Inactivation of the Plasma Protease Inhibitor $\alpha_2$-Macroglobulin by the Antitumor Drug cis-Dichlorodiamineplatinum(II)*

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The plasma protease inhibitor $\alpha_2$-macroglobulin ($\alpha_2$M) was reacted in vitro with cis-dichlorodiamineplatinum(II) (cis-DDP). Following the reaction, $\alpha_2$M demonstrated a significantly decreased ability to bind trypsin as determined by esterase activity assays in the presence of soybean trypsin inhibitor and studies with radiolabeled trypsin. Inactivation of $\alpha_2$M by cis-DDP was not associated with a conversion to the "fast" electrophoretic form, as determined on nondenaturing gels, in contrast to the inactivation of $\alpha_2$M by proteases and certain amine salts. The extent of reaction increased with the elevation of temperature within the thermal stability range of the protein; however, variation of pH within the range 6.82-8.55 had little effect. Binding of $[^{14}]$C)methylamine to $\alpha_2$M was not affected by cis-DDP. The conformational change, however, which normally accompanies this reaction did not occur. It is concluded that the $\alpha_2$M thiolesters are most likely not reactive sites for cis-DDP.

cis-DDP-treated $\alpha_2$M failed to dissociate into quarter subunits under denaturing and reducing conditions, suggesting cross-linking of subunits. This cross-linking may be responsible for locking the $\alpha_2$M quaternary structure into the "slow conformation."

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* cis-Dichlorodiamineplatinum(II) is an uncharged square planar complex with anti-tumor activity (1-4). cis-DDP is relatively inert when dissolved in a salt solution containing chloride at a concentration comparable to that present in the plasma (~100 mM) (5). Some degree of ligand exchange, however, is possible at the chloride sites (6). These chloride sites are probably responsible for the biological reactivity of the molecule since dissociation of the amine groups from the complex does not occur under physiological conditions (6, 7).

cis-DDP-DNA interactions are correlated with anti-tumor activity (5). It has been suggested that cis-DDP may cross-link complementary DNA strands (8). Reactions between cis-DDP and proteins have also been reported. cis-DDP inhibits the intracellular enzymes malate dehydrogenase, liver alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase when studied in vitro (9, 10). Protein sulfhydryl groups react readily with cis-DDP and the presence of these groups at the active site of an enzyme may explain enzyme inactivation (10, 11). By contrast, little is known about the reactivity of plasma proteins with cis-DDP. The plasma clearance of cis-DDP is biphasic, with an initial short phase of rapid clearance followed by a subsequent prolonged slow phase (12). During this second phase, between 65 and 98% of the administered cis-DDP remaining in the plasma is protein-bound. No effort has been made to determine which plasma proteins react with cis-DDP.

$\alpha_2$-Macroglobulin is a plasma protein consisting of four $M_0 = 180,000$ subunits which are considered to be essentially identical (13-15). The protein reacts with numerous proteases representative of all the major protease classes (16). Reactions require active endopeptidase and are essentially irreversible. The active site of the reacting protease may, however, only be inhibited and not totally inactivated (17).

$\alpha_2$M is inactivated by a variety of low molecular weight ammonium analogues (18). For both protease and ammonium analogues, reactions can be followed on nondenaturing gels as an increase in mobility associated with a change in conformation in the $\alpha_2$M protein (18). Recent studies have demonstrated the presence of a reactive thiolester in each of the $\alpha_2$M subunits (19-21). Cleavage of this bond has been implicated in the covalent linkage of both amines and protease to $\alpha_2$M.

$\alpha_2$M function is important in a variety of pathological conditions and disease states (22). Adams et al. (23) have suggested that a neutral protease secreted by activated macrophages has cytolytic activity against various tumors. Plasma protease inhibitors, including $\alpha_2$M, appear to bind and inhibit this protease (24). The presence in $\alpha_2$M of highly labile thiolester linkages and the possible involvement of $\alpha_2$M in physiological anti-tumor function prompted us to study the interaction between this protease inhibitor and cis-DDP.

