Roche, P. A., Jensen, P. E. H., & Pizzo, S. V. (1988) *Biochemistry 27*, 759-764.
Roche, P. A., Moncino, M. D., & Pizzo, S. V. (1989) *Biochemistry 28*, 7629-7636.
Salvesen, G. S., & Barrett, A. J. (1980) *Biochem. J. 187*, 695-701.
Salvesen, G., & Nagase, H. (1989) in *Proteolytic Enzymes: A Practical Approach* (Beynon, R., & Bond, J., Eds.) pp 83-104, IRL Press Ltd., Oxford.
Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453-461.
Sottrup-Jensen, L. (1987) in *The Plasma Proteins* (Putnam, F. W., Ed.) 2nd ed., Vol. 5, pp 191-291, Academic Press, Orlando, FL.
Sottrup-Jensen, L., Peterson, T. E., & Magnusson, S. J. (1980) *FEBS Lett. 121*, 275-279.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 128*, 123-126.
Sottrup-Jensen, L., Stepanik, T. M., Wierzbicki, D. M., Jones, C. M., Londblad, P. B., Kristensen, T., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1983) *Ann. N.Y. Acad. Sci. 421*, 41-60.
Swenson, R. P., & Howard, J. B. (1979) *J. Biol. Chem. 254*, 4452-4456.
Travis, J., & Salvesen, G. S. (1982) *Annu. Rev. Biochem. 52*, 655-709.
Van Leuven, F., Cassiman, J.-J., & Van den Berghe, H. (1981) *J. Biol. Chem. 256*, 9023-9027.

Mechanism of Insulin Incorporation into α_2-Macroglobulin: Implications for the Study of Peptide and Growth Factor Binding†

Charleen T. Chu, David S. Rubenstein, Jan J. Enghild, and Salvatore V. Pizzo*
Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710
Received July 9, 1990; Revised Manuscript Received October 4, 1990

ABSTRACT: In recent years, many studies have suggested a direct role for α_2-macroglobulin (α_2M), a plasma proteinase inhibitor, in growth factor regulation. When coincubated in the presence of either trypsin, pancreatic elastase, human neutrophil elastase, or plasmin, ^{125}I-insulin rapidly formed a complex with α_2M which was >80% covalent. The covalent binding was stable to reduction but abolished by competition with β-aminopropionitrile. Neither native α_2M nor α_2M pretreated with proteinase or methylamine incorporated ^{125}I-insulin. Experiments utilizing α_2M cross-linked with *cis*-dichlorodiammineplatinum(II) indicated that ^{125}I-insulin must be present during α_2M conformational change to covalently bind. A maximum stoichiometry of 4 mol of insulin bound per mole of α_2M and the short half-life of the α_2M intermediate capable of covalent incorporation were consistent with thiol ester involvement. Protein sequence analysis of unlabeled insulin–α_2M complexes, together with results of β-aminopropionitrile competition, confirmed that insulin incorporation occurs via the same γ-glutamyl amide linkage responsible for covalent proteinase and methylamine binding to α_2M. Although intact insulin apparently incorporated through its sole lysine residue on the B chain, we found that isolated A chain also bound covalently to α_2M. Phenyl isothiocyanate derivatization of the N-terminus had no effect on A-chain binding, supporting the possibility of heretofore unreported γ-glutamyl ester linkages to α_2M.

The last 5 years have witnessed an explosion in the number of reports implicating a role for α_2-macroglobulin (α_2M)[1] in the regulation of growth factors. Among numerous other studies, investigations on the binding of purified growth factors to α_2M include work on platelet-derived growth factor (Huang et al., 1984), transforming growth factor β (TGF-β) (O'-Connor-McCourt & Wakefield, 1987; Huang et al., 1988; Danielpour & Sporn, 1990; LaMarre et al., 1990), interleukin-1β (Borth & Luger, 1989), interleukin-6 (Matsuda et al., 1989), and basic fibroblast growth factor (Dennis et al., 1989). Taken as a whole, many of these reports present a number of apparent contradictions, due in part to the complexity of α_2M chemistry.

α_2M is a major plasma proteinase inhibitor that operates through a unique mechanism. Proteolytic cleavage in the "bait region" initiates an electrophoretically detectable conformational change in α_2M, which "traps" the proteinase (Barrett & Starkey, 1973; Salvesen & Barrett, 1980; Feldman et al., 1985). Through a somewhat different mechanism, treatment with small primary amines also results in structural compaction and faster migration through native gel systems. By convention, the native unreacted form is referred to as "slow" α_2M (s-α_2M) and the more compact proteinase- or methylamine-treated form as "fast" α_2M (f-α_2M) (Barrett et al., 1979). The proteinase-induced transition is characterized by cleavage of a reactive β-cysteinyl-γ-glutamyl thiol ester present on each of the four α_2M subunits, and by the appearance of receptor recognition sites that lead to rapid clearance of α_2M–proteinase complexes from the circulation (Ohlsson, 1971a,b; Imber & Pizzo, 1981). Methylamine treatment affects a similar conformational change through direct nucleophilic attack on the

† This work was supported by National Heart, Lung and Blood Institute Grant HL-24066.
* To whom correspondence should be addressed at the Departments of Pathology and Biochemistry, Box 3712, Duke University Medical Center, Durham, NC 27710.

[1] Abbreviations: α_2M, human α_2-macroglobulin; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); DCI, 3,4-dichloroisocoumarin; DDC, diethyldithiocarbamate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNE, human neutrophil elastase; PPE, porcine pancreatic elastase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBE, tris/boric acid/ethylenediaminetetraacetic acid buffer system; TGF-β, transforming growth factor β.

0006-2960/91/0430-1551$02.50/0

thiol ester (Swenson & Howard, 1979; Sottrup-Jensen et al., 1980; Salvesen et al., 1981), exposing the same receptor recognition sites (Imber & Pizzo, 1981). Decay of the thiol esters yields four titratable free thiols, which have been suggested as potential sites of thiol–disulfide interchange (Borth & Lugar, 1989). In addition to being trapped, proteinases containing lysine residues can bind covalently to the glutamyl component of the thiol ester. Covalent binding, however, is not necessary for efficient proteinase inhibition by human α_2M (Salvesen & Barrett, 1980). The biologic role of the evolutionarily conserved α_2M thiol ester is still unclear.

The capacity for α_2M to covalently incorporate proteinases exists in a short-lived state during the transition from slow to fast form. During this "nascent" (Sottrup-Jensen et al., 1981) or "activated" (Salvesen et al., 1981) state, other nonproteolytic amine-containing peptides can also incorporate into the thiol ester. The events following proteolytic activation of α_2M occur very rapidly, making it difficult to study them independent of one another. However, Roche et al. (1988) have utilized platinum cross-linking reagents to isolate a stable "primed" form approximating the nascent intermediate.

Most growth factors are small disulfide-linked multichain proteins. There have been several reports suggesting a role for thiol–disulfide interchange following binding to cell-surface receptors (Clark & Harrison, 1983; Fuchs et al., 1984; Tagaya et al., 1989). In addition, thiol–disulfide interchange may serve as a mechanism for covalent linkage to soluble binding proteins, as has been proposed for α_2M (Huang et al., 1984; Borth & Luger, 1989; Dennis et al., 1989). However, the involvement of multiple disulfide bonds in structural stabilization of both growth factors and α_2M complicates the interpretation of results obtained from global reduction.

In the present study, we examine and characterize the binding of insulin to α_2M. Although there were several early reports suggesting α_2M as a plasma binding protein for insulin [for example in Clausen et al. (1963)], few examined purified materials. Insulin has also been utilized to demonstrate the ability of nonproteolytic proteins to incorporate passively during proteolytic activation of α_2M (Sottrup-Jensen et al., 1981). Moreover, porcine insulin is well suited for probing the many possibilities for growth factor interaction with α_2M. It differs from human insulin by only a single residue. It is a disulfide-linked heterodimer consisting of one chain with a single lysine residue and one without. It possesses a limited number of well-characterized cysteine residues, and the entire protein is small enough to enter the "sprung trap" of f-α_2M to access potential binding sites within. Both intact porcine insulin and the isolated bovine A and B chains are readily available, allowing quantitative analysis. Utilizing insulin as a model, we propose to develop a framework upon which to study growth factor binding to α_2M, which will apply in general to other small proteins.

