Mutation of the Active Site Glutamic Acid of Human Gelatinase A: Effects on Latency, Catalysis, and the Binding of Tissue Inhibitor of Metalloproteinases-1†

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ABSTRACT: Human gelatinase A, a member of the matrix metalloproteinase family, is secreted from cells as the M_r 72 000 latent precursor, progelatinase A. The autolytic removal of an N-terminal propeptide generates the M_r 66 000 active form. Mutants of recombinant progelatinase A, altered such that the proposed active site glutamic acid residue (E^{375}) was replaced by either an aspartic acid (proE^{375}→D), an alanine (proE^{375}→A), or a glutamine (proE^{375}→Q), were purified from medium conditioned by transfected NS0 mouse myeloma cells. Like wild-type progelatinase A, the mutant proenzymes were inactive and could bind tissue inhibitor of metalloproteinases (TIMP)-2 but not TIMP-1 to their C-terminal domains. Their rates of autolytic processing induced by the organomercurial (4-aminophenyl)mercuric acetate, however, were markedly slower and, of the three M_r 66 000 forms so produced, only E^{375}→D displayed any proteolytic activity against either a synthetic substrate (k_{cat}/K_m = 10% that of the wild-type enzyme) or denatured type I collagen (specific activity = 0.9% that of the wild-type enzyme). ProE^{375}→A and proE^{375}→Q could be more rapidly processed to their M_r 66 000 forms by incubation with a deletion mutant of gelatinase A that has full catalytic activity but lacks the C-terminal domain [Δ(418–631)gelatinase A]. These two M_r 66 000 forms displayed low activity on a gelatin zymogram (approximately 0.01% that of the wild-type enzyme) but, like E^{375}→D, were able to bind TIMP-1 with an affinity equal to that of the activated wild-type enzyme. These results confirm the importance of E^{375} in catalysis but indicate that this residue is not involved in either the maintenance of proenzyme latency or the binding of TIMP-1 to the active site.

Gelatinase A (EC 3.4.24.24, 72-kDa gelatinase, type IV collagenase) is a member of the mammalian matrix metalloproteinase family of zinc-dependent enzymes that degrade protein components of the extracellular matrix. This turnover is tightly regulated and is believed to be essential to developmental and wound healing processes. An increase in matrix metalloproteinase activity caused by alterations to the normal control mechanisms has been implicated in the progression of arthritis and cancer (Docherty et al., 1992).

The matrix metalloproteinases share considerable sequence similarity within their functionally defined domains. The N-terminal or catalytic domain contains the conserved sequence HEXXHXXGXXH, and predictions that this sequence forms part of the active site were confirmed recently by the crystal structure of the matrix metalloproteinase fibroblast collagenase (Lovejoy et al., 1994). The three histidine residues, for example, were shown to act as ligands for the catalytic zinc atom. Alteration of the glutamic acid in the homologous sequences of both neutral endopeptidase 24.11 and the matrix metalloproteinase transin (the rat homologue of stromelysin-1) gave rise to catalytically inactive forms of these enzymes (Devault et al., 1988; Sanchez-Lopez et al., 1988), indicating that this residue is also essential for catalysis. Kinetic and crystallographic studies on the bacterial proteinase thermolysin, which contains part of this sequence, suggest that the glutamic acid forms an ion pair with a water molecule that is bound to the zinc. This ionization allows the nucleophilic attack of the water on the carbonyl group of the substrate. The glutamic acid may also donate a proton to the departing amine product (Hangauer et al., 1984). With the exception of matrilysin, all of the matrix metalloproteinases possess a noncatalytic C-terminal domain downstream of the active site, and both gelatinase A and gelatinase B have an additional domain inserted upstream that shares homology with the collagen binding domain of fibronectin (Collier et al., 1988; Wilhelm et al., 1989).

Like the other matrix metalloproteinases, gelatinase A is secreted as a latent precursor (progelatinase A) that is activated by the proteolytic removal of an 80 amino acid N-terminal propeptide. It is proposed that the latency is a result of interactions between the propeptide and constituents of the active site (Van Wart & Birkedal-Hansen, 1990; Park et al., 1991). Once activated, the matrix metalloproteinases can be inhibited by either α_2-macroglobulin or the specific inhibitors, tissue inhibitor of metalloproteinases (TIMP)-1[1] and TIMP-2. The two TIMPs share approximately 40% sequence homology but differ in the kinetics of their binding to the various matrix metalloproteinases. This variation is largely due to interactions between the C-terminal domains of both the proteinase and the inhibitor, which in the case of gelatinase A and TIMP-2 are strong enough to permit the binding of the inhibitor to the latent proenzyme (Willenbrock et al., 1993). TIMP-1 does not bind to progelatinase A but, like TIMP-2,

