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Independent Analysis of Bait Region Cleavage Dependent and Thiolester Bond Cleavage Dependent Conformational Changes by Cross-Linking of α_2-Macroglobulin with *cis*-Dichlorodiammineplatinum(II) and Dithiobis(succinimidyl propionate)†

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ABSTRACT: Treatment of the human plasma proteinase inhibitor α_2-macroglobulin (α_2M) with proteinase results in conformational changes in the inhibitor and subsequent activation and cleavage of the internal thiolester bonds of α_2M. Previous studies from this laboratory have shown that cross-linking the α_2M subunits with *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) prevents the proteinase-induced conformational changes which lead to the activation and cleavage of the internal thiolester bonds of α_2M. In addition, *cis*-DDP treatment prevents the proteinase- or CH_3NH_2-induced conformational changes in α_2M which lead to a "slow" to "fast" change in nondenaturing polyacrylamide gel electrophoresis. In this paper, we demonstrate that treatment of α_2M with dithiobis(succinimidyl propionate) (DSP) also results in cross-linking of the subunits of α_2M with concomitant loss of proteinase inhibitory activity. Although proteinase is not inhibited by DSP-treated α_2M, bait region specific proteolysis of the α_2M subunits still occurs. Unlike *cis*-DDP-treated α_2M, however, incubation of DSP-treated α_2M with proteinase does not prevent the bait region cleavage dependent conformational changes which lead to activation and cleavage of the internal thiolester bonds in α_2M. On the other hand, cross-linking of α_2M with DSP does prevent the conformational changes which trigger receptor recognition site exposure following cleavage of the α_2M thiolester bonds by CH_3NH_2. These conformational changes, however, occur following incubation of the CH_3NH_2-treated protein with proteinase. These results demonstrate that intersubunit cross-linking by either *cis*-DDP or DSP allows the relative contribution of bait region cleavage dependent and thiolester bond cleavage dependent conformational changes in α_2M to be analyzed separately.

Human α_2-macroglobulin (α_2M)[1] is a proteinase inhibitor present in plasma and other body fluids at concentrations up to 3 μM. α_2M functions as a proteinase inhibitor by a mechanism which has been referred to as "proteinase trapping" (Barrett & Starkey, 1973; Barrett et al., 1979). Since that time, numerous investigators have confirmed this hypothesis and have extended it to explain proteinase inhibition by other, nonhuman α-macroglobulin homologues such as the chicken and duck ovostatins (Nagase & Harris, 1983; Nagase et al., 1983; Feldman & Pizzo, 1984a) and the rat proteins α_2M and α_1M (Gonias et al., 1983). Central to the trap hypothesis is the concept that proteolytic cleavage of α_2M at a "bait region" leads to a series of conformational changes in the inhibitor which sterically inhibit the activity of α_2M-bound proteinase (Harpel, 1973; Barrett & Starkey, 1973; Salvesen & Barrett, 1980). One consequence of this conformational change is that receptor recognition sites become exposed on the inhibitor, which allow the proteinase–inhibitor complex to be rapidly taken up and degraded by a number of cell types (Debanne et al., 1975; Van Leuven et al., 1979; Imber & Pizzo, 1981). The unusual properties of α_2M have been extensively reviewed (Pizzo & Gonias, 1984; Sottrup-Jensen, 1987).

Following proteolytic cleavage of α_2M, a series of conformational changes occur in the inhibitor (Barrett et al., 1979; Gonias et al., 1982; Björk & Fish, 1982) which lead to the

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[1] Abbreviations: α_2M, α_2-macroglobulin; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); DSP, dithiobis(succinimidyl propionate); PAGE, polyacrylamide gel electrophoresis; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); α_2M_{DSP}, dithiobis(succinimidyl propionate)-treated α_2M; α_2M_{Pt}, *cis*-dichlorodiammineplatinum(II)-treated α_2M.

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activation and subsequent cleavage of at least two of the four internal thiolester bonds present per mole of α_2M (Sottrup-Jensen et al., 1980; Christensen & Sottrup-Jensen, 1984). It is generally assumed that thiolester bond cleavage, and not bait region cleavage, is the driving force for the dramatic conformational change in α_2M following treatment with proteinases. However, there are a number of studies which are not in accord with this hypothesis. Although many different physicochemical techniques detect nearly identical conformational changes following treatment with proteinases or following direct thiolester bond cleavage by CH_3NH_2, scanning calorimetry routinely detects differences between these two forms of the inhibitor (Cummings et al., 1984). In addition, treatment of rat α_2M and several other nonmammalian α-macroglobulins with CH_3NH_2 does not lead to significant conformational changes in the inhibitor, although this treatment leads to complete thiolester bond cleavage. Finally, ovostatin undergoes a conformational change following proteinase treatment which is similar to the conformational change in human α_2M following treatment with either proteinase or CH_3NH_2 (Feldman & Pizzo, 1984b), but this α-macroglobulin has no thiolester bonds (Nagase et al., 1983). On the basis of these few examples, it is apparent that complex mechanisms are involved in the transduction of conformational changes in α-macroglobulins.

We have demonstrated that intersubunit cross-linking by *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) allows partial dissection of the relative contribution of bait region cleavage and thiolester bond cleavage to conformational changes in α_2M (Gonias & Pizzo, 1981, 1983a; Roche et al., 1988). Specifically, cross-linking with *cis*-DDP prevents the proteinase- or CH_3NH_2-induced conformational changes which lead to the "slow" to "fast" change in nondenaturing polyacrylamide gel electrophoresis (PAGE) described by Barrett et al. (1979). In addition, cross-linking with *cis*-DDP interferes with the proteinase-induced conformational changes which normally lead to the activation and cleavage of the α_2M thiolester bonds. On the other hand, this treatment did not interfere with the CH_3NH_2-induced conformational change which leads to the exposure of the α_2M receptor recognition sites following thiolester bond cleavage. Thus, intersubunit cross-linking by *cis*-DDP allows analysis of thiolester bond cleavage dependent conformational changes in α_2M.

