Ragg, H. (1986) *Nucleic Acids Res. 14*, 1073–1088.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490–6505.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Sie, P., Dupouy, D., Pichon, J., & Boneu, B. (1985) *Lancet 2*, 414–416.
Tollefsen, D. M., & Blank, M. K. (1981) *J. Clin. Invest. 68*, 589–596.
Tollefsen, D. M., Majerus, D. W., & Blank, M. K. (1982) *J. Biol. Chem. 257*, 2162–2169.
Tollefsen, D. M., Pestka, C. A., & Monafo, W. J. (1983) *J. Biol. Chem. 258*, 6713–6716.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tran, T. H., Marbet, G. A., & Duckert, F. (1985) *Lancet 2*, 413–414.
Tran, T. H., Lammle, B., Zbinden, B., & Duckert, F. (1986) *Thromb. Haemostasis 55*, 19–23.
van Dilla, M. A., Deaven, L. L., Albright K. L., Allen, N. A., Aubuchon, M. R., Bartholdi, M. F., Brown, N. C., Campbell, E. W., Carrano, A. J., Clark, L. M., Cram, L. S., Crawford, B. D., Fuscoe, J. C., Gray, J. W., Hildebrand, C. E., Jackson, P. J. Jett, J. H., Longmire, J. L., Lozes, C. R., Luedemann, M. L., Martin, J. C., McNinch, J. S., Meincke, L. J., Mendelsohn, M. L., Meyne, J. Moyzis, R. K., Munk, A. C., Perlman, J., Peters, D. C., Silva, A. J., & Trask, B. J. (1986) *Bio/Technology 4*, 537–552.
Villanueva, G. B. (1984) *J. Biol. Chem. 259*, 2531–2536.
von Heijne, G. (1985) *J. Mol. Biol. 184*, 99–105.
Wallace, R. B., Johnson, M. J., Suggs, S. V., Miyoshi, K., Bhatt, R., & Itakura, K. (1981) *Gene 16*, 21–26.
Witt, I., Kaiser, C., Schrenk, W. J., & Wunderwald, P. (1983) *Thromb. Res. 32*, 513–518.

Intersubunit Cross-Linking by *cis*-Dichlorodiammineplatinum(II) Stabilizes an α_2-Macroglobulin "Nascent" State: Evidence That Thiol Ester Bond Cleavage Correlates with Receptor Recognition Site Exposure†

Paul A. Roche, Poul Erik Hyldgaard Jensen, and Salvatore V. Pizzo*
Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
Received July 16, 1987; Revised Manuscript Received September 18, 1987

ABSTRACT: Treatment of human α_2-macroglobulin (α_2M) with proteinase results in cleavage of the α_2M subunits and subsequently in a conformational change in the inhibitor. This change irreversibly traps the proteinase and is accompanied by the generation of four thiol groups as well as exposure of receptor recognition sites. *cis*-Dichlorodiammineplatinum(II) (*cis*-DDP) causes extensive intersubunit cross-linking of α_2M. Incubation of α_2M or *cis*-DDP-treated α_2M with trypsin results in complete subunit cleavage; however, trypsin treatment of *cis*-DDP-α_2M does not result in a conformational change as determined by nondenaturing polyacrylamide gel electrophoresis (PAGE), receptor recognition site exposure, or appearance of thiol groups from the inhibitor. These results are in marked contrast to previous studies which demonstrated that incubation of *cis*-DDP-treated α_2M with CH_3NH_2 resulted in thiol ester bond cleavage and receptor recognition site exposure. *cis*-DDP-treated α_2M bound only 0.13 mol of ^{125}I-trypsin/mol of *cis*-DDP-α_2M. Incubation of trypsin-treated *cis*-DDP-α_2M with diethyldithiocarbamate (DDC), a potent chelator of platinum compounds, results in the removal of the intersubunit cross-links and completion of the α_2M conformational change as determined by nondenaturing PAGE. Complete receptor recognition site exposure and the appearance of 3.3 thiol groups/mol of α_2M also occur following this treatment. These results demonstrate that cross-linking of α_2M by *cis*-DDP prevents a conformational change in the inhibitor which is necessary for thiol ester bond activation and cleavage. Removal of intersubunit cross-links by incubation with DDC allows the completion of this conformational change. These studies also indicate that there is a strong correlation between thiol ester bond cleavage and exposure of receptor recognition sites on α_2M. It is further suggested that incubation of *cis*-DDP-treated α_2M with trypsin results in a "primed" form of α_2M which is very similar to "nascent" α_2M originally described by Sottrup-Jensen et al. [Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 128*, 127–132]. Incubation of trypsin-treated *cis*-DDP-α_2M with DDC results in the generation of a form of α_2M with complete subunit cleavage and complete thiol ester bond cleavage but essentially no proteinase binding.