Materials and Methods

Materials. Human α_2M was purified by zinc chelate chromatography as previously described (Kurecki et al., 1979; Imber & Pizzo, 1981). Aliquots of α_2M were kept at −20 °C until shortly before use. α_1-Proteinase inhibitor was purified as previously described (Pannell et al., 1974). Porcine pancreatic elastase (PPE), bovine trypsin, porcine insulin, oxidized bovine insulin A and B chains, 3,4-dichloroisocoumarin (DCI), β-aminopropionitrile, blue hide powder, dithiothreitol, sequencer-grade phenyl isothiocyanate, and the substrate *N*-succinyl-AAA-*p*-nitroanilide (for PPE) were obtained from Sigma Chemical Co. (St. Louis, MO). Human neutrophil elastase (HNE) was a gift from Dr. Wieslaw Watorek, University of Georgia, Athens, GA. Human plasmin was from American Diagnostica Inc. (New York, NY). Methylamine, *cis*-dichlorodiammineplatinum(II) (*cis*-DDP), and diethyldithiocarbamate (DDC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The colorimetric substrates H-D-PFR-*p*-nitroanilide (for trypsin) and methoxysuccinyl-AAPV-*p*-nitroanilide (for HNE) were obtained from Helena Laboratories (Beaumont, TX). Protein sequencer-grade trifluoroacetic acid was from Applied Biosystems (Foster City, CA). Poly(vinylidene difluoride) membranes were from Millipore (Bedford, MA). Sephadex G-10 and Sephacryl S-300 HR were obtained from Pharmacia/LKB (Piscataway, NJ). New England Nuclear (Boston, MA) was the source of carrier-free $Na^{125}I$.

Standardization of Proteins. Purified α_2M was >95% native form (s-α_2M), as determined by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) titration of thiols released upon treatment with excess trypsin, buffered in 100 mM Tris-HCl/20 mM $CaCl_2$, pH 8.2 (Ellman, 1959). The value of $\epsilon_{412nm} = 14150\ M^{-1}\ cm^{-1}$ was used (Riddles et al., 1983). In every experiment, the amount of s-α_2M in the starting material and its conversion to f-α_2M by proteinase or methylamine treatment were monitored through nondenaturing 4–20% pore limit gel electrophoresis in a Tris/boric acid/ethylenediaminetetraacetic acid (TBE) buffered system (Manwell, 1977). The total concentration of α_2M was determined in a Shimadzu UV 160U spectrophotometer (Columbia, MD), using $A_{280nm}^{(1\%/1cm)} = 8.93$ (Hall & Roberts, 1978).

Trypsin was active-site-standardized with the direct titrant *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). Standardized trypsin was utilized to determine the active concentration of α_1-proteinase inhibitor, against which the elastases were active-site-titrated (Beatty et al., 1980). The molar ratio of each proteinase relative to α_2M that optimized bait region cleavage was determined by reducing SDS–PAGE, employing the blue hide powder assay to determine the degree of saturation (Barrett et al., 1979).

Insulin Radiolabeling. Porcine insulin and bovine insulin A and B chains were radiolabeled with an Iodobead (Bio-Rad, Richmond, CA), employing conditions recommended by the manufacturer, and desalted on a Sephadex G-10 column. The specific radioactivity of porcine insulin was 5.4–14.5 Ci/mmol, and that of the isolated bovine chains, 1.8–3.0 Ci/mmol. For experiments at physiological concentrations of insulin, the specific radioactivity of the ^{125}I-insulin was increased to 166–217 Ci/mmol. The concentrations were determined by using the following constants: $\epsilon_{280nm} = 5220\ M^{-1}\ cm^{-1}$ for insulin (Praissman & Rupley, 1968), $\epsilon_{276nm} = 3100\ M^{-1}\ cm^{-1}$ for B chain (Nakaya et al., 1967), and $A_{280nm}^{1\%/1cm} = 10.5$ determined from dry weight for A chain. All ^{125}I-labeled proteins were counted in an LKB-Wallac Clinigamma counter 1272 (Piscataway, NJ).

Derivatization of α_2M. Methylamine-derivatized f-α_2M was formed as previously described, with incubation for 10 h to assure completion of reaction (Gonias et al., 1982). Treatment with *cis*-DDP, which allows bait region cleavage to be evaluated separately from conformational change, was performed as previously described (Roche et al., 1988). In brief, a solution of 1.7 mM *cis*-DDP was added to an equal volume of 3 μM α_2M and incubated at 37 °C for 10 h. Unreacted methylamine or *cis*-DDP was dialyzed away, and the derivatized α_2M was evaluated on TBE pore limit gels. To investigate the binding potential of derivatized α_2M, *cis*-DDP–α_2M with PPE or trypsin followed by cross-link reversal either before or concurrent to incubation with ^{125}I-insulin. Reversal

of *cis*-DDP cross-linking was performed by addition of DDC to a final concentration of 10 mM followed by incubation at 37 °C for 10 h. The precipitated platinum salt was removed by microcentrifugation.

Insulin Incorporation into α_2M. s-α_2M or *cis*-DDP derivatives, at concentrations ranging from 0.7 to 3.7 μM, were incubated with a 4–5-fold molar excess of ^{125}I-labeled porcine insulin for 5–15 min at room temperature. To investigate the role of proteinase activity in insulin–α_2M interaction, trypsin, PPE, HNE, or plasmin was added at molar ratios of 1:1 and 2:1 with respect to α_2M. In some cases, α_2M was pretreated with proteinase, followed by inhibition of the proteinase before addition of ^{125}I-insulin. In other cases, active proteinase was present during incubation with ^{125}I-insulin. Inhibition of proteinases was achieved by addition of the active-site-directed serine proteinase inhibitor DCI to a final concentration of 50–100 μM (Harper et al., 1985; Salvesen & Nagase, 1989). Parallel experiments were performed in the presence of 100–150 mM β-aminopropionitrile, a nucleophile that competes for incorporation into the glutamyl residue of the thiol ester (Salvesen et al., 1981). The buffer used in all experiments unless otherwise indicated was 25 mM HEPES/150 mM NaCl, pH 7.4 (HEPES buffer).

Gel Filtration Chromatography. The ^{125}I-insulin–α_2M complex was separated from free ^{125}I-insulin and from free proteinase by gel filtration on a Sephacryl S-300 HR column (55 × 1.5 cm). Fractions of 1.5 mL were collected during isocratic elution with HEPES buffer at a flow rate of 50 mL/h. Protein concentration and radioactivity in each fraction were determined by measuring the absorbance at 280 nm and by γ counting, respectively. The protein peaks were concentrated by lyophilization and evaluated by SDS–PAGE and autoradiography as described below. The level of noncovalent binding was quantified by denaturing the α_2M-containing fractions in 1% SDS followed by centrifugal microfiltration in Centricon 30 microconcentrators (Amicon, Danvers, MA) to isolate the noncovalently bound insulin thus released. The filters were also counted to establish the amount of radioactivity remaining bound to α_2M.

Evaluation of Covalent Insulin Binding by PAGE. The covalent interaction between α_2M and ^{125}I-insulin was examined by PAGE. Samples generated as described above were electrophoresed on 5–15% gradient gels using the glycine/2-amino-2-methyl-1,3-propanediol/HCl buffer system (Bury, 1981). Samples were analyzed under reducing (50 μM dithiothreitol) and nonreducing conditions and on native TBE pore limit gels. All tubes were counted during incubation, before loading, and after loading to assure uniformity. Gels were dried and evaluated by autoradiography, using Kodak X-Omat AR film and Dupont Cronex intensifying screens. Autoradiograms were exposed for 6 h to 1 week.

Quantitative Studies. The stoichiometry of covalent insulin incorporation into α_2M was examined by coincubating molar ratios of ^{125}I-insulin with 1.3 μM α_2M and either HNE or PPE at 2.6 μM for 5 min, in the presence or absence of 150 mM β-aminopropionitrile. Diffusional loss of small peptides from gels can occur during staining (Swank & Munkres, 1971). With insulin, we observed a loss of 30–75% of counts loaded, especially from reduced samples. We therefore modified the procedure used in quantitative studies. Gels were electrophoresed with prestained molecular weight standards (Bio-Rad, Richmond, CA) and dried immediately. Radioactive bands localized by autoradiography were excised, and counted in a γ counter. This resulted in a 92–100% recovery of counts loaded.