The present studies were initiated in order to examine the nature of the conformational changes which occur following treatment of α_2M with proteinases. In order to accomplish this, it was necessary to prevent the conformational changes which normally follow thiolester bond cleavage of α_2M. This was achieved by cross-linking the subunits of native α_2M with the homobifunctional, cleavable cross-linking reagent dithiobis(succinimidyl propionate) (DSP) (Lomant & Fairbanks, 1976). The results of these studies demonstrate that treatment of native α_2M with DSP prevents conformational changes in the inhibitor which normally follow cleavage of the four α_2M thiolester bonds in human α_2M.

Experimental Procedures

Proteins and Reagents. α_2M was purified essentially as described by Imber and Pizzo (1981) with the following modifications. Outdated human plasma (obtained from the Duke University Medical Center Blood Bank) was dialyzed against deionized H_2O for 3 days at 4 °C, and the soluble material was then exhaustively dialyzed against a high-salt NaP_i buffer (0.1 M NaP_i, 0.8 M NaCl, pH 6.5). The dialyzed plasma was applied to a Zn^{2+}-chelate affinity chromatography column in the high-salt NaP_i buffer, and the column was washed in this buffer until the A_{280nm} was <0.05. The affinity column was then washed in a low-salt NaP_i buffer (0.02 M NaP_i, 0.15 M NaCl, pH 6.0) until the A_{280nm} was <0.02, and α_2M was eluted with a buffer of 0.01 M sodium acetate and 0.15 M NaCl, pH 5.0. With this modification, repurification of the α_2M preparation by gel filtration chromatography is generally not required, as the eluted preparation shows no evidence of contaminating proteins as determined by both sodium dodecyl sulfate (SDS)–PAGE and nondenaturing PAGE. The concentration of α_2M was determined from a M_r of 718 000 and an $A_{1\%,280nm}$ of 8.93 (Hall & Roberts, 1978). Bovine trypsin was from Sigma, and human α-thrombin was purified as described by Fenton et al. (1977). This preparation was >95% in the α-form. The active site concentration of each proteinase was determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate as described by Chase and Shaw (1967). Iodination of α_2M was performed with the solid-phase lactoperoxidase–glucose oxidase system (Bio-Rad, Richmond, CA) following the manufacturer's specifications. A specific activity of 70 000 cpm/μg was routinely obtained with no loss of inhibitory activity in the preparation. Sephadex G-25 and DEAE-Sephacel were from Pharmacia-LKB. Twenty-week-old CD-1 mice were obtained from Charles River Laboratories, Raleigh, NC. DSP was from Pierce (Rockford, IL), and 3,4-dichloroisocoumarin was from Boehringer Mannheim Biochemicals. 6-(*p*-Toluidino)-2-naphthalenesulfonic acid (TNS) and *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) were from Sigma, St. Louis, MO. All other reagents were of the highest quality commercially available.

Cross-Linking of α_2M with DSP and cis-DDP. Preliminary experiments demonstrated that the extent of intersubunit cross-linking did not vary when α_2M had been incubated with DSP concentrations ranging from 200 to 5000 μM for 2 h (results not shown). For this reason, the concentration of DSP used in these studies was 200 μM. Stock solutions of DSP were prepared at a concentration of 100 mM in dimethyl sulfoxide and added to α_2M within 30 min. Unless otherwise noted, α_2M (2.8 μM) was treated with 200 μM DSP for 1 h at room temperature in a buffer of 25 mM triethanolamine and 150 mM NaCl, pH 8.0. This buffer was used in all experiments unless otherwise noted. The reaction between α_2M and DSP was stopped by the addition of 20 mM glycine to the incubation. To fully cross-link α_2M with *cis*-DDP, α_2M (2.8 μM) was incubated with 1.67 mM *cis*-DDP for 6 h at 37 °C. These conditions have previously been shown to completely inhibit the proteinase inhibitory activity of α_2M (Gonias & Pizzo, 1981). Free *cis*-DDP was removed by exhaustive dialysis against buffer.

Quantitation of DSP Cross-Links in α_2M. The number of DSP molecules bound to fully cross-linked α_2M was determined as follows. DSP-treated α_2M was reduced and denatured in a solution containing 6 M guanidine, 10 mM dithiothreitol, and 50 mM Tris-HCl, pH 8, for 30 min at 37 °C. Free dithiothreitol was removed by gel filtration chromatography on a Sephadex G-25 column equilibrated in 6 M guanidine and 50 mM Tris-HCl, pH 8. Immediately after elution from the column, free thiol groups were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with an extinction coefficient of 13 600 at 412 nm (Ellman, 1959). Control experiments demonstrated that the extinction coefficient of α_2M decreased by only 5% following complete denaturation in guanidine.

Treatment of α_2M with CH_3NH_2 or Proteinase. α_2M was treated with 0.1 M CH_3NH_2 at pH 8.0 for at least 1 h in order to ensure complete thiolester bond cleavage had occurred.

Since the nucleophile in this reaction is the unprotonated form of the amine, the concentration of CH_3NH_2 in the free base form was used in the calculation of the second-order rate constants for thiolester bond cleavage. Treatment of α_2M with proteinase was performed by the addition of a 2-fold molar excess of active site titrated trypsin or thrombin for 15 min at room temperature. Thiol groups liberated by CH_3NH_2 or proteinase treatment of α_2M or α_2M_{DSP} (2.4 μM) were detected by titration with DTNB as described previously (Ellman, 1959; Roche et al., 1988).

Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE was performed in the Tris/sulfate buffer system described by Neville (1971). Nondenaturing PAGE was performed in the HEPES/imidazole buffer system of McLellan (1982). In order to prevent cleavage of denatured α_2M by active proteinase, 50 μM 3,4-dichloroisocoumarin was added to all samples prior to the addition of SDS sample buffer (Salvesen & Nagase, 1989). The percentage of α_2M subunits cross-linked by DSP or α_2M bait regions cleaved by proteinase was determined by the following procedure. Samples of ^{125}I-α_2M were electrophoresed on the SDS gels, and the position of α_2M subunits was determined by autoradiography. The radioactive bands containing intact, cleaved, or cross-linked subunits were excised and counted for γ-radioactivity in an LKB Model 1272 Clinigamma γ-counter. This procedure, and not densitometry of the Coomassie-stained protein bands, was performed because the intensity of subunit staining progressively decreased with increasing incubation times in DSP (data not shown). This probably occurs because DSP binds to and neutralizes lysine residues on proteins, and staining of protein with Coomassie Blue is diminished when the proteins become less basic (Tal et al., 1985).

TNS Fluorescence. Conformational changes in α_2M were detected by monitoring changes in the uncorrected emission spectra of the fluorescent probe TNS on a Shimadzu Model RF-540 spectrofluorophotometer essentially as described by Strickland and Bhattacharya (1984). TNS (50 μM) was excited at a wavelength of 315 nm, and the emission spectra were scanned from 360 to 560 nm. The excitation and emission slits were 10 nm. The final concentration of α_2M in all experiments was 0.25 μM, and all scans were performed at room temperature (~23 °C). In all experiments, CH_3NH_2 or proteinase was added directly to the sample cuvette containing TNS and α_2M. The effects of dilution on the emission spectra were corrected for by the addition of the appropriate volumes of buffer to the samples.

Miscellaneous Techniques. In vivo plasma elimination studies were performed in CD-1 mice as previously described (Imber & Pizzo, 1981). All experiments were performed in duplicate and the results averaged. We have previously analyzed non-first-order murine clearance curves to obtain the various components of clearance and their individual $t_{1/2}$ values [see, for example, Shifman and Pizzo (1982)]. Such an analysis, however, is not required for the comparison of data as presented in this study. The amount of time required for 50% of the ligand to be removed from the circulation of the mouse is, therefore, reported as the clearance half-life ($t_{1/2}$), even in experiments in which the clearance behavior did not follow first-order kinetics. The proteinase inhibitory activity of α_2M was determined with the macromolecular substrate blue hide powder essentially as described by Barrett et al. (1979). Rates of modification of α_2M by CH_3NH_2 were calculated from the equation $k = (\ln 2)/(t_{1/2}[CH_3NH_2])$, where k is the second-order rate constant for α_2M thiolester bond cleavage and $t_{1/2}$ is the time required for the liberation of two thiol groups. The concentration of CH_3NH_2 present in the free base form ($[CH_3NH_2]$) was determined from a pK_a of 10.43 for CH_3NH_2 (Isenman & Kells, 1982).

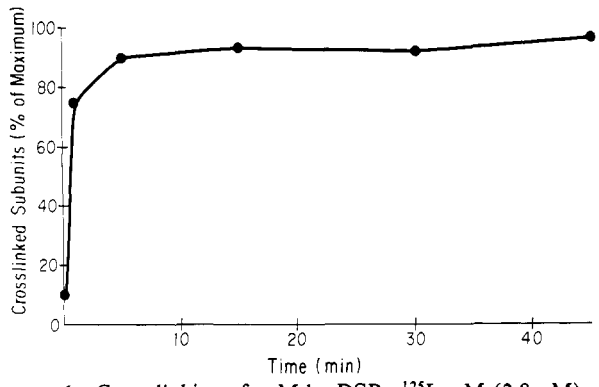

FIGURE 1: Cross-linking of α_2M by DSP. ^{125}I-α_2M (2.8 μM) was treated with 200 μM DSP at room temperature. Aliquots were removed at various times, the reaction was terminated by the addition of 20 mM glycine, and 2-μg aliquots were denatured in 2% SDS and analyzed by SDS-PAGE. The percentage of α_2M subunits cross-linked was calculated as the ratio of the radioactive protein migrating at the position of native α_2M (M_r ~360 000) to the total radioactivity in the aliquot. The maximum amount of cross-linking was designated as the amount of cross-linking following incubation of α_2M with DSP for 2 h. Following this treatment, less than 20% of the protein migrated with M_r ~360 000.

Results

Cross-Linking of α_2M Subunits by DSP. To examine the kinetics of intersubunit cross-linking by DSP, α_2M was incubated with DSP for various times and free DSP quenched with glycine. Figure 1 shows that there was a time-dependent increase in the amount of ^{125}I-α_2M migrating with the mobility of cross-linked subunits (M_r >360 000 in SDS-PAGE). The time required for half-maximal cross-linking of α_2M by DSP ($t_{1/2}$) was ~30 s.

Characterization of α_2M_{DSP}. In order to determine the number of DSP cross-links present in DSP-treated α_2M (α_2M_{DSP}), α_2M was treated with DSP for 5 min, 30 min, and 1 h prior to the addition of glycine. A sample of native α_2M was treated under identical conditions (using glycine hydrolyzed DSP), and DTNB titration detected 23.6 out of a possible 24 thiol groups per α_2M subunit (Sottrup-Jensen et al., 1983). The number of DSP molecules bound to α_2M was calculated on the basis of the difference between the number of thiol groups detected per subunit of α_2M_{DSP} and that per subunit of native α_2M. After incubation with DSP for only 5 min, an additional 5.3 thiol groups per mol of α_2M_{DSP} subunit were detected. This is consistent with the modification of about 6% of the amino groups present in each subunit (Sottrup-Jensen et al., 1983). Incubation for 30 min or 1 h did not change this value significantly (6.4 and 7.7 thiol groups per mol of α_2M_{DSP} subunit at 30 min and 1 h, respectively). Thus a maximum of about 8.5% of the amino groups present in each subunit were modified after 1 h. The extent of the reaction then remained unchanged for at least an additional hour (data not shown). Since DSP is a homobifunctional cross-linking reagent, the binding of 7.7 mol of DSP/mol of α_2M_{DSP} subunit (30.8 mol of DSP/mol of tetramer) indicates that *at most* 15 molecules of DSP are involved in the cross-linking of amino groups in α_2M. Since the extent of derivatization by DSP did not vary significantly over a 2-h period, a standard time for reaction of 1 h was chosen for most studies except where noted in the text.