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**EXPERIMENTAL PROCEDURES**

**Reagents**—Trypsin, purchased from Worthington, was 70% active by active site titration with $p$-nitrophenyl-$p'$-guanidinobenzoate-HCl (25). Soybean trypsin inhibitor, $N^\alpha$-benzyloxyl-$N^\beta$-arginine-$p$-nitroanilide-HCl, and PNPGB were purchased from Sigma. cis-DDP of the highest purity commercially available was purchased from Aldrich, which also supplied the reagents for metal chelation chromatography (26). $[^{14}]$C)methylamine was obtained from Amersham, $[^{12}]$I for protein iodination from New England Nuclear, and lactoperoxidase from Sigma. Electrophoresis reagents were purchased from Bio-Rad. All other reagents were of the best commercial grade available.

$\alpha_2$M Purification—$\alpha_2$M was purified using a modification of the procedure described by Kurecki et al. (27). A detailed description of this purification has been presented elsewhere (28). The final $\alpha_2$M preparation was free of contaminants as determined by NaNiO$_2$ gel electrophoresis. Densitometry scans of nondenaturing gels of the preparation indicated that greater than 90% of the $\alpha_2$M was in the slow conformation (18).

**Radiolabeling of Proteins**—$\alpha_2$M and trypsin were radiolabeled with $[^{12}]$I using the solid state lactoperoxidase method described by David and Reisfeld (29). Radioactivity was measured using a Scien...
The activity of α2M was studied as its ability to bind radiolabeled trypsin. Standardized quantities of protease inhibitor were reacted with cis-DDP for 3 h at 37 °C in 10 mM Tris-HCl, 90 mM NaCl, pH 7.4. Following incubations, all samples were reacted with 115I-trypsin for 5 min and then chromatographed on Sephadex G-150. Two peaks of radioactivity were detected. The first contained labeled trypsin associated with α2M and the second, free trypsin. Binding of trypsin to α2M was calculated from the fraction of radioactivity eluting in the first peak.

The ability of α2M to bind trypsin following incubation with cis-DDP was also studied using the method of Ganrot (30). Following incubation with cis-DDP, each sample was reacted with trypsin at a 5-fold molar excess to the α2M for 5 min. SBTI was then added for 10 min at a 5-fold molar excess to the trypsin. The initial concentration was approximately 0.5 μM in all experiments. Preparations were reacted with the substrate BAPNA for 1 h at 37 °C. Absorbance was then measured.

The validity of the BAPNA assay was evaluated with a series of experiments controlling for possible interaction between cis-DDP and trypsin or SBTI. Samples containing active trypsin were incubated for 15 min at 23 °C with cis-DDP at concentrations equivalent to those present in the α2M assays. An identical series of samples was reacted with trypsin for 5 min and then treated with SBTI at 5-fold the trypsin concentration for 10 min. For both sets of studies, the cis-DDP used was preincubated for 2.5 h in 4.8 mM Tris-HCl, pH 7.4, at both 48 and 100 mM total [Cl−].

Dialysis Experiments—The reversibility of the inactivation of α2M by cis-DDP was tested in the following experiment. Radiolabeled α2M was added to a solution containing 1.22 mM cis-DDP, 90 mM NaCl, and 10 mM Tris-HCl (final volume, 410 μl). Immediately following addition of the cis-DDP, a 20-μl aliquot was removed from the preparation and reacted with trypsin at 5-fold the α2M concentration for 5 min and then excess SBTI for 10 min. Remaining esterase activity was assayed with the substrate BAPNA. The initial reaction mixture was incubated for 2.5 h at 37 °C, at which time a second 20-μl aliquot was removed and treated identically to the first. The remainder of the reaction mixture was then dialyzed against 4 liters of 10 mM Tris-HCl, 90 mM NaCl. Additional 20-μl aliquots were removed for repetitive analysis at 3, 12, and 30 h. α2M concentration was standardized through comparison of the radioactivity associated with each sample. Following removal of each aliquot, 4 liters of fresh dialysis buffer was substituted.

