Proteinase Inhibition by Proform of Eosinophil Major Basic Protein (pro-MBP) Is a Multistep Process of Intra- and Intermolecular Disulfide Rearrangements*

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The metzincin metalloproteinase pregnancy-associated plasma protein A (PAPP-A, pappalysin-1) promotes cell growth by the cleavage of insulin-like growth factor-binding proteins-4 and -5, causing the release of bound insulin-like growth factors. The proteolytic activity of PAPP-A is inhibited by the proform of eosinophil major basic protein (pro-MBP), which forms a covalent 2:2 proteinase-inhibitor complex based on disulfide bonds. To understand the process of complex formation, we determined the status of cysteine residues in both of the uncomplexed molecules. A comparison of the disulfide structure of the reactants with the known disulfide structure of the PAPP-A-pro-MBP complex reveals that six cysteine residues of the pro-MBP subunit (Cys-51, Cys-89, Cys-104, Cys-107, Cys-128, and Cys-169) and two cysteine residues of the PAPP-A subunit (Cys-381 and Cys-652) change their status from the uncomplexed to the complexed states. Upon complex formation, three disulfide bonds of pro-MBP, which connect the acidic propiece with the basic, mature portion, are disrupted. In the PAPP-A-pro-MBP complex, two of these form the basis of both two interchain disulfide bonds between the PAPP-A and the pro-MBP subunits and two disulfide bonds responsible for pro-MBP dimerization, respectively. Based on the status of the reactants, we investigated the role of individual cysteine residues upon complex formation by mutagenesis of specific cysteine residues of both subunits. Our findings allow us to depict a hypothetical model of how the PAPP-A-pro-MBP complex is formed. In addition, we have demonstrated that complex formation is greatly enhanced by the addition of micromolar concentrations of reductants. It is therefore possible that the activity in vivo of PAPP-A is controlled by the redox potential, and it is further tempting to speculate that such mechanism operates under pathological conditions of altered redox potential.

The 1547-residue pregnancy-associated plasma protein A (PAPP-A, pappalysin-1) belongs to the metzincin superfamily of metalloproteinases (1). Known substrates of PAPP-A include insulin-like growth factor-binding proteins (IGFBPs)-4 and -5, which function to inhibit the biological activities of IGF-I and -II, and cleavage by PAPP-A releases bioactive IGF (2, 3). However, the growth-promoting activity of PAPP-A is antagonized by the proform of eosinophil major basic protein (pro-MBP) of 206 residues (4, 5), originally known in its mature form as a cytotoxic protein from the granules of the eosinophil leukocyte (6). Pro-MBP inhibits the proteolytic activity of PAPP-A by the formation of an inactive, 2:2 proteinase-inhibitor complex, denoted PAPP-A-pro-MBP, covalently bound by disulfides (4, 7).

The 500-kDa PAPP-A-pro-MBP complex was first isolated from the circulation of pregnant women (8), and both PAPP-A and pro-MBP were found to be synthesized in the placenta (9). However, PAPP-A and pro-MBP expression has since been demonstrated in several other tissues (10), and data indicate that the proteolytic activity of PAPP-A is involved in a number of normal and pathological processes, such as fetal development (11–13), ovarian follicular development (14), human implantation (15), wound healing (16), atherosclerosis (17), and cancer (18).

In the placenta, PAPP-A and pro-MBP are known to be synthesized by different cell types (9), and about 99% of circulating PAPP-A is present as a tetrameric complex with pro-MBP (5), showing that in vitro complex formation occurs outside the cell. In agreement with this, in vitro formation of the disulfide-bonded PAPP-A-pro-MBP complex after separate synthesis was recently demonstrated and shown to be required for inhibition of the proteolytic activity (4). Other proteinase inhibitors known to bind covalently to their target proteinase are the serpins (19) and α2-macroglobulin (20), but, to the best of our knowledge, there are no prior examples of a proteinase inhibitor, in which the inhibitory mechanism is based on the formation of intermolecular disulfide bonds.

Disulfide bonds in nascent proteins are formed by the oxidation of cysteine residues in the endoplasmic reticulum. Because of the oxidizing nature of the extracellular milieu, disulfides have been considered to be inert. However, several recent reports suggest that the reduction or formation of specific disulfide bonds function in reversible and irreversible switches in the regulation of extracellular protein function. For example, the reduction of specific disulfide bonds in platelet integrin αIIbβ3 causes conformational changes in both integrin subunits and growth factor; IGFBP, insulin-like growth factor-binding protein; ELISA, enzyme-linked immunosorbent assay; NTCB, 2-nitro-5-thiocyanobenzoic acid; mAb, monoclonal antibody; PBS, phosphate-buffered saline; Cya, cysteic acid; RP-HPLC, reversed-phase high performance liquid chromatography.
units and leads to exposure of ligand binding sites (21). Also, the binding of platelets to multimers of the blood protein von Willebrand factor (vWF) is indirectly controlled by thrombospordin-1, which facilitates reversible reduction of vWF intersubunit disulfide bonds (22). Additionally, several animal viruses (e.g. HIV-1 and murine leukemia virus) (23, 24) depend on the rearrangements of specific disulfides for virus-membrane fusion. Two systems of protein regulation based on disulfide switches relate to proteolysis. First, the reduction of two disulfide bonds in the fifth kringle domain of plasmin by phosphoglycerate kinase makes plasmin a substrate for proteolysis (25). Second, the serine proteinase inhibitor, plasminogen activator inhibitor type 2 (PAI-2) uses the disruption or the formation of a disulfide bond to switch between a stable, inhibitory monomeric conformation and a conformation that readily forms inactive polymers (26).