Human α_2-macroglobulin (α_2M)[1] is a plasma proteinase inhibitor composed of four identical 180-kDa subunits that inhibits proteinases from all four classes (Swenson & Howard, 1979; Sottrup-Jensen et al., 1983a; Barrett & Starkey, 1973). The mechanism of proteinase inhibition by α_2M is unique in that cleavage of α_2M at a sequence of residues termed the "bait region", located near the middle of each α_2M subunit, leads to a conformational change in the inhibitor which results in the "trapping" of the proteinase (Barrett & Starkey, 1973; Harpel, 1973). Proteolysis of α_2M at the bait region also leads

† This work was supported by Grants HL-24066 and CA-29589 from the National Institutes of Health.
* Address correspondence to this author at the Department of Pathology, Box 3712.

[1] Abbreviations: α_2M, α_2-macroglobulin; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); *cis*-DDP-α_2M, α_2-macroglobulin treated with *cis*-dichlorodiammineplatinum(II); PAGE, polyacrylamide gel electrophoresis; BPTI, bovine pancreatic trypsin inhibitor; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

0006-2960/88/0427-0759$01.50/0

to activation and cleavage of internal thiol ester bonds in α_2M (Sottrup-Jensen et al., 1980). A similar, but not identical, conformational change is also produced upon reaction of α_2M with CH_3NH_2 because of the cleavage of these bonds (Barrett et al., 1979; Gonias et al., 1982; Bjork & Fish, 1982; Cummings et al., 1984). In human α_2M, this change results in a compacting of the molecule as determined by hydrodynamic studies (Gonias et al., 1982; Bjork & Fish, 1982) and small-angle X-ray scattering techniques (Branegård et al., 1982; Österberg & Malmensten, 1984). The conformational change can be readily demonstrated by a "slow" to "fast" shift in electrophoretic mobility in nondenaturing PAGE (Barrett et al., 1979; Nelles et al., 1980). During the conformational change, receptor recognition sites become exposed on the inhibitor that allow α_2M-proteinase or α_2M-CH_3NH_2 fast forms to bind to cell-surface receptors and clear rapidly from the circulation (Van Leuven et al., 1979; Imber & Pizzo, 1981; Gliemann et al., 1983). In contrast, native α_2M does not bind to the α_2M fast form receptor.

cis-Dichlorodiammineplatinum(II) (*cis*-DDP) is an uncharged square planar complex that has tumoricidal activity, most likely because of cross-linking of complementary strands of DNA in cells (Rosenberg et al., 1969; Roberts & Pascoe, 1972). Reaction of *cis*-DDP with α_2M results in extensive cross-linking of the subunits of α_2M (Gonias & Pizzo, 1981a,b). These cross-links are removed by incubation of *cis*-DDP-treated α_2M with diethyldithiocarbamate (DDC) (Gonias et al., 1984). As a consequence of intersubunit cross-linking, *cis*-DDP-treated α_2M (*cis*-DDP-α_2M) does not show the characteristic slow to fast conformational change after the addition of trypsin (Gonias & Pizzo, 1981a). It was postulated that cross-linking of α_2M by *cis*-DDP locks α_2M in the native or slow conformation. Although there is no conformational change in *cis*-DDP-α_2M following reaction with trypsin, it is not known whether or not α_2M is primed for the conformational change but unable to complete this change because of the intersubunit cross-linking. If this were true, removal of the cross-links should result in completion of the conformational change. In this investigation we show that trypsin-treatment of *cis*-DDP-α_2M does prime the molecule for the conformational change and that removal of the cross-links results in completion of this change. In addition, unlike the reaction of α_2M with trypsin, *cis*-DDP-α_2M does not bind trypsin, and a method is described that results in generation of an α_2M fast form with all four bait regions cleaved but no proteinase bound to the inhibitor.

Experimental Procedures

Materials. Human α_2M was purified as previously described (Kurecki et al., 1979; Imber & Pizzo, 1981). Bovine trypsin, bovine pancreatic trypsin inhibitor (BPTI), Sephacryl S-200, and Sephadex G-25 were obtained from Sigma. The trypsin was 60% active as determined by active-site titration using *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). The concentration of active trypsin was used in all calculations. Carrier-free $Na^{125}I$ was obtained from New England Nuclear. Radioiodination of α_2M and trypsin was performed by the solid-state lactoperoxidase method of David and Reisfeld (1974). Radioiodinated α_2M retained full activity as determined by the ability of α_2M to protect trypsin esterase activity using the method of Ganrot (1966), and trypsin lost less than 10% of its initial activity as determined by active-site titration. Twenty-week-old CD-1 female mice were obtained from Charles River Laboratories, Wilmington, MA. *cis*-DDP and DDC were obtained from Aldrich. All other reagents were of the highest quality available.

Modification of α_2M with cis-DDP and DDC. α_2M (1–2 μM) was allowed to react with *cis*-DDP (1.7 mM) for 10 h at 37 °C in 25 mM HEPES and 150 mM NaCl, pH 7.4. This buffer was used in all experiments unless otherwise noted. Excess *cis*-DDP was removed by extensive dialysis against buffer. The concentrations of α_2M and *cis*-DDP-α_2M were estimated by using $A^{1\%}_{1cm}$ = 8.93 and M_r 718 000 (Hall & Roberts, 1978). In some experiments, *cis*-DDP-treated α_2M was allowed to react with 5 mM DDC for 10 h at 37 °C. Excess DDC was then removed by extensive dialysis. The extent of platinum incorporation in these studies was determined as previously reported (Gonias & Pizzo, 1983a).