RESULTS

Trypsin and SBTI Activity in cis-DDP—Stable activity of both trypsin and SBTI under all imposed conditions is essential for the validity of the α2M Ganrot assay. The esterase activity of trypsin was measured in Table I following incubation with cis-DDP for the length of time required in the α2M assays. It has been suggested that in vivo cis-DDP reactivity is highly dependent on chloride concentration. The reactive species forms through exchange of solvent for Cl− which takes place much more rapidly at cytoplasmic [Cl−] (<5 mm) than at plasma [Cl−] (>100 mM) (5, 23). Since physiologic reaction between drug and α2M would occur in the plasma, a comparison of the nature of the reaction at 48 and 100 mM [Cl−] was important. Table I demonstrates that the trypsin esterase activity associated with the free protease is not affected by the cis-DDP or chloride concentration of the medium to which the trypsin is exposed for 15 min before transfer to BAPNA assay buffer. Table I also demonstrates that the SBTI used in these assays is capable of inhibiting essentially all of the trypsin esterase activity at every tested cis-DDP and chloride concentration.

α2M Activity following Treatment with cis-DDP—Preparations containing identical quantities of α2M were reacted with varying concentrations of cis-DDP at 37 °C for 3, 6, and 8 h. Following incubations, α2M activity assays were performed using the Ganrot method as described under “Experimental Procedures.” A dose- and time-dependent inactivation of α2M in its capacity to bind trypsin with detectable esterase activity is clearly shown at both chloride concentrations (Fig. 1). In consideration of these data, 90 mM NaCl, 10 mM Tris-HCl, pH 7.4, was adopted as a standard reaction medium for all the remaining experiments.

In a second series of experiments, α2M-trypsin complex was formed in the absence of cis-DDP. The preformed complex was then treated with cis-DDP, SBTI, and BAPNA substrate duplicating conditions present in the α2M Ganrot assays. The measured esterase activity of trypsin which was already bound to α2M was unaltered by cis-DDP. This result suggests that the diminished esterase activity demonstrated by cis-DDP-treated α2M in Fig. 1 is a result of a decreased capacity to bind trypsin rather than a loss of esterase activity by bound trypsin.

To further test this hypothesis, cis-DDP-treated α2M was reacted with 115I-trypsin and then subjected to gel filtration on Sephadex G-150. Using similar reaction conditions and gel filtration, it has been demonstrated that as many as 4 mol of 115I-trypsin may chromatograph associated with 1 mol of α2M, although a maximum of 2 of these mol demonstrate retained esterase activity (34). Native α2M which had not been treated with cis-DDP co-chromatographed with essentially 100% of the active trypsin reacted with it at a 2.1:1 molar ratio for 5 min. α2M which had been treated with 1.50 mM cis-DDP for 3 h and then trypsin for 5 min at the same molar ratio chromatographed with less than 0.9 mol of trypsin/mol of inhibitor. Treatments with 60 μM and 600 μM cis-DDP resulted in α2M preparations which chromatographed with 1.8 and 1.4 mol of trypsin, respectively. These data demonstrate that cis-DDP treatment can decrease the ability of α2M to bind trypsin. As expected, after cis-DDP incubation, slightly more 115I-trypsin became associated with the α2M preparation than was measurable by esterase activity assay. Sotrup-Jensen

| [cis-DDP] | [Cl−] | Absorbance 410 nm |
|-----------|-------|------------------|
| 0 m M     | 4.8   | 1.146 0.003      |
| 48 μM     | 4.8   | 1.100 0.006      |
| 302 μM    | 4.8   | 1.204 0.006      |
| 1.87 mM   | 4.8   | 1.180 0.007      |
| 0 μM      | 100   | 1.122 0.006      |
| 48 μM     | 100   | 1.184 0.006      |
| 300 μM    | 100   | 1.214 0.007      |
| 1.87 mM   | 100   | 1.135 0.005      |

Table 1
The effect of cis-DDP on the activities of trypsin and SBTI under conditions present in α2M assays

In series A, the indicated concentrations of cis-DDP were incubated with 48 mM Tris-HCl, pH 7.4, for 2.5 h at 37 °C. NaCl was used to increase the total [Cl−] for the second half of the series. 7.5 μg of active trypsin were then incubated with each preparation for 15 min before addition of the substrate BAPNA. In series B, no SBTI was reacted. Series B duplicates series A except for the addition of SBTI to each solution 5 min into the 15-min trypsin incubation. A15+ was measured following incubation for 1 h with substrate.
culated as the ratio of the
reaction preparation to that of the con-
trol incubated in the absence of cis-DDP.