Experiments at Physiologic Insulin Concentration. To determine whether covalent incorporation occurs under more physiologic conditions, two sets of experiments were done. One micromolar α_2M and 2 μM proteinase were coincubated with concentrations of ^{125}I-insulin ranging from 0.45 to 50 nM. In other cases, increasing molar ratios of proteinase relative to 1.4 μM α_2M were coincubated for 10 min in the presence of 0.5 nM insulin. SDS–PAGE and autoradiography were performed as described above.

Amino Acid Sequence Analysis. Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line 120A phenylthiohydantoin analysis. The instruments were operated as recommended by the manufacturer. The fragments obtained from 100-s incubations of α_2M, HNE, and insulin were separated by SDS–PAGE on 5% gels under reducing conditions. After electrotransfer to a poly(vinylidene difluoride) membrane (Matsudaira, 1987), the 97-kDa band was excised and placed on a polybrene-treated glass fiber filter for sequencing.

Half-Life Experiments. The decay of the proteinase-induced form of α_2M capable of covalently incorporating insulin was followed by addition of a 5-fold excess of ^{125}I-insulin at increasing intervals after treatment of 1 μM with 2 μM PPE. Samples were then incubated for 10 min before the reaction was terminated with 50 μM DCI. The amount of radioactivity incorporated was determined by γ counting, and normalized to the control value obtained from coincubating α_2M, ^{125}I-insulin, and PPE.

The appearance of free thiols after proteinase treatment, an indirect measure of thiol ester cleavage, was monitored by continuous spectrophotometric measurement using DTNB as a reactant (Ellman, 1959). Subsaturating, saturating, and supersaturating amounts of PPE or trypsin (~1.8-, 2-, and 10-fold) were added to 1 mg of α_2M in the presence of 77 μM DTNB in 1.3 mL of 25 mM HEPES, 150 mM NaCl, 1 mM *o*-phenanthroline, and 0.05% NP-40, pH 7.4.

Covalent Incorporation of Insulin A and B Chains. The radiolabeled bovine insulin A or B chains were evaluated for incorporation into α_2M, as described above for intact insulin. In addition, the I_{50}, or concentration of unlabeled competitor required to decrease the binding of ^{125}I-insulin to α_2M by 50% (Cheng & Prosoff, 1973), was determined by competition of labeled insulin against 10–1000-fold molar excesses of each unlabeled chain. The concentration of α_2M in these studies was 0.92 μM, of ^{125}I-insulin, 0.46 μM, and of PPE, 1.8 μM.

In some experiments, phenyl isothiocyanate was first used to block the N-terminus of ^{125}I-labeled A chain at pH 9.8, as previously described (Weiner et al., 1972; Jay, 1984). A portion of the blocked A chain was precipitated with acetone at −20 °C, washed extensively, and lyophilized prior to treatment with trifluoroacetic acid to cleave off the N-terminal amino acid. The insulin A chain was separated from unreacted phenyl isothiocyanate or trifluoroacetic acid, and equilibrated into 50 mM HEPES/150 mM NaCl, pH 7.4, using G-10 spin columns (Salvesen & Nagase, 1989). The purity of the original A-chain preparation was analyzed by C8 reverse-phase HPLC (Aquapore RP-300, Brownlee, Applied Biosystems, Foster City, CA) and protein sequencing. The phenyl isothiocyanate and trifluoroacetic acid treated A chain was sequenced to assess completeness of reaction. Examination of covalent incorporation into α_2M proceeded as described above.

Results

Coincubation of α_2M and ^{125}I-Insulin with Proteinase Necessary for Complex Formation. The conformational forms of α_2M (slow, "nascent", or fast) that were capable of binding

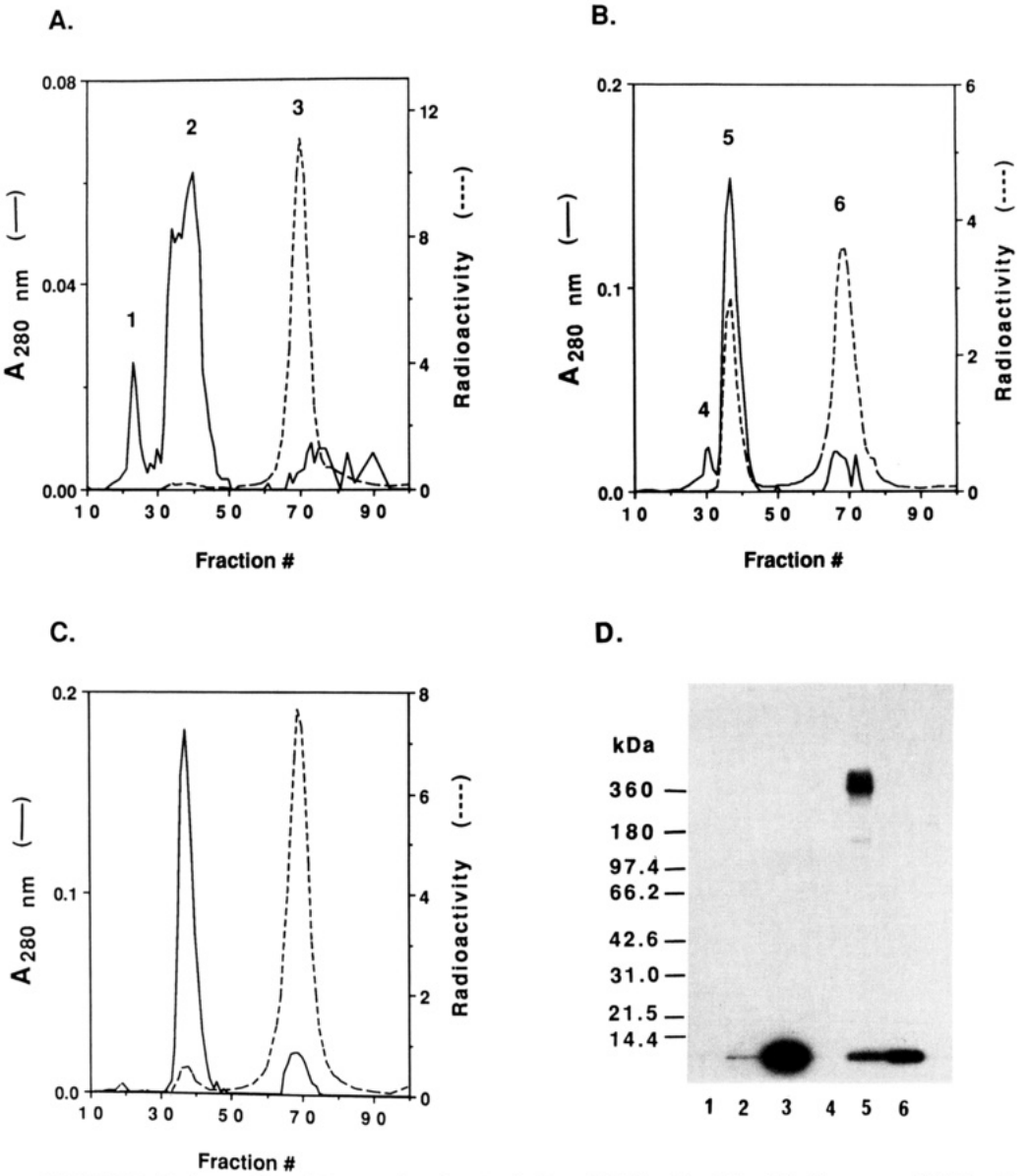

FIGURE 1: Sephacryl S-300 HR elution profiles demonstrating the association of ^{125}I-insulin with α_2M. An excess of ^{125}I-insulin was incubated with α_2M, as described under Materials and Methods, and applied to the column; 1.5-mL fractions were collected. The void volume of the column was 57 mL, and the total volume 105 mL. α_2M eluted in fractions 30–45 (45–68 mL) and free insulin in fractions 60–75 (90–112 mL). The concentration of protein and amount of radioactivity in each fraction were determined. Since preparations of ^{125}I-insulin with different specific activities were used, radioactivity was standardized as the percent of total microcuries loaded. The following temporal conditions were used: (A) incubation of α_2M with ^{125}I-insulin for 10 min; (B) coincubation of α_2M, ^{125}I-insulin, and either PPE or trypsin for 5–10 min, followed by inhibition with DCI; (C) treatment of α_2M with PPE for 10 min, followed by inhibition with DCI, before addition of ^{125}I-insulin for 10 min. Fractions at the indicated peaks were concentrated by lyophilization and analyzed with 5–15% SDS–ammediol gel electrophoresis and autoradiography (D). Molecular weight standards include purified α_2M (360 000, nonreduced; 180 000, reduced) and Bio-Rad low molecular weight standards: phosphorylase *b* (97 400), bovine serum albumin (66 200), ovalbumin (42 600), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