We have recently reported the connectivity of the 188 cysteine residues in the 2:2 PAPP-A-pro-MBP complex (7). We here show that oxidation does not promote complex formation, indicating that the formation of intermolecular disulfide bonds is not a reaction between free thiol groups and molecular oxygen. To understand the process of complex formation, we determined the status of cysteine residues in both of the uncomplexed molecules and further performed mutagenesis of individual cysteines found to change their status upon complex formation.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis—**Mutagenesis of pro-MBP cDNA cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) was carried out by overlap extension PCR (27). In brief, outer primers derived from pcDNA3.1 were: 5'-CCCCCAATTGGACGCAAATGGGCGG-3' (5'-end) and 5'-AGGAAAAGGACATGGGATCGG-3' (3'-end). Internal primers with an overlap of ~22 bp were used to generate mutated fragments that were digested with NheI/XhoI and swapped into the wild-type construct. Constructs encoding pro-MBP mutants (pro-MBP(C515S), pro-MBP(C169S)) with single cysteine residues substituted by serine and one double mutant (pro-MBP(C104S/C107S)) were made. Mutated PAPP-A constructs were obtained using two pBlueScript II SK+ vectors containing PAPP-A cDNA corresponding to residues 1–407 (pBl1-407) and 408–988 (pBl408-988) (1). Mutagenesis was carried out by overlap extension PCR, and the mutated cDNA fragments were swapped into the HindIII/BspEI or the BspEI/KpnI sites of pPA-BspEI (1), a modified version of the PAPP-A wild-type construct, pcDNA3.1-PAPP-A (5), containing a BspEI site at residue 407. Outer primers, derived from pBlueScript II SK+, were: 5'-TAATACGACTCACTATAGGG-3' and 5'-AATATTACACCTGCTAAAGG-3'. Plasmid pBl1–407 was used for the construction of mutant PAPP-A(C381A), and plasmid pBl408-988 was used for mutant PAPP-A(C652A). Protothelysin inactive variants of these two PAPP-A mutants, PAPP-A(C381A/E483Q) and PAPP-A(C652A/E483Q), were also constructed, using PAPP-A mutant E483Q (1) as the template. All PCRs were carried out with Pfu DNA polymerase (Promega), and constructs were verified by sequence analysis. Plasmid DNA for transfection was prepared using the QiAprep Spin Kit (Qiagen). At least two independent clones of each construct were used for further analysis.

**Tissue Culture and Transfection—**Human embryonic kidney 293T cells (293tsA1609neo) (28) were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, nonessential amino acids, and gentamicin (Invitrogen, Life Technologies). Cells were plated onto 6-cm culture dishes, and were transfected 18 h later by calcium phosphate co-precipitation (29) using 10 μg of plasmid DNA. The cells were transfected with either a pro-MBP or a PAPP-A expression vector. After another 48 h, the supernatants were harvested, cleaved by centrifugation, and replaced by serum-free medium (CD293, Invitrogen) for another 48 h to facilitate Western blotting or purification. All PAPP-A mutants expressed at the level of wild-type PAPP-A, and all pro-MBP mutants, except for pro-MBP(C381S) and pro-MBP(C1285S) (4), at the level of wild-type pro-MBP, as determined by ELISA.

**In Vitro Formation of the PAPP-A-pro-MBP Complex—**The PAPP-A-pro-MBP complex was formed by mixing culture supernatants from 293T cells transfected separately with PAPP-A or pro-MBP cDNAs. The concentrations of PAPP-A and pro-MBP in the reaction mixtures were 20 and 200 nM, respectively, reflecting physiological concentrations (30). The mixtures were incubated at 37 °C, and samples were taken out and frozen at defined time points between 0 and 48 h. In some reactions, defined concentrations of hydrogen peroxide (H₂O₂), reduced glutathione (GSH), dithiothreitol, or β-mercaptoethanol were added. Complex formation was visualized by Western blotting, and/or quantitatively monitored by an ELISA specific for the complex (see below).

**Western Blotting—**Visualization of PAPP-A and pro-MBP was done by Western blotting following separation by SDS-PAGE in 3–8% precast Tris acetate gels (Invitrogen). After electrophoresis, the protein was blotted onto a polyvinylidene difluoride membrane and blocked in 2% skimmed milk powder diluted in 50 mM Tris, 500 mM sodium chloride, 0.1% Tween 20, pH 9.0 (TST). After washing and equilibration in TST, the membrane was incubated with primary antibodies (mAb 234-2 for PAPP-A or mAb 234-10 for pro-MBP), diluted to 1 μg/ml in TST containing 2% skimmed milk powder, and incubated for 1 h at room temperature. Incubation with peroxidase-conjugated secondary antibodies (P0260, DAKO), diluted in TST containing 2% skimmed milk powder, was done for 0.5 h at room temperature. The blots were developed using enhanced chemiluminescence (ECL Plus, Amersham Biosciences), and images were captured with a KODAK Image Station 1000.