B, BAPNA were then added to each solu-
tion as described under “Experimental
Procedures.” Per cent activity was cal-
culated by the activity assay described
above. In no experiment was protein
inactivation correlated with conversion to
the fast electrophoretic form (Fig. 2). This
is in direct contrast to the result obtained with reactive amine
salts (18).

The altered ability of cis-DDP-treated a2M to complex
protease was then studied as reduced capacity to change
electrophoretic conformation. Eight solutions containing 0.5
μM a2M were incubated with cis-DDP at concentrations be-
tween 2.7 μM and 1.67 mM for 3 h. All 8 samples were reacted
with trypsin for 5 min at a trypsin to a2M molar ratio of 5 and
then excess SBTI. Nondenaturing gels of these solutions were
scanned by a densitometer. Peaks were automatically resolved
and fractional protein content was determined. Fig. 2 shows
four of the eight samples. At a cis-DDP concentration of 6.8
μM, minimal difference in electrophoretic pattern is demon-
strated by a2M as compared to untreated protein. At 42 μM
cis-DDP, an observable fraction of the a2M fails to increase in
mobility from slow to fast. At 1.67 mM cis-DDP the broad
a2M electrophoretic band contains essentially no component
of fast mobility.

Densitometric analysis of these experiments is shown in
Fig. 3. A shoulder of mobility intermediate between the “slow”
and “fast” form is first detected at 17 μM cis-DDP. The propor-
tion of a2M electrophoresing at slower mobilities in-
creases with increasing cis-DDP concentrations. The inability
of cis-DDP-treated a2M to convert to the “fast” conformation
following trypsin treatment is consistent with its failure to
bind trypsin. The possibility, however, that cis-DDP affects
the a2M structure in a manner which interferes with confor-
mational change cannot be excluded.

CBB-R-250 dye binding is directly proportional to the mass
of protein in a band for any given protein within the concen-
tration and mobility ranges applied to nondenaturing gels in
this study (35). The relative dye binding capacities of native
“slow” a2M, protease complexed “fast” a2M, and a2M follow-
ing cis-DDP treatment were determined in a series of experi-
ments in which equal masses of each type of protein were
electrophoresed. Following gel staining, densitometric meas-
urements of bound dye were essentially identical for each of
the three a2M species (data not shown). These data permitted
direct interpretation of the densitometry scans in Fig. 3 as
representing actual protein content in the electrophoretic
bands. In Fig. 4, cis-DDP incubation concentration is plotted
against the percentage of a2M which retains the capacity to
bind trypsin and convert to the fast mobility conformation.

The Behavior of cis-DDP-treated a2M on NaDodSO4
Gels—a2M was incubated with cis-DDP and applied to
denaturing gels (Fig. 5). Before application, solutions were re-
acted with 4 mg/ml of dithiothreitol, 1% NaDodSO4, for 40
min at 37 °C. The possibility of interaction between unreacted
cis-DDP and dithiothreitol was recognized. The NaDodSO4,
dithiothreitol incubation media, however, contained dithio-
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Fig. 3. Densitometric analysis of the ability of cis-DDP-treated $\alpha_2$M to convert to the "fast" electrophoretic form following reaction with trypsin. Samples were treated with cis-DDP for 3 h followed by reaction with trypsin and SBTI as described in Fig. 2. Electrophoresis proceeded toward the anode from left to right. Scans are shown for incubations containing no cis-DDP (a), 17 $\mu$M cis-DDP (b), 106 $\mu$M cis-DDP (c), 267 $\mu$M cis-DDP (d), 665 $\mu$M cis-DDP (e), and 1.67 mM cis-DDP (f). Peak positions correlate with distance of migration for all scans.

Fig. 4. Summary of densitometry data. Incubation of $\alpha_2$M with cis-DDP, followed by reaction with trypsin and SBTI, was performed as described in Fig. 2. Peaks from gel scans were automatically resolved and integrated and the percent protein migrating with a mobility equivalent to that of the fast conformation is plotted against initial cis-DDP incubation concentration.

