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Hepatocyte Receptors for Antithrombin III-Proteinase Complexes

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The in vivo clearance of antithrombin III-proteinase complexes occurs via a specific and saturable pathway located on hepatocytes. We now report studies of the catabolism of antithrombin III-proteinase complexes in vitro using rat hepatocytes in primary culture. Antithrombin III-thrombin and trypsin complexes were prepared and purified to homogeneity. Ligand uptake by hepatocytes was concentration, temperature, and time dependent. Initial rate studies were performed to characterize the maximum rate of uptake, V, and apparent Michaelis constant K_{app}. These studies yielded a V of 12.8 fmol/mg cell protein/min and a K_{app} of 144 nM for antithrombin-trypsin complexes. Competition experiments with antithrombin III, antithrombin III-proteinase complexes, α2-macroglobulin-methylamine, asialoorosomucoid and the neoglycoproteins, fucosyl-bovine serum albumin (BSA), N-acetylglucosaminyl-BSA, and mannosyl-BSA indicated that only antithrombin III-proteinase complexes were recognized by the hepatocyte receptor. Uptake studies were performed at 37°C with ^{125}I-antithrombin III-trypsin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in conjunction with autoradiography. These studies demonstrate time-dependent uptake and degradation of the ligand to low molecular weight peptides. In addition, there was a time-dependent accumulation of a high molecular weight complex of ligand and a cellular protein. This complex disappeared when gels were performed under reducing conditions.

Key words: antithrombin III, thrombin, receptor-mediated endocytosis, protease regulation, hepatocyte receptors

The clearance from the mammalian circulation of a number of proteins is mediated by specific receptors. Receptor systems recognizing various glycoproteins, α2-macroglobulin-proteinase complexes, insulin, and hemoglobin-haptoglobin complexes have been extensively characterized both in vivo and in vitro using isolated cell [1–9]. We have recently shown that the in vivo clearance of antithrombin III-thrombin (ATIII-thrombin) complexes occurs via a specific and saturable pathway located on hepatocytes that recognizes the inhibitor-proteinase complex [10,11].

In the present report the catabolism of ATIII-proteinase complexes was studied in vitro using rat hepatocytes in primary culture. Cultured hepatocytes were chosen

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to allow repair of any membrane damage that may have occurred during isolation, since some receptors are sensitive to the collagenase treatment employed to isolate cells [6,9]. Our studies show that ATIII-trypsin complexes are catabolized by liver cells in a specific, saturable, and concentration- and temperature-dependent manner. In addition, we demonstrate the formation of a disulfide interchange product between ligand and a cellular protein.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), lactoperoxidase, and p-nitrophenyl guanidino benzoate were from Sigma. Collagenase and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin were from Worthington. This trypsin was 60% active by active titration [12]. $Na^{125}I$, carrier free, was obtained from New England Nuclear. Human ATIII, thrombin, α2-macroglobulin (α2M) and α2M-methylamine were prepared as previously described [3,10,11,13].Orosomucoid was a gift from the American National Red Cross and asialoorosomucoid was prepared by the method of Schmid et al [14]. The neoglycoproteins were prepared from bovine albumin (BSA) by the method of Stowell and Lee [15]. The characterization of these particular preparations has been reported [3]. Clearance studies were performed as previously described using an *in vivo* mouse system [3].

Methods

Preparation of ATIII-trypsin complex. The ATIII-trypsin complex was prepared by reacting 5 mg of ATIII with 6 mg of trypsin in a total volume of 4 ml, of 0.02 M HEPES, pH 8.0. The reactants were incubated at 25°C for 30 sec, and the reaction was terminated by lowering the pH to 5.5 with 0.1 M HCl. The mixture was immediately applied to a Sephacryl S-200 (Pharmacia) column (50 × 2.0 cm) equilibrated at 4°C with 0.02 M NaCl, 0.05 M sodium acetate, pH 5.5 and 1.5 ml fractions were collected at a flow rate of 9 ml/hr. The purity and integrity of the peak fractions were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% gels and a 0.1 M Tris, 0.1 M borate, 0.1% SDS running buffer, pH 8.6. The samples were boiled for 60 sec in sample buffer containing 20% glycerol, 0.05 M Tris, 0.05 borate, 0.5% SDS, and electrophoresed at 4 mA for 2 hr. The complexes were iodinated by the lactoperoxidase method of David and Reisfeld [16] and resolved from free ^{125}I on a Sephadex G25 column (15 × 0.5 cm) and were used for experiments within a 24 hr of labeling. The lead edge of the peak was pooled and the ^{125}I-ATIII-trypsin was examined by SDS-PAGE and autoradiography (as described below). By this technique, the ^{125}I-ATII-trypsin complex was homogeneous with no detectable free iodine or other contaminants noted on the gel.