**Time-linked Immunoassay—**PAPP-A and pro-MBP concentrations were measured by sandwich ELISAs, in which polyclonal rabbit anti-(PAPP-A-pro-MBP) (31) was used for capture, and monoclonal antibodies against PAPP-A (234-2) (32) or pro-MBP (234-10) (33) followed by peroxidase-conjugated anti-mouse IgG (P0260, DAKO) were used for detection. The wells were blocked by incubation with phosphate-buffered saline (PBS) containing 2% bovine serum albumin. Antibodies were diluted in PBS with 0.01% Tween-20 (PBST) and 1% bovine serum albumin. PBST was used for washing. The PAPP-A-pro-MBP complex formation was monitored over time using a complex-specific double monomolecular ELISA: The PAPP-A specific monoclonal antibody VRPA-1A² was used as the catching antibody, and the PAPP-A-pro-MBP complex was detected with biotinylated, pro-MBP-specific monoclonal antibody VRPM-5A³ followed by incubation with peroxidase-conjugated avidin (P0347, DAKO). In the latter assay, sample dilution and washing after sample incubation were carried out using PBST to which 500 mM sodium chloride was added, to avoid noncovalent association. The amount of complex formed was expressed as a percentage of the amount of complex formed upon completion of the reaction (100%). Dilution series of the PAPP-A-pro-MBP complex purified from pregnancy serum (31) were used to establish standard curves.

**Detection of IGFBP-4 Proteolytic Activity—**Proteolytic activity of PAPP-A-containing samples was measured as described (3), following incubation with pro-MBP or pro-MBP mutants for 48 h, as indicated. In brief, PAPP-A (0.6 nM, 0.12 μg/ml, corresponding to a ~30-fold dilution of the sample), 125I-labeled IGFBP-4 (10 nM, 0.30 μg/ml), and IGF-II (50 nM, 0.35 μg/ml) (DSL) in 50 mM Tris, 100 mM sodium chloride, 1 mM calcium chloride, pH 7.5 were incubated at 37 °C. Samples of the reaction mixtures were taken out at time points from 0 to 30 min and separated by non-reducing SDS-PAGE (10–20% Tris-glycine gels). The degree of cleavage was assessed by measuring band intensities with a PhosphorImager (Molecular Dynamics) and plotted as a function of time after subtraction of background (3).

**Reduction of Cysteine Connectivity by S-Cyanation—**²Nitro-5-thio-glucosamine (NTGB, Sigma) specifically reacts with free cysteines at pH 8.0, and the peptide bond is subsequently cleaved N-terminal to the cyanylated cysteine at pH 9.0 (34). To avoid interference by fragments originating from PAPP-A autoproteolysis (1), the S-cyanation experiments were carried out using proteolytically inactive PAPP-A, mutant E483Q (1), or inactive PAPP-A variants, PAPP-A(C381A/E483Q), and PAPP-A(C652A/E483Q). Culture supernatant with pH adjusted to 8.0 by the addition of 3 M Tris, pH 8.0 was incubated with 2 mM NTGB for 30 min at 37 °C. N-Ethylmaleimide was then added to the mixture to a final concentration of 20 μM, the pH was raised to 9.0 by the addition of 3 M Tris, pH 9.0, and the sample was incubated for 3 V. Rodacker, M. Overgaard, K. Mortensen, H. Sperling-Petersen, and C. Ovrig, unpublished data.

²The numbering of the 1547-residue mature PAPP-A polypeptide is used in this report. Glu-1 of mature PAPP-A corresponds to position 81 of prepro-PAPP-A (Swiss-Prot accession number Q13219). The numbering of the 222-residue prepro-MBP polypeptide (Swiss-Prot accession number P13727) is used in this report.
16 h at room temperature. NTGC cleavage profiles were visualized by Western blotting using polyclonal anti-PAPP-A (31) following separation by reducing SDS-PAGE (10–20%). The sizes of the resulting fragments were compared with a well-characterized fragment of PAPP-A autoproteolysis, representing PAPP-A residues 1–386 (1), to C-terminally truncated, recombinant PAPP-A variants (representing PAPP-A residues 1–950 (mutant PA1–950) and 1–1129 (mutant PA1–1129) (35), or to an N-terminally truncated PAPP-A variant representing residues 1133–1547 (mutant PA1133–1547).4

Amino Acid Analysis—Amino acids were quantified by cation exchange after hydrolysis of peptide bonds at 110 °C for 18 h in 6 M HCl, 0.1% phenol, 5% thiglycolic acid (36). For detection of chromatographic fractions containing disulfide-bonded peptides, paired and unpaired cysteine residues were oxidized to cysteic acid (Cya) by incubation of lyophilized samples with performic acid prepared by reacting hydrogen peroxide (30%) with formic acid (1:9 v/v) on ice for 10 min. Following incubation on ice for 2 h, the samples were lyophilized again and then hydrolyzed.

Generation of Pro-MBP Peptides and Chromatographic Separation—Recombinant pro-MBP (1 mg), purified by affinity chromatography (4), was digested with thermolysin (type X protease, Sigma) for 18 h at 55 °C using an enzyme:substrate ratio of 1:25 (w/w). Thermolytic peptides were separated by reversed-phase high-pressure liquid chromatography (RP-HPLC) on a 4 × 250 mm column packed with Nucleosil C18 100–5 (Macherey-Nagel). Gradients were formed from 0.1% (v/v) trifluoroacetic acid (solvent A) and 0.075% (v/v) trifluoroacetic acid in C18 100–5 (Macherey-Nagel). Gradients were acquired with a Voyager-DE PRO matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instrument (Applied Biosystems). Samples were prepared by co-crystallization of the peptide with a-cyano-4-hydroxycinnamic acid (Sigma) and of intact peptides were acquired by on-target reduction using dithiothreitol. Spectra were obtained by averaging 50–100 single shot spectra and were externally calibrated. Edman degradation was performed on an Applied Biosystems 477A sequencer equipped with an on-line HPLC. For sample loading, isolated peptides or protein (50–200 pmol) were pipetted onto Polybrene-coated glass filters.