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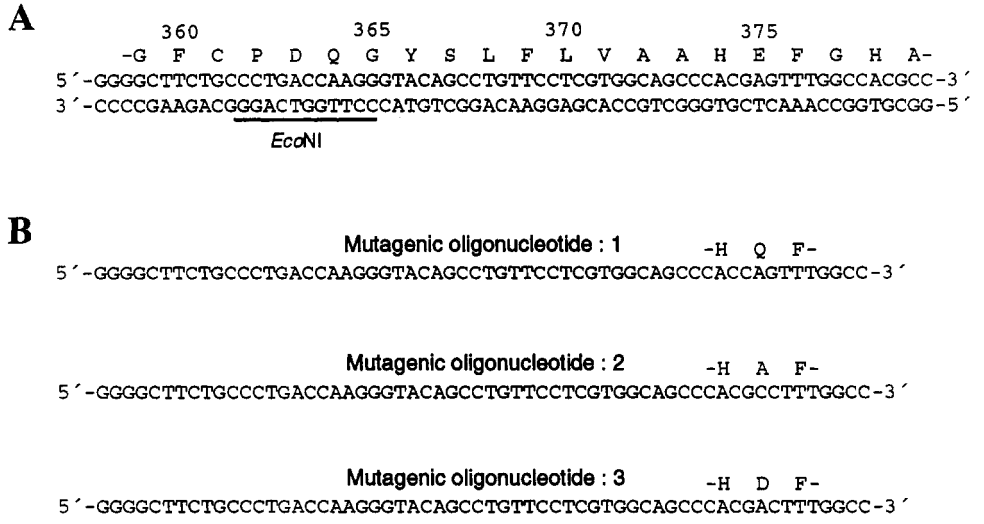

FIGURE 1: (A) DNA sequence encompassing the putative zinc-binding region (active site) of progelatinase A. The *Eco*NI restriction site used in the construction of the mutants is indicated. (B) The three mutagenic oligonucleotides used to generate the E^{375} mutants of progelatinase A.

it will bind to activated gelatinase A, even when the enzyme has no C-terminal domain (Murphy et al., 1992; Fridman et al., 1992). The likelihood that TIMP-1 and TIMP-2 bind to the enzyme's active site is supported by the fact that TIMP-1 competes with synthetic inhibitors that interact with the catalytically essential zinc (Lelièvre et al., 1990). In this study we used site-directed mutagenesis to replace the active site glutamic acid of progelatinase A (E^{375}) with either an aspartic acid, an alanine, or a glutamine residue. The role of E^{375} in the maintenance of proenzyme latency and active enzyme catalysis and TIMP-1 binding was investigated by comparing the properties of the mutants with those of wild-type gelatinase A.

MATERIALS AND METHODS

Materials. Recombinant human wild-type progelatinase A, Δ(418–631)progelatinase A, TIMP-1, and TIMP-2 were purified from medium conditioned by the relevant transfected mouse myeloma cells as previously described (Crabbe et al., 1993; Murphy et al., 1991; Willenbrock et al., 1993). All chromatography materials used were supplied by Pharmacia Ltd. (U.K.). The synthetic substrate, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (McaPLGLDpaAR), was a generous gift from Dr. C. G. Knight (Strangeways Research Laboratory, Cambridge, U.K.), and ^{14}C-labeled, denatured rat type I collagen (gelatin) was prepared as previously described (Murphy et al., 1981).

Construction and Expression of E^{375} Mutants. The wild-type progelatinase A expression vector pEE12-GL A (Murphy et al., 1992) was used as a template for PCR amplification, and the mutations were generated by using the oligonucleotide primer 5′-CCAGCTTCAGGTAATAGG-3′ in conjunction with three mutagenic oligonucleotides encoding sequences that convert E^{375} into either D, A, or Q (Figure 1). In order to reconstruct the full-length mutant gelatinase A cDNAs, the amplified products were digested with *Eco*NI and *Kpn*I and the resultant 480 bp fragments ligated into pSP65 that contained the 5′ and 3′ flanking sequences of gelatinase A. The sequences of the PCR-generated fragments were shown by the dideoxy chain-termination method to correspond to that of wild-type progelatinase A altered as required at E^{375}. Each full-length coding sequence was then inserted into the vector pEE12 for expression in the mouse myeloma NS0 cells as previously described (Murphy et al., 1992). The three mutants were all, therefore, secreted as proenzymes (termed $proE^{375}\rightarrow D$, $proE^{375}\rightarrow A$, and $proE^{375}\rightarrow Q$), which, when purified, were converted by autolytic processing to the M_r 66 000 forms that lack the propeptide (termed $E^{375}\rightarrow D$, $E^{375}\rightarrow A$, and $E^{375}\rightarrow Q$).

Mutant Progelatinase A Purification. Conditioned medium containing the secreted progelatinase A mutant was incubated for 16 h at 4 °C with gelatin Sepharose 4B (25 mL of gelatin Sepharose/L of medium). The gelatin Sepharose was then collected and washed with 500 mL of buffer A (25 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5) containing 1 M NaCl before bound protein was eluted using buffer A containing 1 M NaCl and 10% (v/v) DMSO. This solution was desalted into buffer A containing 30 mM NaCl by Sephadex G-25 gel filtration and analyzed by SDS–polyacrylamide gel electrophoresis and gelatin zymography to establish the purity of the progelatinase A mutants. This revealed that $proE^{375}\rightarrow A$ and $proE^{375}\rightarrow Q$ were purified by the above method but that the $proE^{375}\rightarrow D$ sample contained a low level of contaminating progelatinase B. This was removed by passing the solution through a column of Con A Sepharose that binds only the glycosylated progelatinase B (Wilhelm et al., 1989). The concentration of the purified progelatinase A mutants was determined by absorbance at A_{280} using $\epsilon = 122\,800\ M^{-1}cm^{-1}$ (Crabbe et al., 1993).