Isolation and maintenance of hepatocytes. Male Fischer 344 rats (Charles River Breeding Laboratories) weighing between 120 and 250 g were used for preparation of hepatocytes. Isolation of parenchymal hepatocytes was performed using a two-step collagenase perfusion technique of Seglen [17] as modified by Jirtle et al [18]. The viability of isolated cells was 80–95%, as judged by trypan blue exclusion with greater than 95% of the preparation as single cells. The hepatoctyes were diluted to 1 million cells/ml in Leibovitz L-15 medium (Gibco) supplemented with 10% fetal calf serum, insulin, 10^{-7} M, and gentamicin, 50 μg/ml. Collagen coated 35 mm

diameter culture dishes (Falcon Plastics) were then plated with 1 ml of cell suspension and incubated at 37°C for 2 h [19]. At that time, the medium was changed omitting the fetal calf serum. The plated cells were incubated in this medium overnight and used for experiments.

Uptake and degradation experiments. The cells were equilibrated for 1 h with L-15 medium containing insulin, gentamicin, 0.02 M HEPES (pH 7.5), and 10 mg/ml BSA. The medium was aspirated, and 0.75 ml of medium containing radiolabeled ligand was added. The cells were incubated under various conditions for various periods of time. The reaction was terminated by aspirating the solution and washing the cells four times with 1 ml of cold Earle's balanced salt solution containing Ca^{+2}, Mg^{+2} (Gibco), and 0.02 M HEPES, pH 7.5. The cells were dissolved using a solution of 0.2 M NaOH and 2.5% SDS for 1 hr and counted in a gamma counter. Cell proteins were assayed using the Peterson assay [20] and radioactivity normalized to cell protein.

In some studies binding was performed with 15 nM ^{125}I-AT-trypsin at 37°C for time intervals between 0 and 7 hr. The supernatants were aspirated and the cells washed four times with large volumes of 50 mM HEPES, 150 mM NaCl, pH 7.4. The cells were solubilized in 2.5% SDS for 1 hr. Both supernatants and solubilized cell fractions were subjected to SDS-PAGE [21] in the presence and absence of β-mercaptoethanol.

In addition, supernatant culture medium taken from hepatocytes in culture for 24 hr was incubated with 15 nM ^{125}I-ATIII-trypsin for 7 hr at 37°C, and the samples were subjected to SDS-PAGE as described above. The gels were processed to prevent loss of peptides as follows: (1) 50% methanol, 10% acetic acid for 30 min; (2) 5% methanol, 7% acetic acid for 30 min; (3) 10% glutaraldehyde for 30 min; (4) four washes in water followed by staining with Coomassie brilliant blue in the usual manner. All gels were then subjected to autoradiography employing Kodak XAR-2 film (Cardinal X-ray, Winston-Salem, NC) with an image intensifying screen for various time periods at −70°C.

RESULTS

In vivo clearance of ATIII-trypsin complexes. ATIII-trypsin complexes, purified as described above, were greater than 95% pure (Fig. 1, inset). SDS-PAGE of this complex after incubation at 37°C for 7 hr showed neglible breakdown of the complex, in contrast to the results of Wong et al [2], who found that complex dissociated with a half-life of 15 min. The preformed complexes were labeled with a specific radioactivity of between 50 and 200 cpm/fmol and approximately 10^{-10} mol of complex was injected per mouse. The trypsin complex cleared rapidly from the circulation with a half-life of 5 min (Fig. 1), which is comparable to the 2-min half-life for purified ATIII-thrombin complex [10]. Simultaneous injection of labeled ATIII-trypsin with 1,000-fold molar excess of unlabeled complex resulted in a diminished rate of clearance, half-life of 11 min, indicating a saturable clearance process.