RESULTS

Reducant, but Not Oxidant Promotes Covalent Complex Formation—To understand the process of PAPP-A-pro-MBP complex formation, we first analyzed the ability of reducing and oxidizing agents to influence the reaction. Culture supernatants from human embryonic kidney 293T cells transfected separately with PAPP-A and pro-MBP cDNA, were mixed, incubated, separated by nonreducing SDS-PAGE, and analyzed by Western blotting using a PAPP-A-specific antibody. The incubations were carried out for 1 h in the absence (lane 1) or in the presence of H2O2 (100 μM) (lane 2), or GSH (100 μM) (lane 3). PAPP-A was also incubated (1 h) in the absence of pro-MBP and in the presence of GSH (100 μM) (lane 4), and PAPP-A and pro-MBP were incubated for 48 h in the presence of GSH (100 μM) (lane 5). A, a similar experiment using a pro-MBP-specific antibody, C, the amount of complex formed, relative to fully complexed PAPP-A (100%), after 1 h of incubation as a function of increasing concentrations of GSH (black columns) or H2O2 (white columns). An ELISA using a PAPP-A-specific antibody for catching and a pro-MBP-specific antibody for detection was used to monitor the PAPP-A-pro-MBP complex formation. The results are averages of four independent experiments. Vertical lines indicate S.D.

MBP complex formed after 1 h was measured quantitatively using an ELISA specific for the complex (Fig. 1C). Incubation of PAPP-A and pro-MBP in the presence of 10 μM GSH caused a 4-fold increase in the amount of complex formed, and incubation with 100 μM GSH led to an 8-fold increase. In the presence of 1 mM GSH, all PAPP-A was found in the complexed form after 1 h of incubation. Similar results were obtained using the reductants dithiothreitol and β-mercaptoethanol (data not shown). Interestingly, the presence of oxidant appeared to inhibit the process (Fig. 1C), as did concentrations of GSH higher than 5 μM (data not shown).

PAPP-A-pro-MBP Complex Formation Is Not Catalyzed by Residues of the Cys–Xxx–Xxx–Cys Motif—The Cys–Xxx–Xxx–Cys motif comprises the active site of enzymes involved in disulfide

4 H. Boldt and C. Oxvig, unpublished data.
exchange reactions, including protein-disulfide isomerase (PDI) and thioredoxin (37), but has also been reported to function as a redox switch in a number of extracellular proteins unrelated to protein folding, as in integrin αIIbβ3 and murine leukemia virus Env (24, 38). Recombinant pro-MBP migrates in nonreducing SDS-PAGE as a monomer (4), but since it exists as a disulfide-bonded dimer in the PAPP-A-pro-MBP complex through cysteine residues Cys-104PM and Cys-107PM (7), these residues are necessarily involved in exchange reactions during complex formation. To test the hypothesis that pro-MBP residues Cys-104PM and Cys-107PM, which are part of a Cys-Xxx-Xxx-Cys motif, function to catalyze complex formation, we constructed a plasmid encoding the mutant pro-MBP(C104S/C107S), in which both cysteines are replaced by serine residues. However, the ability of mutant pro-MBP(C104S/C107S) to form a covalent complex with PAPP-A or to inhibit its proteolytic activity could not be distinguished from wild-type pro-MBP (4), we also conclude that these residues do not function to catalyze complex formation.

**PAPP-A-pro-MBP Complex Formation Occurs Independently of Pro-MBP Dimerization**—To exclude that the above results are caused by the absence of dimerization of wild-type pro-MBP in the recombinant PAPP-A-pro-MBP complex, we examined the stoichiometry of mutant PAPP-A(C1130A), in complex with wild-type pro-MBP. Unlike wild-type PAPP-A, which is secreted as a dimer, mutant PAPP-A(C1130A) is secreted as a monomer, because the single disulfide bridge between the PAPP-A subunits is absent (7). As expected, the PAPP-A(C1130A) mutant migrated at 200 kDa (Fig. 2C, lane 1). In contrast, the PAPP-A(C1130A)-pro-MBP complex migrated as the wild-type 2:2 covalent complex at 500 kDa (Fig. 2C, lane 2), sufficiently formed via pro-MBP dimerization. Thus, this experiment unequivocally shows that the pro-MBP dimer is formed in the recombinant complex.

**Deduction of Cysteine Connectivity in Uncomplexed PAPP-A**—To describe the events leading to the formation of the tetrameric PAPP-A-pro-MBP complex, knowledge of the status of cysteine residues in both of the reactants and in the product is required. The status of cysteines in the PAPP-A-pro-MBP complex, a total of 188, was recently delineated (7), but it would not be feasible to determine the status of cysteines in the uncomplexed PAPP-A dimer by a direct method. Instead, we used an indirect approach based on chemical modification of unpaired cysteine residues by S-cyanation with NTCB. S-Cyanylation of exposed cysteines at pH 8 and subsequent spontaneous chemical cleavage of the peptide bond N-terminal to the modified cysteine at pH 9, results in the formation of an N-terminal fragment and C-terminal 2-iminothiazolidine-4-carboxyli (ITC) peptide derivative (34). Hence, the number of fragments and their sizes in the NTCB cleavage profile of PAPP-A will be indicative of the number and location of free cysteines. Previously characterized fragments and truncated variants of the 1547-residue recombinant PAPP-A subunit were used to estimate the location of the NTCB cleavage sites, and candidate cysteines were probed by mutagenesis: If a given cysteine residue is free, a mutation into alanine eliminates one cleavage site, causing two fragments to disappear from the NTCB cleavage profile. Alternatively, if the cysteine residue is engaged in disulfide pairing, substitution into alanine results in one additional reactive cysteine, adding fragments to the cleavage profile. At the sites of modified cysteines, only partial cleavage, typically less than 10% is observed (39, 40). Thus, all bands observed are N- or C-terminal fragments, as fragments arising from two cleavages within the same polypeptide chain are quantitatively negligible.