Treatment of α_2M with Trypsin. α_2M or *cis*-DDP-α_2M was allowed to react with a 3-fold molar excess of trypsin for 15 min at 22 °C. In some experiments, the trypsin was then inactivated by incubation with a 25-fold molar excess of BPTI for 15 min. α_2M–trypsin or *cis*-DDP-α_2M-trypsin complexes were purified by gel filtration chromatography on Sephacryl S-200 and used within 3 days.

Electrophoresis. Nondenaturing PAGE was performed in 5% gels using a HEPES/imidazole buffer system (McLellan, 1982). Samples of α_2M (5 μg) were incubated with running buffer for 5 min prior to electrophoresis at 5 W for 6 h. Previous studies have shown that α_2M, *cis*-DDP-α_2M, and α_2M–trypsin have essentially identical dye-binding capacities, and for this reason the intensity of an α_2M band is directly proportional to the amount of protein in that band (Gonias & Pizzo, 1981a). SDS–PAGE was performed by using the Tris/sulfate system of Neville (1971). Samples of α_2M (10 μg) were incubated with upper reservior buffer containing 2% SDS and 65 mM dithiothreitol at 37 °C for 1 h prior to electrophoresis in a 5% gel. In samples containing active proteinase, a 2-fold molar excess of BPTI over proteinase was added 5 min prior to denaturation. The gel was stained with Coomassie Brilliant Blue R-250 and scanned by automatic integrating densitometry as previously described (Roche & Pizzo, 1987).

Titration of Thiol Groups of α_2M. The appearance of thiol groups from α_2M or *cis*-DDP-α_2M was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). The appearance of thionitrobenzoate anion was monitored at 410 nm in a Shimadzu split-beam UV–vis recording spectrophotometer using ϵ_{410nm} = 13 600 M^{-1} cm^{-1} (Habeeb, 1972). α_2M or *cis*-DDP-α_2M (1–2 μM) was treated with DTNB (400 μM) in buffer, and the absorbance at 410 nm was monitored for 5 min. A 3-fold molar excess of trypsin was then added to the sample and reference cuvettes, and the absorbance at 410 nm was monitored for 15 min. In some experiments, trypsin-treated *cis*-DDP-α_2M that had been allowed to react with DDC was desalted on a column of Sephadex G-25 prior to thiol group titration, since DDC alone is highly reactive with DTNB.

Binding of ^{125}I-Trypsin to α_2M. α_2M or *cis*-DDP-α_2M (1–2 μM) was allowed to react with a 3-fold molar excess of ^{125}I-trypsin for 15 min at 22 °C. In some experiments, the trypsin was inactivated by the addition of a 25-fold molar excess of BPTI. Free ^{125}I-trypsin was separated from α_2M-bound ^{125}I-trypsin by gel filtration chromatography on a column of Sephacryl S-200. In other experiments, ^{125}I-trypsin-treated *cis*-DDP-α_2M was allowed to react with 5 mM DDC for 6 h at 37 °C. Excess DDC was then removed by gel filtration chromatography on Sephadex G-25. The molar ratio of trypsin bound to α_2M or *cis*-DDP-α_2M was determined by using the known specific activity of the trypsin preparation (13 000 cpm/pmol) and the following parameters: trypsin M_r

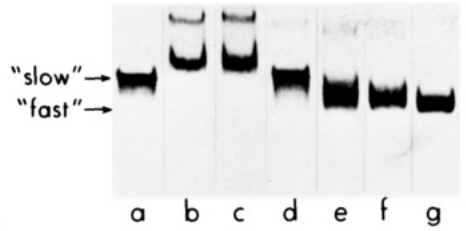

FIGURE 1: Electrophoretic mobility of α_2M slow form (lane a). α_2M treated with *cis*-DDP alone (lane b) and before (lane c) treatment with trypsin. DDC added to *cis*-DDP-α_2M (lane d) or trypsin-treated *cis*-DDP-α_2M (lane e). *cis*-DDP-α_2M incubated with DDC prior to treatment with trypsin (lane f). Mobility of α_2M–trypsin fast form (lane g).

23 800; α_2M, $A^{1\%}_{1cm}$ = 8.93, M_r 718 000 (Hall & Roberts, 1978); α_2M–trypsin, $A^{1\%}_{1cm} \sim 9.0$.

Plasma Elimination of α_2M. Plasma elimination studies of α_2M were performed on 20-week-old CD-1 female mice. A preparation of radioiodinated α_2M or *cis*-DDP-α_2M (1 μg in a volume of 400 μL) was injected into the lateral tail veins of the mice as previously described (Imber & Pizzo, 1981). Briefly, clearance from the circulation was monitored by repetitive blood sampling from the retroorbital venous plexus. The amount of radioactivity in a sample withdrawn within 10 s of injection was defined as the initial ligand concentration, and the radioactivity in subsequent aliquots was represented as a percentage of this first measurement.