insulin were examined, by using Sephacryl S-300 HR gel filtration chromatography to separate insulin–α_2M complexes from free insulin. Elution profiles derived from incubations of ^{125}I-insulin with s-α_2M demonstrated a slight amount of radioactivity eluting with α_2M in fractions 30–45 (Figure 1A). The presence of proteinase in the incubation mixture resulted in a marked shift in radioactivity to α_2M-containing fractions (Figure 1B). Summation of the counts in fractions comprising the peaks demonstrated that only 1.95 ± 0.25% of the counts loaded bound to s-α_2M. Coincubation of α_2M and insulin with proteinase resulted in a >15-fold increase in binding to 32 ± 4.6%. Pretreating α_2M with proteinase before addition of insulin reduced the binding to 2.85 ± 0.25% (Figure 1C). Analysis of the indicated peaks by SDS–PAGE and autoradiography (Figure 1D) demonstrated that the interaction between s-α_2M and ^{125}I-insulin was nonconvalent (lane 2), with all the radioactivity migrating in the position of free insulin (lanes 3 and 6). Incubation of α_2M with ^{125}I-insulin and proteinase resulted in both covalent and noncovalent association, with most of the α_2M–^{125}I-insulin complex stable to denaturation and boiling (lane 5). Quantitation of the noncovalent contribution to binding through denaturation and ultrafiltration, as described under Materials and Methods, demonstrated that noncovalent interaction accounted for only 16–17% of bound insulin in samples displaying both covalent and noncovalent interaction.

Characterization of Covalent Insulin Incorporation into α_2M. The conditions required for covalent incorporation of ^{125}I-insulin were examined further by reducing and nonreducing SDS–PAGE and by native pore limit gel electropho-

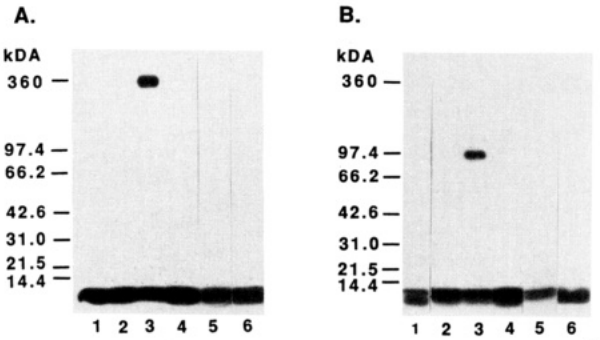

FIGURE 2: Autoradiograms demonstrating covalent binding of ^{125}I-insulin to α_2M. Composite photographs were formed from autoradiograms of (A) nonreduced and (B) reduced 5–15% polyacrylamide gels of samples prepared as described under Materials and Methods. The following conditions were employed during incubation: (1) ^{125}I-insulin alone; (2) s-α_2M + ^{125}I-insulin; (3) s-α_2M + ^{125}I-insulin + PPE (coincubation); (4) methylamine-treated f-α_2M + ^{125}I-insulin + PPE; (5) PPE-treated f-α_2M + ^{125}I-insulin; and (6) s-α_2M + ^{125}I-insulin + PPE (coincubation), in the presence of 150 mM β-aminopropionitrile. Molecular weight standards are as described in Figure 1.

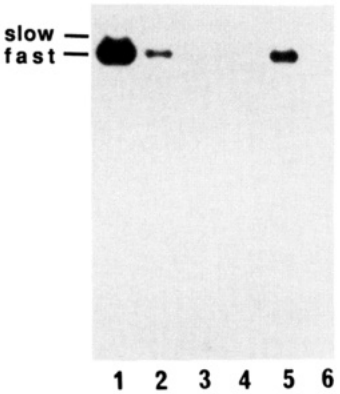

FIGURE 3: Autoradiogram of a native 4–20% TBE pore limit gel examining the relative abilities of native, *cis*-DDP-cross-linked, bait region cleaved, and conformationally fast forms of α_2M to incorporate ^{125}I-insulin. The following incubation conditions were employed: (1) coincubation of α_2M, ^{125}I-insulin, and PPE; (2) α_2M treated with *cis*-DDP and DDC prior to coincubation with ^{125}I-insulin and PPE; (3) *cis*-DDP-treated α_2M incubated with ^{125}I-insulin; (4) *cis*-DDP–α_2M coincubated with ^{125}I-insulin and PPE; (5) *cis*-DDP–α_2M treated first with PPE, followed by inhibition with DCI, before coincubation with ^{125}I-insulin and DDC; and (6) *cis*-DDP–α_2M, treated with PPE, followed by inhibition with DCI and reversal of cross-linking with DDC, before the final incubation with ^{125}I-insulin. The difference in band intensity reflects unequal amounts of material applied to the gel, secondary to α_2M loss during dialysis. Positions of migration for s-α_2M for f-α_2M (methylamine-treated) are indicated. See Materials and Methods for details.

resis. Following coincubation of α_2M, ^{125}I-insulin, and proteinase, radiolabeled insulin appeared in the 360-kDa band corresponding to the disulfide-linked α_2M half-molecule when electrophoresed under nonreducing conditions (Figure 2). Electrophoresis after reduction of the sample demonstrated that insulin specifically incorporated into the upper band of the characteristic Coomassie-stained doublet (~97 kDa on the autoradiogram) formed from bait region cleaved α_2M (Barrett et al., 1979; Salvesen & Barrett, 1980). α_2M-associated radioactivity always migrated as f-α_2M during pore limit electrophoresis (Figure 3). Use of either trypsin, PPE, HNE, or human plasmin, at 1:1 or 2:1 molar ratios relative to α_2M, all yielded comparable results, with the amount of insulin incorporation paralleling the amount of bait region cleavage (data not shown).

Neither s-α_2M nor preformed f-α_2M, derived from proteinase or methylamine treatment, associated appreciably with

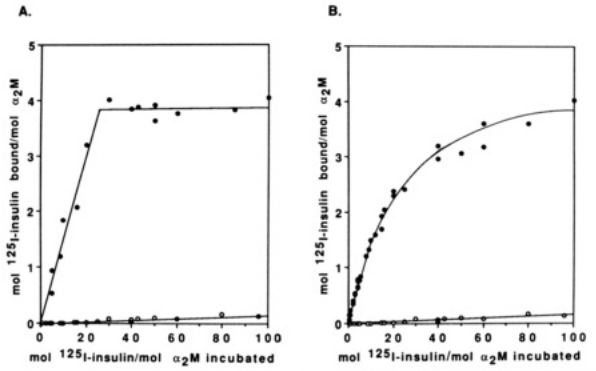

FIGURE 4: Stoichiometry of covalent ^{125}I-insulin binding to α_2M. Increasing molar ratios of ^{125}I-insulin to α_2M were incubated in the presence of (A) HNE and (B) PPE. Covalent binding was examined in the presence (○) and absence (●) of β-aminopropionitrile. ^{125}I-insulin bound to α_2M was separated from unbound ^{125}I-insulin by electrophoresis, and subjected to γ counting for quantitative determination as described under Materials and Methods.

^{125}I-insulin. With proteinase-derived f-α_2M, it was necessary to ensure complete inhibition of the proteinase before addition of insulin. In preliminary studies using preformed f-α_2M, we observed a small amount of insulin incorporation, especially with use of trypsin to generate f-α_2M. This made it difficult to differentiate a low level of binding to f-α_2M from binding to a residual population of s-α_2M still undergoing reaction with uninhibited proteinase. Thus, we investigated the conditions needed for complete proteinase inhibition, particularly since DCI is 13–50 times less efficient in inhibiting trypsin than the elastases (Harper et al., 1985). Following treatment of α_2M with PPE, the time allowed for DCI reaction with the proteinase before addition of ^{125}I-insulin and the final DCI concentration were systematically varied. These studies established that preformed f-α_2M did not incorporate insulin (data not shown). Thus, formation of α_2M–insulin complexes required the presence of insulin during activation of α_2M by proteinase.

