We have shown previously that all three fibronectin type II modules of gelatinase A contribute to its gelatin affinity. In the present investigation we have studied the structure and module-module interactions of this gelatin-binding domain by circular dichroism spectroscopy and differential scanning calorimetry. Comparison of the $T_m$ values of the thermal transitions of isolated type II modules with those of bimodular or trimodular proteins has shown that the second type II module is significantly more stable in the trimodular protein coll 123 ($T_m = 44 \, ^\circ C$) than in the single-module protein coll 2 ($T_m = 42 \, ^\circ C$) or in the bimodular proteins coll 23 ($T_m = 47 \, ^\circ C$) and coll 12 ($T_m = 48 \, ^\circ C$). Analysis of the enthalpy changes associated with thermal unfolding of the second type II module suggests that it is stabilized by domain-domain interactions in coll 123. We propose that intimate contacts exist between the three tandem type II units and they form a single gelatin-binding site. Based on the three-dimensional structures of homologous metalloproteinases and type II modules, a model is proposed in which the three type II units form an extension of the substrate binding cleft of gelatinase A.

Proteolytic degradation of constituents of the extracellular matrix and basement membranes plays an important role in tissue restructuring processes such as those accompanying cell migration, morphogenesis, wound healing, angiogenesis, and tumor invasion (Liotta et al., 1991). The proteinases implicated in tumor invasion include components of the urokinase receptor/urokinase/plasminogen system and several members of the metalloproteinase family (He et al., 1989; Matrisian, 1992; Vassalli and Pepper, 1994). Enzymes capable of degrading type IV collagen are crucial for tumor metastasis as this collagen is a major component of basement membranes which have to be penetrated during migration of tumor cells. Significantly, secretion of type IV collagenases is well correlated with metastasis and transformation (Librach et al., 1991; Stetler-Stevenson et al., 1993).

Studies on the primary structures of 72-kDa and 92-kDa type IV collagenases (gelatinase A and B) have revealed that they contain a catalytic domain, a hemopexin-like domain, and three tandem homology units closely related to the type II domains of fibronectin (Collier et al., 1988; Wilhelm et al., 1989). The hemopexin-like domain of gelatinase A has been shown to be involved in modulation of its activity by TIMPs, the tissue inhibitors of metalloproteinases (Murphy et al., 1992; Fridman et al., 1992). This region is also required for the activation of progelatinase A by a membrane-associated activator, suggesting that it may interact with some component of the cell membrane (Murphy et al., 1992; Vassalli and Pepper, 1994).

Studies on the substrate specificity of gelatinases have shown that they are able to degrade type IV, type V, type VII, and type X collagens, fibronectin, elastin, and all types of denatured collagens (Murphy et al., 1991; Senior et al., 1991; Matrisian, 1992). Gelatinases are unique among metalloproteinases in having pronounced affinity for denatured collagens. Recently it was shown that the gelatin-binding sites of gelatinases reside in their fibronectin-related regions. Recombinant proteins corresponding to the fibronectin-related regions of gelatinase A and B were found to have high affinity for gelatin (Bánya and Patthy, 1993; Collier et al., 1992; Bánya et al., 1994). Conversely, recombinant gelatinase A lacking the fibronectin type II units was shown to be devoid of affinity for gelatin or type I and type IV collagens, indicating that the fibronectin-like domain is the sole site of collagen binding (Murphy et al., 1994; Allan et al., 1995).

The functional significance of the gelatin-binding site is suggested by the observation that although deletion of this domain does not affect the catalytic properties of gelatinase A on small synthetic substrates (Ye et al., 1995), activity on gelatin is drastically reduced and the cleavage pattern of type IV collagen is altered (Murphy et al., 1994; Ye et al., 1995). On the basis of these observations, it has been proposed that the fibronectin-like domain of gelatinase A specifically orients the enzyme on type I gelatin or type IV collagen, thus enhancing the rate of substrate cleavage (Murphy et al., 1994).

To clarify the role of the collagen-binding domain, we have initiated studies to define its interaction with substrates. In a previous paper (Bánya et al., 1994), we described the expression of type II domains of the fibronectin-related region of gelatinase A in Escherichia coli and the characterization of their gelatin-binding properties. We have shown that although each of the three type II domains binds gelatin, proteins containing all three tandem type II domains of gelatinase A have significantly higher affinity than any of the constituent units: all three type II units contribute to gelatin binding. In the present work, we have studied the collagen-binding domain by circular dichroism spectroscopy and differential scanning calorimetry to elucidate the structure and interactions of the three type II modules.
**Gelatin-binding Site of 72-kDa Gelatinase**

**FIG. 1.** Circular dichroism spectra of DEL-galcoll 3 (---), DEL-galcoll 123 (-- - - ), coll 123 (--), and reduced-alkylated DEL-galcoll123 (--- - - -). Spectra were obtained in 10 mM Tris-HCl, pH 8.0 at 25 °C.