RESULTS

Nondenaturing PAGE of cis-DDP-Treated α_2M. The reaction of *cis*-DDP with α_2M results in extensive cross-linking of α_2M subunits, and subsequent incubation with DDC removes these cross-links (Gonias et al., 1984). Figure 1 shows that in nondenaturing PAGE the mobility of α_2M following incubation with *cis*-DDP was decreased. It is unlikely that *cis*-DDP (itself an uncharged molecule) would cause an increase in the net charge of the protein, and we therefore attribute the change in electrophoretic mobility to a slight increase in the Stokes radius of α_2M. No aggregated α_2M was observed when *cis*-DDP-treated α_2M was analyzed by high-performance size-exclusion chromatography (results not shown). However, faint bands of decreased mobility were observed by PAGE following treatment of α_2M with *cis*-DDP. These bands have been observed previously (Gonias & Pizzo, 1981a), and it is possible that they represent a small amount of cross-linked α_2M dimers or higher molecular weight forms. Incubation of *cis*-DDP-α_2M with DDC led to an increase in the electrophoretic mobility of the protein to a position identical with that of native α_2M. This treatment also resulted in the disappearance of the bands of very slow mobility.

When the *cis*-DDP-α_2M derivative was assayed for platinum, a value of 32.0 mol platinum/mol of inhibitor was obtained. This is equivalent to eight *cis*-DDP molecules incorporated per subunit. DDC treatment removed greater than 95% of the platinum, consistent with previous observations from this laboratory (Gonias et al., 1984).

Treatment of α_2M with trypsin results in the characteristic slow to fast shift in the electrophoretic mobility of α_2M in nondenaturing PAGE (Barrett et al., 1979). In contrast, *cis*-DDP-α_2M did not undergo any change in electrophoretic mobility following reaction with excess trypsin (Figure 1). Incubation of trypsin-treated *cis*-DDP-α_2M with DDC resulted in a dramatic increase in the electrophoretic mobility of the protein to the position of α_2M–trypsin fast form. Similarly, DDC treatment of *cis*-DDP-α_2M followed by the addition of trypsin resulted in a nearly identical shift in electrophoretic mobility. This result is notable in that it shows that *cis*-DDP and DDC treatment of α_2M did not interfere with the ability of α_2M to undergo its typical conformational change. Similarly, Gonias et al. (1984) has shown that when DDC was incubated with *cis*-DDP-α_2M, protein cross-linking and inactivation were totally prevented. The results of these studies are in agreement with previously published results and suggest that *cis*-DDP acts as a lock to the conformation of α_2M (Gonias & Pizzo, 1981a). In addition, they demonstrate that incubation of *cis*-DDP-α_2M or trypsin-treated *cis*-DDP-α_2M with DDC removes the constraints which hold the molecule in that particular conformation.

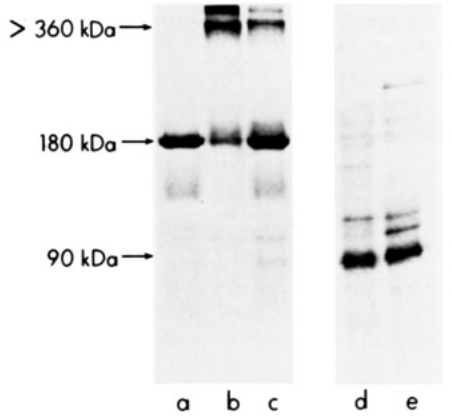

FIGURE 2: α_2M or *cis*-DDP-treated α_2M analyzed by SDS–PAGE under reducing conditions. α_2M was treated with *cis*-DDP and DDC as described under Experimental Procedures. α_2M or *cis*-DDP-α_2M was treated with trypsin before or after incubation with DDC. (Lane a) α_2M; (lane b) *cis*-DDP-α_2M; (lane c) *cis*-DDP-α_2M incubated with DDC; (lane d) trypsin-treated *cis*-DDP-α_2M incubated with DDC; (lane e) trypsin-treated α_2M.

Table I: Percentage of α_2M Migrating as 180-kDa Subunits following Treatment with *cis*-DDP[a]

α_2M treatment	180-kDa subunits (%)	α_2M treatment	180-kDa subunits (%)
none	97	trypsin	2
cis-DDP	22	*cis*-DDP + trypsin + DDC	8
cis-DDP + DDC	96	*cis*-DDP + DDC + trypsin	13

[a] The gel shown in Figure 2 was analyzed by automatic integrating densitometry. Ten micrograms of α_2M or *cis*-DDP-α_2M was applied to each lane. The percentage of α_2M migrating at the position of 180-kDa subunits was taken as the ratio of the area of the peak corresponding to the 180-kDa subunit to the total area of the protein bands in the scan. The peak at the dye front is free trypsin and is not considered in the calculations.

Reducing SDS–PAGE of cis-DDP-Treated α_2M. SDS–PAGE of α_2M and *cis*-DDP-α_2M with and without trypsin treatment was performed and is shown in Figure 2. The results of automatic integrating densitometry of the Coomassie Brilliant Blue R-250 stained gel are shown in Table I. Figure 2 confirms that the reaction of *cis*-DDP with α_2M resulted in extensive intersubunit cross-linking of α_2M. Only 22% of the protein migrated at the position of the 180-kDa subunit following treatment with *cis*-DDP, whereas 97% of the protein migrated at this position in untreated α_2M. Incubation of *cis*-DDP-α_2M with DDC almost completely removed these cross-links, and 96% of the protein now migrated at the position of the 180-kDa α_2M subunit.