DSP-treated α_2M was also subjected to ion exchange chromatography to assess the homogeneity of the preparation.

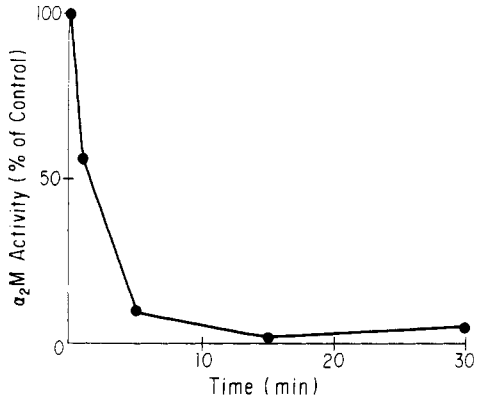

FIGURE 2: Inactivation of α_2M by DSP. α_2M was treated with 200 μM DSP as described in Figure 1. Aliquots of 15 μg were removed from the incubation at various times, and the cross-linking reaction were terminated by the addition of glycine. One microgram of bovine trypsin was then added to each aliquot, followed 5 min later by the trypsin substrate blue hide powder. The ability of α_2M to inhibit proteolysis of the substrate (α_2M activity) was expressed as the percentage of the activity in the DSP-treated aliquots relative to that of a 15-μg sample of α_2M which had been treated with glycine-hydrolyzed DSP. Control experiments demonstrated that glycine-hydrolzyed DSP had no effect on α_2M activity.

α_2M was treated with DSP for 1 h, excess DSP hydrolyzed by glycine, and the preparation exhaustively dialyzed against buffer (25 mM triethanolamine and 150 mM NaCl, pH 8.0). The material was then applied to a column of DEAE-Sephacel (.5 × 3.0 cm). Under these conditions, untreated α_2M was not adsorbed to the matrix. The adsorbed α_2M_{DSP} was eluted by the use of a 40-mL linear gradient of 25 mM triethanolamine, pH 8, containing from 0.15 to 1 M NaCl to a flow rate of 20 mL/h. The α_2M_{DSP} preparation eluted from the column as a single, symmetrical peak at a salt concentration of 0.30 M (data not shown). These results suggest that the preparation consisted of a relatively homogeneous population of α_2M_{DSP} molecules.

Proteinase Inhibitory Activity of α_2M_{DSP}. The effect of DSP on the ability of α_2M to inhibit the activity of trypsin was examined with the macromolecular substrate blue hide powder. α_2M was treated with DSP for various times, free DSP again quenched with glycine, and the trypsin inhibitory activity assayed. Figure 2 shows that there was a time-dependent loss of proteinase inhibitory activity following treatment of α_2M with DSP. The time required for half-maximal inhibition of the initial activity of α_2M following DSP treatment ($t_{1/2}$) was ~90 s. The similarity between this value and the $t_{1/2}$ for the cross-linking of α_2M subunits by DSP ($t_{1/2}$ ~ 30 s) suggests that inactivation of α_2M is a result of the intersubunit cross-linking of α_2M by DSP. Although the reaction has not been as extensively characterized kinetically, the published data suggest that inactivation of α_2M which has been treated with *cis*-DDP (α_2M_{Pt}) is also a result of intersubunit cross-linking of the inhibitor (Gonias & Pizzo, 1981, 1983a; Gonias et al., 1984).

CH_3NH_2-Induced Thiolester Bond Cleavage of α_2M_{DSP}. Since cleavage of the internal thiolester bonds also results in a loss of proteinase inhibitory activity by α_2M, experiments were performed to determine if treatment with DSP resulted in the modification of the internal thiolester bonds in α_2M. Figure 3 demonstrates that there was essentially no difference in the rate of thiolester bond cleavage following treatment of either α_2M or α_2M_{DSP} with CH_3NH_2. In each case, the second-order rate constant for thiol appearance was 3.1 M^{-1} s^{-1} at 23 °C and pH 8.0. This value is in reasonable agreement with previously published values of 13.8 M^{-1} s^{-1} at 25 °C

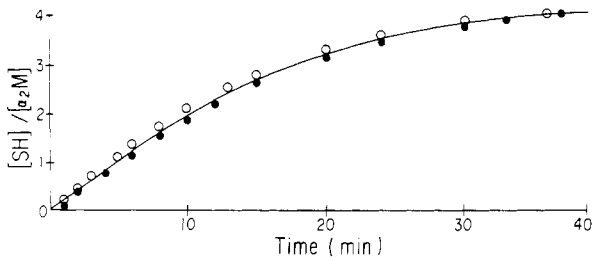

FIGURE 3: CH_3NH_2-induced thiol group appearance in α_2M or α_2M_{DSP}. α_2M (●) or α_2M_{DSP} (○) was incubated with 0.1 M CH_3NH_2 at a final protein concentration of 2.4 μM. Free thiol groups were detected by titration with DTNB, and the data are plotted as a time course of moles of thiol generated per mole of α_2M. The second-order rate constant for the appearance of thiol groups (k) was calculated from a half-life for thiol appearance ($t_{1/2}$) of 600 s and the relationship $k = (\ln 2)/(t_{1/2}[CH_3NH_2])$ as described under Experimental Procedures.

