescence intensity of the protein whose S-S bonds were progressively cleaved by *cis*-DDP and sulfitolysis. The relative fluorescence quenching due to metal binding was greater than that resulting

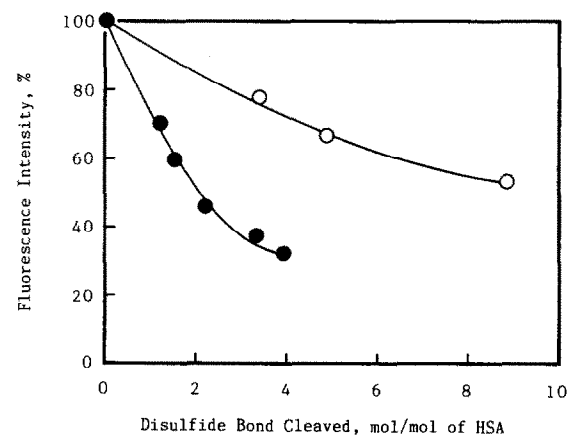

Fig. 2. Relative fluorescence intensity changes of HSA as a function of S-S bonds cleaved by *cis*-DDP (●) and by Na_2SO_3 (○). HSA: 2 μM in pH 7.4 phosphate buffer with 0.1 M NaCl. Temperature: 25°C. Excitation at 300 nm and emission at 350 nm. The relative fluorescence intensity of native HSA was taken as 100%.

from sulfitolysis: the 50% decrease in intensity was generated by two bond cleavages in the metal binding, while the same degree of decrease requires about eight bond cleavages in sulfitolysis. According to empirical rules (Freifelder, 1976), the tryptophan residue is considered to be exposed to a more polar environment with increasing S-S bond cleavage. Try-214 is located in domain II which contains six S-S bonds (Dugaiczyk et al., 1982). Although the S-S bonds which are predominantly involved in these reactions are unknown, the results suggest that the order of susceptibility of the S-S bonds which are influenced by *cis*-DDP binding is different from that in sulfitolysis.

Fig. 3 shows changes in the UV difference spectra, measured at 225 nm for the same proteins as indicated in Fig. 2. This change is characterized as being a measure of the denaturation of albumin when a positive difference is seen in the neighborhood of 230 nm (Glazer and Smith, 1960; Wetlaufer, 1962). The spectra showed clear positive differences compared to the native protein as a function of S-S bond cleavage. The magnitude of the change in the difference spectrum caused by metal binding was greater than that induced by sulfitolysis. This result also suggests that the mode of reaction of the cleavage is different

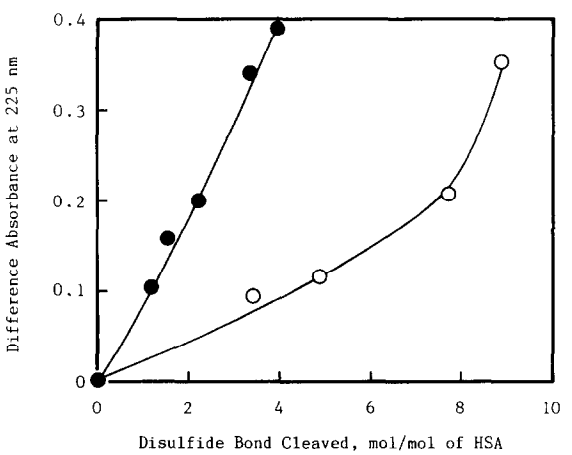

Fig. 3. Difference absorbance changes of HSA as a function of S-S bonds cleaved by *cis*-DDP (●) and by Na_2SO_3 (○). HSA: 5 μM in pH 7.4 phosphate buffer with 0.1 M NaCl. Wavelength: 225 nm. Temperature: 25°C.

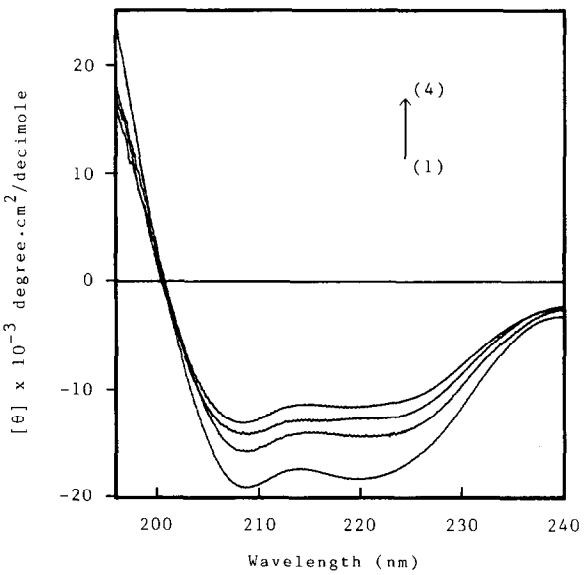

Fig. 4. CD spectral changes of HSA with different numbers of S-S bonds cleaved by *cis*-DDP. Number of bonds cleaved: (1) 0, (2) 1.18, (3) 2.20, (4) 3.93. HSA: 1 μM in pH 7.4 phosphate buffer with 0.1 M NaCl.

between the two treatments. These difference spectra appear to reflect conformational changes of the peptide bonds in addition to microenvironmental perturbations of aromatic amino acid residues (Glazer and Smith, 1960; Wetlaufer, 1962).