Figure 2 and Table I show that treatment of α_2M with trypsin resulted in the cleavage of 98% of the α_2M subunits at the bait region into two polypeptides of $M_r \sim 90000$. Some α_2M migrates in higher molecular weight bands due to the

Table II: Appearance of Thiol Groups from α_2M or *cis*-DDP-Treated α_2M[a]

α_2M treatment	thiol groups/mol of α_2M	*cis*-DDP-α_2M treatment	thiol groups/mol of α_2M
none	0	none	0
trypsin	3.9	trypsin	0
		trypsin, followed by incubation with DDC	3.3

[a] α_2M or *cis*-DDP-α_2M was treated with DTNB, and the change in absorbance at 410 nm was observed for 15 min. Similar experiments were performed following treatment of α_2M or *cis*-DDP-α_2M with trypsin. In experiments in which DDC was added, gel filtration was employed to remove free reagent prior to thiol titration.

Table III: Binding of Radioiodinated Trypsin to α_2M or *cis*-DDP-Treated α_2M[a]

protein	mol of ^{125}I-trypsin bound/mol of α_2M: without BPTI	with BPTI
α_2M	1.8	1.6
cis-DDP-α_2M	0.13	0.14
cis-DDP-α_2M, followed by incubation with DDC	0.88	0.80

[a] α_2M or *cis*-DDP-α_2M was incubated with a 3-fold molar excess of ^{125}I-trypsin for 15 min. In some experiments, DDC was incubated with trypsin-treated *cis*-DDP-α_2M. Experiments were performed in the presence and absence of a 25-fold molar excess of BPTI prior to gel filtration chromatography.

formation of ϵ-lysyl–γ-glutamyl cross-links between trypsin and the Glx residue of the cleaved thiol ester bond (Salvesen & Barrett, 1981; Sottrup-Jensen et al., 1981). The determination of the extent of *cis*-DDP-α_2M bait region cleavage by trypsin using SDS–PAGE was complicated by the fact that most of the α_2M was still cross-linked by *cis*-DDP following treatment with trypsin. Incubation of *cis*-DDP-α_2M with DDC, however, resulted in the removal of the intersubunit cross-links and revealed that trypsin cleaved essentially all four bait regions of *cis*-DDP-α_2M. In addition, DDC treatment of *cis*-DDP-α_2M followed by the addition of trypsin resulted in a similar extent of bait region cleavage. This study indicates that *cis*-DDP and DDC treatment produced no irreversible changes in the α_2M molecule which rendered it more or less susceptible to bait region cleavage by trypsin. It should be pointed out that in these experiments trypsin was inactivated after reaction with α_2M or *cis*-DDP-α_2M by the addition of BPTI, thereby ensuring that any bait region cleavage occurred before incubation of *cis*-DPP-α_2M with DDC.

Thiol Group Titration of cis-DDP-Treated α_2M. Studies were performed to correlate changes in electrophoretic mobility in nondenaturing PAGE with the appearance of thiol groups in α_2M and *cis*-DDP-α_2M, and the results of these studies are summarized in Table II. In agreement with previous studies (Sottrup-Jensen et al., 1980), native α_2M did not contain any free thiols, but treatment with excess trypsin resulted in the appearance of 3.9 thiol groups/mol of α_2M as a consequence of the cleavage of four internal thiol ester bonds. Like native α_2M, *cis*-DDP-treated α_2M did not contain any free thiols. This is consistent with the previous observation that *cis*-DDP-treated α_2M was still capable of incorporating 3.6 mol of $[^{14}C]H_3NH_2$/mol of *cis*-DDP-α_2M (Gonias & Pizzo, 1981a). Unlike native α_2M, however, treatment of *cis*-DDP-α_2M with trypsin did not result in an increase in thiol appearance. We hypothesized that intersubunit cross-linking of α_2M by *cis*-DDP prevented the conformational change in trypsin-treated *cis*-DDP-α_2M required for activation and cleavage of the internal thiol ester bonds. Preliminary experiments were performed to examine the time course of thiol appearance during incubation of trypsin-treated *cis*-DDP-α_2M with DDC. These studies demonstrated that there was a rapid generation of thiols after the addition of DDC to trypsin-treated *cis*-DDP-α_2M which was complete within 15 min. However, quantification of thiol appearance was impossible due to generation of thionitrobenzoate anion by the reaction of DDC with DTNB. For this reason, free DDC was removed by gel filtration chromatography, and an end-point determination of thiol groups revealed that 3.3 thiol groups had appeared per mole of α_2M.