Fig. 5. NaDodSO$_4$-gel electrophoresis of cis-DDP-treated $\alpha_2$M. $\alpha_2$M was reacted with cis-DDP for 4 h at 37 °C. Samples were then incubated in NaDodSO$_4$-dithiothreitol and subjected to electrophoresis under denaturing conditions. The cis-DDP incubation concentrations for a through g were 0, 7.9 $\mu$m, 27 $\mu$m, 90 $\mu$m, 302 $\mu$m, 753 $\mu$m, and 1.88 mM, respectively. 7.9 $\mu$m, a faint band of very slow mobility is visible. At 90 $\mu$m, a second band of even slower mobility becomes apparent. At higher drug concentrations, significant quantities of protein electrophorese in these two regions. These two bands correspond to those obtained by Harpel (37) when $\alpha_2$M was subjected to NaDodSO$_4$-gel electrophoresis without prior reduction and most likely represent half and whole molecules of $\alpha_2$M. Further evidence that these bands represent half and whole molecules is provided by comparison to the electrophoretic patterns observed by Barrett et al. (18) when $\alpha_2$M was treated with glutaraldehyde. These data demonstrate that cis-DDP prevents dissociation of the $\alpha_2$M quarter subunit under strongly denaturing and reducing conditions. It is highly likely that this effect of cis-DDP results from the formation of cross-linking coordinate covalent bonds between the two reactive sites on the drug and separate subunits of $\alpha_2$M.

Two additional bands of faster mobility than the $\alpha_2$M quarter subunits are present in the untreated control of Fig. 5. These well described bands result from incubation of $\alpha_2$M at temperatures of 37 °C or above in denaturing buffer (38, 39). The diminished intensity of these bands following cis-DDP treatment parallels the decrease in protein mass electrophoresing as resolved quarter subunits.

The Effect of Temperature and pH—Identical preparations of $\alpha_2$M were incubated at various temperatures for 2.5 h both with and without a standard concentration of cis-DDP (Fig. 6). Activity assays using the substrate BAPNA were then performed. In the absence of drug, the activity of the native protein is relatively stable for 2.5 h over a range of increasing

Dithiothreitol at 16 times the cis-DDP molarity for even the sample with the highest drug content. Adequate dithiothreitol was therefore available for complete reduction of the disulfide bonds present in $\alpha_2$M (36, 37). The effect of such reduction should be conversion of $\alpha_2$M to quarter subunits (13) and essentially total conversion was achieved in the absence of cis-DDP. At a cis-DDP incubation concentration as low as
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Fig. 6. Temperature dependence of cis-DDP-induced inactivation of αM. Samples of αM were incubated in duplicate, with and without 1.25 mM cis-DDP, for 2.5 h at the temperatures indicated. αM assays were then performed at room temperature and standard pH using trypsin, SBTI, and the substrate BAPNA. The open bar graphs demonstrate results of the controls without cis-DDP. The hatched bars show measurements for the identical αM samples following cis-DDP exposure. The percentage within each graph represents the activity of the reacted αM relative to the control at the given temperature.

Fig. 7. pH dependence of the inactivation of αM by cis-DDP. αM was incubated at 37°C for 2.5 h in solutions of 90 mM NaCl, 10 mM Tris-HCl at the final pH values noted. Samples were prepared in duplicate. One contained 1.67 mM cis-DDP (hatched bar) and one did not (open bar). αM activity assays using the substrate BAPNA were conducted for all samples. Percentages noted represent activity of the treated αM samples relative to the controls of identical pH.

temperatures to 52°C. αM preparations treated with cis-DDP lost the capacity to protect trypsin esterase activity in a manner which was highly temperature-dependent. At 4°C, the cis-DDP-treated αM retained 94% of the activity of the untreated control while at 52°C only 1% of the relative activity was retained.

The pH dependence of the cis-DDP-αM interaction is studied in Fig. 7. Native αM was relatively stable at 37°C for 2.5 h throughout the tested pH range of 6.85-8.55. Significant inactivation of αM by cis-DDP occurred at each pH tested. Although alteration in reactivity with pH was small, reaction seemed to take place optimally near neutrality.

Kinetic Aspects of the Ability of cis-DDP-treated αM to Bind Trypsin—At room temperature, binding of trypsin to αM is rapid. The possibility that treatment of αM with cis-DDP results in “sluggishly” reacting αM is explored in Fig. 8. Identical cis-DDP-treated samples of αM were incubated with trypsin for between 2 and 60 min before addition of excess SBTI. Following 2 min of trypsin reaction time, the αM preparation demonstrated 86% less trypsin esterase activity than the untreated control. A 10-min incubation resulted in a preparation with slightly greater activity; however, nearly an 80% loss was maintained through 1 h, at which time apparent equilibrium had been reached.