**FIG. 2.** Alignment and secondary structure of the fibronectin type II domains of several proteins. The secondary structure predicted by the PHD program (Rost and Sander, 1994): the secondary structure predicted by the PHD program (Rost and Sander, 1994). In the present work, the N-terminal peptides of the DEL-galcoll proteins (1 22% linearpolyacrylamidegradualslabgels (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue G-250.

**FIG. 3.** Unfolding of DEL-galcoll 1 (*), DEL-galcoll 2 (\(\odot\)), DEL-galcoll 3 (\(\triangle\)), and DEL-galcoll 123 (\(\Delta\)) with guanidine HCl (A) and urea (B). Changes in the CD of the proteins were monitored at 224 nm in 10 mM Tris-HCl buffer, pH 8.0 at 25 °C.

and lyophilized. SDS-polyacrylamide gel electrophoresis analysis of the elastase-digested recombinant proteins (coll proteins) indicated a 5-kDa reduction in molecular mass compared with that of DELgalcoll proteins (data not shown). Sequence analyses have shown that elastase cleavage occurred at Ala-34 of the \(\beta\)-galactosidase region of the DELgalcoll fusion proteins.

Reduced-alkylated DELgalcoll 123 was prepared as follows: DELgalcoll 123 (0.025 mM) was dissolved in 0.1 M guanidinium HCl, 5 mM EDTA, 50 mM dithiothreitol, pH 8.0, and the solution was incubated at 25 °C for 30 min. The reduced-denatured protein was alkylated with iodoacetic acid (110 mM). The alkylated protein was desalted by gel filtration on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate, pH 8.0, and the protein was lyophilized. For some experiments, reduction-alkylation of DELgalcoll 123 was carried out in a similar way, except that guanidinium HCl was omitted from the reaction mixture.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins—**Recombinant proteins DELgalcoll 1, DELgalcoll 2, DELgalcoll 3, DELgalcoll 12, DELgalcoll 23, and DELgalcoll 123 containing different segments of the collagen-binding site of human gelatinase A and a 37-amino acid-long N-terminal peptide derived from the \(\beta\)-galactosidase moiety of the expression vector (Bánya, et al., 1991) were produced in E. coli. The properly folded, functionally homogeneous recombinant DELgalcoll proteins were isolated by binding to gelatin-Sepharose 4B columns and elution with a 0–8 M urea gradient as described previously (Bánya, et al., 1994). We have shown previously that the \(\beta\)-galactosidase moiety may be removed by limited elastase digestion, whereas the regions containing the properly folded type II domains are resistant to elastase (Bánya, et al., 1994). In the present work, the N-terminal peptides of the DELgalcoll fusion proteins were removed as follows: the DELgalcoll proteins (1 mg/ml) were dissolved in 0.1 M ammonium bicarbonate, pH 8.0 buffer and were incubated with 0.01 mg/ml elastase (Serva) at 25 °C for 60 min. Reaction was arrested with 2 mM phenylmethylsulfonyl fluoride (Serva), the digest was applied to a gelatin-Sepharose 4B column, and the bound protein was eluted with a urea gradient. Protein eluted at the same urea concentration as the starting material was pooled, dialyzed against 0.1 M Tris-HCl, pH 8.0 at 25 °C.

**Circular Dichroism Spectroscopy—**CD spectra were measured over the range of 190–250 nm by using a JASCO J-720 spectropolarimeter thermostatted with a Neslab RT-100 water bath. The CD spectropolarimeter and the optical cells were calibrated with recrystallized d-10-camphorsulfonic acid. The measurements were carried out in 1-mm path length cells and protein solutions of approximately 0.5–1.0 mg/ml. All spectra were measured at 25 °C with a 16-s time constant and a scan rate of 1.0 nm/s.
mains were predicted by the method of Chou and Fasman (1978). Calorimetric measurements were carried out by the procedure of Rost and Sander (1994) using the PHD program.

Protein Modeling—Homology modeling of the catalytic domain and type II domains of gelatinase A was carried out at the Glaxo Institute for Molecular Biology SA using the Swiss-Model Automated Protein Modelling Service (Pertsch, 1995). Protein structure prediction by the Swiss-Model is based on the principle that homologous proteins with a high sequence similarity are characterized by distinct structural similarity. Swiss-Model first searches the Brookhaven Protein Data Bank for proteins that show a significant sequence similarity to the target protein. The framework structure of the model is produced by aligning the target sequence with the selected template sequences using a combination of sequence alignment tools and three-dimensional superposition. Gaps in the framework structure are then filled by structural similarity searches through the Brookhaven Data Bank (Pertsch, 1995). In the present work, the catalytic domain of gelatinase A was modeled using the coordinates of fibroblast collagenase, MMP-1 (Lovejoy et al., 1994a, 1994b; Spurlino et al., 1994), neutrophil collagenase, MMP-8 (Stans et al., 1994), and stromelysin, MMP-3 (Goody et al., 1994). The three type II domains of gelatinase A were modeled using the coordinates of the second type II domain of PDC-109 (Constantine et al., 1992).