The NTCB cleavage profile of PAPP-A was visualized by Western blotting using polyclonal anti-PAPP-A following separation by reducing SDS-PAGE. Six bands were observed, indicating the presence of three unpaired cysteines in uncomplexed PAPP-A (Fig. 3A). Considering their migration, we conclude that the uppermost band (band 1) pairs with the lower band (band 6). Accordingly, band 2 pairs with band 5, and band 3 with band 4.

Band 6 migrated close to PAPP-A fragment 1–386 (Fig. 3B, lanes 1 and 2), suggesting Cys-381PA as a candidate free cysteine, as it binds to pro-MBP in the PAPP-A-pro-MBP complex (7). We consequently expressed a PAPP-A mutant, PAPP-A(C381A), with this cysteine substituted into alanine. Upon
NTCB treatment of PAPP-A(C381A), bands 1 and 6 were absent from the cleavage profile (Fig. 3C, lane 1), demonstrating that these bands originated from cleavage at Cys-381PA.

Band 3 migrated slightly faster than PAPP-A fragment 1–950 (Fig. 3C, lanes 2 and 3), leaving at least 14 cysteine residues in the sequence stretch from approximately residue 600–950 as possible candidates (7). As Cys-652PA also binds to the pro-MBP subunit in the PAPP-A-pro-MBP complex, we probed this residue by the expression of mutant PAPP-A(C652A). Upon NTCB cleavage, bands 3 and 4 were absent from its profile (Fig. 3D), demonstrating that Cys-652PA is unpaired in uncomplexed PAPP-A.

Band 2 comigrated with a PAPP-A variant representing residues 1–1129 (Fig. 3C, lanes 3 and 4), and band 5 comigrated with a C-terminal PAPP-A fragment of residues 1133–1547 (Fig. 3B, lanes 2 and 3), pointing at Cys-1130PA as a possible candidate. In the NTCB cleavage profile of mutant PAPP-A(C1130A), bands 2 and 5 were not observed (data not shown), demonstrating that cleavage did occur at Cys-1130PA, which in wild-type PAPP-A is responsible for dimerization through the Cys-1130PA-Cys-1130PA disulfide bond (7). However, a minor fraction of wild-type PAPP-A (about 10%) is observed to migrate as a monomer in nonreduced Western blots (data not shown), indicating that, in this fraction, Cys-1130PA is unpaired and particular susceptible to S-cyanlation. We have now unequivocally shown that the NTCB cleavage profile of wild-type PAPP-A originates from cleavage at Cys-381PA, Cys-652PA, and Cys-1130PA. In the PAPP-A-pro-MBP complex, Cys-381PA and Cys-652PA form intermolecular disulfide bonds to pro-MBP residues Cys-51PA and Cys-169PA, respectively (Fig. 4).

The Disulfide Structure of Uncomplexed pro-MBP—As the pro-MBP subunit is smaller, we used a direct approach to determine the status of pro-MBP cysteine residues. Prior to purification of pro-MBP by affinity chromatography (4), potentially free cysteine residues were modified by alkylation with iodoacetamide. The mass spectrum of the purified protein showed a broad peak with an average mass of 28 kDa, representing the pro-MBP monomer, and short, broad peaks at 14 and 56 kDa, representing M2H⁺ and 2MH⁺ species, respectively (Fig. 5). By SDS-PAGE, reduced pro-MBP migrated at 38 kDa, while non-reduced pro-MBP migrated at 36 kDa indicating the presence of disulfide bonds (Fig. 5, gel inset).

The alkylated pro-MBP was treated with performic acid, and the content of Cya was determined by amino acid analysis to 9.9 ± 0.3 residues per molecule. No difference in Cya content was found for pro-MBP modified by iodoacetamide in the presence of 8 M urea and EDTA after purification. We conclude that 10 cysteine residues of alkylated pro-MBP were susceptible to performic acid oxidation and therefore engaged in disulfide bonding.

A thermolytic digest of pro-MBP was then separated by reversed-phase high-pressure liquid chromatography (RP-HPLC) (Fig. 6). The eluate was screened for carbamidomethylated cysteine (CAM-Cys) and the content of Cya following performic acid oxidation, and selected fractions were analyzed by mass spectrometry, as detailed in the legend of Fig. 7. The isolated peptides were also subjected to N-terminal sequence analysis (not shown).

A total of five disulfide bonds were located in uncomplexed pro-MBP (Table I): Cys-51PA-Cys-169PA, Cys-89PM-Cys-128PM, Cys-104PM-Cys-107PM, Cys-125PM-Cys-220PM, and Cys-197PM-Cys-212PM (Table I). In striking contrast, only two of these intramolecular disulfide bonds, Cys-125PM-Cys-220PM and Cys-197PM-Cys-212PM, are present in the pro-MBP subunit of the PAPP-A-pro-MBP complex (Fig. 4B). Thus, in the course of complex formation, the three pro-MBP disulfide bonds Cys-51PA-Cys-169PA, Cys-89PM-Cys-128PM, and Cys-104PA-Cys-107PM, which all connect the acidic propiece with the basic MBP region, are disrupted.

The Effect of Cysteine Substitutions on the PAPP-A-pro-MBP Complex Formation—We have now established a set of cysteines residues in both PAPP-A and pro-MBP that all change their status during the process of complex formation (Fig. 4, A and B). Based on this, we analyzed the role of individual cysteine residues upon complex formation by mutagenesis of specific cysteine residues in both subunits.