Stoichiometry of ^{125}I-Trypsin Binding to cis-DDP-Treated α_2M. When α_2M was reacted with excess trypsin, approximately 1.8 mol of trypsin became bound per mole of α_2M as determined by gel filtration chromatography of α_2M reacted with an excess of ^{125}I-trypsin (Table III). Inactivation of trypsin with BPTI did not result in a significant decrease in binding. Similar experiments using *cis*-DDP-α_2M revealed that 0.13 mol of trypsin become bound per mole of α_2M. Additional incubation with BPTI resulted in essentially no change in trypsin binding to *cis*-DDP-α_2M. These results are in general agreement with those obtained by Gonias and Pizzo (1981a), who found in preliminary studies that less than 0.9 mol of trypsin was bound following incubation with *cis*-DDP-α_2M. Thus, trypsin cleaves the bait regions of α_2M without becoming bound to the inhibitor. This is similar to the "nonproductive" cleavage of α_2M bait regions by excess proteinase previously reported (Roche & Pizzo, 1987). When trypsin-treated *cis*-DDP-α_2M was incubated with DDC, 0.88 mol of trypsin became bound per mole of *cis*-DDP-α_2M. Inactivation of trypsin with BPTI prior to incubation with DDC and gel filtration chromatography decreased the amount of binding to 0.80 mol of trypsin/mol of *cis*-DDP-α_2M. Thus, approximately 1 mol of trypsin became bound per mole of *cis*-DDP-α_2M following removal of the intersubunit cross-links by DDC.

Plasma Elimination of cis-DDP-Treated ^{125}I-α_2M. As a further probe of the conformation of *cis*-DDP-α_2M following reaction with trypsin, plasma clearance studies were performed. Native α_2M clears very slowly from the circulation of mice, whereas α_2M–trypsin exhibits a clearance half-life of only 3–4 min (Imber & Pizzo, 1981). In contrast to the rapid clearance of CH_3NH_2-treated *cis*-DDP-α_2M (Gonias & Pizzo, 1983b), trypsin-treated *cis*-DDP-α_2M exhibited a clearance half-life of greater than 45 min (Figure 3). Subsequent incubation with DDC resulted in rapid clearance from the circulation with a half-life of 4 min. The biphasic nature of the clearance curve may reflect incomplete receptor recognition exposure of a subpopulation of α_2M that still contains bound *cis*-DDP (Pizzo et al., 1986). These results demonstrate that trypsin-treated *cis*-DDP-α_2M is capable of interacting with α_2M fast form cell-surface receptors only after incubation with DDC.

Discussion

Previous studies from this laboratory suggest that α-macroglobulins undergo three distinct changes in conformation upon reaction with CH_3NH_2. These include a change in secondary structure, which is observed by circular dichroism spectroscopy (Feldman & Pizzo, 1984), a further change in secondary structure, resulting in exposure of receptor recognition sites (Feldman et al., 1984; Strickland et al., 1984), and finally a large change in conformation characterized by the slow to fast shift in electrophoretic mobility in nondenaturing PAGE (Barrett et al., 1979). With trypsin these conforma-

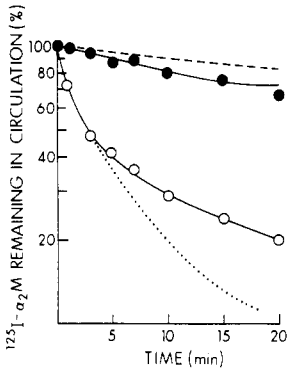

FIGURE 3: In vivo plasma elimination of α_2M or *cis*-DDP-α_2M. In each experiment, 1×10^6 cpm of ^{125}I-α_2M was injected into the mouse. The clearance curves of α_2M slow form (---) and α_2M–trypsin fast form (···) are shown for comparison. α_2M was treated with *cis*-DDP prior to addition of trypsin and was injected into the mouse before (●) and after (○) incubation with DDC. The clearance rate of *cis*-DDP-α_2M was essentially identical with that of native α_2M slow form (results not shown).

tional changes are so rapid that generally only the slow to fast change is observed. In every species of α-macroglobulin yet studied, the slow to fast conformational change correlates with a compacting of the molecule as determined by hydrodynamic studies (Gonias et al., 1982; Bjork & Fish, 1982; Dangott & Cunningham 1982; Nishigai et al., 1985).

There have been extensive studies on the interaction of α_2M with *cis*-DDP. The interaction of α_2M–trypsin or α_2M–CH_3NH_2 fast forms with *cis*-DDP results in protein cross-linking and loss of receptor binding (Gonias & Pizzo, 1981a,b; Pizzo et al., 1986). Studies have demonstrated that *cis*-DDP reacts with one or more methionyl residues at or near the receptor recognition site of fast form α_2M (Pizzo et al., 1986). Reaction of *cis*-DDP with α_2M slow form results in extensive protein cross-linking; however, *cis*-DDP does not react with the receptor recognition site methionyl residues because these residues are not exposed in this conformational form of α_2M. Reaction of *cis*-DDP-α_2M with CH_3NH_2 results in incorporation of CH_3NH_2 into the cleaved thiol ester and complete receptor recognition. This species does not, however, undergo slow to fast conformation change (Gonias et al., 1981a; Gonias & Pizzo, 1983b).