In vitro hepatocyte uptake of ATIII-trypsin complexes. Time-dependent uptake was studied by incubating cells with 1 to 15 nM concentrations of ATIII-trypsin over 7 hr (Fig. 2). Uptake of ligand was concentration dependent, and a steady state was achieved by 3 hr at the lower concentrations of ligand. Background binding to collagen-coated plates was less than 10%. ATIII-trypsin complexes, 5 nM, were incubated with

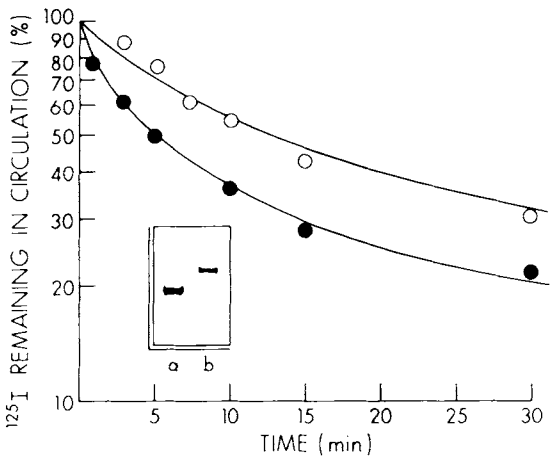

Fig. 1. Clearance of intravenously injected ATIII-trypsin and competition with unlabeled complexes. ^{125}I-labeled ATIII-trypsin complexes were injected into mice an blood samples were obtained at intervals. ^{125}I-ATIII trypsin, 10^{-10} mol (●); simultaneous injection of ^{125}I-ATIII-trypsin with 1,000-fold molar excess of unlabeled complex (○). Each point is the mean of three experiments. Inset is a 7.5% SDS-PAGE, which shows purified ATIII in gel a and purified ATIII-trypsin in gel b. Samples were processed as described in Materials and Methods.

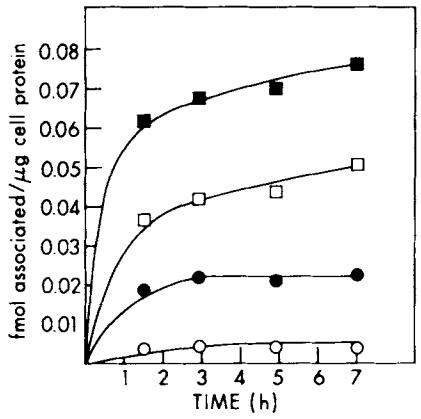

Fig. 2. Uptake of ^{125}ATIII-trypsin at various concentrations as a function of time. The concentrations employed were 1 nM (○), 5 nM (●), 10 nM (□), 15 nM (■). All points were performed in duplicate.

hepatocytes at 37°C, 25°C, and 4°C over a period of 6 hr (Fig. 3). The cell-associated radioactivity at 25°C and 37°C rose rapidly to a plateau, as seen in the concentration studies. At 4°C, the cell-associated radioactivity was significantly lower than at the other temperatures, suggesting the importance of temperature-dependent endocytosis.

Uptake of ATIII-trypsin and ATIII-thrombin in the presence of competitive doses of unlabeled ATIII-trypsin. The 2-hr uptake of 1 nM ATIII-trypsin was studied with increasing concentrations of unlabeled ligand (Fig. 4). The uptake showed a prominent concentration dependence with increasing doses of unlabeled

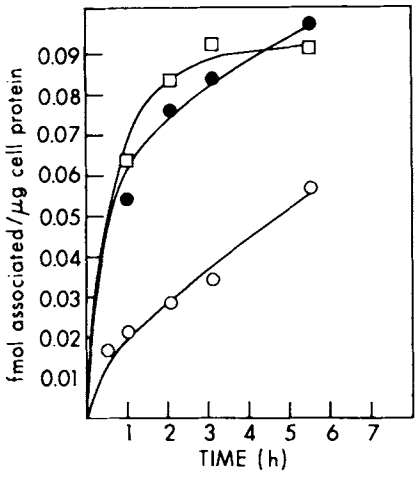

Fig. 3. Uptake of 5 nM ^{125}I-ATIII-trypsin at various temperatures. The temperatures studied were 4°C (○), 25°C (●), 37°C (□). All points were performed in duplicate.

