A Conserved Region in α-Macroglobulins Participates in Binding to the Mammalian α-Macroglobulin Receptor†

Jan J. Enghild, Ida B. Thøgersen, Paul A. Roche, and Salvatore V. Pizzo*
Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
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ABSTRACT: Efforts to characterize the receptor recognition domain of α-macroglobulins have primarily focused on human α_2-macroglobulin (α_2M). In the present work, the structure and function of the α-macroglobulin receptor recognition site were investigated by amino acid sequence analysis, plasma clearance, and cell binding studies using several nonhuman α-macroglobulins: bovine α_2M, rat α_1-macroglobulin (α_1M), rat α_1-inhibitor 3 (α_1I_3), and proteolytic fragments derived from these proteins. Each α-macroglobulin bound to the murine peritoneal macrophage α-macroglobulin receptor with comparable affinity ($K_d \sim 1$ nM). A carboxyl-terminal 20-kDa fragment was isolated from each of these proteins, and this fragment bound to α-macroglobulin receptors with K_d values ranging from 10 to 125 nM. The amino acid identity between the homologous carboxyl-terminal 20-kDa fragments of human and bovine α_2M was approximately 90%, while the overall sequence homology between all carboxyl-terminal fragments studied was 75%. The interchain disulfide bond present in the human α_2M carboxyl-terminal 20-kDa fragment was conserved in bovine α_2M and rat α_1I_3, but not in rat α_1M. The clearance of each intact α-macroglobulin–proteinase complex was significantly retarded following treatment with *cis*-dichlorodiammineplatinum(II) (*cis*-DDP). *cis*-DDP treatment, however, did not affect receptor recognition of purified carboxyl-terminal 20-kDa fragments of these α-macroglobulins. A carboxyl-terminal 40-kDa subunit, which can be isolated from rat α_1M, bound to the murine α-macroglobulin receptor with a K_d of 5 nM. Treatment of this subunit with *cis*-DDP resulted in an increase in the K_d from 5 to 50 nM, similar to the affinity of the isolated carboxyl-terminal 20-kDa fragment of rat α_1M. It is concluded from these studies that the platinum-sensitive component of the α_2M receptor recognition site is located in the carboxyl-terminal region of the protein. However, this region is distinct from the location of the remainder of the α-macroglobulin receptor recognition site which is contained in the carboxyl-terminal 20-kDa fragment.

The α-macroglobulins are a homologous group of high molecular weight proteins which inhibit the activity of endopeptidases from all four catalytic classes [for reviews, see Travis and Salvesen (1983), Roberts (1986), and Sottrup-Jensen (1987)]. The molecular weight of α-macroglobulin subunits is approximately 180 000, and the subunits are arranged to form tetramers in bovine α_2M (Dangott & Cunningham, 1982), human α_2M (Swenson & Howard, 1979), and rat α_1M; however, each rat α_1M subunit is processed to form approximately 160- and 40-kDa chains which are held together by a disulfide bond (Gordon, 1976; Geiger et al., 1987). Unlike other inhibitors of the α-macroglobulin group, the homologous rat protein α_1I_3 is monomeric (Esnard & Gauthier, 1980). The inhibitory mechanism of the α-macroglobulins involves limited proteolysis and is usually followed by cleavage of an internal β-cysteinyl-γ-glutamyl thiol ester bond. These events trigger a conformational change which appears to physically entrap the proteinase molecule(s) (Barrett & Starkey, 1973; Gonias et al., 1982) and results in the appearance of determinants on the inhibitor which are recognized by cell surface receptors [see Pizzo and Gonias (1984) for a review]. Subsequent to receptor binding, the inhibitor is rapidly removed from the circulation by receptor-mediated endocytosis (Kaplan & Nielsen, 1979a,b; Imber & Pizzo, 1981).

Several approaches have been employed in an effort to characterize the receptor recognition site of human α_2M. Studies employing monoclonal antibodies and limited proteolysis suggested that a lysine residue is involved in receptor recognition of human α_2M (Marynen et al., 1982; Van Leuven et al., 1986b; Sottrup-Jensen et al., 1986). Chemical modification experiments with H_2O_2 and *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) suggested that there is an oxidation-sensitive residue present in the α_2M receptor binding site (Gonias & Pizzo, 1981; Pizzo et al., 1986). A monoclonal antibody, 7H11D6, has been produced which specifically recognizes the platinum-sensitive epitope of this site (Isaacs et al., 1988; Roche et al., 1988). This monoclonal antibody was employed to generate an anti-idiotypic antibody which binds to the α-macroglobulin receptor with extremely high affinity (Isaacs et al., 1988). Recent reports have shown that the carboxyl-terminal 20-kDa region of human α_2M competes for the binding of α_2M–proteinase complexes to the α-macroglobulin receptor present on hepatocytes (Sottrup-Jensen et al., 1986), fibroblasts (Van Leuven et al., 1986a,b), and macrophages (Roche et al., 1988). These results indicate that at least part of the receptor recognition site is located in this region of the molecule.

Studies of the carboxyl-terminal 20-kDa fragment of human α_2M, however, indicated that it does not contain the *cis*-DDP-reactive component of the receptor recognition site (Isaacs et al., 1988; Roche et al., 1988). Preliminary observations suggested that monoclonal antibody 7H11D6 binds to a complex of several polypeptide chains of $M_r \sim 55\,000$ designated p55 (Roche et al., 1988). However, the affinity of the antibody for p55 was very poor ($I_{50} \sim 2000$ nM). In the present study, we have isolated the carboxyl-terminal region from bovine α_2M, rat α_1M, and rat α_1I_3 in order to investigate the nature of receptor recognition of nonhuman

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* Correspondence should be addressed to this author.

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α-macroglobulins as well as the relationship between the *cis*-DDP-reactive component and the remainder of the receptor recognition site. Clearance experiments in mice, in vitro binding studies with murine peritoneal macrophages, and a receptor recognition site specific monoclonal antibody were employed to study receptor recognition of these α-macroglobulins. Furthermore, the amino acid sequence of the bovine carboxyl terminus was established to permit comparison of these sequences for human, bovine, and rat α-macroglobulins.

