An analysis of the conformational changes that accompany the activation and inhibition of gelatinase A

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Abstract The latent precursors of the matrix metalloproteinases (MMPs) are converted by (4-aminophenylmercuric)acetate to active forms that lose their propeptide as a result of autolysis. C.D. and an active site mutant of progelatinase A (MMP2) were used to demonstrate that, although propeptide removal is accompanied by a decrease in the enzyme's β-sheet content, the initial activation is achieved with only minor modifications to the conformation. Mixing activated gelatinase A with the natural inhibitor, TIMP-1, resulted in conformational changes that were absent when a synthetic inhibitor was used. The relevance of these results to MMP activation and inhibition is discussed.

Key words: Gelatinase A; Activation; TIMP-1; Inhibition

1. Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases that contribute to the turnover of the extracellular matrix [1]. Interest in the mechanisms that regulate their activity has been stimulated by the proposed role of MMPs in mediating a variety of clinical disorders [2]. The MMPs are secreted from cells as inactive proenzymes whose latency is maintained by an approximately 80 amino acid N-terminal propeptide. Once activated the MMPs are subject to inhibition by one of the three known tissue inhibitors of metalloproteinases (TIMP-1, -2 or -3) [3].

The physiological mechanism of proenzyme activation is most likely to be the removal of the propeptide by serine proteinase or active MMP catalysed hydrolysis [4–6]. The same end-result can be achieved, however, by treatment of the proenzymes with organomercurials such as 4-(aminophenylmercuric)acetate (APMA) [7] and the study of this convenient in vitro system of MMP activation has led to an increased understanding of how proenzyme latency is controlled. The organomercurials bind to a conserved propeptide cysteine and thereby break an interaction that would otherwise exist between this residue and the active site zinc [8–10]. The interaction plays a key role in maintaining proenzyme latency because it prevents the zinc from recruiting a water molecule essential to the catalytic mechanism. Once the active site is liberated there follows a series of autolytic cleavages to the proenzyme that serve to remove the propeptide. The initial cleavage is an intramolecular event because the rate of proenzyme disappearance is not dependent upon its own starting concentration [11–12]. This supports the concept that the addition of APMA must in the first instance generate an active MMP that still possesses a complete propeptide. It has been proposed that the initial activation requires significant alterations to the conformation of the proenzyme [10] and this report sets out to directly demonstrate the existence and extent of any changes that might occur. Gelatinase A (EC 3.4.24.24.; MMP2; 72 kDa gelatinase) is used as a representative member of the family and we have overcome the difficulty of deciding whether a conformational alteration to the proenzyme is the result of activation or the accompanying loss of the propeptide by studying the effect of APMA on $proE^{375}\rightarrow A$. This is an active site mutant of progelatinase A that possesses only 0.01% of the normal proteolytic activity after activation and so displays a correspondingly reduced rate of propeptide removal [13]. We have also used C.D. to investigate whether or not any substantial conformational alteration accompanies the inhibition of gelatinase A by either TIMP-1 or a low molecular mass synthetic inhibitor.

2. Materials and methods

2.1. Materials

Recombinant forms of human progelatinase A, (Δ418–631)progelatinase A, $proE^{375}\rightarrow A$, and human TIMP-1 were purified and their concentrations determined as previously described [13–15]. A peptide hydroxamate derivative that is a reversible inhibitor of MMPs (CT989) was supplied by the Chemistry Department of Celltech Therapeutics Ltd. It has a K_i against gelatinase A of 10 pM [16 (Ref no. 31)].

2.2. Proenzyme activation

Progelatinase A and (Δ418–631)progelatinase A were fully activated by their incubation with 1 mM APMA for at least 2 h at 23°C. The APMA was removed from activated (Δ418–631)gelatinase A by gel filtration using Sephadex G-25. The propeptide-lacking mutant ($E^{375}\rightarrow A$) was generated by incubating 20 μM $proE^{375}\rightarrow A$ with 5 μM activated (Δ418–631)gelatinase A and 1 mM APMA at 37°C for 105 min. $E^{375}\rightarrow A$ was then isolated using heparin Sepharose CL-6B as previously described [13]. All reactions were performed in 25 mM Tris/HCl, 30 mM NaCl, 10 mM $CaCl_2$, pH 7.5 (buffer A).

2.3. Enzyme assay

Gelatinase A activity was assayed by following the increase in fluorescence that accompanied hydrolysis of the synthetic substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3diaminopropionyl]-Ala-Arg-NH_2 (McaPLGLDpaAR) as previously described [17–18] and with a gelatinase A concentration in the assay of 100 pM.