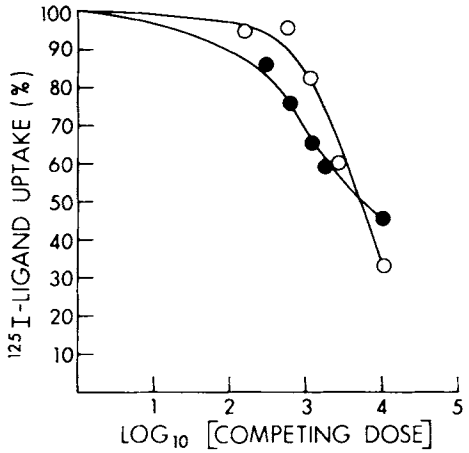

Fig. 4. Concentration-dependent competition of ^{125}I-ATIII-trypsin and ^{125}I-ATIII-thrombin uptake. ^{125}I-ATIII-trypsin, 1 nM, or ^{125}I-ATIII-thrombin were incubated for 2 hr with increasing doses (one-to 10,000-fold molar excess) of unlabeled ATIII-trypsin. The uptake in the absence of unlabeled ATIII-trypsin was taken as 100%. The symbols employed are ^{125}I-ATIII-trypsin (●), ^{125}I-ATIII-thrombin (○).

ligand, and a characteristic S-shaped log dose-response curve. ATIII-thrombin uptake was studied in a similar manner. Unlabeled ATIII-trypsin also inhibited the uptake of the thrombin complex. This cross-competition indicates that both complexes are metabolized by the same saturable process.

Initial rate studies of the uptake of ATIII-trypsin complexes. Initial rate studies were performed to characterize the maximum rate of uptake and the apparent Michaelis constant. Preliminary studies showed that the initial rates were constant over the first 45 min. A concentration velocity curve is shown in Figure 5. This curve

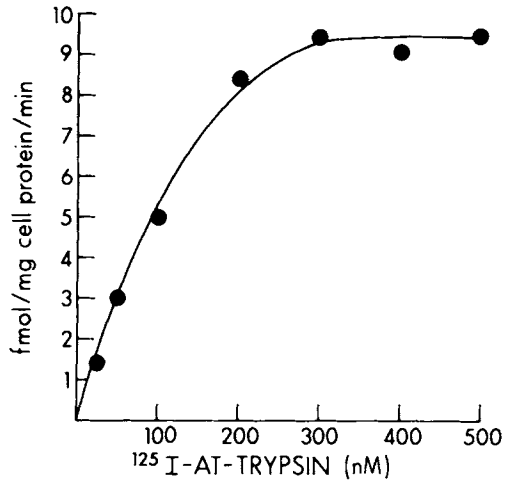

Fig. 5. Initial velocity of uptake of ATIII-trypsin. Increasing concentrations of ATIII-trypsin were incubated with hepatocytes for 30 min, and the cells were processed for counting. All points were done in duplicate. Nonspecific uptake with 1,000-fold molar excess of unlabled complex was subtracted.

has the expected hyperbolic shape of a receptor-mediated saturable process. The V and K_{app} were fit to the equation $v = VS/K_{app} + S$ using the Gauss-Newton method for nonlinear regression [23]. The fitted values were V = 12.8 fmol/mg/min and K_{app} = 144 nM. Nonspecific uptake using 1,000-fold molar excess of unlabeled complex was substracted.

Initial uptake in the presence of other ligands with known specificity. Initial velocity experiments were performed with 1,000-fold molar excess of ATIII, ATIII-trypsin, α2M-methylamine, asialoorosomucoid, mannosyl-BSA, N-acetylglucosaminyl-BSA, and fucosyl-BSA. The results are shown in Table I. Large molar excesses of these ligands had a modest effect on the uptake as compared to unlabeled ATIII-trypsin, which generally inhibits 50–75% of the uptake, the uninhibited ligand being viewed as nonspecific uptake. The studies show that the uptake of ATIII-trypsin is not mediated by the receptors for α2M or glycoproteins.