Materials and Methods

Chemicals and Reagents. TPCK-treated trypsin, soybean trypsin inhibitor, *Staphylococcus aureus* V8 proteinase, guanidine hydrochloride, iodoacetamide, dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and HEPES were from Sigma (St. Louis, MO). Iodo[^{14}C]acetamide was from Amersham (Arlington, IL). DEAE-Sephacel, Cibacron Blue Sepharose CL-4B, Superose 12, Sephadex G-25, and Ultrogel AcA-22 were from Pharmacia/LKB (Rockville, MD). Reversed-phase HPLC columns Vydac C18 (5 μm) and Microsorb (3 μm) C18 "short-one" were from the Separation Group (Hesperia, CA) and Rainin (Emeryville, CA), respectively. Reagents and solvents for the gas/liquid and pulsed liquid phase sequencers were from Applied Biosystems Inc. (Foster City, CA). HPLC-grade tetrahydrofuran, 2-propanol, and acetonitrile were from Burdick and Jackson Laboratories (Muskegon, MI). Twenty-week-old female CD-1 mice were from Charles River Laboratories (Raleigh, NC). *cis*-DDP was from Aldrich (Milkwakee, WI). Earle's balanced salt solution and RPMI-1640 media were from Gibco (Grand Island, NY). Thioglycolate-elicited murine peritoneal macrophages were provided by Dr. Dolph O. Adams, Duke University Medical Center. Fresh frozen human plasma was from the Duke University Medical Center Blood Bank. Bovine plasma and rat plasma were obtained from Pel-Freez Biologicals (Rogers, AR).

Protein Purification. Human α_2M was purified as described by Kurecki et al. (1979), with modifications as previously reported (Imber & Pizzo, 1981). Bovine α_2M was purified as follows. Bovine plasma was extensively dialyzed against deionized water at 4 °C. Following removal of the insoluble material by centrifugation, the plasma was applied to an affinity column of Cibacron Blue Sepharose CL-4B in 50 mM Tris-HCl/50 mM NaCl, pH 7.4. The fractions which contained proteinase inhibitory activity were then subjected to anion-exchange chromatography on DEAE-Sephacel in 20 mM sodium phosphate and eluted with a linear gradient from 10 to 200 mM NaCl. The active fractions were further purified by gel filtration chromatography on Ultrogel AcA-22 in 20 mM HEPES/100 mM NaCl, pH 7.4. Rat α_1M and α_1I_3 were purified essentially as described by Lonberg-Holm et al. (1987). The purity of the preparations was examined by SDS–PAGE and by amino-terminal sequence analysis as described below. Monoclonal antibody 7H11D6 was a gift from Dr. Dudley Strickland, American Red Cross Biomedical Research and Development, Rockville, MD. The characterization of the antibody has recently been published (Issacs et al., 1988).

Isolation of Carboxyl-Terminal Fragment. The fragments were isolated essentially as described for human α_2M (Sottrup-Jensen et al., 1986) and identified by amino-terminal sequence analysis. The carboxyl-terminal 40-kDa subunit from rat α_1M was prepared by reduction with DTT and alkylation with iodoacetamide in 6 M guanidine hydrochloride, 100 mM Tris-HCl, and 1 mM EDTA, pH 8.3 (Hirs, 1967a,b). The carboxyl-terminal 40-kDa subunit was isolated on a Superose 12 column in the Tris/guanidine buffer described above. After the purification, it was dialyzed into 25 mM HEPES/100 mM NaCl, pH 7.4, to remove the denaturant.

Enzymatic Digestion. Bovine carboxyl-terminal fragment, 100 nmol, was digested using trypsin, chymotrypsin, or *S. aureus* V8 proteinase. The enzyme:substrate ratio was 1:50, and the digestions were performed for 4 h at 37 °C in 100 mM NH_4HCO_3. For some experiments, the fragment was reduced and alkylated with iodo[^{14}C]acetamide prior to digestion with trypsin or *S. aureus* V8 proteinase.

Peptide Separation. The peptides were separated immediately an a Vydac C18 reverse-phase HPLC column (0.4 cm × 20 cm) using two LKB 2150 HPLC pumps and a LKB 2152 controller to form a gradient in A (0.1% trifluoroacetic acid) and B [trifluoroacetic acid/water/acetonitrile 0.1:9.9:90 (v/v)]. Where necessary, peptides were repurified on a Microsorb C18 "short-one" reverse-phase HPLC column using the chromatography system described above except that acetonitrile was substituted with 2-propanol. The peptides were detected at 214 nm using an LKB 2140 diode array detector, collected manually in polypropylene tubes, and stored at −20 °C.

Amino Acid Composition and Sequence Analysis of Peptides. Peptides, 1–2 nmol, were hydrolyzed in gas phase for 24 h at 110 °C, essentially as described by Meltzer et al. (1987). The hydrolyzed samples were dried in a Speed Vac concentrator (Savant), and the amino acid composition of the hydrolysates was determined in a Beckman 6300 high-performance amino acid analyzer with sodium citrate buffers provided by the manufacturer. Peptide or proteins, 200–500 pmol, were sequenced by automated Edman degradation in an Applied Biosystems 470A gas/liquid-phase sequencer or an Applied Biosystems 477A pulsed liquid phase sequencer with on-line PTH analysis using an Applied Biosystems 120A HPLC. The instruments were operated as recommended by the manufacturer. The "off line" PTH derivatives from the Applied Biosystems 470A were dissolved in 10% aqueous acetonitrile and separated on an Applied Biosystems reverse-phase column (2.1 × 22 cm) in a Hewlett-packard 1090 HPLC system, using a slight modification of the sodium acetate/tetrahydrofuran/acetonitrile/dimethylphenylthiourea gradient recommended by Applied Biosystems Inc.

Protein Concentrations. Protein concentrations were calculated by using the following constants: human and bovine α_2M, $A^{1\%,1cm} = 8.93$, $M_r = 718K$ (Hall & Roberts, 1978; Feldman et al., 1984); rat α_1M, $A^{1\%,1cm} = 9.8$ $M_r = 746K$; rat α_1I_3, $A^{1\%,1cm} = 7.5$, $M_r = 200K$ (Esnard & Gauthier, 1985).