Progelatinase A Activation. Wild-type progelatinase A and $proE^{375}\rightarrow D$ were activated by incubation with 1 mM 4-(aminophenyl)mercuric acetate (APMA) at 23 °C. $ProE^{375}\rightarrow A$ and $proE^{375}\rightarrow Q$ were converted to active M_r 66 000 forms by incubation with Δ(418–631)gelatinase A, a deletion mutant of the wild-type enzyme that lacks the C-terminal domain but has full catalytic activity (Murphy et al., 1992). Briefly, 5.5 μM solutions of either $proE^{375}\rightarrow A$ or

[1] Abbreviations: TIMP, tissue inhibitor of metalloproteinases; $proE^{375}\rightarrow D$, progelatinase A mutant with E^{375} replaced by D (also $proE^{375}\rightarrow A$ and $proE^{375}\rightarrow Q$); $E^{375}\rightarrow D$, same as $proE^{375}\rightarrow D$ except without the N-terminal propeptide (amino acids 1–80; also $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$); McaPLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2; APMA, (4-aminophenyl)mercuric acetate; Δ(418–631)gelatinase A, deletion mutant of gelatinase A lacking amino acids 418–631 (C-terminal domain); SDS, sodium dodecyl sulfate.

proE^{375}→Q were incubated at 37 °C for 16 h in the presence of 0.9 μM APMA-activated Δ(418–631)gelatinase A, and the mixture was applied to a heparin Sepharose CL-6B column. Δ(418–631)gelatinase A fails to bind to this matrix and was washed from the column with buffer A containing 30 mM NaCl before the bound proteins, which included activated E^{375}→A or E^{375}→Q, were eluted using buffer A containing 0.6 M NaCl.

Enzyme Assay. Gelatinase A activity was routinely assayed by following the increase in fluorescence that accompanied hydrolysis of the synthetic substrate, McaPLGLDpaAR, as previously described (Knight et al., 1992; Willenbrock et al., 1993). The concentration of active E^{375}→D was determined by titration against purified TIMP-1 of known concentration as previously described (Crabbe et al., 1992) and assuming a 1:1 stoichiometry of interaction. The k_{cat}/K_m of E^{375}→D was measured at a substrate concentration of 0.5 μM (i.e., [S] $\ll K_m$). Activity against gelatin was assayed using ^{14}C-labeled denatured rat type I collagen as previously described (Murphy et al., 1981) except that the reaction was performed at 23 °C.

Kinetic Analysis of Data. The value of the rate constant for the association of the active E^{375}→D/TIMP-1 inhibitory complex (k_{on}) was determined by addition of TIMP-1 (1–2 nM) to a reaction mixture containing active enzyme (0.5 nM) and substrate (0.5 μM). The reaction was followed continuously until a steady-state velocity was reached, and progress curves were analyzed by using the Enzfitter program (Leatherbarrow, 1987) and the equation:

$$P = v_s t + [(v_0 - v_s)(1 - \gamma)/\lambda\gamma]\ln[(1 - \gamma e^{\lambda t})/(1 - \gamma)] \quad (1)$$

in which P is the product concentration, v_0 and v_s are the initial and steady-state velocities, respectively, and γ and λ are parameters described previously (Williams et al., 1979; Willenbrock et al., 1993). The value of k_{on} was calculated by using the equation:

$$k_{on} = \lambda/[(E_t + I_t)^2 - 4E_tI_t]^{1/2} \quad (2)$$

in which E_t and I_t are the total enzyme and inhibitor concentrations.

The K_d values for the binding of TIMP-1 to proE^{375}→A and to E^{375}→A and E^{375}→Q activated by Δ(418–631)gelatinase A were determined in competition experiments. TIMP-1 (0.5 nM) was added to a reaction mixture containing substrate (0.5 μM), wild-type active gelatinase A (0.025 nM), and various concentrations of the relevant mutant. The reaction was followed until a steady-state velocity could be measured, and the results were analyzed by using the equations (Stone et al., 1987):

$$K_d = (F_t - FI)(I_t - EI - FI)/FI \quad (3)$$

$$FI = I_t - E_t(1 - v_s/v_0) + K_i(1 - v_0/v_s) \quad (4)$$

I_t, E_t, and F_t are the total concentrations of TIMP-1, active wild-type gelatinase A, and mutant gelatinase A, respectively, *EI* and *FI* are the concentrations of the TIMP-1 complex with wild-type and mutant gelatinase A, respectively, v_0 is the initial rate in the absence of TIMP-1, and v_s is the final steady-state rate.

TIMP-2 Binding. The three mutants of progelatinase A were compared with wild-type proenzyme and Δ(418–631)progelatinase A in their ability to form complexes with TIMP-2 using an immunoassay. Increasing concentrations of progelatinase A were added to a fixed concentration of TIMP-2 and the mixtures applied to microwell plates coated with MAC93, a mouse monoclonal antibody raised against recombinant human TIMP-2. Bound material was detected using sheep antiserum raised against TIMP-2, donkey anti-sheep IgG–peroxidase, and H_2O_2/tetramethylbenzidine. The formation of progelatinase A/TIMP-2 complexes was revealed by a decrease in the TIMP-2 signal (A_{630nm}) because the combination of the two anti-TIMP-2 antibodies used in this assay binds free inhibitor significantly better than complexed inhibitor (results not shown).

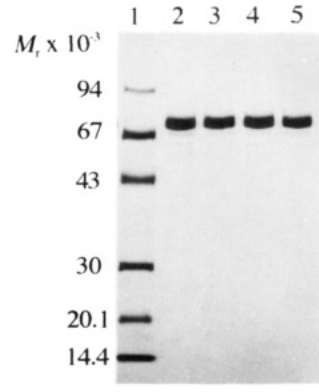

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the purified recombinant progelatinase A mutants. Lane 1, marker proteins; lane 2, wild-type progelatinase A; lane 3, proE^{375}→A; lane 4, proE^{375}→D; lane 5, proE^{375}→Q.