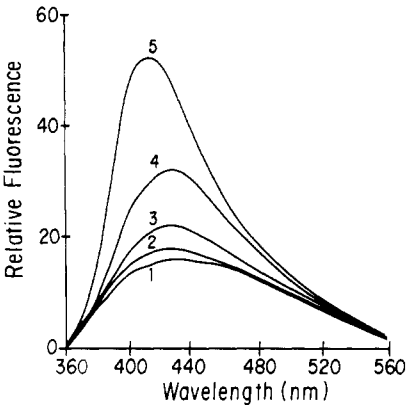

FIGURE 4: Emission spectra of TNS in the presence of α_2M_{DSP}. α_2M or α_2M_{DSP} (0.25 μM) was incubated with 50 μM TNS and the emission spectrum of the fluorescent probe obtained as described under Experimental Procedures. The plotted spectra are as follows: (1) α_2M_{DSP}; (2) CH_3NH_2-treated α_2M_{DSP}; (3) CH_3NH_2-treated α_2M_{DSP} following incubation with thrombin; (4) CH_3NH_2-treated α_2M_{DSP} following incubation with trypsin; (5) CH_3NH_2-treated native α_2M.

(Larsson & Bjork, 1984) and 11.6 M^{-1} s^{-1} at 30 °C (Strickland & Bhattacharya, 1984). This demonstrates not only that the thiolester bonds were intact following DSP treatment but that DSP treatment had not rendered these bonds unusually reactive toward CH_3NH_2.

CH_3NH_2-Induced Conformational Changes in α_2M_{DSP}. Since the rates of reaction of α_2M and α_2M_{DSP} with CH_3NH_2 were essentially identical, experiments were performed to determine if this treatment led to similar conformational changes in α_2M and α_2M_{DSP}. For these studies, the conformational change in α_2M_{DSP} was monitored by the fluorescence probe TNS. This technique was chosen due to its extreme sensitivity in detecting conformational alterations in α_2M (Strickland & Bhattacharya, 1984). Figure 4 shows that the emission spectrum of TNS changes dramatically following treatment of native α_2M with CH_3NH_2, confirming that this treatment leads to a dramatic conformational change in α_2M. In contrast, the emission spectrum of α_2M_{DSP} changed very little following treatment with CH_3NH_2. Table I demonstrates that the intensity of emitted fluorescence of TNS in the presence of CH_3NH_2-treated α_2M_{DSP} was only 4% that of native α_2M–CH_3NH_2, suggesting that CH_3NH_2 treatment led to a very minor change in the conformation of α_2M_{DSP}. Following addition of trypsin to CH_3NH_2-treated α_2M_{DSP}, the intensity of emitted fluorescence of TNS increased to 43% that of native α_2M–CH_3NH_2, whereas the addition of thrombin to CH_3NH_2-treated α_2M_{DSP} resulted in an increase in the

Table I: Effects of Proteinase on the Conformation of CH_3NH_2-Treated α_2M_{DSP}[a]

proteinase	conformational change, F/F_{max} (%)
none	4
thrombin	11
trypsin	43

[a] The extent of the conformational change induced by treatment of CH_3NH_2-treated α_2M_{DSP} with proteinase (F/F_{max}) was determined by changes in the emission spectra of the fluorescent probe TNS. The data were obtained from the emission spectra shown in Figure 4. The intensity of emitted TNS fluorescence at 430 nm following incubation of CH_3NH_2-treated α_2M_{DSP} with proteinase (F) is expressed as a percentage of the intensity of emitted TNS fluorescence of CH_3NH_2-treated native α_2M (F_{max}). DSP alone had no effect on the emission spectra of TNS in the presence or absence of native α_2M or CH_3NH_2-treated native α_2M (results not shown).

Table II: Bait Region Cleavage of α_2M or α_2M_{DSP}[a]

proteinase	α_2M (%)	α_2M_{DSP} (%)	CH_3NH_2-treated α_2M_{DSP} (%)
none	4	7	4
thrombin	49	67	73
trypsin	78	93	94

[a] Determined by excision and counting of ^{125}I-labeled α_2M subunits following reducing SDS–PAGE as described under Experimental Procedures. The amount of cleaved ^{125}I-α_2M subunits migrating with M_r ~90 000 and the covalent adducts with proteinase (M_r ~110 000 and M_r >180 000) is expressed as a percentage of the total ^{125}I-α_2M present in the sample. Since 20 mM glycine was present in the incubation with proteinase, the contribution of covalent adducts with proteinase constituted less than 20% of the total cleaved subunit radioactivity.

intensity of emitted fluorescence of TNS to a value 11% that of native α_2M–CH_3NH_2.

As expected, very similar results were obtained when the conformational changes of CH_3NH_2-treated α_2M_{DSP} were analyzed by nondenaturing PAGE (results not shown). Nondenaturing PAGE is not the ideal technique to probe the conformational alterations in α_2M_{DSP}, however, since quantitation of the magnitude of the conformational changes would be very difficult. Furthermore, as demonstrated by Strickland et al. (1988), very good correlation exists between conformational changes detected by nondenaturing PAGE and those detected by changes in the emission spectrum of the fluorescent probe TNS.

SDS–PAGE of CH_3NH_2-Treated α_2M_{DSP}. Since the thiolester bonds in α_2M_{DSP} had been cleaved in the experiments described above, experiments were performed to determine if the conformational changes which had occurred were the result of proteolytic cleavage of the bait regions of α_2M_{DSP}. Table II demonstrates that incubation of CH_3NH_2-treated α_2M_{DSP} with trypsin leads to almost complete bait region cleavage. In contrast, only 67% of the α_2M_{DSP} bait regions had been cleaved following incubation of CH_3NH_2-treated α_2M_{DSP} with thrombin. Essentially identical results were obtained following incubation of these proteinases with α_2M_{DSP} which had not been pretreated with CH_3NH_2. Table II also reveals that there was consistently greater bait region cleavage in preparations of α_2M_{DSP} than in preparations of native α_2M; however, these differences were relatively minor. These results demonstrate that although the bait regions of α_2M_{DSP} were still accessible to proteinase following treatment with CH_3NH_2, they were less accessible to the proteinase thrombin than they were to the proteinase trypsin.