Protein Radioiodination. Proteins were labeled with ^{125}I by the solid-state lactoperoxidase method of David and Reisfeld (1974). Under the labeling conditions employed, α-macroglobulins retain full proteinase inhibitory and receptor binding activity (Imber & Pizzo, 1981).

Plasma Clearance Studies. ^{125}I-Labeled α-macroglobulin–proteinase complexes were injected into the lateral tail vein of female CD-1 mice. Blood, 25 μL, was collected from the retroorbital venous plexus at various times and counted for γ radioactivity. The initial ligand concentration was defined as the radioactivity in an aliquot removed 5–10 s after injection, and the radioactivity remaining in the circulation was expressed as a fraction of this initial value (Imber & Pizzo, 1981).

Preparation of α_2M–Methylamine. Human α_2M was treated with 200 mM methylamine in 50 mM Tris–HCl/100 mM NaCl, pH 8.2, overnight and dialyzed into 50 mM HEPES/100 mM NaCl, pH 7.4.

Binding Studies. In vitro binding studies were performed essentially as described by Roche et al. (1988). Briefly, monolayers of thioglycolate-elicited mouse peritoneal macrophages were plated at a density of 10^6 cells/well in 24-well culture plates and were allowed to adhere for 2 h at 37 °C. The monolayers were then washed and equilibrated in Earle's balanced salt solution containing 1% BSA and 10 mM HEPES, pH 7.4 (binding buffer), at 4 °C. The cells were then incubated with various concentrations of unlabeled competing ligand containing 0.3 nM ^{125}I-labeled human α_2M-methylamine. After 6 h, the cells were washed 3 times with binding buffer, harvested in 0.1 M NaOH, and counted to determine cell-associated radioactivity. Nonspecific binding was determined by incubation in the presence of 5 mM EDTA. All experiments were performed at least 5 times and the results averaged. In all experiments shown, the standard deviation was less than ±2%. The I_{50}, as defined by Cheng and Prosoff (1973), is the amount of ligand required to decrease the specific binding of ^{125}I-labeled human α_2M-methylamine by 50%. Under the conditions employed in these studies, the K_d was calculated according to the relationship $I_{50} = 2K_d$ (Roche et al., 1988).

Radioimmunoassay Using Monoclonal Antibody 7H11D6. Monoclonal antibody 7H11D6 was used in competitive radioimmunoassays as described by Roche et al. (1988). Briefly, antibody 7H11D6 was added to microtiter plates, 10 nM in 100 μL of 25 mM Hepes/150 mM NaCl, pH 7.4 (HBS), and incubated for 24 h at 4 °C. The wells were washed with 2 × 150 μL of cold HBS, and residual binding sites were blocked by adding HBS containing 1% BSA (RIA buffer). Various concentrations of unlabeled competing ligand were added in RIA buffer containing 5 nM ^{125}I-labeled human α_2M-methylamine. After incubation at 4 °C for 24 h, the wells were washed 3 times with 200 μL of RIA buffer, removed, and counted for γ radioactivity. Nonspecific binding was determined by the addition of a 1000-fold molar excess of unlabeled human α_2M-methylamine in the incubation and represented less than 20% of the total binding. The I_{50} was determined as described above.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS)-PAGE was performed in 5–15% linear gradient gels (10 cm × 10 cm × 1 mm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury (1981). The gels were stained with Coomassie Briliant Blue R-250 and destained with dilute methanol/acetic acid in a conventional manner.

Results

Characterization of the α-Macroglobulin Receptor Binding Domain. It has been reported that a carboxyl-terminal 20-kDa fragment liberated by limited proteolysis of human α_2M is recognized by the α-macroglobulin receptor (Van Leuven et al., 1986a,b; Sottrup-Jensen et al., 1986; Roche et al., 1988). When treated with papain, bovine α_2M, rat α_1M, and rat α_1I$_3$ were degraded to produce a number of fragments. When subjected to gel filtration on Sephacryl S-200, a void volume peak and a low molecular weight peak were obtained. In contrast to human α_2M (Roche et al., 1988), no peak equivalent to p55 was obtained from any of the nonhuman α-macroglobulins studied (data not shown). The low molecular weight fragment from these three α-macroglobulins each consisted of a single polypeptide chain of $M_r \sim 20\,000$, as determined by SDS-PAGE (Figure 1). Amino-terminal sequence analysis identified only a single amino acid at each cycle of analysis. The carboxyl-terminal 20-kDa fragment of bovine α_2M gave multiple bands on the gel, probably due to carbohydrate heterogeneity, an observation also made with the fragment derived from human α_2M (Sottrup-Jensen et al., 1986). The amino-terminal sequences of the purified carboxyl-terminal fragments are shown in Figure 2. The carboxyl-terminal 40-kDa subunit of rat α_1M was purified as described under Materials and Methods. Amino-terminal sequence analysis of this subunit was identical with the sequence recently published (Sottrup-Jensen, 1987). The first 20 amino acids corresponded to residues Asp-1199–Gln-1218 in the human sequence.

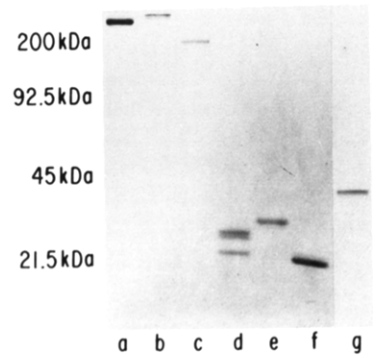

FIGURE 1: SDS-PAGE in a linear 5–15% gradient gel system of the different α-macroglobulins used and derived fragments. Lane a, bovine α_2M; b, rat α_1M; c, rat α_1I$_3$. The 20-kDa carboxyl-terminal fragments are shown in lanes d (bovine α_2M), e rat α_1M, and f (rat α_1I$_3$). The 40-kDa subunit of rat α_1M is shown in lane g. The gel was stained with Coomassie Brilliant Blue R-250. All of the samples were studied under nonreducing conditions.