In the present investigation, we used *cis*-DDP as a cross-linking reagent to lock α_2M in the native conformation. Addition of trypsin to *cis*-DDP-α_2M resulted in bait region cleavage without thiol ester bond activation, proteinase binding, receptor recognition site exposure, or a change in electrophoretic mobility in nondenaturing PAGE. Subsequent removal of the intersubunit cross-links with DDC resulted in completion of the conformational change in α_2M as determined by thiol ester bond activation and cleavage, receptor recognition site exposure, and a complete slow to fast conformational change. Thus, these results suggest that in human α_2M there is a conformational change following bait region cleavage which normally leads to activation of the thiol ester bonds, but this change is prevented by the intersubunit cross-linking of α_2M by *cis*-DDP. This proteolytically primed conformational form may resemble the nascent α_2M proposed and described by Sottrup-Jensen et al. (1981). The conformational change required for thiol ester bond activation must be very subtle, since it was not detected by ^{1}H NMR of the α_2M bait region following treatment with trypsin (Gettins & Cunningham, 1987). This might represent a slight change in the polypeptide backbone of α_2M that labilizes the thiol ester bond located 15–20 Å from the bait region (Feldman et al., 1985; Feldman & Pizzo, 1987). From these studies it is concluded that bait region cleavage of α_2M is insufficient to cause receptor recognition site exposure. These results confirm the hypothesis that receptor recognition site exposure is dependent upon thiol ester bond cleavage (Roche & Pizzo, 1987). It should be pointed out, however, that thiol groups themselves are not part of the receptor recognition site of α_2M (Gonias et al., 1981b; Van Leuven et al., 1982).

A model of α_2M structure proposed by this laboratory suggests that α_2M is composed of two adjacent proteinase binding domains (Feldman et al., 1985). It was proposed that the slow to fast conformational change observed in nondenaturing PAGE is the result of compaction of the space between the two domains and not a change in the secondary structure of the molecule. Since trypsin or CH_3NH_2 treatment of *cis*-DDP-α_2M does not result in a slow to fast conformational change, we conclude that the *cis*-DDP cross-linking sites are at or near the interface between the domains. For *cis*-DDP to cross-link, the functional centers must be no more than 2.8 Å apart (Roberts & Pascoe, 1972), and it is therefore unlikely that *cis*-DDP cross-links α_2M subunits along the external surface of the protein. The placement of the *cis*-DDP cross-linking sites in this region of the molecule is attractive, since it has been suggested that the receptor recognition sites of α_2M are also located near this region. It has also been proposed by Sottrup-Jensen et al. (1983b) that the bait region and the thiol ester bonds are located deep in the proteinase binding pocket, and for this reason the interface between adjacent domains is thought of as a critical site in the structure and function of α_2M.

Treatment of *cis*-DDP-α_2M with trypsin followed by gel filtration chromatography and incubation with DDC results in the generation of a new form of α_2M, a species with complete bait region cleavage, thiol ester bond cleavage, receptor recognition site exposure, but no bound proteinase. This molecule is, in effect, an "empty trap". The preparation of empty traps may prove to be useful in studying the exact spacial arrangement between cleaved bait regions and the thiol ester site.

REFERENCES

Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724.

Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979) *Biochem. J. 181*, 401–418.

Bjork, I., & Fish, W. N. (1982) *Biochem. J. 207*, 347–356.

Branegård, B., Österberg, R., & Sjöberg, B. (1982) *Eur. J. Biochem. 122*, 663–666.

Chase, T., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508–514.

Cummings, H., Pizzo, S. V., Strickland, D. K., & Castellino, F. J. (1984) *Biophys. J. 45*, 721–724.

Dangott, L. J., & Cunningham, L. W. (1982) *Biochem. Biophys. Res. Commun. 107*, 1243–1251.

David, G. S., & Reisfeld, R. A. (1974) *Biochemistry 13*, 1014–1021.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Feldman, S. R., & Pizzo, S. V. (1984) *Biochem. Biophys. Res. Commun. 123*, 771–777.

Feldman, S. R., & Pizzo, S. V. (1987) *Semin. Thromb. Hemostasis* 12, 223–227.

Feldman, S. R., Gonias, S. L., Ney, K. A., Pratt, C. W., & Pizzo, S. V. (1984) *J. Biol. Chem. 259*, 4458–4462.

Feldman, S. R., Gonias, S. L., & Pizzo, S. V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5700–5704.

Ganrot, P. A. (1966) *Clin. Chim. Acta 14*, 493–501.