SDS-PAGE and autoradiographic analysis of uptake and degration of ^{125}I-ATIII-trypsin complexes by hepatocytes. Figure 6 is an autoradiograph derived from an SDS-PAGE of solubilized hepatocytes incubated with 15 nM ^{125}I-ATIII-trypsin for up to 7 hr at 37°C. This study demonstrates time-dependent accumulation of radiolabel near the dye front. In addition, there is a time-dependent accumulation of a high molecular weight complex near the top of the gel. This band plateaus after 90 min of incubation (Fig. 6A). When the same samples were electrophoresed under reducing conditions, the high molecular weight component is greatly diminished and there is an increased amount of radiolabel near the dye front as well as intact ATIII-trypsin complex (Fig. 6B). There are several possible explanations for the presence of radiolabel at the dye front. This label could represent free ^{125}I or 125I-labeled peptides. The ^{125}I-ATIII-trypsin preparation when electrophoresed at a concentration at least 100-fold higher than ligand in any experimental lane demonstrated no evidence of label at the dye front. It is concluded that the label at the dye

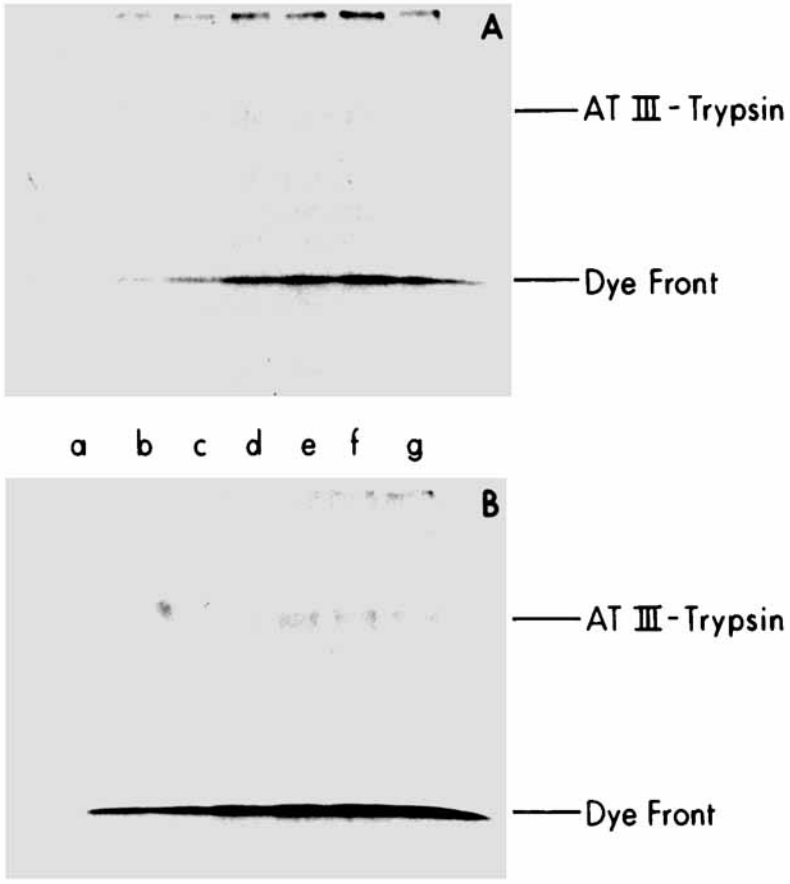

Fig. 6. SDS-PAGE and autoradiography of cell-associated ligand. A) SDS-PAGE under nonreducing conditions at 0(a), 30(b), 60(c), 90(d), 180(e), 300 (f), and 420 min (g). B) SDS-PAGE under reducing conditions at the same time points. The position of the ATIII-trypsin complex was determined by electrophoresis of ^{125}I-ATIII-trypsin complexes. Identical sample loads were applied to each gel lane.

TABLE I. Inhibition of ATIII-Trypsin Uptake by Various Ligands*

Ligand	% of initial uptake ± SD
α2M-methylamine	73 ± 13
Asialoorosomucoid	98 ± 15
Fucosyl-BSA	79 ± 8
Mannosyl-BSA	89 ± 9
N-acetyl glucosaminyl-BSA	94 ± 17
ATIII	85 ± 14
ATIII-trypsin	45 ± 5

*Radiolabeled ATIII-trypsin, 1 nM, was incubated for 30 min with 1,000-fold molar excess of the above ligands. The cells were then processed as described in Materials and Methods.

front resulted from the action of the hepatocytes on the ^{125}I-ATIII-trypsin complex. This label could represent free ^{125}I arising from deiodination of the ligand or ^{125}I-labeled peptides arising from degradation of the ligand after internalization. The latter hypothesis is strongly supported by several lines of evidence. If this label were free ^{125}I, the intensity of the label would be equivalent on reduced and nonreduced samples electrophoresed at the same protein load. However, this is not the case, as clearly demonstrated by the data presented in Figure 6A, B. Moreover, the intensity of the band at the dye front was greatly reduced when the gels were not fixed in glutaraldehyde prior to processing (data now shown). Free ^{125}I would diffuse out of the gels equally well in fixed or nonfixed gels in contrast to peptides. It is concluded that this material represents radiolabeled peptides derived from degradation of internalized ligand.