Cross-linking experiments with *cis*-DDP were designed to isolate events relating to proteinase activity from subsequent thiol ester exposure and conformational change, α_2M that was treated with *cis*-DDP followed by DDC, returning it to the "native" conformation, was able to incorporate ^{125}I-insulin during coincubation with PPE (Figure 3, lane 2). *cis*-DDP–α_2M (analogous to s-α_2M) did not incorporate ^{125}I-insulin either in the presence or in the absence of PPE (lanes 3 and 4). However, bait region cleaved *cis*-DDP–α_2M, which is primed for conformational change, did incorporate ^{125}I-insulin present during reversal of cross-links (lane 5). If conformational change of the primed molecule was permitted before addition of ^{125}I-insulin (analogous to incubation with preformed f-α_2M), no incorporation was seen (lane 6).

Stoichiometric and Temporal Aspects of Insulin Binding to α_2M. A maximum stoichiometry of ~4 mol of insulin bound/mol of α_2M (or one molecule of insulin per α_2M subunit) was found after treatment with either HNE or PPE (Figure 4). However, roughly 4 times as much insulin was required to achieve maximum incorporation when using PPE, which associates covalently with α_2M itself (Salvesen & Barrett, 1980). The presence of β-aminopropionitrile effectively prevented covalent insulin binding, suggesting a similar mechanism to that of covalent proteinase incorporation.

A time course indicated that covalent association was complete within 2 min. No further increases in radioactive band intensity were seen after 90 min of incubation (data not shown). In addition, results from incubations involving pre-

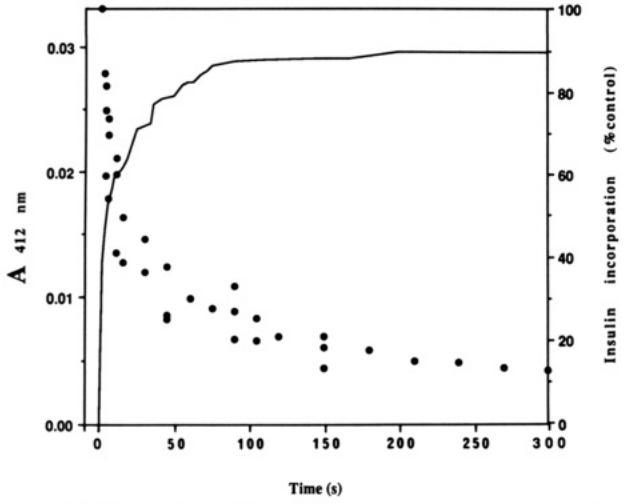

FIGURE 5: Comparison of free thiol appearance to the decay of the α_2M state capable of covalent binding. The time course of thiol appearance (solid line) after addition of a twofold molar excess of PPE to s-α_2M was monitored with continuous spectrophotometric measurement in the presence of DTNB. Time zero was defined by resumption of continuous monitoring after addition of proteinase, which occurred no more than 7 s after its interruption. The decay of a short-lived α_2M form capable of covalently incorporating ^{125}I-insulin was studied by adding ^{125}I-insulin, at rapid intervals, to incubations of α_2M and PPE. Amount of incorporation (filled circles) was expressed as percent control binding as described under Materials and Methods. Time zero was defined as the moment of PPE addition to α_2M.

formed f-α_2M suggested that a transient form of α_2M is needed for covalent incorporation of ^{125}I-insulin. Accordingly, the half-life of the form of α_2M capable of incorporating ^{125}I-insulin was investigated. Figure 5 shows that the insulin binding potential of proteinase-"activated" α_2M disappeared rapidly with time, in parallel to the proteinase-induced appearance of free thiol groups detectable by DTNB. The apparent half-life of this process was about 7 s, although some insulin was still covalently incorporated when added 5 min after treatment with proteinase. These results support an association between an intact thiol ester and the capability for incorporating insulin.

Binding of Insulin at Physiologic Concentrations. After characterization of the covalent incorporation of insulin to α_2M using supraphysiologic concentrations of ^{125}I-insulin, experiments were performed using a range of ^{125}I-insulin concentrations analogous to those found in human plasma. Covalent incorporation of ^{125}I-insulin into α_2M, when coincubated with PPE or HNE, was seen with initial insulin concentrations as low as 0.45 nM (Figure 6). Prolonged incubations or exposure times often resulted in the presence of radioactivity in minor bands of lower molecular weight than the 97-kDa doublet characteristic of bait region cleaved α_2M, as illustrated here. We believe these arose from further proteolysis of the 97-kDa fragment following the initial bait region cleavage, an interpretation supported by unpublished sequence analysis of fragments generated by limited proteolysis in our laboratory.

Binding to α_2M at nanomolar insulin concentrations occurred regardless of the proteinase used during coincubation. Binding at physiologic insulin concentrations also occurred regardless of the proteinase:α_2M ratio used and paralleled the amount of bait region cleavage achieved (Figure 7). Like that seen at higher insulin concentrations, insulin incorporation was abolished by competition with β-aminopropionitrile (results not shown).

Confirmation of the Binding Site through Sequence Analysis. Sequence analysis of the 97-kDa proteolytic fragment, derived from coincubation of α_2M with unlabeled insulin and HNE, yielded two sequences: the N-terminal sequence of a thiol ester containing fragment formed from α_2M bait region cleavage, and FXNQHLXGSHLVEA. This sequence

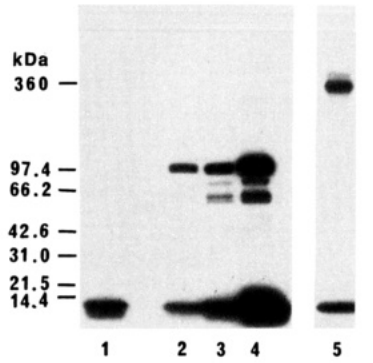

FIGURE 6: Covalent incorporation of ^{125}I-insulin to α_2M at physiologic insulin concentrations. This autoradiogram of a reduced polyacrylamide gel depicts incorporation of insulin into α_2M following coincubation with PPE. The concentrations of ^{125}I-insulin present in the coincubation were 0.45 nM (lane 2), 0.9 nM (lane 3), and 4.5 nM (lane 4). Also shown for comparison is an autoradiogram of a lane from a nonreducing gel depicting the results of a coincubation analogous to that of lane 2 (lane 5). Lane 1 depicts a control incubation of 0.45 nM ^{125}I-insulin alone.

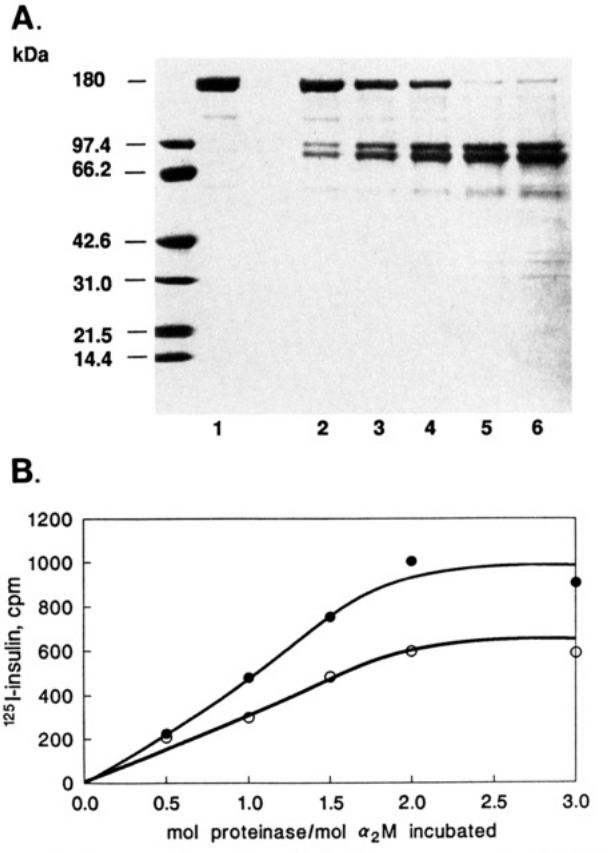

FIGURE 7: Incorporation of insulin during treatment of α_2M with varying molar ratios of proteinase. Physiologic concentrations of α_2M and ^{125}I-insulin were incubated with increasing molar ratios of PPE as described under Materials and Methods. The samples were analyzed by SDS–PAGE. (A) Coomassie blue staining of a reduced gel showing increasing amounts of α_2M bait region cleaved material at 97 kDa and decreasing amounts of intact subunits at 180 kDa. Ratios of PPE/α_2M used were 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 1.5 (lane 4), 2 (lane 5), and 3 (lane 6). Molecular weight standards shown on the left are as described in Figure 1. (B) The radioactivity contained in the α_2M bands after excision from reduced (O) and nonreduced (●) gels was plotted against the molar ratios of proteinase used to achieve bait region cleavage.