Gel Electrophoresis. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed using precast 10–20% polyacrylamide gradient gels (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). Samples to be analyzed typically contained 1 μg of protein and were prepared by boiling in standard running buffer (Laemmli, 1970) which included 2% (v/v) β-mercaptoethanol and 30 mM EDTA. Completed gels were stained for protein using Coomassie Blue R-250 and quantitated as required using a computing densitometer Model 300A (Molecular Dynamics, Sunnyvale, CA). Gelatin zymography was performed using 10% SDS–polyacrylamide gels containing 0.6 mg/mL gelatin as previously described (Hibbs et al., 1985; Crabbe et al., 1993). The size and intensity of the zones of clearing produced on zymograms by activated enzyme were also measured by densitometry in order to compare gelatin degrading activities.

N-Terminal Amino Acid Sequencing. Sequencing was performed by the Edman degradation of samples electroblotted from SDS–polyacrylamide gels onto poly(vinylidene difluoride) membranes as previously described (Crabbe et al., 1992).

RESULTS

Purification and Analysis of the Progelatinase A Mutants. All three progelatinase A mutants were purified to >95% homogeneity as determined by reduced SDS–polyacrylamide gel electrophoresis where they comigrated with wild-type progelatinase A at the expected M_r of 72 000 (Figure 2). The N-terminal amino acid sequence of proE^{375}→A was found to be APSPIIKFPG, which is identical to that of naturally secreted progelatinase A (Collier et al., 1988). None of the three proenzyme mutants had any catalytic activity against McaPLGLDpaAR. The effect of the E^{375} substitutions upon the overall conformation of progelatinase A was adjudged to be minimal because the relative molecular masses and rates of appearance of the fragments of proE^{375}→Q generated by trypsin digestion were similar to those of the wild-type proenzyme (Figure 3). None of the fragments will have been

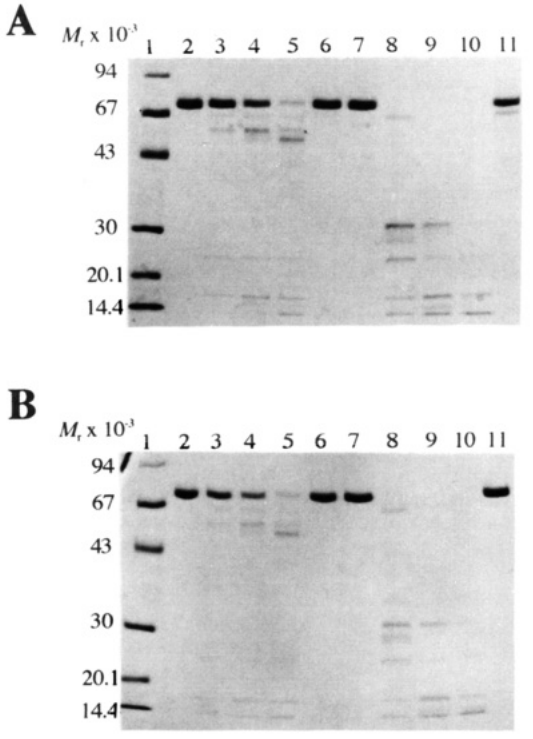

FIGURE 3: Trypsin digestion of wild-type progelatinase A and proE^{375}→Q. (A) Wild-type progelatinase A and (B) proE^{375}→Q at a concentration of 2 μM were incubated with bovine trypsin (0.4 μM) at 23 °C in either the absence (lanes 2–6) or presence (lanes 7–11) of 28 mM EDTA. At the time points indicated, aliquots from each incubation were removed and the trypsin was inactivated with 4 mM phenylmethanesulfonyl fluoride before their analysis by SDS–polyacrylamide gel electrophoresis. Lane 1, marker proteins; lanes 2 and 7, 0 h; lanes 3 and 8, 2 h; lanes 4 and 9, 8h; lane 5 and 10, 24 h. The samples run in lanes 6 and 11 are the 24-h time points taken from separate incubations performed in the absence of trypsin.

generated by an autolytic cleavage because trypsin is unable to catalyze wild-type progelatinase A activation (Okada et al., 1990). The addition of EDTA accelerated trypsin digestion, indicating that, like the wild-type enzyme, the mutant utilizes metal cations (Ca^{2+} and Zn^{2+}) in order to maintain its correct conformation. ProE^{375}→A and proE^{375}→D gave similar patterns of trypsin digestion (results not shown). The TIMP-2 immunoassay used in these studies binds free TIMP-2 better than it does TIMP-2 complexed with progelatinase A. Increasing the concentration of any of the three progelatinase A mutants in the assay reduced the TIMP-2 signal to the same extent as did wild-type proenzyme (Figure 4), thereby demonstrating that alteration of E^{375} did not affect the ability of the proenzyme to bind TIMP-2 . The inability of Δ(418–631)progelatinase A to elicit any significant decrease in the TIMP-2 signal confirms that the inhibitor binds to the C-terminal domain of progelatinase A.