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Abbreviations: MMP, matrix metalloproteinase; APMA, (4-aminophenylmercuric)acetate; TIMP, tissue inhibitor of metalloproteinases; (Δ418–631)progelatinase A, deletion mutant of progelatinase A lacking amino acids 418–631 (C-terminal domain); $proE^{375}\rightarrow A$, active site mutant of progelatinase A; McaPLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2.

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2.4. C.D.

C.D. spectra of samples presented in Buffer A were recorded at 23°C on a JASCO J-600 spectropolarimeter using cells of path lengths 0.02 or 0.05 cm. Molar ellipticity values were calculated using a value of 114 for the mean residue weight. Analysis of the C.D. spectra for secondary structure content was performed using the CONTIN procedure [19].

2.5. SDS-PAGE

Samples taken for analysis were immediately boiled in standard running buffer containing 2% (v/v) 2-mercaptoethanol and 30 mM EDTA and run on precast 10–20% polyacrylamide gels (Daichii, Tokyo). Protein was visualised using Coomassie Brilliant Blue R-250.

3. Results

3.1. APMA-induced activation of progelatinase A

The results in Fig. 1a illustrate how the activation of progelatinase A by APMA is accompanied by a decrease in its molecular mass. At the start of the incubation progelatinase A displayed <0.1% of maximum activity and a molecular mass of 72 kDa. Full activity was achieved after approximately 2 h, at which point the proenzyme had been converted to a 66 kDa form that, according to previous studies, lacks the 80 amino acid N-terminal propeptide [12]. 50% of maximum activity was achieved at a time (~20 min) when approximately half of the proenzyme had been processed. No decrease in molecular mass occurred if the incubation with APMA was performed in the presence of excess quantities of TIMP-1 or the synthetic inhibitor, CT989 (results not shown). An active site mutant of progelatinase A, which has an alanine residue as a replacement for the catalytically essential glutamic acid (proE^{375}→A), has been expressed and purified [13]. The addition of 1 mM APMA was, on its own, unable to alter the position of migration of proE^{375}→A, which remained at 72 kDa throughout a 2 h incubation (Fig. 1b). The 66 kDa form that lacks the propeptide (E^{375}→A) could be generated, however, by a cleavage catalysed by APMA-activated (Δ418–631)gelatinase A. This reaction proceeded at a relatively slow rate that was significantly enhanced if 1 mM APMA was added at the start of the incubation. A 2 h preincubation with APMA did not increase the rate any further but complete propeptide removal could be achieved within 2 h if the incubation temperature was set to 37°C (results not shown). The (Δ418–631)gelatinase A had already been activated to its maximum, so it is most likely that the APMA exerted its effect by increasing the susceptibility of the proE^{375}→A propeptide to intermolecular cleavage. The rate of propeptide loss was still approximately 6 times slower than that displayed by the wild type proenzyme. This presumably reflects the fact that the wild type proenzyme is cleaved by an intramolecular reaction [12], which tends to raise the effective substrate concentration [20].

3.2. Analysis by C.D. of progelatinase A activation

The results in Fig. 2 show that, prior to APMA addition, the C.D. spectrum of progelatinase A is nearly identical to that of proE^{375}→A. This agrees with previously obtained results, which demonstrated that the alteration of this glutamic acid residue had little or no effect on the conformation of the proenzyme [13]. A 2 h incubation of the two proenzymes at 23°C made no difference to their C.D. spectra (results not shown). Analysis of the progelatinase A spectrum over the range 240–205 nm by the CONTIN procedure [19] yielded the following estimates of secondary structure: 0% α-helix, 61% β-sheet and