Reversibility of Inactivation— αM treated with 1.25 mM cis-DDP for 2.5 h was dialyzed extensively as described under “Experimental Procedures.” The ability of the αM to bind trypsin and preserve esterase activity was tested before and after reaction with cis-DDP as well as at various stages of dialysis. Approximately an 80% loss in activity was initially observed. Up to 30 h of dialyses failed to reverse this inactivation. There remained over this time period either tightly bound platinum, inhibiting activity of many of the αM molecules, or some degree of irreversible protein modification.

The Reactivity of the Thiolester in cis-DDP-treated αM— Native αM may covalently bind up to 4 mol of methylamine per mol of protein (40). The methylamine-reactive site is the labile thiolester present in each αM subunit (19, 21). Reaction with methylamine induces a conformational change in αM producing the electrophoretic “fast” form (18). αM was incubated for 3 h in solutions containing 50 μM, 555 μM, and 1.85 mM cis-DDP. Following extensive dialysis, each preparation was reacted for 40 min with 200 mM [14C]methylamine and again dialyzed exhaustively. The binding of methylamine to untreated αM was calculated at 3.6 mol/mol. Calculated methylamine binding ratios for αM treated with 50 μM, 555 μM, and 1.85 mM cis-DDP were 3.6, 3.8, and 3.9, respectively. Treatment with cis-DDP, therefore, did not interfere with the reaction between methylamine and the αM thiolestes. This strongly suggests that cis-DDP-αM interaction does not involve αM thiolester bonds.

αM was treated with different concentrations of cis-DDP for 3 h and then dialyzed thoroughly. Each solution was then made 200 mM in methylamine for 60 rain before application on a nondenaturing gel. cis-DDP treatment clearly inhibits the methylamine-reactive site is the labile thiolester present in each αM subunit (19, 21). Reaction with methylamine induces a conformational change in αM producing the electrophoretic “fast” form (18). αM was incubated for 3 h in solutions containing 50 μM, 555 μM, and 1.85 mM cis-DDP. Following extensive dialysis, each preparation was reacted for 40 min with 200 mM [14C]methylamine and again dialyzed exhaustively. The binding of methylamine to untreated αM was calculated at 3.6 mol/mol. Calculated methylamine binding ratios for αM treated with 50 μM, 555 μM, and 1.85 mM cis-DDP were 3.6, 3.8, and 3.9, respectively. Treatment with cis-DDP, therefore, did not interfere with the reaction between methylamine and the αM thiolestes. This strongly suggests that cis-DDP-αM interaction does not involve αM thiolester bonds.

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Inhibition of conformational change was demonstrated in this experiment following reactions equivalent to those which resulted in the maximal incorporation of [\(^{14}\)C]methylamine into \(\alpha_2M\) as described above. The inability of \(\alpha_2M\) molecules to convert to the "fast" conformation in Fig. 9 may not, therefore, be attributed to decreased binding of methylamine to \(\alpha_2M\) as will be discussed below.

**Discussion**

The studies presented in this paper have demonstrated altered function of the plasma protease inhibitor \(\alpha_2M\) following incubation with cis-DDP. This altered function was demonstrated following reaction for 3 h with 17 \(\mu M\) cis-DDP in an experiment testing the ability of drug-treated \(\alpha_2M\) to undergo conformational change following reaction with trypsin. Time course studies using the Ganoth technique (30) indicated that maximal inactivation of \(\alpha_2M\) by cis-DDP was probably not achieved until after 8 h and definitely not by 3 h. In addition, a physical alteration in \(\alpha_2M\) resulting from incubation with 7.9 \(\mu M\) cis-DDP for 4 h was demonstrated using NaDodSO\(_4\)-gel electrophoresis. It is suggested that these in vitro data may be representative of in vivo plasma chemistry occurring during some of the more intensive therapeutic regimens using cis-DDP (2).