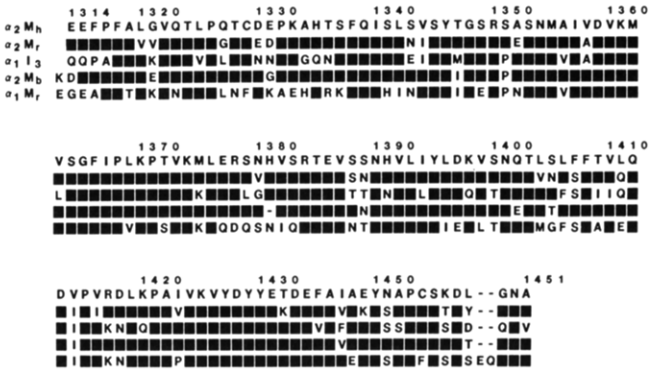

FIGURE 2: Alignment of the carboxyl-terminal fragment from human α_2M (Sottrup-Jensen et al., 1984), rat α_2M, rat α_1I$_3$ (Schweizer et al., 1987; Braciak et al., 1988; Aiello et al., 1988), bovine α_2M, and rat α_1M (Eggersten and Fey, personnel communication). Gaps were introduced to align the carboxyl-terminal amino acids and to indicate the deletion of residue 1381 in the bovine carboxyl-terminal fragment. A solid box indicates that the amino acid is identical with the human sequence. The numbering is according to the human α_2M amino acid sequence (Sottrup-Jensen et al., 1984).

Receptor Recognition of the Carboxyl-Terminal 20-kDa Fragments. The receptor binding of each fragment was examined by the reversible competitive macrophage binding assay using human ^{125}I-α_2M-methylamine as described above. The human α_2M, bovine α_2M, and rat α_1M carboxyl-terminal 20-kDa fragments bound to the murine macrophage α-macroglobulin receptor with a K_d of ~125 nM (Table I). This is comparable to the K_d of 200 nM previously reported for the binding of the human α_2M carboxyl-terminal fragment to the α-macroglobulin receptor on fibroblasts (Van Leuven et al., 1986b), hepatocytes (Sottrup-Jensen et al., 1986), and macrophages (Roche et al., 1988). Suprisingly, the carboxyl-terminal 20-kDa fragment of rat α_1I$_3$ bound with a K_d value of only 10 nM. Therefore, it is possible to obtain a carboxyl-terminal 20-kDa fragment from these three nonhuman α-macroglobulins which contains at least part of the α-ma-

Table I: Macrophage Binding of α-Macroglobulin–Proteinase Complexes and Carboxyl-Terminal Fragments of α-Macroglobulins[a]

	K_d (nM)		
	inhibitor–proteinase complex	20-kDa fragment	40-kDa fragment
human α_2M	0.5	125	
bovine α_2M	0.8	125	
rat α_1M	1.0	125	5[b]
rat α_1I_3	0.7	10	

[a] The carboxyl-terminal 20-kDa fragments and the 40-kDa subunit of rat α_1M were purified to apparent homogeneity as described under Materials and Methods. The ability of the various preparations to bind to the murine peritoneal macrophage receptor was studied in a displacement assay employing ^{125}I-labeled human α_2M–methylamine as previously described (Roche et al., 1988). [b] Rat α_1M monomers consist of two chains of 160 and 40 kDa held together by a disulfide bond (Gordon, 1976). The 40-kDa subunit was purified to apparent homogeneity as described under Materials and Methods. None of the other α-macroglobulins are processed to yield a monomer consisting of more than one polypeptide chain.

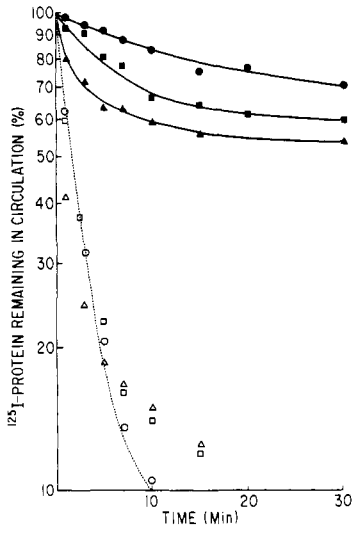

FIGURE 3: Clearance of ^{125}I-α-macroglobulins before and after *cis*-DDP treatment. Rat α_1M–trypsin (▲), rat α_1I_3–chymotrypsin (■), and bovine α_2M–trypsin (●) were treated with 1.7 mM *cis*-DDP and injected into the lateral tail vein of a mouse as described under Materials and Methods. The clearance rates of untreated rat α_1M–trypsin (△), rat α_1I_3–chymotrypsin (□), and bovine α_2M–trypsin (○) are also indicated and are essentially the same as that of human α_2M–trypsin (···).

croglobulin receptor recognition site.

Receptor Recognition of the Rat α_1M Carboxyl-Terminal 40-kDa Subunit. Rat α_1M is unique in that its polypeptide chain is processed intracellularly after synthesis, producing two chains of approximately 160 and 40 kDa which are held together by disulfide bonds (Gordon, 1976; Geiger et al., 1987). Efforts were made to obtain the carboxyl-terminal 40-kDa subunit by reduction, alkylation, and subsequent gel filtration. It was found that under nondenaturing conditions the carboxyl-terminal 40-kDa fragment remained associated with the 160-kDa subunit (data not shown). Therefore, the chains were separated and purified in guanidine hydrochloride. The resultant, presumably denatured, carboxyl-terminal 40-kDa subunit was dialyzed to remove the denaturant. This preparation, when studied in the receptor binding assay, demonstrated a very high affinity, $K_d \sim 5$ nM, not significantly different than the K_d of intact rat α_1M–proteinase complexes (Table I).