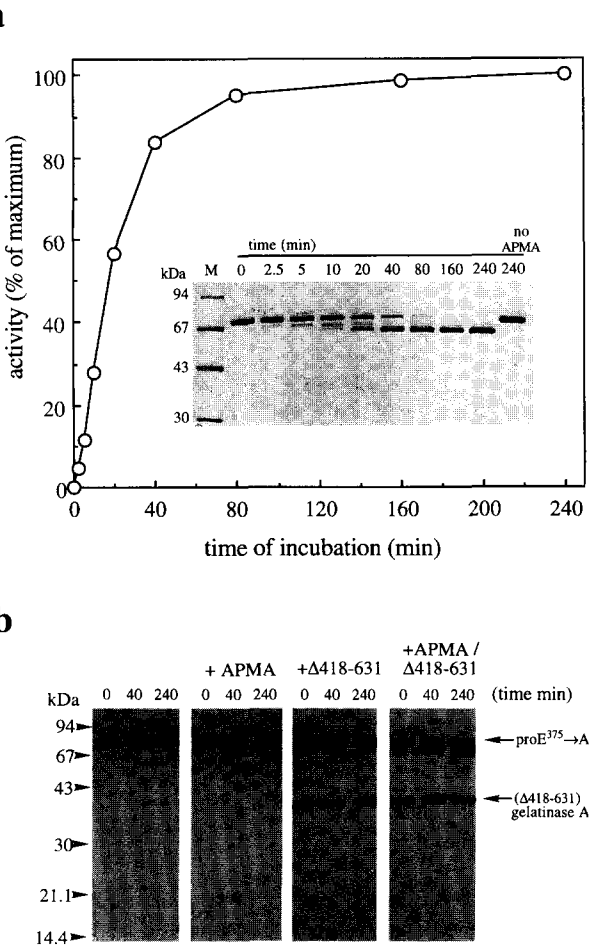

Fig. 1. The removal of the propeptide from progelatinase A. (a) Progelatinase A (2 μM) was incubated with 1 mM APMA at 23°C and, at selected time points, an aliquot was removed and assayed for proteolytic activity as described in section 2. The same aliquots were also analysed by SDS-PAGE (inset). Molecular mass marker proteins were run in lane M. (b) ProE^{375}→A (2 μM) was incubated at 23°C with either no additions, 1 mM APMA, 2 μM activated (Δ418–631)gelatinase A or 1 mM APMA + 2 μM activated (Δ418–631)gelatinase A as indicated. At set time points, aliquots from each incubation were removed and analysed by SDS-PAGE.

39% remainder. These values should be treated with caution in view of the relatively low molar ellipticities and the restricted range of wavelengths over which the analysis was carried out. The C.D. spectrum of wild type progelatinase A displayed an progressive alteration upon addition of APMA that mirrored the gain in enzymic activity and the loss of the propeptide (Fig. 2a). The removal of APMA and propeptide fragments by gel filtration had no effect on the final spectrum (results not shown). According to the CONTIN analysis over the range 240–205 nm, the change in the spectrum corresponds to a decrease in the β-sheet content (to 52%) and an increase in the remainder (to 48%) but, again, these figures must be treated with caution. In contrast, the C.D. spectrum of proE^{375}→A was not altered during the 2 h incubation with APMA (Fig. 2b). Thus the transition of proE^{375}→A from a latent to an 'active' state and the increased susceptibility of its propeptide to autolysis were not accompanied by any significant changes to the secondary structure. The alterations observed with the wild