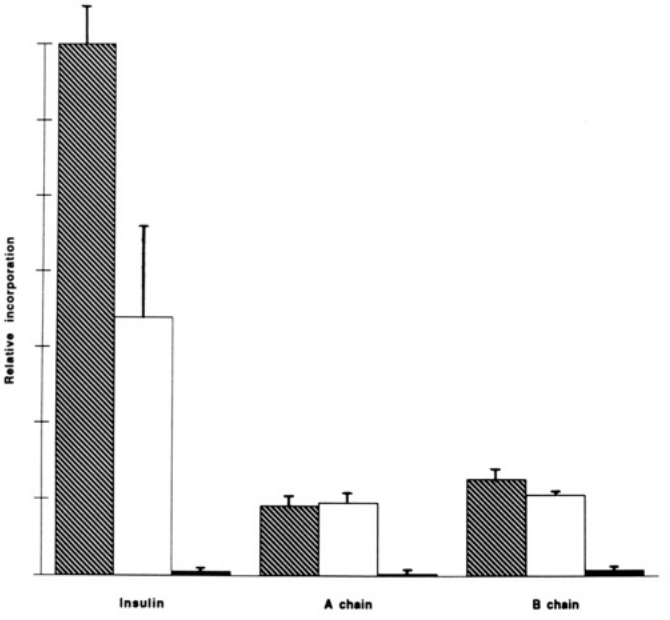

FIGURE 8: Bar graph depicting relative incorporation of native insulin, isolated A chain, and isolated B chain to α_2M. Three- to fivefold molar excesses of A chain and B chain relative to α_2M were used during coincubation with PPE. For each insulin "type", the number of moles bound per mole of α_2M loaded on the gel was calculated from counting protein bands excised from polyacrylamide gels. These values were divided by the molar ratios incubated, resulting in values equivalent to the initial slope of the graphs in Figure 4. The amount of incorporation derived from nonreduced samples (hatched bars), samples undergoing reduction before analysis by SDS–PAGE (white bars), or samples incubated in the presence of 100 mM β-aminopropionitrile (black bars) is shown. The specific activities of the original labeled materials were used regardless of whether or not samples had undergone reduction. Error bars depict one standard deviation above the mean.

corresponds to the N-terminal of the B chain, indicating that incubation of native insulin with α_2M and proteinase resulted in covalent attachment of only the insulin B chain.

Binding Experiments Using Isolated Insulin A and B Chains. To further investigate the role of the insulin lysine residue in covalent attachment to α_2M, isolated insulin A and B chains were examined for binding. Each isolated chain was capable of competing with insulin incorporation during coincubation, displaying I_{50} values of 73 μM (A chain) and 46 μM (B chain) under the conditions described under Materials and Methods. Radiolabeled A and B chains also incorporated directly into α_2M when coincubated with PPE, but not in the presence of β-aminopropionitrile (Figure 8). However, the isolated chains appeared to be much less reactive than intact insulin, with incorporation of about one-seventh than seen with intact insulin. Unlike α_2M complexes with native insulin, in which a decrease in bound radioactivity of about 50% was observed upon reduction, complexes involving the isolated chains were unaffected by reduction. This supports the hypothesis that the loss of radioactivity from α_2M–^{125}I-insulin complexes upon reduction results from the loss of the labeled nonbinding insulin chain, rather than loss of a population of insulin linked through disulfides to α_2M.

The unexpected binding of the isolated A chain, which lacks ϵ-amino groups, was investigated further. Sequence analysis of commercial A chain revealed no contaminating peptides. To investigate the possible involvement of the N-terminus, phenyl isothiocyanate treatment was employed to quantitatively derivatize the insulin A chain α-amino group. To control for any unanticipated effect of derivatization on binding, a portion of the sample underwent treatment with trifluoroacetic

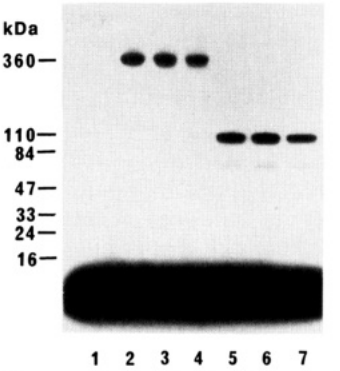

FIGURE 9: Autoradiogram showing binding of insulin A chain to α_2M following modification of the N-terminus. Insulin A chain was modified as described under Materials and Methods. ^{125}I-labeled A chain (lanes 2 and 5), phenyl isothiocyanate derived ^{125}I-labeled A chain (lanes 3 and 6), and trifluoroacetic acid treated ^{125}I-labeled A chain (lanes 4 and 7) were coincubated with α_2M and PPE before analysis with SDS–PAGE under reducing (lanes 5–7) and nonreducing (lanes 1–4) conditions. ^{125}I-Labeled A chain alone was loaded in lane 1. Molecular weight standards include purified α_2M (360 000) and Bio-Rad prestained standards: phosphorylase *b* (110 000), bovine serum albumin (84 000), ovalbumin (47 000), carbonic anhydrase (33 000), soybean trypsin inhibitor (24 000), and lysozyme (16 000).

acid to cleave off the phenylthiocarbamyl N-terminal amino acid, exposing a new primary amine. Sequence analysis of the derivatized material revealed that the reaction was 90% complete. As shown in Figure 9, derivatization of the α-amino group had no effect on the level of binding. These results indicate that amine groups do not mediate the binding of isolated insulin A chain to α_2M.

Discussion

The family of thiol ester containing plasma proteins includes α_2M, pregnancy zone protein, and the complement proteins C3 and C4 (Sottrup-Jensen, 1987). Like the complement proteins, α_2M, with respect to its well-characterized mechanism of proteinase inhibition, requires proteolytic cleavage for activation. Proteinases cleave α_2M in the bait region, initiating a series of events that result in the trapping and inhibition of the attacking proteinase (Barrett & Starkey, 1973; Salvesen & Barrett, 1980). Covalent proteinase binding to the thiol ester occurs during the short-lived nascent state that occurs following bait region cleavage and before completion of the slow to fast form transition (Sottrup-Jensen et al., 1981; Salvesen et al., 1981). With attainment of the fast form, the "closed trap" of α_2M no longer has the potential to interact with proteinases (Saunders et al., 1971; Gonias & Pizzo, 1983). The interaction of insulin with α_2M followed a similar pattern: insulin must be present during treatment of s-α_2M with proteinase for significant covalent and noncovalent association (Figures 1 and 2).

This observation contrasts that of previous studies, which report association of growth factors with untreated, presumably native, α_2M (Huang et al., 1984, 1988; O'Connor-McCourt & Wakefield, 1987; Dennis et al., 1989; Matsuda et al., 1989; Danielpour & Sporn, 1990). Only a few studies addressed the conformational state of growth factor bound α_2M by native gel electrophoresis, reporting migration of the growth factor–α_2M complex as f-α_2M (Huang et al., 1988; Borth & Luger, 1989; Danielpour & Sporn, 1990). Some investigators have suggested α_2M trapping as a possible mechanism for noncovalent association with TGF-β without examining the means of activating the "trap" (O'Connor-McCourt & Wakefield, 1987; Danielpour & Sporn, 1990). In addition, Borth and Luger (1989) reported the need for proteinase or methylamine treatment of α_2M for cytokine incorporation.

Because of the rapid clearance of f-α_2M, and its apparent involvement as an immune modulator (Hubbard et al., 1981; Hoffman et al., 1987), it is crucial to fully characterize the conformational state of α_2M to which growth factors are binding before any hypotheses on biological significance can be broached.

Several previous studies of α_2M–growth factor interaction that did not examine coincubation in the presence of proteinases focused on noncovalent association (Dennis et al., 1989; O'Connor-McCourt & Wakefield, 1987; Danielpour & Sporn, 1990). Under analogous conditions, when we incubated insulin and s-α_2M alone, the slight binding that occurred was entirely noncovalent (Figure 1). However, virtually all endogenous serum TGF-β was shown to be covalently bound to α_2M by O'Connor-McCourt and Wakefield (1987), who were unable to replicate this binding in their incubations of purified α_2M and TGF-β. However, they did not examine the effect of proteinases on TGF-β binding. We found that when a saturating (2-fold) amount of proteinase was coincubated with equimolar ratios of α_2M and insulin, more than 80% of bound insulin was covalently attached. It has been suggested that the covalent incorporation observed in incubations of α_2M and TGF-β was a labeling artifact (Huang et al., 1988). This is not the case with insulin, where N-terminal sequence analysis demonstrated that unlabeled insulin incorporates covalently in the same manner as labeled insulin.