CD study

As significant structural changes of HSA were suggested from the results shown above, we estimated the contents of the secondary structure from CD spectral changes. Figs 4 and 5 show CD spectra of HSA with different numbers of S-S bonds cleaved by *cis*-DDP binding and sulfitolysis, respectively. We found that, with increasing numbers of S-S bonds cleaved, the positive band at 195 nm decreased and a broad negative band with a minimum at 208 nm increased.

Figs 6 and 7 depict the relative contents of α-helix, β-sheet, β-turn and random coil of the corresponding proteins, where the NRMSD value of each fit was less than 0.1. Native HSA was found to have a content of 50.5% α-helix, 8.6% β-sheet, 2.6% β-turn and 38.3% random coil. It should be noted that the random coil and β-turn

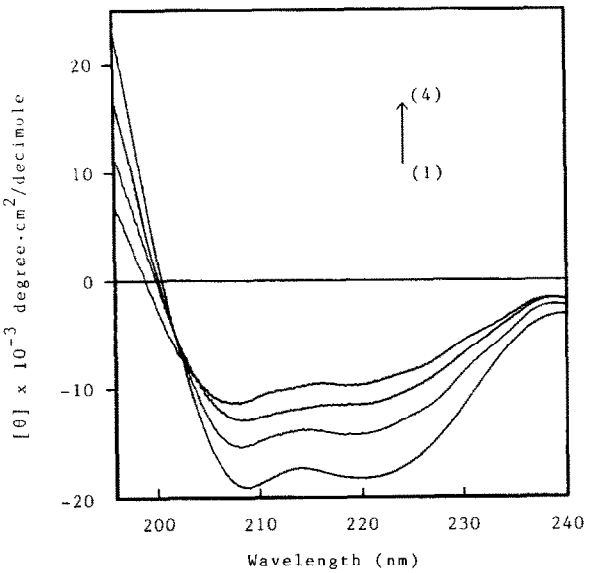

Fig. 5. CD spectral changes of HSA with different numbers of S-S bonds cleaved by Na_2SO_3. Number of bonds cleaved: (1) 0, (2) 3.39, (3) 4.88, (4) 8.86. HSA: 1 μM in pH 7.4 phosphate buffer with 0.1 M NaCl.

contents remained almost unchanged with increasing cleavage of S-S bonds regardless of metal binding and sulfitolysis. The data obtained in sulfitolysis are consistent with those reported for

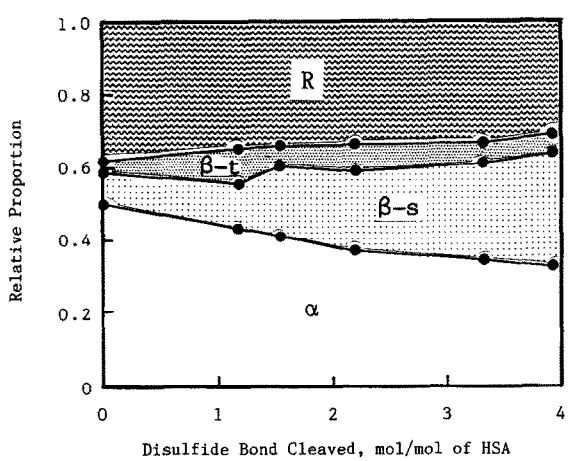

Fig. 6. Relative contents of α-helix (α), β-sheet (β-s), β-turn (β-t) and random coil (R) of HSA as a function of S-S bonds cleaved by *cis*-DDP.

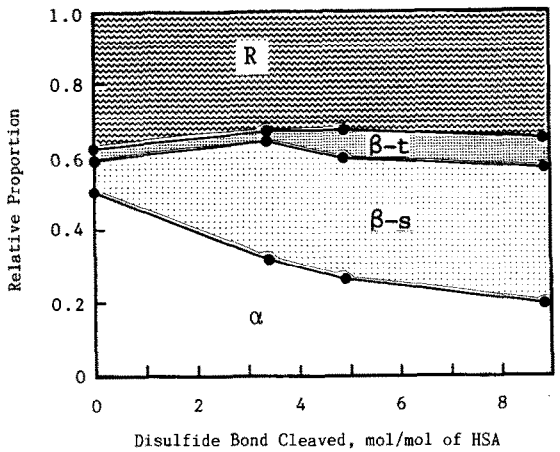

Fig. 7. Relative contents of α-helix (α), β-sheet (β-s), β-turn (β-t) and random coil (R) of HSA as a function of S-S bonds cleaved by Na_2SO_3.

bovine serum albumin (BSA) (Kella et al., 1988). These results suggest that the *cis*-DDP binding reactions (corresponding to up to four cleavages) and sulfitolysis (up to eight 8 cleavages) did not bring about denaturation in the same sense as that which is generally characterized by an increase in random coil content. In addition, the α-helix content decreased with increasing numbers of bonds cleaved, this decrease corresponding approximately with an increase in the β-sheet alone. However, to our surprise, we found that the tendency toward a decrease due to metal binding was quite similar to that as a result of sulfitolysis, despite the considerable differences observed in fluorescence and UV difference measurements even up to the cleavage of four S-S bonds.

Although a reasonable explanation for these unexpected results could not be determined, possibly due to the multiple contributions involved, it is probable that the cleavage of S-S bonds without a denaturant would exert a relatively minor effect on the secondary structure of HSA, if the sequence of cleavage occurred in a different order, however, changes in the microenvironment of a specific region would be largely affected by the cleaving agents used.

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