Activation of the Progelatinase A Mutants. ProE^{375}→D was maximally activated after 24 h of incubation at 23 °C in the presence of APMA: a reduced rate in comparison with that of the wild-type proenzyme, which took between 2 and 4 h to fully activate at this temperature (Figure 5A). Analysis by SDS–polyacrylamide gel electrophoresis revealed that, as with the wild-type enzyme (Okada et al., 1990), the increase in activity occurred alongside cleavage of the M_r 72 000 proE^{375}→D to the M_r 66 000 form that lacks the propeptide (E^{375}→D) (Figure 5B). The conversion appears to have proceeded via the formation of a transient M_r 69 000 intermediate. A number of inactive lower M_r fragments were also generated. The fragment migrating at M_r 31 000 is likely

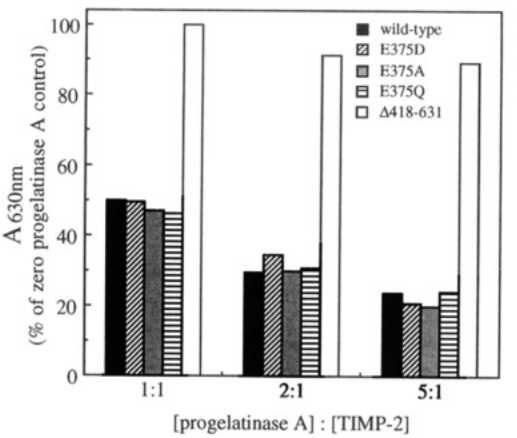

FIGURE 4: TIMP-2 binding to progelatinase A. The formation of progelatinase A/TIMP-2 complexes was measured by using the TIMP-2 immunoassay described in Materials and Methods. Complex formation results in a decrease in the TIMP-2 signal (A_{630nm}). Results are presented as a percentage of the value obtained with no progelatinase A present.

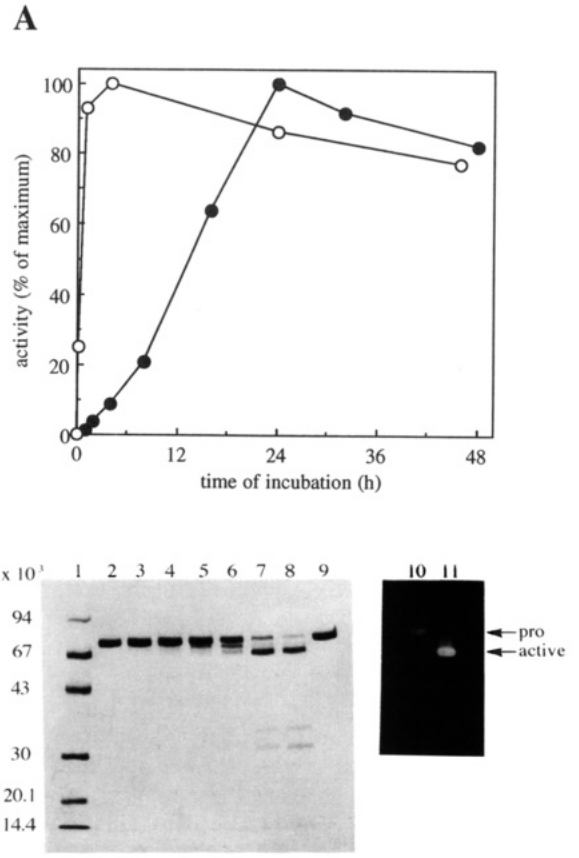

FIGURE 5: Activation of wild-type progelatinase A and proE^{375}→D. (A) Wild-type progelatinase A (○) and proE^{375}→D (●) were incubated at a concentration of 2.7 μM in the presence of 1 mM APMA at 23 °C. At the indicated time points, aliquots were removed and assayed for activity using McaPLGLDpaAR. Results are presented as a percentage of the two maximum activities obtained. (B) Samples taken during the APMA activation of proE^{375}→D were analyzed by SDS–polyacrylamide gel electrophoresis. Lane 1, marker proteins; lane 2, 0 h; lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 8 h; lane 7, 24 h; lane 8, 48 h; lane 9, 48 h, no APMA. Lanes 10 and 11 are the 0- and 48-h samples taken from the proE^{375}→D activation analyzed by gelatin zymography. The positions of migration of wild-type progelatinase A and its M_r 66 000 active form are as indicated.

to be the gelatinase A C-terminal domain (amino acids 415–631) that is produced as a result of a series of secondary autolytic cleavages within the N-terminal domain (Howard et al., 1991; Strongin et al., 1993; Crabbe et al., 1993). E^{375}→D

was the only active species visible on the zymogram after 48 h of incubation, so its concentration could be determined at this time point by active site titration with TIMP-1 (results not shown). The result was used to calculate the catalytic efficiency (k_{cat}/K_m) of $E^{375}\rightarrow D$ using McaPLGLDpaAR as substrate. The value obtained, 5.7×10^4 M^{-1} s^{-1}, is 10 times lower than the k_{cat}/K_m of activated wild-type gelatinase A against the same substrate (Murphy et al., 1992). Similarly, the specific activity of $E^{375}\rightarrow D$ against ^{14}C-labeled gelatin was found to be 11 μg of gelatin degraded/(min·μmol of enzyme) compared with a value of 1260 μg/(min·μmol of enzyme) for wild-type gelatinase A.