The role of proteinases in the formation of α_2M–insulin complexes was examined by using serine proteinases that differed in size, specificity, and ability to cross-link to α_2M subunits. Trypsin and PPE both rapidly cleave and form covalent cross-links with α_2M, while HNE is unable to form cross-links despite rapidly cleaving bait regions (Enghild et al., 1989). Plasmin, which is large and forms 1:1 complexes with α_2M, is able to rapidly cleave only two bait regions followed by slower cleavage of the other two (Roche & Pizzo, 1987, 1988). In addition, cross-linking platinum reagents were utilized to isolate the effects of proteinase activity on insulin binding from those of conformational change itself.

Reversible cross-linking of s-α_2M by *cis*-DDP prevents proteinase-induced conformational change while allowing bait region cleavage (Roche et al., 1988). Removal of cross-links by DDC then allows the conformational change to occur, resulting in f-α_2M indistinguishable from that derived from proteinase treatment alone (Roche et al., 1988). Thus, *cis*-DDP treatment, when used prior to proteinase treatment, can be utilized to create a stable form of α_2M which is primed to undergo transition to the nascent state (Roche et al., 1988). The proteinase can then be removed or inactivated, before platinum removal by DDC initiates conformational change and exposure of the thiol ester. Experiments using *cis*-DDP-derivatized α_2M showed that bait region cleavage alone was insufficient for incorporation of ^{125}I-insulin, nor was incorporation dependent upon the mere presence of an active proteinase (Figure 3, lane 4). Instead, the covalent interaction between ^{125}I-insulin and α_2M was dependent upon the presence of ^{125}I-insulin during the conformational change from slow to fast form (lanes 5 and 6). This indicates that the presence of proteinases is required solely to drive conformational change of α_2M to a state that allows incorporation of nucleophiles.

These results suggested a mechanism of binding that was dependent on the changes occurring during conformational change, namely, exposure of the thiol ester, trap closure, and appearance of receptor recognition sites. The fact that preformed f-α_2M failed to incorporate significant amounts of insulin argues against a role for receptor recognition epitopes. In analogy with proteinases, closure of the trap may result in an increase in noncovalent incorporation, despite the small size of insulin (<6 kDa), and the thiol ester may mediate covalent binding.

The dependence of covalent incorporation upon exposure of the thiol ester raises the possibility of binding to either the cysteinyl component of the glutamyl component. The covalent complex between insulin and α_2M was stable to reduction and its formation sensitive to competition by β-aminopropionitrile; this provided evidence for involvement of the glutamyl residue (Figures 2, 4, 6, 7, and 8). The migration pattern of the fragment containing ^{125}I-insulin on SDS–PAGE was reminiscent of the pattern seen in the ϵ-lysyl-γ-glutamyl cross-linking of proteinases (Salvesen & Barrett, 1980). Stoichiometric characterization of insulin incorporation into α_2M revealed a maximum of four molecules bound per α_2M molecule (Figure 4), which was consistent with the number of thiol esters. In addition, these studies suggested competition for insulin binding by PPE, but not by HNE, which lacks lysine groups (Sinha et al., 1987; Enghild et al., 1989). It is of particular interest to note that the incorporation of up to 2 mol of insulin/mol of α_2M was not affected by the choice of proteinase (Figure 4). Proteinases possessing lysine groups tend to bind to α_2M with a maximum stoichiometry of 2:1, leaving two available thiol esters for reaction with other molecules. Sequence analysis confirmed the site of covalent linkage between insulin and α_2M, and demonstrated the actual attachment of only the lysine-containing B chain.

Several investigators in the past have proposed thiol–disulfide interchange as a mechanism for growth factor binding (Huang et al., 1984; Dennis et al., 1989; Borth & Luger, 1989), yet only the latter study presented a consistent mechanism for binding. Borth and Luger's (1989) work on interleukin-1β, performed at pH 8.0, indicated a requirement for proteinase or methylamine treatment, presumably to affect the appearance of free thiols. They showed that preformed f-α_2M incorporated interleukin-1β equally well as s-α_2M that had been coincubated with interleukin-1β and proteinase. This is consistent with a mechanism of binding which involves free thiols, rather than nucleophilic attack on an intact thiol ester. The other studies, however, did not address the fact that s-α_2M has no free thiols (Huang et al., 1984), nor did they explain only a 51% loss of radioactivity from the complex with reduction (Dennis et al., 1989).

The loss of radioactive label upon reduction, however, can also result from loss of a labeled nonbinding chain from dimeric disulfide-linked growth factors. Sequence analysis of the insulin–α_2M complex demonstrated that only the B chain is covalently linked. Reduction resulted in a 50% decrease in radioactivity (Figures 7 and 8), attributable to the fact that both insulin chains contain the same number of tyrosine residues available for radiolabeling. When isolated insulin A or B chain was used, all the bound radioactivity was resistant to reduction (Figure 8).

Characteristic of proteinase interaction with α_2M is the inhibition of binding following treatment of α_2M with methylamine (Salvesen et al., 1981). This results either from premature methylamine-induced trap closure or from modification of the glutamyl residue following nucleophilic attack by methylamine on the thiol ester. The reaction of methylamine with α_2M is a slow process, which may account for the inconsistent effect on covalent proteinase binding when methylamine is coincubated with proteinase and s-α_2M (Chen et al., 1990). Thus, pretreatment with methylamine is necessary to completely inhibit proteinase binding. As predicted

by the proposed ϵ-lysyl-γ-glutamyl mechanism of insulin association with α_2M, methylamine-pretreated α_2M failed to incorporate any insulin (Figure 2).

The observation that f-α_2M demonstrated impaired insulin incorporation may be attributed either to steric hindrance or to involvement of a transient α_2M intermediate. The small size of insulin should render it capable of diffusing through the closed trap arms to reach the thiol ester site (Barrett & Starkey, 1973). Competition with proteinases for the same binding site occurred only at higher insulin concentrations (Figure 4). Although the trypsin- or elastase-induced transformation occurs extremely rapidly, the specific appearance of free thiol groups during the transformation can be spectrophotometrically followed. The decay of the nascent α_2M state capable of incorporating insulin was investigated and found to parallel the generation of free thiols following proteinase treatment (Figure 5). Thus, the temporal characteristics of insulin incorporation are consistent with incorporation during slow to fast transformation. Given the proposed mechanism of covalent insulin incorporation, this provides another estimate of the half-life of thiol ester decay upon activation or exposure by proteinase-induced conformational change. The half-life of the α_2M intermediate capable of incorporating insulin is 7 s, which is in agreement with several estimates of thiol ester decay (Sottrup-Jensen et al., 1981; Sottrup-Jensen, 1987). The rapid drop in binding potential followed by a more gradual decline is compatible with the possibility of two rates of proteolytic activation of α_2M proposed by Christensen and Sottrup-Jensen (1984).

In normal human plasma, insulin typically exists in concentrations ranging from 0.08 to 1.1 nM, using the equivalence factor of 24 IU in 1 mg (Butt, 1967; Yalow & Bersen, 1960). In obese diabetics, peak insulin concentrations can exceed 3.6 nM (500 microcounts/mL) (Yalow et al., 1965). After characterization of the covalent binding of α_2M and insulin at supraphysiologic concentrations, parallel experiments using physiologic concentrations demonstrated similar β-aminopropionitrile-sensitive, reduction-resistant incorporation (Figures 6 and 7). In addition, the low reactivity of isolated B chains when compared to native insulin indicated that the presence of a lysine residue is not sufficient for maximum incorporation into α_2M (Figure 8). This suggests a conformational requirement for insulin incorporation which favors native insulin over isolated B chains.

Under physiological conditions, subsaturating levels of active proteinase relative to α_2M are likely. Our data suggest that even at low levels of proteinase and insulin, the moment the bait region of an α_2M molecule is cleaved and the thiol ester exposed, it becomes available for reaction with insulin, which proceeds very rapidly before the reactive form decays (Figure 5). Thus, under subsaturating conditions, the level of covalent insulin binding directly reflected the extent of α_2M bait region cleavage (Figure 7), irrespective of proteinase type.