Effect of cis-DDP on Receptor Recognition. *cis*-DDP binds to human α_2M and greatly decreases its receptor recognition

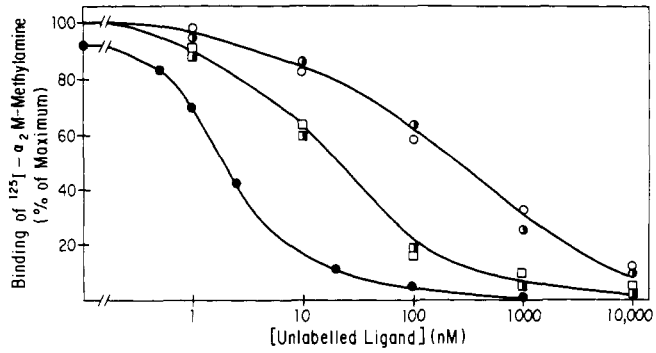

FIGURE 4: In vitro binding to macrophages of bovine α_2M and rat α_1I_3 carboxyl-terminal 20-kDa fragment before and after reaction with *cis*-DDP. The carboxyl-terminal 20-kDa fragments were obtained by papain treatment of the native proteins as described under Materials and Methods. A displacement assay was employed as described under Materials and Methods. The probe studied was ^{125}I-labeled human α_2M–methylamine. The ability of proteinase complexes of bovine α_2M (●) and rat α_1I_3 (data not shown) to prevent binding of ^{125}I-labeled human α_2M–methylamine is comparable to that of human α_2M (Roche et al., 1988). The ability of the bovine α_2M carboxyl-terminal 20-kDa fragment (○) to prevent binding of ^{125}I–labeled human α_2M–methylamine is compared to the effect of the same preparation treated with *cis*-DDP (◑). Similar studies are shown in which rat α_1I_3 carboxyl-terminal 20-kDa fragment is studied before (□) and after (◧) treatment with *cis*-DDP.

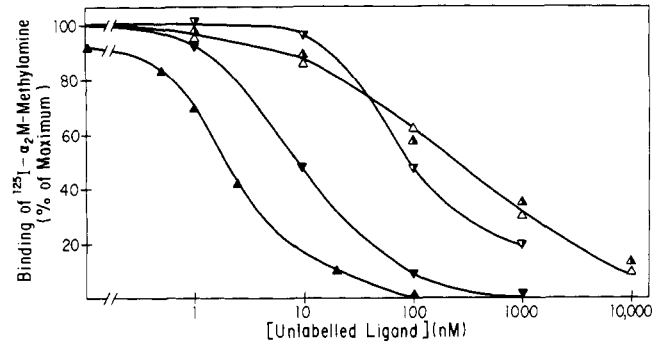

FIGURE 5: In vitro binding to macrophages of rat α_1M, rat α_1M carboxyl-terminal 40-kDa subunit, and carboxyl-terminal 20-kDa fragment. The ability of rat α_1M–proteinase complex (▲) to prevent binding of ^{125}I-labeled human α_2M is comparable to that of human α_2M–methylamine (Roche et al., 1988). The preparation of the carboxyl-terminal 40-kDa subunit (▼) is compared to the carboxyl-terminal 20-kDa proteolytic fragment (△). As can be seen, *cis*-DDP treatment altered the receptor binding activity of the carboxyl-terminal 40-kDa subunit (▼) but not the carboxyl-terminal 20-kDa fragment (△).

by the macrophage receptor (Gonias & Pizzo 1981; Pizzo et al., 1986). Rat α-macroglobulin– and bovine α_2M–proteinase complexes were treated with 1.7 mM *cis*-DDP as previously described for human α_2M and analyzed for receptor binding in the mouse clearance model. *cis*-DDP markedly decreased the rate of clearance of the intact α-macroglobulin–proteinase complexes (Figure 3). *cis*-DDP treatment of the carboxyl-terminal fragments, however, did not result in any detectable decrease in binding to the receptor as determined by in vitro binding assays (Figures 4 and 5). It is thus apparent that the effect of *cis*-DDP on the receptor binding is not related to the carboxyl-terminal 20-kDa fragment of α-macroglobulins, consistent with recent studies which indicate that a second domain contains the platinum-sensitive part of the α_2M receptor recognition site (Roche et al., 1988; Isaacs et al., 1988).

The high affinity of the rat α_1M carboxyl-terminal 40-kDa subunit suggested that it might contain the entire receptor recognition site, including the *cis*-DDP-sensitive component

(Pizzo et al., 1986; Isaacs et al., 1988; Roche et al., 1988). In order to test this hypothesis, the carboxyl-terminal 40-kDa subunit of rat α_1M was treated with *cis*-DDP and employed in an in vitro receptor competition assay (Figure 5). The affinity of the platinum-treated subunit was decreased as demonstrated by a change in the K_d from 5 to 50 nM.

Previous work in collaboration with the laboratory of Dr. Dudley Strickland produced a monoclonal antibody, 7H11D6, which binds specifically to the *cis*-DDP-sensitive component of the human α_2M receptor recognition site (Isaacs et al., 1988; Roche et al., 1988). Preliminary observations (Roche et al., 1988) indicated that a complex of several polypeptides obtained by papain digestion of α_2M of M_r ~55 000 (p55) bound to monoclonal antibody 7H11D6 with low affinity (I_{50} ~ 2000 nM) but that the carboxyl-terminal 20-kDa fragment bound extremely poorly ($I_{50} \gg$ 10 000 nM). To determine if the platinum-reactive epitope for monoclonal antibody 7H11D6 is present in p55, we studied the binding of human p55 to monoclonal antibody 7H11D6 before and after treatment with 1.7 mM *cis*-DDP. Platinum treatment did not alter the binding of p55 to monoclonal antibody 7H11D6 (I_{50} ~ 2000 nM). It is concluded that the binding of p55 to the monoclonal antibody 7H11D6 cannot involve the platinum-sensitive epitope of the human α_2M receptor recognition site. Since monoclonal antibody 7H11D6 does not bind to rat α_1M (data not shown), it was not possible to employ this antibody to probe receptor recognition of this protein.