As a further control, supernatants from the cell-binding study were examined as described above, and there was no evidence of ligand degradation. Also, when 15 nM of ^{125}I-ATIII-trypsin was incubated with conditioned medium, obtained from 24-hr hepatocyte cultures, there was no evidence of ligand degradation (data not shown). These studies indicate that the peptides found within the hepatocytes (Fig. 6) resulted from degradation of the ^{125}I-ATIII-trypsin complexes after binding and endocytosis.

DISCUSSION

Antithrombin III is an effective inhibitor of trypsin, and virtually all coagulation serine proteinases except factor VII [22,24,25].The removal from the circulation of ATIII-thrombin complexes is accomplished by a recently described receptor-mediated process located on hepatocytes [10,11]. Previous studies from this laboratory have demonstrated that proteinase inhibitor-proteinase complexes clear in vivo and bind to cells in vitro independently of the proteinase [3,10,11,26–28]. Receptor recognition apparently depends on conformational changes induced in the inhibitor by proteinase binding [29–32]. Such conformational change presumably expresses or properly orients receptor recognition sites present on the inhibitor. In order to extend these observations to another proteinase that is readily available, ATIII-trypsin complexes were prepared and purified. Trypsin complexes were stable over the time course of the experiments. They cleared rapidly from the circulation with clearance inhibited by a molar excess of unlabeled complexes, confirming the receptor-mediated clearance of ATIII-trypsin complexes and validating their use as an in vitro probe.

In vitro studies were performed using rat hepatocytes in primary culture. Primary cultured hepatocytes were chosen to allow time for the membrane and metabolic lesions induced by the isolation procedure to be repaired. It is well known that the insulin receptor and hemoglobin-haptoglobin receptors are injured by the isolation procedure [6,9]. Primary cultured hepatocytes have a reasonably intact metabolism and do not leak enzymes, with the exception of lactic acid dehydrogenase [33,34].

The studies described in this manuscript demonstrate that cultured hepatocytes specifically bind and degrade ATIII-trypsin in a saturable and temperature- and time-dependent manner. Uptake of the ATIII-thrombin complexes also was concentration dependent (data not shown) and cross-competed by the ATIII-trypsin complex. The uptake of AT-proteinase complexes was minimally effected by large doses of unrelated ligands, confirming the uniqueness of this pathway, first proposed as a result of in vivo studies [10].

Our data are in conflict with those of Bauer et al [35], who reported that ATIII-trypsin was not bound, internalized or degraded to a significant degree. Their findings could result from using freshly isolated hepatocytes whose receptors had been damaged in the isolation procedure. The results also could result from the labeling procedure, chloramine T, which is known to severely damage some proteins and change their capacity for binding [36,37]. Finally, the purity of their ligand was not explicitly documented by electrophoresis and might have contained partly degraded complex or unreacted material.

SDS-PAGE and autoradiography demonstrated degradation of the ^{125}I-ATIII-trypsin by the hepatocytes. In addition, this study provides direct evidence for the formation of a disulfide interchange product between the ligand and a cellular protein. In this regard, a similar observation has been made with insulin which undergoes disulfide interchange with the insulin receptor [38]. The identity of the cellular protein participating in disulfide interchange is unknown. This protein presumably is carried into the lysozomes with ligand, since it is associated in disulfide linkage with peptides derived from the ^{125}I-ATIII-trypsin complex.

Fuchs et al demonstrated that the trypsin complexes of the homologous inhibitor α_1-proteinase inhibitor are cleared and endocytosed by the same ATIII-proteinase receptor on hepatocytes [11]. While ATIII has no free cysteine, α_1-proteinase inhibitor contains a free cysteine residue that readily forms mixed disulfides [39]. It is intriguing to speculate on the potential role of this highly reactive cysteine residue in formation of similar disulfide interchange products during uptake of α_1-proteinase inhibitor-proteinase complexes. This question is under investigation.

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