Receptor Recognition of α_2M_{DSP}. In vivo plasma elimination studies were used as another probe of the conformation changes in α_2M_{DSP}, namely, those which result in the exposure of the receptor recognition sites in α_2M. Figure 5 demonstrates

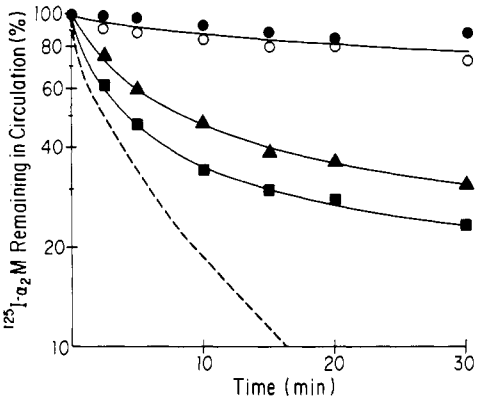

FIGURE 5: Plasma elimination of α_2M_{DSP}. Fifteen micrograms of ^{125}I-α_2M_{DSP} (●) or CH_3NH_2-treated ^{125}I-α_2M_{DSP} incubated with buffer (○), thrombin (▲), or trypsin (■) was injected in the lateral tail vein of a mouse. The percentage of ^{125}I-labeled α_2M remaining in the circulation is plotted as a function of time. The rate of plasma elimination of trypsin-treated native ^{125}I-α_2M (--) is shown for comparison.

that there was essentially no differences in the extent of receptor recognition of α_2M_{DSP} before or after treatment with CH_3NH_2. In each case, there was only minimal interaction of the injected ligand with the α-macroglobulin receptor ($t_{1/2} \gg 60$ min). By contrast, incubation of CH_3NH_2-treated α_2M_{DSP} with trypsin resulted in the rapid elimination of the ligand from the circulation of the mouse with a half-life of only 4 min, a value which is similar to that of untreated α_2M–trypsin ($t_{1/2} \sim 3$ min). Although incubation with thrombin led to an increase in the rate of clearance of CH_3NH_2-treated α_2M_{DSP} ($t_{1/2} \sim 10$ min), the clearance rate was not as rapid as that observed following incubation of CH_3NH_2-treated α_2M_{DSP} with trypsin. Essentially identical results were obtained in experiments in which α_2M was treated with DSP for only 5 min prior to the addition of CH_3NH_2 (data not shown).

In contrast to the results obtained following treatment of α_2M_{DSP} with CH_3NH_2, treatment of α_2M_{Pt} with CH_3NH_2 leads to conformational changes in the inhibitor which result in receptor recognition site exposure and plasma elimination of the ligand (Gonias & Pizzo, 1983a). The clearance of this ligand displayed higher order kinetics, and the half-life of plasma elimination was 20 min. However, when α_2M_{Pt} was incubated with DSP for 1 h prior to treatment with CH_3NH_2, receptor recognition of the ligand was significantly retarded ($t_{1/2} \sim 70$ min, data not shown). These results demonstrate that DSP prevents conformational changes in α_2M_{Pt} which normally lead to receptor recognition site exposure following treatment with CH_3NH_2.

Proteinase-Induced Thiolester Bond Cleavage in Cross-Linked α_2M. When native α_2M is incubated with thrombin or trypsin, titration with DTNB reveals that two or four thiolester bonds per mole of inhibitor are cleaved, respectively (Roche & Pizzo, 1988). Similarly, Figure 6 demonstrates that when α_2M_{DSP} was treated with thrombin, two thiolester bonds were cleaved after 20 min of incubation. When α_2M_{DSP} is treated with trypsin, however, titration with DTNB revealed that only two thiolester bonds were cleaved. Identical results were obtained following treatment of α_2M_{DSP} with porcine pancreatic elastase (titration data not shown). As in native α_2M, the rate of thiolester bond cleavage following treatment of α_2M_{DSP} with thrombin is slower than the rate of thiolester bond cleavage following treatment with trypsin (Steiner et al., 1985). Figure 6 also shows that the addition of CH_3NH_2 to the samples after 20-min incubation with proteinase led to the

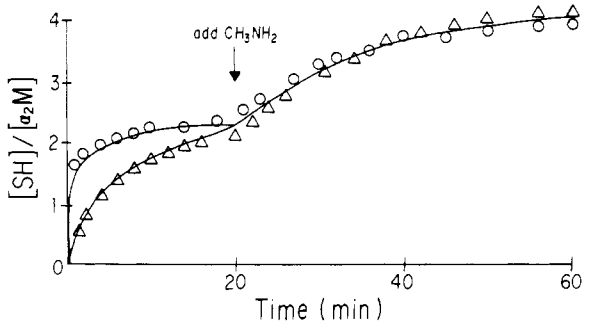

FIGURE 6: Proteinase-induced thiol group appearance in α_2M or α_2M_{DSP}. α_2M_{DSP} (2.4 μM) was incubated with a 2-fold molar excess of thrombin (△) or trypsin (○), and free thiol groups were detected by titration with DTNB. The data are plotted as moles of thiol generated per mole of α_2M and are shown as a function of time. After 20 min, a 1:50 dilution of 5 M CH_3NH_2 was added to the sample (final $[CH_3NH_2]$ = 0.1 M).

appearance of all four thiol groups, demonstrating that the uncleaved thiolester bonds were accessible to CH_3NH_2 in proteinase-treated α_2M_{DSP}.