It took several days of incubation at 37 °C for APMA to convert either $proE^{375}\rightarrow A$ or $proE^{375}\rightarrow Q$ into their M_r 66 000 forms ($E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$) and the M_r 31 000 fragment (Figure 6A). This contrasts with the wild-type enzyme, which is maximally activated after 5 min of incubation with APMA at 37 °C (Okada et al., 1990). No detectable activity against McaPLGLDpaAR was generated during the APMA-induced conversion of these mutants, and both $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ were inactive on the zymogram (Figure 6C). We have previously demonstrated that, in the absence of APMA, an autolytic cleavage catalyzed by active, wild-type gelatinase A can also remove the propeptide from progelatinase A (Crabbe et al., 1993). In this study, active Δ(418–631)gelatinase A was used to remove the propeptides of $proE^{375}\rightarrow A$ and $proE^{375}\rightarrow Q$ because it possesses the same specific activity as that of the wild-type enzyme (Murphy et al., 1992) and has the advantage that after the reaction it can be separated from full-length gelatinase A by heparin Sepharose CL-6B chromatography (Crabbe et al., 1993). When incubated with active Δ(418–631)gelatinase A (M_r =36 000 with an M_r 22 000 breakdown product) for 16 h at 37 °C, approximately half of the $proE^{375}\rightarrow A$ or $proE^{375}\rightarrow Q$ originally present was converted to $E^{375}\rightarrow A$ or $E^{375}\rightarrow Q$ (Figure 6B). A small quantity of the M_r 31 000 fragment was also produced. The N-terminal amino acid sequence of $E^{375}\rightarrow A$ was found to be YNFFP, which is identical to that of APMA-activated wild-type gelatinase A and indicates that cleavage at N^{80}–Y^{81} had occurred (Stetler-Stevenson et al., 1989). Analysis by SDS–polyacrylamide gel electrophoresis suggested that heparin Sepharose CL-6B chromatography had removed all of the Δ(418–631)gelatinase A, but the more sensitive gelatin zymogram revealed that approximately 1% of the starting activity remained (Figure 6C). The background Δ(418–631)gelatinase A activity prevented the accurate quantitation of any activity possessed by $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ using the McaPLGLDpaAR assay, but the zymogram clearly shows that both possessed some gelatin degrading activity when produced by this method (approximately 0.01% of that displayed by APMA-activated wild-type gelatinase A). The possibility that this zymogram activity was from contaminating mouse progelatinase A secreted by the myeloma cells was discounted by the fact that incubation of either $proE^{375}\rightarrow A$ or $proE^{375}\rightarrow Q$ with 1 mM APMA for 24 h at 23 °C failed to produce any active bands on a zymogram (results not shown). These conditions will fully activate any wild-type progelatinase A but will not affect the mutant proenzymes.

The Binding of TIMP-1 to the Activated Gelatinase A Mutants. TIMP-1 is only able to bind to gelatinase A lacking the propeptide, so, although the methods described above failed to convert all of the mutant proenzyme present, they did give rise to just one potential TIMP-1 binding species. This enabled us to study the kinetics of the interaction of TIMP-1 with the activated mutants and compare the results with those obtained