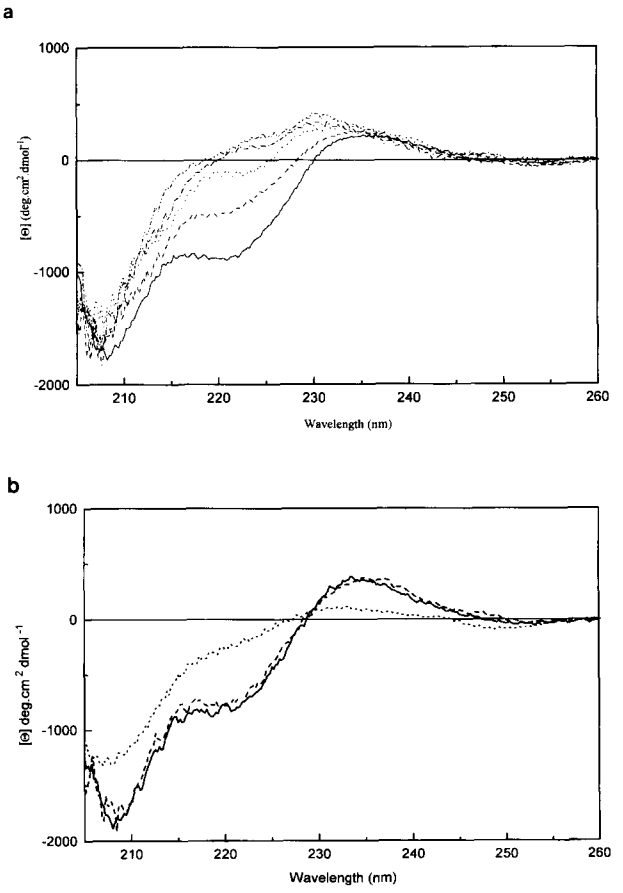

Fig. 2. C.D. analysis of progelatinase A activation. APMA (1 mM) was added to 6 μM solutions of either progelatinase A (a) or proE$^{375}\rightarrow$A (b). In (a) the spectra were recorded before the addition of APMA (solid line) and 30 min (dashed line), 60 min (dotted line), 90 min (dash-dot-dash line) and 120 min (dash-dot-dot-dash line) after the addition of APMA. In (b) the spectra were recorded before the addition of APMA (solid line) and 120 min after the addition of APMA (dashed line). The C.D. profile of a 3 μM solution of E$^{375}\rightarrow$A is also given (dotted line).

type proenzyme must simply have been the result of propeptide removal. In accordance with this proposal, the C.D. profile of E$^{375}\rightarrow$A was similar to the spectra of APMA-treated wild type enzyme (Fig. 2b). It is not an exact match but this might be because the purification method employed did not remove the low levels of contaminating breakdown products that are also generated during the incubation with APMA and (Δ418–631)gelatinase A [13].

3.3. Analysis by C.D. of gelatinase A inhibition

Conformational changes that might accompany gelatinase A inhibition were also studied by C.D. This was done by subtracting the C.D. spectrum of TIMP-1 from the spectrum of an equimolar mixture of TIMP-1 and gelatinase A and comparing the results obtained with those of a control where buffer A replaced inhibitor. There was no difference between the spectra when inhibitor was added to progelatinase A (Fig. 3a); an expected result because the latent proenzyme is unable to interact with either TIMP-1 [13] or synthetic inhibitors [21]. The spectrum of activated wild type gelatinase A and TIMP-1, however, is significantly different to that of the buffer control (Fig. 3b). This result does not indicate as to whether the implied structural change was the result of an alteration to the inhibitor, the enzyme or a combination of both. It is not integral to the process of gelatinase A inhibition, however, because the C.D. spectrum of activated wild type gelatinase A in the presence of excess CT989 was no different from that of the buffer control (results not shown). The spectrum of TIMP-1 (Fig. 3c) is broadly similar to that described previously [22].

4. Discussion

The results which show that the activation of progelatinase A is accompanied by conformational changes detectable by C.D. are similar to those obtained in a study of the APMA-induced activation of prostromelysin-1 [10]. By using an active site mutant, however, we have been able to demonstrate that these changes are not a consequence of the proenzyme converting from a latent to an active state but occur instead as a result of the concomitant dissolution of the propeptide. It is also shown that gelatinase A inhibition can be achieved without the conformational alterations that occur upon TIMP-1 addition.

The crystal structure of progelatinase A has not yet been solved. Even so, the estimates of its secondary structure as determined by C.D. (high β-sheet and negligible α-helix content) are only partly in accord with the recently published crystal structures of porcine fibroblast collagenase [23] and a deletion mutant of human prostromelysin 1 [24]. The C-terminal domain of collagenase is predominantly β-sheet but both structures have regions of α-helix in their catalytic domains that are made up of sequences similar to those found in human gelatinase A. Estimates of α-helix content using the CONTIN procedure are sensitive to the levels of noise in the region between 200 and 190 nm so the discrepancy is probably due to our inability to obtain good quality C.D. data below 205 nm. Our results also suggest that the propeptide of progelatinase A contains some β-sheet. This would make it markedly different to the propeptide of prostromelysin-1, which has 3 short α-helices but no β-sheet [24].

Although APMA destroys the cysteine–zinc interaction of the proenzyme, this event is, on its own, insufficient for activation [10]. An additional contribution is required and it is possible that the organomercurial also disrupts interactions involving amino acids situated upstream of the conserved cysteine [25]. These alterations do not affect the far U.V. C.D. spectrum of the proenzyme but they nevertheless serve to both liberate the active site and perturb the propeptide's conformation such that it becomes subject to rapid autolytic cleavage. It is only after the propeptide has been at least partially removed that the hydrolysis of exogenous substrates can be detected. This is not because access to the active site is still blocked as TIMP-1 and the zinc-chelating inhibitor CT989 were able to prevent APMA-induced autolysis. A more likely explanation is that the propeptide's enforced proximity to the active site will ensure that it is at a considerably higher localised concentration than other substrates [20], which will tend to be excluded from hydrolysis. This also explains why the initial cleavage of the wild type proenzyme is by an intra- and not an intermolecular autolytic event. APMA-induced MMP activation is different,