Primary Structure of the Bovine Carboxyl-Terminal Fragment. Since sequence data were not available for bovine α_2M, the complete amino acid sequence of the bovine α_2M carboxyl-terminal 20-kDa fragment was determined by Edman degradation of peptides generated by trypsin, chymotrypsin, and *S. aureus* V8 proteinase. All tryptic peptides were sequenced through the carboxyl terminus, and the chymotrypsin and *S. aureus* V8 peptides were used to identify the order of the tryptic peptides. The sequence of the complete bovine carboxyl-terminal fragment and the location of the various peptides are shown in Figure 6. Asn-1401 in human α_2M is glycosylated, as is the corresponding Asn in the bovine carboxyl-terminal fragment. Another carbohydrate-containing Asn residue was detected in the bovine carboxyl terminus. His-1381 present in the human α_2M is deleted in the bovine sequence, thus creating a second potential glycosylation site at Asn-1380 (Figure 6). This Asn was glycosylated as determined by low recovery of PTH-Asn and the presence of glucosamine in the amino acid composition trace. Furthermore, this residue is located in the Asn-Xxx-Ser/Thr consensus sequence (Marhsall & Neuberger, 1964).

Conservation of the Disulfide Bridge in the α-Macroglobulin Receptor Binding Domain. The disulfide bridge in human α_2M Cys-1329–Cys-1444 is conserved in the bovine α_2M carboxyl-terminal fragment. This was confirmed by two independent techniques. First, a peptide was isolated from unreduced tryptic and *S. aureus* V8 proteinase digests and sequenced with the disulfide bond intact. Second, the tryptic peptide was reduced, alkylated with iodo[^{14}C]acetamide, and rechromatographed on HPLC. The two resulting peptides were subjected to sequence analysis, and the PTH derivatives were counted in a scintillation counter for ^{14}C incorporation.

To identify the isolated carboxyl-terminal fragment from rat α_1I_3 and rat α_1M, amino-terminal sequence analysis was performed. Amino-terminal sequence analysis of the rat α_1I_3 fragment did not release any detectable PTH derivative in cycle 16; however, after reduction and ^{14}C alkylation, a radioactive PTH derivative was recovered. The mRNA encoding

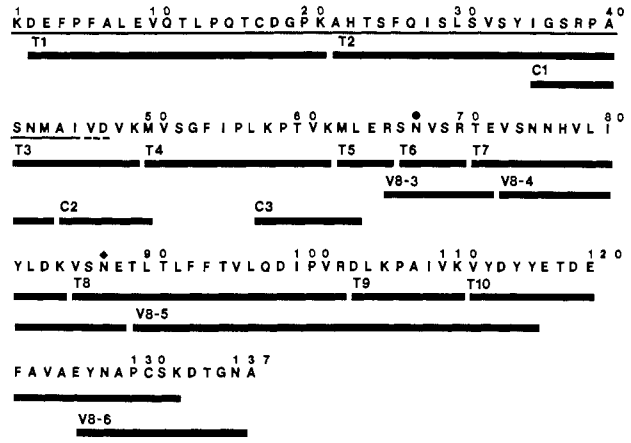

FIGURE 6: Amino acid sequence analysis of bovine α_2M carboxyl-terminal fragment. The sequence is given in one-letter code (IUPAC–IUB Commission on Biochemical Nomenclature, 1968). The prefix "T" corresponds to the peptides generated by trypsin and "C" by chymotrypsin, and "V8" denotes peptides generated by digestion of the carboxyl-terminal fragment with *S. aureus* V8 proteinase. The line shows the results of amino-terminal sequence analysis of undigested material. The bars indicate the various peptides used to establish the amino acid sequence. The position of the two glucosamine-based carbohydrate groups is indicated by the solid diamonds.

α_1I_3 contains only two codons for cysteine in this region of the molecule (Braciak et al., 1988; Aiello et al., 1988). Therefore, we conclude that the disulfide bridge is conserved in this protein, although it is possible that the cysteinyl residue in the carboxyl terminus of rat α_1I_3 forms a low molecular weight mixed disulfide with glutathione or cysteine as seen with human α_1-proteinase inhibitor (Jeppson et al., 1978). Amino-terminal sequence analysis of the rat α_1M carboxyl-terminal fragment resulted in the identification of 35 amino acids, and excessive (50 cycles) amino acid sequence analysis of reduced and ^{14}C-alkylated material did not release any radioactive amino acids. This indicates that the disulfide bridge is not conserved in rat α_1M, a finding which is supported by the lack of codons for cysteine in this region of the protein (Eggertsen and Fey, personal communication).

Discussion

Human α_2M, bovine α_2M, rat α_1M, and rat α_1I_3 are removed very slowly from the murine circulation ($t_{1/2} >$ 1 h). Following reaction with proteinase, however, these inhibitors undergo a conformational change which reveals a site recognized by cellular receptors [see Pizzo and Gonias (1984) for a review; Gliemann & Sottrup-Jensen, 1987]. Consequently, the inhibitor–proteinase complex is rapidly removed from circulation ($t_{1/2}$ ~ 4 min). The results of in vitro receptor binding studies presented previously and in the present work demonstrate that each α-macroglobulin–proteinase complex binds to the same receptor and that the binding affinity of each ligand for the mammalian α-macroglobulin receptor is essentially identical.

When treated with *cis*-DDP, receptor recognition of human α_2M is greatly decreased (Gonias & Pizzo, 1981;; Pizzo et al., 1986). No previous studies have examined the question of whether a *cis*-DDP-sensitive site is generally present in α-macroglobulins. The results of in vivo plasma elimination studies reported here clearly demonstrate that receptor recognition of the different α-macroglobulins was similarly affected following treatment with *cis*-DDP. These studies demonstrate that a *cis*-DDP-reactive component is present in the receptor recognition site of all α-macroglobulins studied.