Two of the cysteines engaged in an intramolecular disulfide bond of pro-MBP, Cys-51PM and Cys-169PM, form intermolecular disulfide bonds with PAPP-A cysteines Cys-381PA and Cys-652PA, respectively, when complexed (Fig. 4). We expressed and analyzed two substitution mutants, in which this disulfide was disrupted: Pro-MBP(C169S), in which Cys-51PM is unpaired, was unable to form a covalent complex and inhibit the proteolytic activity of PAPP-A (Fig. 8, A and B and Table II). In contrast, pro-MBP(C51S), in which Cys-169PM is unpaired, formed a covalent complex severalfold faster than wild-type pro-MBP (Fig. 8A).

Additional cysteine residues, here shown to change their status upon complex formation were analyzed in a similar manner (Table II). Pro-MBP(C89S) and pro-MBP(C128S) formed a covalent complex at a rate similar to wild-type pro-MBP, indicating that disruption of the Cys-89PM-Cys-128PM disulfide does not occur prior to the formation of the first intermolecular disulfide bond between a PAPP-A and a pro-MBP subunit. PAPP-A(C381A) also formed the complex at a rate similar to wild-type PAPP-A, while the ability of PAPP-A(C652A) to form a covalent complex was several fold reduced.

The Formation of One Intermolecular Disulfide Bond outside the Proteolytic Domain Inhibits the Proteolytic Activity of PAPP-A—Our findings suggest that the first covalent interaction between PAPP-A and pro-MBP is the formation of the Cys-169PM-Cys-652PA disulfide bond. To determine the effect of
this disulfide bond on the proteolytic activity of PAPP-A, we compared the proteolytic activity with the degree of covalent complex formation for PAPP-A incubated with wild-type pro-MBP or pro-MBP(C51S). The degree of inhibition and the degree of covalent complex formed correlated well for both the wild-type PAPP-A and the PAPP-A(pro-MBP(C51S) complex (Fig. 8, C and D), suggesting that the formation of the Cys-169PM-Cys-652PA intermolecular disulfide bond leads to immediate inhibition of the proteolytic activity of PAPP-A, independent of the formation of the Cys-51PM-Cys-381PA disulfide. This finding indicates that the inhibitory mechanism of pro-MBP is based on the formation of one disulfide bond between the PAPP-A and pro-MBP subunits, located ~70 residues outside the proteolytic domain (1) (Fig. 4A).

**DISCUSSION**

We here show that formation of a covalent PAPP-A-pro-MBP complex is accelerated by low concentrations of reductant, but
Concentrations of GSH comparable to the level in blood plasma (10–20 μM) (41) enhanced the rate of PAPP-A/pro-MBP complex formation about 5-fold, while 1 mM GSH enhanced the rate 10-fold (Fig. 1C). Our findings indicate that the redox potential of the local environment in tissues may function to control PAPP-A/pro-MBP complex formation and hence, in turn, IGF activity. Importantly, this may be relevant under pathological conditions, in which the redox potential is altered.

To delineate the events leading to the formation of the PAPP-A/pro-MBP complex, we first analyzed the cysteine connectivity of the reactants, uncomplexed PAPP-A and pro-MBP. By S-cyanation of unpaired cysteine residues in wild-type PAPP-A and subsequent chemical cleavage N-terminal to the modified cysteine, we showed that uncomplexed PAPP-A contains reactive cysteine residues. Using recombinant PAPP-A fragments to evaluate the size of the cleavage fragments and further

FIG. 7. Mass spectrometric analysis of disulfide-bonded peptides. Fractions containing disulfide-bonded peptides, identified as cysteic acid following oxidation by performic acid, were analyzed by MALDI-TOF mass spectrometry. Mass values (m/z) represent MH⁺ species. A, the mass spectrum of fraction 38 showed a peak of 1129.47 Da, representing the peptide TL-1, 197WSRGH-Cys-212PM. B, fraction 73 contained a triple peptide of 2301.08 Da, TL-2, in which 17LTCPCEEEDT, 12FTCRRC, and 1ICTSY are held together by two disulfide bonds. The disulfide bonds underwent spontaneous fragmentation one by one and the resulting double peptides gave rise to the peaks 1267.91 and 1819.08 Da, respectively, corresponding to the peptide masses of partially reduced TL-2. To determine its disulfide connectivity, TL-2 was further digested with trypsin and analyzed. Two peaks, TR-1 (1008.36 Da) and TR-2 (1311.41 Da), resulting from cleavage C-terminal to Arg-126PM were observed (inserted spectrum), revealing Cys-89PM-Cys-128PM connectivity. C, TL-3 (3394.80 Da) of fraction 80 represented peptides 34TLPEDEETQEMEETPCR and 16ITGSGRCRR, connected by Cys-51PM-Cys-169PM. The disulfide bond underwent spontaneous fragmentation, and gave rise to a peak of 1005.62 Da (not visible in the shown spectrum range) and a peak of 2392.18 Da, corresponding to the peptide masses of reduced TL-3. D, TL-4 (1293.74 Da) of fraction 94 contained the peptide 99VGIPGQTCRY, in which Cys-104PM and Cys-107PM form an intrachain disulfide bridge.