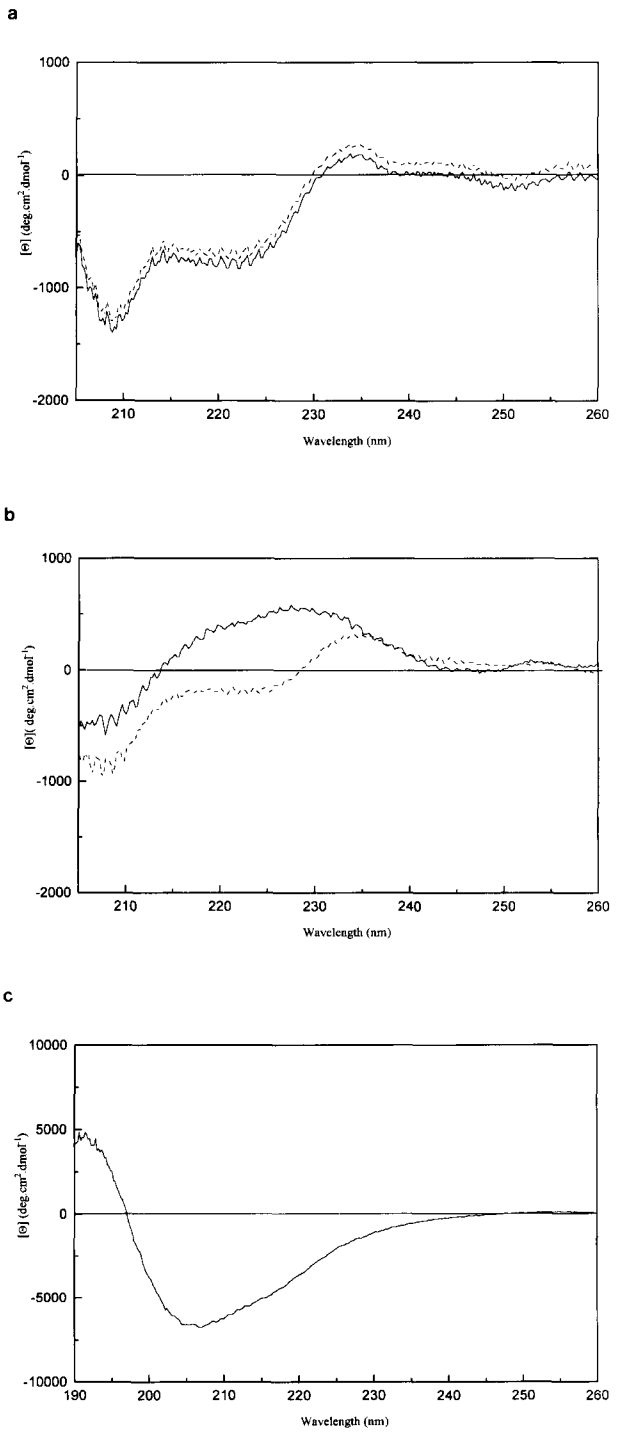

Fig. 3. C.D. analysis of gelatinase A inhibition by TIMP-1. Progelatinase A (a) and activated gelatinase A (b) were incubated for 1 h at 4°C with either an equal concentration (3 μM) of TIMP-1 (dashed line) or the equivalent volume of buffer A (solid line) before being analysed by C.D. The spectra of the TIMP-1 containing samples are corrected for the contribution of TIMP-1 alone (shown as (c)). For clarity in (a) the corrected spectrum of the TIMP-1-containing sample has been offset by +100 deg·cm^2·dmol^{-1}.

therefore, to that brought about by serine proteinases, where propeptide removal is the cause, rather than an effect, of activation [4]. It need not be physiologically irrelevant, however, because the existence of biological systems that activate the MMPs in a manner analogous to the organomercurials cannot be ruled out.

The precise mechanism by which the TIMPs inhibit the MMPs has not yet been elucidated. It is known that they interact with the active site [26] and studies using deletion mutants of the TIMPs and MMPs have shown that secondary interactions occur between other sites of the enzyme and inhibitor that serve to increase the association rate constant [27–28]. The data presented in this report support the view that inhibition by the TIMPs is a complex, multifactorial process because no structural changes detectable by C.D. were observed when CT989 was used to inhibit gelatinase A. Detailed structural studies of the TIMP complex using, for example, X-ray crystallography will be required in order to define the interactions and conformational changes more precisely.

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