Reaction between \(\alpha_2M\) and an active endopeptidase is dependent upon proteolytic cleavage of a susceptible site in \(\alpha_2M\), termed the "bait region" (16). A covalent linkage may be formed between protease and inhibitor; however, this bond is not essential for the inhibitory capacity of \(\alpha_2M\) (41). Conformational change within \(\alpha_2M\), however, is most likely essential to the completion of the irreversible reaction (34). \(\alpha_2M\) demonstrated decreased ability to bind radioactively labeled trypsin following reaction with cis-DDP. This result is consistent with some form of "bait region" modification. Alternatively this result may be explained as failure of the \(\alpha_2M\) molecule to change conformation and trap reacting protease.

Many proteins are completely unaffected by high concentrations of cis-DDP (10). Although other amino acids may react with cis-DDP, it has been suggested that sensitivity of a protein to the drug depends upon that protein having an essential sulfhydryl group at its active site (10, 11). \(\alpha_2M\) possesses no free sulfhydryl groups (21). The labile thiolesters present in \(\alpha_2M\) were explored as alternative reaction sites. cis-DDP failed to inhibit reaction of the subunit thiolesters with [\(^{14}\)C]methylamine. These data are most consistent with lack of interaction between cis-DDP and the \(\alpha_2M\) thiolesters. If, indeed, reaction occurs at these sites, it occurs without significantly altering the thiolester chemistry. This is considered unlikely.

Cis-DDP is a bifunctional reagent in which the two active sites are relatively close together. Attempts in this laboratory to use cis-DDP as a general cross-linking reagent for otherwise dissociable polypeptide chains have been unsuccessful for a variety of proteins. When \(\alpha_2M\) was reacted with cis-DDP, a significant fraction of the protein failed to dissociate into half-subunits under denaturing and reducing conditions. This apparent subunit cross-linking may occur by either of two mechanisms. \(\alpha_2M\) subunits are bridged into half-molecules by disulfide bonds while noncovalent interactions bind the half-molecules together (37). Cis-DDP may react by a two-step mechanism with an intersubunit disulfide bond resulting in a potentially dithiothreitol-resistant cross-link at the disulfide bond site. Alternatively, previously independent amino acids on separate subunits may be cross-linked by cis-DDP. Only this second mechanism can account for subunits which remain associated as units greater than pairs (Fig. 5). It therefore seems likely that at least some and perhaps all of the apparent cross-linking in Fig. 5 represents novel interchain bonds.

The conformational change that occurs in \(\alpha_2M\) following reaction with either methylamine or endopeptidase most likely involves repositioning of the subunits relative to each other (18). Cis-DDP-treated \(\alpha_2M\) incorporated [\(^{14}\)C]methylamine essentially to saturation while a significant fraction of the protein did not demonstrate conformational change. Cis-DDP, therefore, appears to be capable of locking in the quaternary structure associated with the "slow conformation" of \(\alpha_2M\) during the reaction with methylamine. It would seem consistent that this effect should be duplicated during reaction with protease. Failure of \(\alpha_2M\) to change conformation, or "close the trap," may, indeed, totally explain the trypsin binding data. It is difficult to further address this point, however, with the data collected thus far. Initial attack on \(\alpha_2M\) by a reacting protease or amine most likely occurs at different regions of the \(\alpha_2M\) (20). The possibility, therefore, that conformational change is initiated by slightly different mechanisms during these two reactions cannot be excluded. If this were the case, cis-DDP might inhibit a triggering signal during one reaction and not during the other.

The reactivity of cis-DDP has been correlated with the exchange of chloride leaving groups for water molecules in solution. Rosenberg (5) has postulated that the difference between plasma and cytoplasmic chloride content accounts for the ability of the drug to convert to an active mono or dihydroxy species only inside the cell. Evidence has been presented in this study for reaction between cis-DDP and \(\alpha_2M\) at 100 \(mM\) [Cl\(^{-}\)]. The possible reaction between cis-DDP and plasma proteins possessing major physiological function cannot, therefore, be discounted.

Throughout this study, reaction with trypsin has been applied as a standard experiment probing \(\alpha_2M\) protease interaction. \(\alpha_2M\) has been shown to bind proteases from all of the major protease classes (16). Whether the loss of \(\alpha_2M\) activity is specific to its trypsin binding capacity or whether this inactivation generalizes to other proteases is currently under investigation.

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