**TABLE I**

| Peptide | Fraction | Amino acid sequence | Cysteine pairing | Observed mass |
|---------|----------|---------------------|-----------------|--------------|
| TL-1    | 38       | 197WSRGH-Cys-212PM  | Cys-197PM-Cys-212PM | 1129.47 (0.03) |
| TL-2    | 73       | 17LTCPCEEEDT, 12FTCRRC, 1ICTSY | Cys-89PM-Cys-128PM, Cys-125PM-Cys-220PM | 2301.08 (0.16) |
| TL-3    | 80       | 34TLPEDEETQEMEETPCR and 16ITGSGRCRR | Cys-51PM-Cys-169PM | 3394.80 (0.31) |
| TL-4    | 94       | 99VGIPGQTCRY | Cys-104PM-Cys-107PM | 1293.74 (0.09) |
| TL-5    | 49       | 14YHQCSCS | Cys-147PM | 826.42 (-0.09) |
| TL-6    | 59       | 149GHCVALCTRGGYWRRAHCLRR | Cys-197PM-Cys-212PM | 2583.12 (0.13) |
| TR-1    | 73       | 12FTCR, 21ICTSY | Cys-125PM-Cys-220PM | 1008.36 (-0.08) |
| TR-2    | 73       | 17LTCPCEEEDT, 127RC | Cys-89PM-Cys-128PM | 1311.41 (-0.12) |

*Peptides denoted TL originate from thermolytic digestion of proMBP. Peptides denoted TR originate from additional tryptic digestion (of peptide TL-2).

The numbers correspond to the RP-HPLC fractions analyzed by mass spectrometry in Fig. 7.

The first residue of each sequence is numbered. Cysteine residues are shown in bold.

Numbers indicate pairing of cysteine residues of the isolated peptides. An asterisk indicates that the given cysteine residue is carbamidomethylated.

Experimentally determined masses (m/z) of MH⁺ species are given with the deviations from the calculated peptide masses indicated in parentheses.

Variants of TL-1 with one or both histidines modified by iodoacetamide were found in fractions 34 and 35.

A variant of TL-3 was found in fraction 65 with the methionine modified by iodoacetamide.

A shorter variant of this peptide, 99VGIPGQTCRY, was found in fraction 88.

TL-6 is a longer variant of TL-1 and contains a carbamidomethylated cysteine.
Disulfide Rearrangement in Proteinase Inhibition by pro-MBP

The disruption of the disulfide bond Cys-51_pM-Cys-169_pM and the formation of the interchain disulfide Cys-169_pM-Cys-652_pA occur at an early stage of complex formation. A, complex formation by the incubation of wild-type pro-MBP (filled circles), pro-MBP(C51S) (filled squares), or pro-MBP(C169S) (filled triangles) with wild-type PAPP-A. The amount of complex formed was measured over time by an ELISA specific for the complex. The experiment shown was repeated several times with similar results. B, measurement of IGFBP-4 proteolytic activity of PAPP-A preincubated (48 h) with mock medium (open circles), wild-type pro-MBP (filled circles), pro-MBP(C51S) (filled squares), or pro-MBP(C169S) (filled triangles). The experiment shown was repeated several times with similar results. C, wild-type PAPP-A/pro-MBP complex formation rate (filled circles) compared with the degree of inhibition of PAPP-A proteolytic activity (open bars). The amount of complex formed and the degree of inhibition are expressed relative to the values obtained after 48 h of incubation. The data shown are an average of four independent experiments; vertical lines indicate S.D. D, a similar set of experiments comparing complex formation between PAPP-A and pro-MBP(C51S) (filled squares) with the degree of inhibition of proteolytic activity (open bars).

TABLE II

The effect of cysteine substitutions on PAPP-A/proMBP complex formation

| PAPP_A^a | ProMBP^b | Complex formed after 1 h % |
|----------|----------|---------------------------|
| Wild-type | Wild-type | 10 ± 2.0 |
| Wild-type | C51S | 43 ± 2.3 |
| Wild-type | C169S | 0.2 ± 0.1 |
| Wild-type | C652A | 11 ± 1.5 |
| Wild-type | C128S | 10 ± 1.7 |
| C381A | Wild-type | 11 ± 2.1 |
| C381A | C51S | 40 ± 3.0 |
| C381A | C169S | 0.4 ± 0.3 |
| C652A | Wild-type | 0.9 ± 0.6 |
| C652A | C51S | 2.3 ± 0.5 |
| C652A | C169S | 0.2 ± 0.2 |

^a Variant of PAPP-A used in complex formation experiment.
^b Variant of proMBP used in complex formation experiment.

The extracellular formation of disulfide-bonded complexes has been studied for the interaction of thrombospondin and vitronectin with thrombin-antithrombin III (44–46). The proposed mechanism, based on the disulfide structures of the reactants, is the isomerization of a free cysteine in thrombospondin or vitronectin with a disulfide bridge in thrombin, leading to the formation of an intermolecular disulfide bond. We found that the target cysteines in PAPP-A are unpaired, whereas the reacting cysteines of pro-MBP are connected to each other by a disulfide bond (Fig. 4A and B). However, a comparison of the disulfide structure of the reactants with that of the PAPP-A/pro-MBP complex, shows that six cysteine residues of the pro-MBP subunit (Cys-51_pM, Cys-89_pM, Cys-104_pM, Cys-128_pM, and Cys-169_pM) and two cysteine residues of the PAPP-A subunit (Cys-381_pA and Cys-652_pA) change their status from the uncomplexed to the complexed states (Fig. 4A and B). Thus, the covalent changes during formation of the PAPP-A/pro-MBP complex are far more extensive than expected considering the status of the reactants. The covalent binding of pro-MBP to PAPP-A serves to inhibit its proteolytic activity. However, the extensive rearrangement of disulfide bonds upon complex formation likely causes conformational changes, which may not be related directly to the control of proteolysis.