The potential biologic relevance of hormone or growth factor interactions with α_2M is particularly interesting considering the development variation in serum α_2M levels. Human α_2M attains its peak serum concentration between 28 weeks of gestation and 1 year of age, dropping gradually to reach adult levels by 25–30 years of age (Ganrot & Scherstên, 1967; Gitlin & Gitlin, 1975). During puberty, serum α_2M concentrations stabilize at about twice that of adult levels, before beginning to fall again in the late teenage years (Ganrot & Scherstên, 1967). During pregnancy, the levels increase to 140–160% that seen in sex- and age-adjusted controls, and there are great increases in the structurally and functionally related dimeric pregnancy zone protein (Ganrot & Bjerre, 1967; Sottrup-Jensen, 1987). These are all stages of life characterized by increased metabolic demands, which require regulation.

Although complement proteins incorporate proteins into their thiol esters via ester linkages (Dodds & Law, 1988), work on α_2M has focused on the formation of amide bonds through lysine residues. Our results demonstrated incorporation of native insulin into α_2M solely through the lysine-containing B chain. However, the isolated A chain was also capable of covalent incorporations (Figure 8), although 7 times more A chain was required to produce a level of binding comparable to that of intact insulin. Competition between the isolated A chain and insulin or β-aminopropionitrile suggested a role for the glutamine residue of the α_2M thiol ester. The A chain was shown to be free from contaminants by sequence analysis, and covalent incorporation into α_2M was not affected by blocking the A-chain N-terminus with phenyl isothiocyanate (Figure 9); therefore, binding could not be attributed to contamination or to the N-terminal α-amino group. This suggests the possibility of ester-type linkages involving other amino acids as suggested earlier by Sottrup-Jensen (1987).

In this report, we have characterized the mechanism and temporal dependence of insulin incorporation into α_2M. Insulin forms an ϵ-lysyl-γ-glutamyl linkage to α_2M during proteinase-induced conformational change, demonstrating no evidence of disulfide interaction. Since the conformational states of α_2M vary vastly in terms of inhibitory potential, capacity for covalent or noncovalent binding, and biologic destination, particular attention was directed at quantifying the amount of α_2M in each state both before and after experimental treatment. We have also discussed other possible mechanisms of peptide incorporation in general, as well as providing evidence supporting the unexplored possibility of ester linkages with the glutamyl residue of the thiol ester. We believe that this study may provide a framework upon which to base future studies of α_2M–growth factor interaction.

Registry No. Insulin, 9004-10-8.

References

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

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

Beatty, K., Bieth, J., & Travis, J. (1980) *J. Biol. Chem. 255*, 3931–3934.

Borth, W., & Luger, T. A. (1989) *J. Biol. Chem. 264*, 5818–5825.

Bury, A. F. (1981) *J. Chromatogr. 213*, 491–500.

Butt, W. R. (1967) *Hormone Chemistry*, D. Von Nostrand Co., London.

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

Chen, B. J., Yuan, A. I., Wang, D., & Feinman, R. D. (1990) *Biochemistry 29*, 3361–3365.

Cheng, Y., & Prosoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099–3108.

Christensen, U., & Sottrup-Jensen, L. (1984) *Biochemistry 23*, 6619–6626.

Clark, S., & Harrison, L. C. (1983) *J. Biol. Chem. 258*, 11434–11437.

Clausen, J., Gjedde, F., & Jørgensen, K. (1963) *Proc. Soc. Exp. Biol. Med. 112*, 778–781.

Danielpour, D., & Sporn, M. B. (1990) *J. Biol. Chem. 265*, 6973–6977.

Dennis, P. A., Saksela, O., Harpel, P., & Rifkin, D. B. (1989) *J. Biol. Chem. 264*, 7210–7216.

Dodds, A. W., & Law, S. K. A. (1988) *Complement 5*, 89–97.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 72–77.
Enghild, J. J., Salvesen, G., Thøgersen, I. B., & Pizzo, S. V. (1989) *J. Biol. Chem. 264*, 11428–11435.
Feldman, S. R., Gonias, S. L., & Pizzo, S. V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5700–5704.
Fuchs, H. E., Michalopoulos, G. K., & Pizzo, S. V. (1984) *J. Cell. Biochem. 25*, 231–243.
Ganrot, P. O., & Bjerre, B. (1967) *Acta Obstetr. Gynecol. Scand. 46*, 126–137.
Ganrot, P. O., & Scherstén, B. (1967) *Clin. Chim. Acta 15*, 113–120.
Gitlin, D., & Gitlin, J. D. (1975) in *The Plasma Proteins: Structure, Function, and Genetic Control* (Putnam, F. W., Ed.) Vol. II, pp 263–319, Acadmic Press, New York.
Gonias, S. L. & Pizzo, S. V. (1983) *J. Biol. Chem. 258*, 14682–14685.
Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306–314.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 171*, 27–38.
Harper, J. W., Hemmi, K., & Powers, J. C. (1985) *Biochemistry 24*, 1831–1841.
Hoffman, M. R., Pizzo, S. V., & Weinberg, J. B. (1987) *J. Immunol. 139*, 1885–1890.
Huang, J. S., Huang, S. S., & Deuel, T. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 342–346.
Huang, S. S., O'Grady, P., & Huang, J. S. (1988) *J. Biol. Chem. 263*, 1535–1541.
Hubbard, W. J., Hess, A. D., Hsia, S., & Amos, D. B. (1981) *J. Immunol. 126*, 292–299.
Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134–8139.
Jay, D. G. (1984) *J. Biol. Chem. 259*, 15572–15578.
Kurecki, T., Kress, L., & Laskowski, M. (1979) *Anal. Biochem. 99*, 415–420.
LaMarre, J., Wollenberg, G. K., Gauldie, J., & Hayes, M. A. (1990) *Lab. Invest. 62*, 545–551.
Manwell, C. (1977) *Biochem. J. 165*, 487–495.
Matsuda, T., Hirano, T., Nagasawa, S., & Kishimoto, T. (1989) *J. Immunol. 142*, 148–152.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Nakaya, K., Horinishi, H., & Shibata, K. (1967) *J. Biochem. 61*, 345–351.
O'Connor-McCourt, M. D., & Wakefield, L. M. (1987) *J. Biol. Chem. 262*, 14090–14099.
Ohlsson, K. (1971a) *Acta Physiol. Scand. 81*, 269–272.
Ohlsson, K. (1979b) *Biochim. Biophys. Acta 236*, 84–91.
Pannell, R., Johnson, D., & Travis, J. (1974) *Biochemistry 13*, 5439–5445.
Praissman, M., & Rupley, J. A. (1968) *Biochemistry 7*, 2431–2445.
Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49–60.
Roche, P. A., & Pizzo, S. V. (1987) *Biochemistry 26*, 486–491.
Roche, P. A., & Pizzo, S. V. (1988) *Arch. Biochem. Biophys. 267*, 285–293.
Roche, P. A., Jensen, P. E. H., & Pizzo, S. V. (1988) *Biochemistry 2*, 759–764.
Salvesen, G. S., & Barrett, A. J. (1980) *Biochem. J. 187*, 695–701.
Salvesen, G., & Nagase, H. (1989) in *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J., & Bond, J. S., Eds.) pp 83–104, Oxford University Press, Oxford.
Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Saunders, R., Dyce, B. J., Vannier, W. E., & Haverback, B. J. (1971) *J. Clin. Invest. 50*, 2376–2383.
Sinha, S. L., Watorek, W., Karr, S., Gives, J., Bode, W., & Travis, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2228–2232.
Sottrup-Jensen, L. (1987) in *The Plasma Proteins: Structure, Function, and Genetic Control* (Putnam, F. W., Ed.) Vol. V, pp 191–291, Academic Press, New York.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 128*, 123–126.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.
Swenson, R. P., & Howard, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N., Arai, K., Yokota, T., Wakasugi, H., & Yodoi, J. (1989) *EMBO J. 8*, 757–764.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247*, 3242–3251.
Yalow, R. S., & Berson, S. A. (1960) *J. Clin. Invest. 39*, 1157–1175.
Yalow, R. S., Glick S. M., Roth, J., & Berson, S. A. (1965) *Ann. N.Y. Acad. Sci. 131*, 357–373.