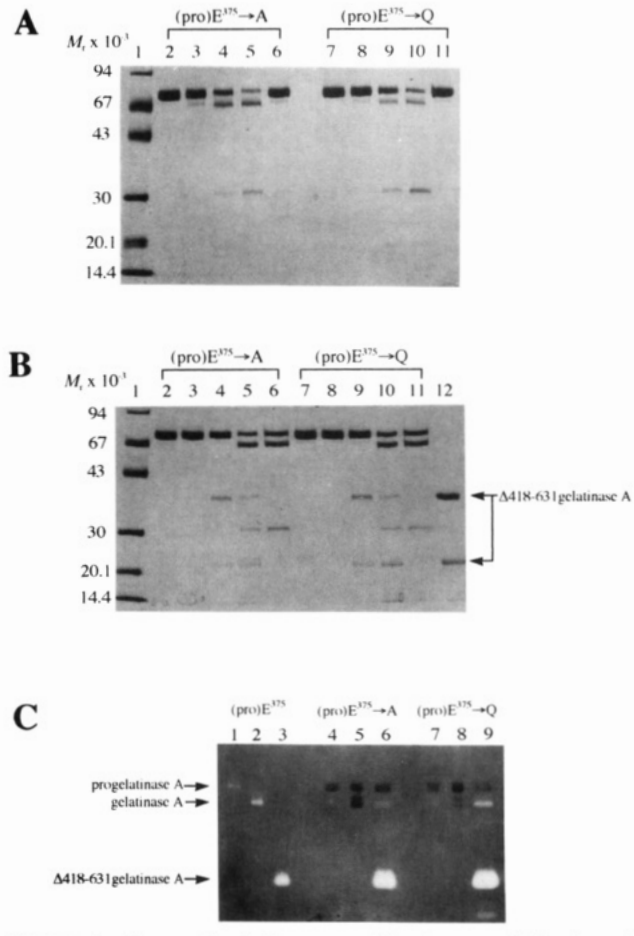

FIGURE 6: Removal of the propeptide from $proE^{375}\rightarrow A$ and $proE^{375}\rightarrow Q$. (A) 7.1 μM $proE^{375}\rightarrow A$ (lanes 2–6) and $proE^{375}\rightarrow Q$ (lanes 7–11) were incubated at 37 °C with 1 mM APMA. Aliquots from each incubation were removed at the following time points: lane 1, marker proteins; lanes 2 and 7, 0 h; lanes 3 and 8, 24 h; lanes 4 and 9, 96 h; lanes 5 and 10, 168 h. The samples run in lanes 6 and 11 are the 168-h time points taken from separate incubations performed in the absence of APMA. (B) $ProE^{375}\rightarrow A$ (lanes 2–6) and $proE^{375}\rightarrow Q$ (lanes 7–11) were incubated at 37 °C for the times indicated in either the presence or absence of APMA-activated Δ(418–631)gelatinase A and then purified as described in Materials and Methods. Lane 1, marker proteins; lanes 2 and 7, 0 h; lanes 3 and 8, 16 h; lanes 4 and 9, 0 h plus Δ(418–631)gelatinase A; lanes 5 and 10, 16 h plus Δ(418–631)gelatinase A; lanes 6 and 11, 16 h plus Δ(418–631)gelatinase A after heparin Sepharose CL-6B chromatography; lane 12, Δ(418–631)gelatinase A. (C) Gelatin zymogram of wild-type and mutant gelatinase A samples. Lane 1, 30 pg of wild-type progelatinase A; lane 2, 30 pg of APMA-activated wild-type gelatinase A; lane 3, 30 pg APMA-activated Δ(418–631)gelatinase A; lane 4, 100 ng of $proE^{375}\rightarrow A$; lane 5, 1 μg of $proE^{375}\rightarrow A$ incubated with 1 mM APMA for 196 h at 37 °C; lane 6, 300 ng of $proE^{375}\rightarrow A$ incubated with Δ(418–631)gelatinase A for 16 h at 37 °C and purified by heparin Sepharose CL-6B; lane 7, 100 ng of $proE^{375}\rightarrow Q$; lane 8, 1μg of $proE^{375}\rightarrow Q$ incubated with 1 mM APMA for 196 h at 37 °C; lane 9, 300 ng of $proE^{375}\rightarrow Q$ incubated with Δ(418–631)gelatinase A for 16 h at 37 °C and purified by heparin Sepharose CL-6B.

with the wild-type enzyme (Willenbrock et al., 1993). TIMP-2 could not be similarly studied because its ability to also bind to the residual proenzyme and the M_r 31 000 fragment (Strongin et al., 1993) will distort the results. Equations 1 and 2 were used to determine the k_{on} for the interaction of TIMP-1 with $E^{375}\rightarrow D$ and gave a value of $(3.38 \pm 0.11) \times 10^6$ M^{-1} s^{-1}, which is similar to that obtained with activated wild-type enzyme [$(3.2 \pm 0.2) \times 10^6$ M^{-1} s^{-1}]. The low activity of $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ produced by Δ(418–631)gelatinase A prevented the determination of their TIMP-1 k_{on} values but did allow approximate K_ds to be obtained using competition

Table 1: Effect of Competition by Latent or Activated Mutant Gelatinase A on the Inhibition of Wild-Type Gelatinase A by TIMP-1[a]

	mutant gelatinase A (nM)[b]	v_s (pM/s)	K_d (pM)[c]
	0	0.08	
proE^{375}→A	0.54	0.1	
	5.4	0.09	
E^{375}→A	0.8	1.94	6.0
	1.2	2.24	4.0
	1.6	2.52	5.2
	2.0	2.68	2.2
E^{375}→Q	0.8	0.78	6.8
	1.2	0.94	12.3
	1.6	1.49	10.1
	2.0	1.65	11.8

[a] Competition experiments were performed as described in Materials and Methods. [b] ProE^{375}→A concentration was determined by A_{280nm} measurement. E^{375}→A and E^{375}→Q were formed by incubation of the mutant proenzymes with Δ(418–631)gelatinase A as described in Materials and Methods and their concentrations determined by densitometric gel scanning of the semipurified solutions. [c] Data were analyzed using eqs 3 and 4 described in Materials and Methods. The initial rate (v_0) was 4.40 pM/s for data obtained in the presence of proE^{375}→A, 5.40 pM/s for the E^{375}→A data set, and 4.84 pM/s for the E^{375}→Q data set.

experiments with wild-type enzyme. The results in Table 1 are corrected for the background activity caused by the contaminating Δ(418–631)gelatinase A and show that proE^{375}→A did not interact with TIMP-1 but that TIMP-1 bound to both E^{375}→A and E^{375}→Q with a K_d in the low picomolar range. To obtain these values, it was assumed that the approximate K_i of TIMP-1 for the wild-type enzyme was 2 pM (Murphy et al., 1992; Willenbrock et al., 1993).

DISCUSSION

Three mutant forms of recombinant progelatinase A possessing a single amino acid substitution at the active site residue E^{375} were purified from medium conditioned by transfected mouse myeloma cells. The alteration to an aspartic acid residue will maintain the negative charge at the enzyme's active site, but its position will be retracted by approximately 1.4 Å because of the loss of a methylene group. The negative charge will be removed by the alanine and glutamine substitutions, although the latter residue will closely match the space-filling characteristics of the original amino acid. Characterization of the mutants centered on determining the effects of these substitutions on proenzyme latency and, following removal of the N-terminal propeptide, on catalytic activity and ability to bind TIMP-1.

It has been proposed that the latency of the proenzyme forms of the matrix metalloproteinases results from an interaction between the active site zinc and the cysteine residue located within the conserved propeptide sequence PRCG(V/N)PD (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990). Latency is lost upon dissolution of this interaction, which allows water to replace the cysteine as the fourth zinc ligand. In accordance with this model, site-directed mutagenesis of the propeptide sequence of transin and human fibroblast collagenase showed that cysteine mutants were susceptible to spontaneous activation (Park et al., 1991; Windsor et al., 1991). Mutants with alterations to the arginine residue immediately N-terminal to the cysteine were equally unstable and led to the proposal that this residue is also involved, possibly interacting via a salt bridge with the active site glutamic acid. If this were true, the proE^{375}→A and proE^{375}→Q mutants described in this paper should possess an incorrectly folded propeptide, but the similarity of their patterns of trypsin digestion as compared with that of the wild-type proenzyme suggests that their conformations are not radically different. In addition, all three mutant proenzymes were fully latent, and proE^{375}→A failed to bind TIMP-1 unless its propeptide was first removed. We conclude, therefore, that E^{375} is not involved in maintaining proenzyme latency.