One aim of the present study was to define the relationship of the *cis*-DDP-sensitive component of the receptor recognition

site and to compare the sequences of the carboxyl-terminal 20-kDa fragments from several nonhuman α-macroglobulins. The only published study, however, indicates that the *cis*-DDP-sensitive component of the receptor recognition site is not located in the carboxyl-terminal 20-kDa fragment (Roche et al., 1988). This region appears to contain the human α_2M receptor recognition site (Van Leuven et al., 1986a,b; Sottrup-Jensen et al., 1986; Roche et al., 1988). Since receptor binding of nonhuman α-macroglobulins is highly conserved (Feldman et al., 1984; Feldman & Pizzo, 1984, 1985), sequence studies of the carboxyl-terminal fragments, if obtainable from nonhuman species, should yield evidence for highly conserved regions. To investigate the receptor binding ability of the different α-macroglobulins, fragments responsible for at least part of the receptor binding ability were isolated. Digestion of human α_2M with papain at pH 4.5 resulted in cleavage and release of a carboxyl-terminal 20-kDa fragment with receptor binding ability ($K_d \sim 200$ nM) (Van Leuven et al., 1986a,b; Sottrup-Jensen et al., 1986; Roche et al., 1988). In the current study, the same approach was used for the nonhuman α-macroglobulins, and cleavage occurred within three amino acids in all four proteins studied. These data suggest that this region of the molecule is both highly exposed during the cleavage reaction and most likely has a very similar conformation. The purified carboxyl-terminal 20-kDa fragments of bovine α_2M and rat α_1M bound to the α-macroglobulin receptor with the same affinity as the human α_2M carboxyl-terminal 20-kDa fragment ($K_d \sim 125$ nM), while the carboxyl-terminal 20-kDa fragment of rat α_1I_3 bound with a K_d of 10 nM. We can only speculate as to why this fragment binds with a 12.5-fold increase in affinity when compared to other α-macroglobulins, and other studies will be needed to clarify this behavior.

The amino acid sequences of the human α_2M (Sottrup-Jensen et al., 1984), rat α_2M (Gehring et al., 1987), rat α_1I_3 (Schweizer et al., 1987; Braciak et al., 1988; Aiello et al., 1988), and rat α_1M (Eggersten and Fey, personal communication) have been established. The amino acid sequence of bovine α_2M is not known; however, the complete amino acid sequence of the bovine α_2M carboxyl-terminal 20-kDa receptor binding fragment has been deduced and is included in this paper. The α-macroglobulins studied show extensive sequence identity in the carboxyl-terminal region. The amino acid sequence identity between the human α_2M and bovine α_2M carboxyl-terminal 20-kDa domains was greater than 90%, and the overall sequence identity between all of the carboxyl-terminal 20-kDa domains studied was 75%. High-stringency diagonal plots of the various carboxyl-terminal fragments were performed by using a log-odds score of 110, a window of 10 amino acids with 9 matches, and a κ-tuple of 1 (Wilbur & Lipman, 1983). By this analysis, the various carboxyl-terminal fragments revealed two segments of extraordinarily high identity corresponding to residues 1359–1376 and 1424–1435 in the human amino acid sequence (Figure 2), suggesting that the primary receptor recognition domain is located in one or both of these regions.

The Cys-1329–Cys-1444 disulfide bridge in human α_2M was conserved in bovine α_2M and rat α_1I_3 but was not conserved in rat α_1M. It has been proposed that the interchain disulfide bond is required for binding of the human α_2M carboxyl-terminal 20-kDa fragment to the receptor (Sottrup-Jensen et al., 1986). Presumably, in the human fragment, this bond is necessary to maintain the conformation around the primary receptor recognition site. It is likely that the conformation of the fragment obtained from rat α_1M is stabilized in a similar configuration by noncovalent interactions. The absence of disulfides in rat α_1M is somewhat surprising since the integrity of this link in human α_2M is thought to be essential for receptor binding (Sottrup-Jensen et al., 1986). However, it is possible that the two phenylalanine residues substituting for the cysteines may stack, thus stabilizing a similar conformation.

With rat α_1M, it is possible to obtain a larger carboxyl-terminal subunit of $M_r \sim 40000$. The disulfide bridge between the 160-kDa and the 40-kDa subunits was readily reduced under nondenaturing conditions as determined by SDS–PAGE, but the subunit remained associated with the inhibitor even after pretreatment with methylamine or proteinase. However, the subunit was isolated under denaturing conditions, and the renaturated domain bound to the α-macroglobulin receptor with a K_d of 5 nM. This represents a 25-fold increase in affinity relative to the carboxyl-terminal 20-kDa fragment of the parent protein. This result suggests that although the carboxyl-terminal 20-kDa fragment of rat α_1M does bind to the α-macroglobulin receptor, it does not contain all the receptor binding information located in the carboxyl-terminal region of the protein.

In order to pursue this question, the carboxyl-terminal 40-kDa subunit of rat α_1M was treated with *cis*-DDP. The affinity of the platinum-treated protein decreased to 50 nM, representing a 10-fold decrease in binding affinity of the subunit for the receptor. Thus, the affinity of the *cis*-DDP-treated carboxyl-terminal 40-kDa subunit is very similar to that of the carboxyl-terminal 20-kDa fragment obtained by papain treatment of human α_2M, bovine α_2M, and rat α_1M. Moreover, treatment of intact human α_2M–methylamine with *cis*-DDP reduces the K_d for receptor binding from 0.5 nM to approximately 100 nM (Roche et al., 1988). When these studies are taken together, they suggest that the carboxyl-terminal 40-kDa region of the α-macroglobulin studied contains the entire receptor recognition site. The *cis*-DDP-sensitive component must lie toward the amino terminus of this region of the subunit, while the remainder of the receptor recognition site is located further toward the carboxyl-terminal end of the subunit in the region designated as the 20-kDa fragment (Sottrup-Jensen et al., 1986; Van Leuven et al., 1986a,b; Roche et al., 1988).

Preliminary studies with monoclonal antibody 7H11D6 suggested that it bound to an α_2M proteolytic fragment, $M_r \sim 55000$, which consists of a pair of noncovalently associated polypeptides. None of the chains of so-called p55 are part of the carboxyl-terminal 20-kDa fragment of α_2M (Roche et al., 1988). Monoclonal antibody 7H11D6 shows some binding activity toward p55 (Roche et al., 1988). However, the affinity was very poor ($I_{50} \sim 2000$ nM). From these initial studies, it was felt that p55 might contain the *cis*-DDP-sensitive epitope of human α_2M. However, in the present work, we show that *cis*-DDP-treated p55 has the same affinity ($I_{50} \sim 2000$ nM) as the non-platinum-derivatized fragment. Since *cis*-DDP treatment of intact human α_2M–methylamine abolishes antibody binding, it must be concluded from these studies that binding of p55 to monoclonal antibody 7H11D6 cannot involve the platinum-sensitive epitope. It is possible that binding of the antibody to p55 occurs because p55 contains a sequence which is partially homologous to the actual epitope.