When samples of α_2M_{Pt} were treated with either trypsin or thrombin, titration with DTNB did not demonstrate the appearance of any thiol groups on the inhibitor (titration data not shown). Subsequent addition of 0.1 M CH_3NH_2 to the preparation did result in cleavage of the thiolester bonds, however, and titration with DTNB demonstrated that 3.8 thiol groups per mole of α_2M_{Pt} appeared after 90 min of incubation. Interestingly, the second-order rate constant for thiol group appearance following treatment of α_2M_{Pt} with CH_3NH_2 was half that of native α_2M following treatment with CH_3NH_2 (1.5 M^{-1} s^{-1} for α_2M_{Pt} versus 3.1 M^{-1} s^{-1} for native α_2M). This may reflect *cis*-DDP-induced conformational changes in the proximity of the thiolester bonds in α_2M_{Pt}.

Discussion

A previous study from this laboratory demonstrated that intersubunit cross-linking with *cis*-DDP prevents bait region cleavage dependent conformational changes in α_2M and thus allowed analysis of thiolester bond cleavage dependent conformational changes in the inhibitor (Roche et al., 1988). In the present investigation, the experiments were designed in the hope of gaining insight into the mechanism of bait region cleavage dependent conformational changes in α_2M. Since these changes are difficult to dissociate from those changes induced by cleavage of the thiolester bonds in α_2M, it was necessary to modify α_2M in a manner which would prevent thiolester bond cleavage dependent conformational changes yet still allow bait region cleavage dependent conformational changes. Fortunately, treatment of native α_2M with DSP allowed such an analysis.

When the subunits of α_2M are cross-linked with DSP, complete cleavage of the internal thiolester bonds by CH_3NH_2 does not result in a major conformational change in the inhibitor. This was demonstrated by the findings that thiolester bond cleavage does not result in (a) significant changes in the TNS emission spectrum of α_2M_{DSP}, (b) changes in the electrophoretic mobility of α_2M_{DSP} in nondenaturing PAGE, (c) changes in the exposure of the receptor recognition sites of α_2M_{DSP}, and (d) changes in the succeptibility of the bait regions of α_2M_{DSP} to proteolytic cleavage by trypsin or thrombin. These results are in dramatic contrast to those obtained following treatment of native α_2M with CH_3NH_2. In this case, the bait regions are not highly succeptible to proteolytic cleavage (Gonias & Pizzo, 1983b; Roche & Pizzo, 1987), and the conformation of the inhibitor is dramatically altered as determined by changes in the TNS emission spectrum (Strickland & Bhattacharya, 1984), mobility in nondenaturing PAGE (Barrett et al., 1979), and exposure of the receptor recognition sites (Imber & Pizzo, 1981). These results demonstrate quite conclusively that treatment with DSP effectively prevents thiolester bond cleavage dependent conformational changes in α_2M.

Although the conformation of α_2M_{DSP} is not significantly altered following complete thiolester bond cleavage by CH_3NH_2, the conformation of CH_3NH_2-treated α_2M_{DSP} can be altered by cleavage of the bait regions in the protein. These changes were detected by changes in the TNS emission spectrum and nondenaturing PAGE. These results are very similar to those obtained following treatment of bovine α_2M with CH_3NH_2. In this case, the protein undergoes complete thiolester bond cleavage, although there is no "slow" to "fast" change or change in the TNS emission spectrum until the bait regions of the inhibitor are cleaved by proteinase (Feldman et al., 1984; Strickland et al., 1984). In addition, the results obtained following incubation of CH_3NH_2-treated α_2M_{DSP} with the proteinases trypsin and thrombin demonstrate that the magnitude of the conformational change (as determined by changes in the TNS emission spectrum and rate of in vivo plasma elimination) can be roughly correlated with the extent of bait region cleavage in the complex. That is to say, incubation of CH_3NH_2-treated α_2M_{DSP} with thrombin (which results in cleavage of only 73% of the α_2M subunits) leads to less dramatic conformational changes than those observed following incubation with trypsin (which results in cleavage of 94% of the α_2M subunits).

Treatment with excess thrombin usually results in the cleavage of only two bait regions and two thiolester bonds in α_2M (Steiner et al., 1985; Roche & Pizzo, 1988), while treatment with excess trypsin usually results in cleavage of all four bait regions and all four thiolester bonds (Sottrup-Jensen et al., 1980). Although under certain reaction conditions it is possible to cleave only two bait regions with the concomitant exposure of all four thiol groups (Christensen & Sottrup-Jensen, 1984), to our knowledge conditions have not been described which lead to cleavage of all four bait regions and the exposure of only two thiol groups, as was demonstrated in these studies. These results may be due to the artificial nature of the DSP cross-links in α_2M_{DSP}, or they may be evidence for a fundamental asymmetry in the structure of α_2M_{DSP}. Experiments are now being performed to address these issues, but analysis is difficult since proteinase treatment leads to both bait region and thiolester bond cleavage. In any event, these results demonstrate that unlike cross-linking by *cis*-DDP intersubunit cross-linking by DSP does not prevent the conformational changes which lead to activation and cleavage of the internal thiolester bonds following cleavage of the bait regions in α_2M.