Gettins, P., & Cunningham, L. W. (1986) *Biochemistry 25*, 5011-5017.
Gliemann, J., Larsen, T. R., & Sottrup-Jensen, L. (1983) *Biochem. Biophys. Acta 756*, 230-237.
Gonias, S. L., & Pizzo, S. V. (1981a) *J. Biol. Chem. 256*, 12478-12484.
Gonias, S. L., & Pizzo, S. V. (1981b) *Biochim. Biophys. Acta 678*, 268-274.
Gonias, S. L., & Pizzo, S. V. (1983a) *J. Biol. Chem. 258*, 5764-5769.
Gonias, S. L., & Pizzo, S. V. (1983b) *Ann. N.Y. Acad. Sci. 421*, 457-471.
Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306-314.
Gonias, S. L., Oakley, A. C., Walther, P. J., & Pizzo, S. V. (1984) *Cancer Res. 44*, 5764-5770.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457-464.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27-38.
Harpel, P. C. (1973) *J. Exp. Med. 138*, 508-521.
Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134-8139.
Kurecki, T., Kress, L. F., & Laskowski, M. (1979) *Anal. Biochem. 99*, 415-420.
McLellan, T. (1982) *Anal. Biochem. 126*, 94-99.
Nelles, L. P., Hall, P. K., & Roberts, R. C. (1980) *Biochim. Biophys. Acta 623*, 45-56.
Neville, D. M., Jr. (1971) *J. Biol. Chem. 246*, 6328-6334.
Nishigai, M., Osada, T., & Ikai, A. (1985) *Biochim. Biophys. Acta 831*, 236-241.
Österberg, R., & Malmensten, B. (1984) *Eur. J. Biochem. 143*, 541-544.
Pizzo, S. V., Roche, P. A., Feldman, S. R., & Gonias, S. L. (1986) *Biochem. J. 238*, 217-225.
Roberts, J. J., & Pascoe, J. M. (1972) *Nature (London) 235*, 282-284.
Roche, P. A., & Pizzo, S. V. (1987) *Biochemistry 26*, 486-491.
Rosenberg, B., van Camp, L., Trosko, J. E., & Mansour, V. H. (1969) *Nature (London) 222*, 385-386.
Salvesen, G. S., Sayers, C. S., & Barrett, A. J. (1981) *Biochem. J. 195*, 453-461.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275-279.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 128*, 127-132.
Sottrup-Jensen, L., Stepanik, T. M., Wierzbicki, D. M., Jones, C. M., Londblad, P. B., Kristensen, T., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1983a) *Ann. N.Y. Acad. Sci. 421*, 41-60.
Sottrup-Jensen, L., Hansen, H. F., & Christensen, U. (1983b) *Ann. N.Y. Acad. Sci. 421*, 188-208.
Strickland, D. K., Steiner, J. P., Feldman, S. R., & Pizzo, S. V. (1984) *Biochemistry 23*, 6679-6685.
Swenson, R. P., & Howard, J. B. (1979) *J. Biol. Chem. 254*, 4452-4456.
Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1979) *J. Biol. Chem. 254*, 5155-5160.
Van Leuven, F., Marynen, P., Cassiman, J.-J., & Van den Berghe, H. (1982) *Biochem. J. 203*, 405-411.

Ca^{2+} Channel Inhibitors That Bind to Plant Cell Membranes Block Ca^{2+} Entry into Protoplasts†

Annick Graziana,‡ Michel Fosset,§ Raoul Ranjeva,‡ Allistair M. Hetherington,‖ and Michel Lazdunski*,§

Centre de Biochimie du Centre National de la Recherche Scientifique, Parc Valrose, 06034 Nice Cedex, France, and Centre de Physiologie Végétale, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France

Received June 22, 1987; Revised Manuscript Received August 28, 1987

ABSTRACT: Ca^{2+} channel inhibitors of the phenylalkylamine and of the diphenylbutylpiperidine series, as well as other blockers such as bepridil, inhibit $^{45}Ca^{2+}$ influx into carrot protoplasts. The corresponding plasma membranes have a high density (120 pmol/mg of protein) of sites for the phenylalkylamine (−)-[^{3}H]-desmethoxyverapamil (K_d = 85 nM). For 10 different Ca^{2+} channel inhibitors, there was a good correlation between efficacy of blockade of $^{45}Ca^{2+}$ influx into protoplasts and efficacy of binding of the ^{3}H-ligand to membranes. Specific binding sites for the tritiated 1,4-dihydropyridine blocker (+)PN 200-110 could not be identified, and no blockade of Ca^{2+} influx was observed with several molecules in this series such as (+)PN 200-110, nifedipine, or nitrendipine.

Ca^{2+} is an important intracellular mediator for metabolic and developmental events in plants (Hepler & Wayne, 1985; Elliott, 1986). Ca^{2+}-mediated processes in plants include polarized growth, mitosis and cytokinesis, cytoplasmic streaming, physiological responses to red and blue lights, gravitropism, and physiological responses to plant growth substances.

Voltage-dependent Ca^{2+} channels have been shown to play a very important role in nerve, cardiac, and muscle cells to couple excitation to contraction and to secretion (Tsien, 1983; Reuter, 1983; Baker & Knight, 1984). On the other hand, Ca^{2+} channel blockers are a very important class of cardiovascular drugs (Henry, 1980; Nayler & Horowitz, 1983). They include chemically distinct series of molecules such as 1,4-dihydropyridines [nitrendipine, nifedipine, (+)PN

† This work was supported by the Centre National de la Recherche Scientifique.
* Author to whom correspondence should be addressed.
‡ Centre National de la Recherche Scientifique.
§ Université Paul Sabatier.
‖ Present address: Department of Biological Sciences, University of Lancaster, Bailrigg, Lancaster LA1 4YQ, U.K.

0006-2960/88/0427-0764$01.50/0