In summary, the results of the present and past work suggest that the α-macroglobulin receptor recognition site is completely contained within a region located approximately 250 amino acid residues upstream from the carboxyl terminus of each α-macroglobulin subunit. Treatment of α-macroglobulins with

papain separates the platinum-sensitive epitope of the recognition site from the remainder of the site located in the carboxyl-terminal 20-kDa region of the molecule.

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Registry No. *cis*-DDP, 15663-27-1; α_1-proteinase inhibitor 3, 88943-21-9.

REFERENCES

Adams, D. O. (1979) *Methods Enzymol. 58*, 494–506.
Aiello, L. P., Shia, M. A., Robinson, G. S., Pilch, P. F., & Farmer, S. R. (1988) *J. Biol. Chem. 263*, 4013–4022.
Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–724.
Bjork, I., & Jornvall, H. (1986) *FEBS Lett. 205*, 87–91.
Braciak, T. A., Northemann, W., Hudson, G. O., Shiels, B. R., Gehring, M. R., & Fey, G. (1988) *J. Biol. Chem. 263*, 2999–4012.
Bury, A. F. (1981) *J. Chromatogr. 213*, 491–500.
Dangott, L. J., & Cummingham, L. W. (1982) *Biochem. Biophys. Res. Commun. 107*, 1243–1251.
David, D. S., & Riesfeld, R. A. (1974) *Biochemistry 13*, 1014–1021.
Delain, E., Barray, M., Tapon-Bretaudiere, J., Pochon, F., Marynen, P., Cassiman, J.-J., Van den Berghe, H., & Van Leuven, F. (1988) *J. Biol. Chem. 263*, 2981–2989.
Esnard, F., & Gauthier, F. (1980) *Biochim. Biophys. Acta 614*, 553–563.
Feldman, S. R., & Pizzo, S. V. (1984) *Arch. Biochem. Biophys. 235*, 267–275.
Feldman, S. R., & Pizzo, S. V. (1985) *Biochemistry 24*, 2569–2575.
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*, 5700–5702.
Gehring, M. R., Shiels, B. R., Northemann, W., Maarten, H. L., de Bruijn, H. L., Kan, C. C., Chain, A. C., Noonan, D. J., & Fey, G. H. (1987) *J. Biol. Chem. 262*, 446–454.
Geiger, T., Trans-Thi, T. A., Decker, K., & Heinrick, P. C. (1987) *J. Biol. Chem. 262*, 4975–4977.
Gliemann, J., & Sottrup-Jensen, L. (1987) *FEBS Lett. 221*, 55–60.
Gonias, S. L., & Pizzo, S. V. (1981) *Biochim. Biophys. Acta 678*, 268–274.
Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306–314.
Gordon, A. H. (1976) *Biochem. J. 159*, 643–650.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27–38.
Hirs, C. H. W. (1967a) *Methods Enzymol. 11*, 197–199.
Hirs, C. H. W. (1976b) *Methods Enzymol. 11*, 325–329.
Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134–8139.
Isaacs, I. J., Steiner, J. P., Roche, P. A., Pizzo, S. V., & Strickland, D. K. (1988) *J. Biol. Chem. 263*, 6709–6714.
Kaplan, J., & Nielsen, M. L. (1979a) *J. Biol. Chem. 254*, 7323–7328.
Kaplan, J. & Nielsen, M. L. (1979b) *J. Biol. Chem. 254*, 7329–7335.
Kurecki, T., Kress, L., & Laskowski, M. (1979) *Anal. Biochem. 99*, 415–420.
Marshall, R. D., & Neuberger, A. (1964) *Biochemistry 3*, 1596–1600.
Meltzer, N. M., Tous, G. I., & Stein, S. (1987) *Anal. Biochem. 160*, 356–361.
Pizzo, S. V., & Gonias, S. L. (1984) in *The Receptors* (Conn, P. C., Ed.) Vol. 1, pp 178–212, Academic Press, New York.
Pizzo, S. V., Roche, P. A., Feldman, S. R., & Gonias, S. L. (1986) *Biochem. J. 238*, 217–225.
Roberts, R. C. (1986) in *Reviews in Hematology*, Vol. II, pp 129–224, PJD Publications Ltd., Westbury, NY.
Roche, P. A., Strickland, D. K., Enghild, J. J., & Pizzo, S. V. (1988) *J. Biol. Chem. 263*, 6715–6721.
Schweizer, M. Takabayashi, K., Geiger, T., Laux, T., Biermann, G., Buhler, G., Gauthier, F., Roberts, L. M., & Heinrich, P. C. (1987) *Eur. J. Biochem. 164*, 375–381.
Sottrup-Jensen, L. (1987) in *The Plasma Proteins* (Putnam, F. W., Ed.) Vol. V, pp 191–291, Academic Press, New York.
Sottrup-Jensen, L., Stepanic, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lønblad, P. B., Magnusson, S., & Petersen, T. E. (1984) *J. Biol. Chem. 259*, 8318–8327.
Sottrup-Jensen, L., Gliemann, J., & Van Leuven, F. (1986) *FEBS Lett. 205*, 20–24.
Swenson, R. P., & Howard, J. B. (1979) *J. Biol. Chem. 254*, 4452–4456.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.
Van Leuven, F., Marynen, P., Cassiman, J.-J., & Van den Berghe, H. (1986a) *J. Biol. Chem. 261*, 6933–6937.
Van Leuven, F., Marynen, P., Sottrup-Jensen, L., Cassiman, J.-J., & Van den Berghe, H. (1986b) *J. Biol. Chem. 261*, 11369–11373.
Wilbur, W. J., & Lipman, D. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 726–730.