The set of cysteine residues here shown to change their status, enabled us to probe the role of individual cysteine residues in the process of complex formation by mutagenesis. We found that disruption of the pro-MBP disulfide Cys-51_pM-Cys-169_pM positions the free cysteines so that Cys-169_pM and Cys-201_pM are free in uncomplexed PAPP-A (Fig. 4A). In addition, pro-MBP contains three disulfide bonds, Cys-51_pM-Cys-169_pM, Cys-89_pM-Cys-128_pM, and Cys-104_pM-Cys-107_pM, which anchor the acidic propiece to the basic MBP domain, leaving only cysteines, Cys-147_pM and Cys-201_pM, unpaired. In striking contrast, five cysteine residues are unpaired in mature MBP purified from the granules of eosinophil leukocytes (Fig. 4B).
readily interacts with Cys-652PA, as the rate of complex formation for pro-MBP(C51S) was accelerated 4-fold. In contrast, the position of Cys-51PM is not favorable for initial interaction with Cys-381PA as the rate of complex formation for pro-MBP(C169S) was reduced severalfold compared with the wild-type (Fig. 8A). The substitution of additional pro-MBP cysteine residues had no effect on the rate of complex formation suggesting that the initial changes of pro-MBP during complex formation are the disruption of the disulfide bond Cys-51PM-Cys-381PA and the reaction of Cys-169PM with Cys-652PA (Table II).

Micromolar levels of reductant may function to reduce disulfide bonds particularly susceptible as a consequence of protein-protein interactions between PAPP-A and pro-MBP. A priori, we are unable to predict the location of such disulfides in either the PAPP-A or the pro-MBP subunit. However, it is tempting to speculate that the redox potential of the disulfide bond Cys-51PM-Cys-169PM is altered upon the initial interaction between PAPP-A and pro-MBP, as the effect of substituting cysteine residue Cys-51PM with a serine residue, thereby disrupting the disulfide bond, are similar to the effect of micromolar amounts of reductant. A similar effect of reductant and substitution of cysteine residues has been observed with integrin αIIbβ3, in which disruption of specific disulfide bonds in the cysteine-rich domain of the β-subunit caused the exposure of ligand binding sites (47–49). Thus, analogously with this, the covalent interaction between PAPP-A and pro-MBP may require the disruption of specific disulfide bonds. Such disruption may be favored by the initial interaction between the subunits.

Unlike PAPP-A, uncomplexed pro-MBP is a monomer, but is present in the PAPP-A-pro-MBP complex as a dimer based on two disulfides, Cys-104PM-Cys-104PM and Cys-107PM-Cys-107PM. Upon complex formation, these two bridges form from the intra-chain disulfide Cys-104PM-Cys-107PM, present in each pro-MBP monomer. Because mutant pro-MBP(C104S/C107S) forms a fully inhibited 2:2 complex with PAPP-A at a rate similar to the rate for wild-type pro-MBP (Fig. 2A and B), we conclude that the process of pro-MBP dimerization occurs independently of other disulfide rearrangements. Importantly, however, dimerization of pro-MBP does require an interaction between PAPP-A and pro-MBP, as pro-MBP does not form dimers in the absence of PAPP-A. The presence of intermediate PAPP-A-pro-MBP 2:1 complexes (Fig. 1A, lane 3) shows that a pro-MBP subunit, once complexed to PAPP-A, is able to dimerize with a second pro-MBP subunit. Additionally, we have ruled out that Cys-104PM and Cys-107PM function to catalyze disulfide bonding between PAPP-A and pro-MBP, although they conform to the Cys-Xxx-Xxx-Cys motif, known from the active site of enzymes involved in disulfide exchange reactions (37).

Based on our findings, we have depicted the formation of the PAPP-A-pro-MBP complex in a hypothetical model (Fig. 9). We suggest that first, the pro-MBP disulfide bond Cys-51PM-Cys-169PM is broken causing exposure of a reactive Cys-169PM, which then forms the first interchain disulfide, Cys-169PM-Cys-652PA (step 1). Upon disruption of the pro-MBP disulfide Cys-89PM-Cys-128PM, Cys-51PM forms the second interchain disulfide, Cys-51PM-Cys-381PA (step 2). Last, independently of these events, pro-MBP dimerizes through formation of the two interchain bonds, Cys-104PM-Cys-107PM and Cys-107PM-Cys-107PM, from two Cys-104PM-Cys-107PM intrachain disulfides (step 3). This process may involve a hypothetically catalytic activity of the Cys-Xxx-Xxx-Cys motif, to which these residues belong. Importantly, the total redox balance upon complex formation is maintained, as each bond broken is accompanied by another bond formed. In vivo, this series of disulfide bond rearrangements may be mediated by a specific or a nonspecific catalyst, e.g., a disulfide reductase or a low molecular weight thiol compound.

In conclusion, we have determined the status of cysteine residues in the reactants, uncomplexed PAPP-A and pro-MBP. A total of six pro-MBP and two PAPP-A cysteine residues have been shown to change their status upon formation of the covalent PAPP-A-pro-MBP complex. Based on the status of the reactants, we investigated the role of individual cysteine residues upon complex formation by mutagenesis of specific cysteine residues, and we have proposed a model of how the PAPP-A-pro-MBP complex is formed. We have also found that the rate of complex formation is greatly influenced by the addition of micromolar concentrations of reductants. It is therefore possible that the activity in vivo of PAPP-A is controlled by the redox potential, and it is further tempting to speculate that such mechanism operates under pathological conditions of altered redox potential to control the biological activity of IGF.

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