Mutation of the corresponding glutamic acid residue of the matrix metalloproteinase transin to a glycine has already been described, but the characterization of the resulting protein was limited by the proenzyme's inability to be activated by APMA (Sanchez-Lopez et al., 1988). APMA activation of the matrix metalloproteinases is dependent upon an initial intramolecular autocatalytic processing event (Okada et al., 1988), so the loss of the catalytically essential glutamic acid will hinder propeptide removal. Even when the matrix metalloproteinases are activated by other endoproteinases such as trypsin, an essential component of the process is a final autolytic cleavage to produce the mature active enzyme (Nagase et al., 1990; Crabbe et al., 1992). This problem cannot be circumvented by the expression of N-terminally truncated mutants that lack the propeptide because in our hands this strategy gave rise to incorrectly folded and inactive proteins. In order to study the role of the corresponding glutamic acid residue in gelatinase A, we therefore made the conservative substitution to an aspartic acid, which we reasoned would allow sufficient activity for APMA-induced autolysis to occur. This proved to be correct, with the pattern of proE^{375}→D activation by APMA being similar to that of wild-type progelatinase A except that it took approximately 10 times longer to occur. Once formed, E^{375}→D proceeded to lose its activity at a rate comparable to that of the wild-type enzyme, suggesting that autolysis does not determine the rate of gelatinase A inactivation as it does activation. Active site titration using TIMP-1 demonstrated that the reduced activity of E^{375}→D as compared with the wild-type enzyme was due to a decrease in the catalytic efficiency of the enzyme rather than the production of inactive forms. It is unlikely that substrate binding had been affected because we have shown in separate studies that CT90.435, a low-M_r synthetic inhibitor based on the substrate cleavage site of collagenase and containing a zinc binding ligand (Crabbe et al., 1992), had the same K_i against E^{375}→D as it did against the wild-type enzyme (results not shown). Instead, we believe that a reduction in k_{cat} was responsible, indicating that the spacing of the negative charge within the active site is crucial for maximum turnover of substrate. This agrees with the conclusions drawn on mutational analysis of the corresponding active site glutamic acid of neutral endopeptidase 24.11 (Devault et al., 1988) although, in this case, the E→D substitution gave rise to a catalytically inactive protein. The specific activity of E^{375}→D on denatured type I collagen (gelatin) was less than 1% of wild-type enzyme activity, but comparisons between this substrate and McaPLGLDpaAR are complicated by the fact that, unlike the synthetic substrate assay, the gelatin assay relies upon cleavage at several sites.

Results demonstrating that the k_{on} of the TIMP-1/E^{375}→D interaction is the same as that obtained using activated wild-type enzyme suggested either that E^{375} is not involved in the binding of TIMP-1 or that a negative charge is required but its spacing within the active site is relatively unimportant. In order to discriminate between these two possibilities, we analyzed the binding of TIMP-1 to E^{375}→A and E^{375}→Q. ProE^{375}→A and proE^{375}→Q were expected to be incapable

of performing any autolytic processing, but their incubation at 37 °C in the presence of APMA surprisingly resulted in cleavage to the standard lower M_r forms. Their rate of conversion was extremely slow and was undetectable when the incubation was performed at 23 °C, but it nevertheless indicates that these mutants do possess a degree of activity. The activity could not be otherwise detected, but this was probably due to enzyme inactivation occurring during the prolonged incubation. In order to speed up the formation of $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$, we took advantage of the fact that the propeptide can also be removed by a single cleavage at the N^{80}–Y^{81} peptide bond catalyzed by active gelatinase A (Crabbe et. al., 1993). The concentration dependence of this reaction meant that micromolar levels of both Δ(418–631)gelatinase A and either $proE^{375}\rightarrow A$ or $proE^{375}\rightarrow Q$ were required for there to be a relatively rapid rate of cleavage. It is unlikely that better than 50% conversion to the M_r 66 000 form can be achieved using this method because Δ(418–631)gelatinase A gradually inactivates during the incubation and the M_r 66 000 form is itself converted to the M_r 31 000 fragment. The low but detectable activity of $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ produced by this method suggests that the zinc-bound water molecule can still get ionized when the negative charge at residue 375 is absent. It is possible that another residue nearby can act as a general base and will, to a limited degree, take over the role of E^{375}.

TIMP-1 could be competed off active gelatinase A by increasing concentrations of the low-M_r synthetic inhibitor (results not shown), suggesting that TIMP-1 must in some way interact with the enzyme's active site. The fact that TIMP-1 binds to both $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ with approximately the same affinity as it does the wild-type active enzyme suggests, however, that the glutamic acid residue and its negative charge are not involved in this process. It is also an indication that the amino acid substitutions did not significantly alter the conformation of the active site. The details of how TIMP-1 binds remain unknown, but it is possible, for example, that it is able to interact with the substrate binding pocket. The resolution of this issue will probably require the determination of the tertiary structure of an enzyme/TIMP complex.

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