When the thiolester bonds of α_2M_{Pt} are cleaved by treatment with CH_3NH_2, the modified protein undergoes a series of conformational changes which result in almost complete receptor recognition site exposure (Gonias & Pizzo, 1983a; this investigation). In addition, a very similar form of α_2M can be generated by treatment of α_2M with CH_3NH_2 in the presence of the thiol group cyanylating reagent dinitrophenyl thiocyanate (Van Leuven et al., 1982; Björk, 1985). As with α_2M_{Pt}, cyanylated α_2M–CH_3NH_2 does not undergo the slow to fast conformational change but does undergo a limited conformational change which leads to receptor recognition site exposure. Unlike intersubunit cross-linking by *cis*-DDP or cyanylation of the liberated thiol groups, however, cross-linking

by DSP does prevent the conformational changes in α_2M which trigger receptor recognition site exposure following thiolester bond cleavage. In addition to preventing the exposure of the receptor recognition sites following CH_3NH_2 treatment of native α_2M, DSP treatment also prevents the exposure of the receptor recognition sites following CH_3NH_2 treatment of α_2M_{Pt}. This result demonstrates once again that *cis*-DDP and DSP have very different cross-linking properties. Interestingly, subsequent treatment of CH_3NH_2-treated α_2M_{DSP} with proteinase releases the conformational "lock" and allows transduction of the conformational changes which trigger receptor recognition site exposure.

These studies can contribute to our understanding of the mechanisms of proteinase-induced conformational changes in the α-macroglobulin homologue chicken ovostatin. In this protein, there are no thiolester bonds and no receptor recognition sites (Nagase et al., 1983; Feldman & Pizzo, 1984a). Following treatment with proteinase, however, ovostatin does undergo a dramatic conformational change which leads to proteinase inhibition. In fact, the changes in the circular dichroism spectrum of ovostatin following treatment with proteinase are identical with those observed following treatment of human α_2M with proteinase (Feldman & Pizzo, 1984b). This demonstrates that bait region cleavage alone can result in conformational changes which are sufficient to inhibit proteinases even without thiolester bond cleavage.

The present studies are also relevant to the mechanism of proteinase inhibition by rat α_2M. Although treatment with CH_3NH_2 results in the cleavage of all four thiolester bonds in this α-macroglobulin, the modified protein is not receptor recognized and is still capable of proteinase binding (Gonias et al., 1983). In a manner analogous to the results obtained in this investigation, incubation of CH_3NH_2-treated rat α_2M with proteinase results in a slow to fast change in nondenaturing PAGE, and the modified protein is recognized by the mammalian α-macroglobulin receptor with high affinity. Thus, treatment of human α_2M with DSP creates a "rat α_2M analogue" which can be used to study the conformational changes in the native protein.

It is evident from these studies that conformational changes occurs on many levels when α_2M reacts with proteinase. Previous studies from this and other laboratories have suggested and described a number of such levels of conformational change [reviewed in Feldman et al. (1985)]. Primarily on the basis of data obtained from circular dichroism studies, we predicted at least three levels of conformational change in α-macroglobulins. These include those changes resulting from (a) increased interactions of the pairs of subunits constituting the proteinase binding sites (demonstrated by increases in electrophoretic mobility in nondenaturing PAGE, (b) "trap" closure (demonstrated by circular dichroism changes and loss of proteinase inhibitory activity), and (c) subtle changes which expose receptor recognition sites (demonstrated by in vivo and in vitro binding studies). Previous studies of α_2M conformation, however, did not provide insights into the mechanisms of conformational change in ovostatin, which does not contain thiolester bonds, nor the unexplained data obtained following CH_3NH_2 treatment of rat or bovine α_2M. On the basis of the data presented in this investigation, we suggest that cleavage of the bait region itself drives the major conformational changes seen with most α-macroglobulins. Cleavage of thiolester bonds may in some cases be sufficient to induce a similar conformational change in the inhibitor (as with human α_2M or rat α_1M); however, in most α-macroglobulins (including those of frog, chicken, cow, and rat) cleavage of bait regions is essential for induction of the complete conformational change which leads to proteinase trapping and receptor recognition site exposure.

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Reaction of Proteinases with α_2-Macroglobulin: Evidence for Alternate Reaction Pathways in the Inhibition of Trypsin†

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ABSTRACT: Titration experiments were employed to measure the binding stoichiometry of α_2M for trypsin at high and low concentrations of reactants. These titration experiments were performed by measuring the SBTI-resistant trypsin activity and by direct binding measurements using ^{125}I-labeled trypsin. The binding stoichiometry displayed a marked dependence upon protein concentration. At high α_2M concentrations (micromolar), 2 mol of trypsin are bound/mol of inhibitor. However, at low α_2M concentrations (e.g., 0.5 nM), only 1.3 mol of trypsin were bound/mol of inhibitor. Sequential additions of subsaturating amounts of trypsin to a single aliquot of α_2M also resulted in a reduction in the final binding ratio. A model has been formulated to account for these observations. A key element of this model is the observation that purified 1:1 α_2M-proteinase complexes are not capable of binding a full mole of additional proteinase [Strickland et al. (1988) *Biochemistry 27*, 1458-1466]. The model predicts that once the 1:1 α_2M-proteinase complex forms, this species undergoes a time-dependent conformational rearrangement to yield a complex with greatly reduced proteinase binding ability. According to this model, the ability of α_2M to bind 2 mol of proteinase depends upon the association rate of the second enzyme molecule with the binary (1:1) complex, the enzyme concentration, and the rate of the conformational alteration that occurs once the initial complex forms. Modeling experiments suggest that the magnitude of the rate constant for this conformational change is in the order of 1-2 s^{-1}.

α_2-Macroglobulin (α_2M)[1] is a large (M_r = 718 000) plasma proteinase inhibitor that contains four identical subunits and has the ability to inhibit proteinases from all subclasses (Jones et al., 1972; Barrett & Starkey, 1973; Hall & Roberts, 1978; Sottrup-Jensen et al., 1984). This molecule is a member of a class of proteins that include pregnancy zone protein (PZP) and complement components C3 and C4. In addition to regions of conserved sequence among these proteins, all of them contain one or more internal β-cysteinyl-γ-glutamyl thiolester

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[1] Abbreviations: α_2M, α_2-macroglobulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PZP, pregnancy zone protein; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; S-2222, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

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