RESULTS

The far UV CD spectra of DELgalcoll123 (containing the entire fibronectin-related part of human gelatinase A), as well as the CD spectra of recombinant proteins containing single type II modules are characterized by maxima at 224 nm and minima at 198 nm (Fig. 1). Removal of the β-galactosidase fusion peptides had no major effect on the CD spectra of these proteins (cf. DELgalcoll123 and coll123 in Fig. 1). Analysis of the CD spectra has shown that the fibronectin-related domain of gelatinase A consists of about 32% β-sheet, 19% β-turn with no detectable α helix. Recombinant proteins coll1, coll2, and coll3 were estimated to contain 30%, 30%, and 31% β-sheet, 28%, 27%, and 23% β-turn structures, respectively.

The estimated secondary structures of type II domains of gelatinase A are in good agreement with the predictions based on their homology with the second type II domain of PDC-109. The solution structure of the latter protein has been solved by NMR spectroscopy (Constantine et al., 1992), and these studies have revealed the presence of two short antiparallel β-sheets connected by β-turns, with no evidence for α helix. Secondary structure predictions by the method of Chou and Fasman

Table I

|             | Domain 1 | Domain 2 | Domain 3 |
|-------------|----------|----------|----------|
| DELgalcoll1| 0.64 ± 0.8 | 0.57 ± 0.7 | 0.87 ± 0.7 |
| coll1      |           |          |          |
| DELgalcoll2| 4.40 ± 0.5 | 4.37 ± 0.7 | 4.45     |
| coll2      |           |          |          |
| DELgalcoll3| 7.99 ± 0.6 | 8.20 ± 0.9 | 8.18 ± 0.8 |
| coll3      |           |          |          |
| DELgalcoll12| 5.89 ± 0.7| 4.79 ± 0.3 | 4.78 ± 0.7 |
| coll12     |           |          |          |
| DELgalcoll23| 6.08 ± 0.8| 4.77 ± 0.6 | 4.78 ± 0.7 |
| coll23     |           |          |          |
| DELgalcoll13| 6.00 ± 0.9| 7.61 ± 0.8 | 7.61 ± 0.8 |
| coll13     |           |          |          |
| DELgalcoll123| 6.24 ± 0.8| 5.22 ± 0.5 | 5.75 ± 0.5 |
| coll123    |           |          |          |
| DELgalcoll123| 6.02 ± 0.7| 5.37 ± 0.5 | 5.71 ± 0.5 |
| coll123*   | 6.32 ± 0.8| 5.62 ± 0.3 | 7.39 ± 0.7 |

The abbreviation used is: MMP, matrix metalloproteinase.
(1978) also suggested the presence of β-turns and β-sheets but no α helix in fibronectin type II domains (Patthy et al., 1984). This conclusion was confirmed and extended in the present work for type II modules of gelatinase A and B as well as PDC-109 (Esch et al., 1983), BSP A3 (Seidah et al., 1987), mannos6-phosphate receptor (Morgan et al., 1987), blood coagulation factor XII (McMullen and Fujikawa, 1985), hepatocyte growth factor activator (Miyazawa et al., 1993), mannos receptor (Taylor et al., 1990), and phospholipase receptor (Ishizaki et al., 1994) using the multiple alignment-based method of Rost and Sander (1994). In agreement with our earlier findings (Patthy et al., 1984), the type II modules of these proteins are predicted to have four short β-strands in the vicinity of their four half-cystines and an additional β-strand is predicted in the N-terminal extension that is present only in some of the type II units (Fig. 2). It is noteworthy that the predicted β-strands align with the four β-strands determined experimentally for PDC-109 domain 2. The CD spectra of type II modules are similar to those of the related kringle domains inasmuch as they also have characteristic maxima near 225 nm (Castellino et al., 1986). In accordance with the homology of kringles and type II domains (Patthy et al., 1984), both are characterized by the presence of antiparallel β-sheets and β-turns (Constantine et al., 1992).

The maxima at 224 nm are characteristic of the ordered, native structure of the type II modules of the gelatin-binding domain. For example, denaturation of DELβgalcoll 1, DELβgalcoll 2, and DELβgalcoll 3 to denaturant-induced unfolding is markedly different: the midpoints of unfolding with guanidinium hydrochloride are 0.3 M, 1.6 M, and 4.8 M for DELβgalcoll 2, DELβgalcoll 1, and DELβgalcoll 3, respectively. The order of sensitivity of type II domains to urea-induced unfolding is similar: the midpoints of unfolding with this denaturant are 1.7 M and 6.5 M for DELβgalcoll 2 and DELβgalcoll 1, respectively. The most stable DELβgalcoll 3 is not unfolded even in 8 M urea.

It may be pointed out that unfolding of DELβgalcoll 123 with guanidinium HCl occurs in three steps, the midpoints of these steps coincide with unfolding of the individual type II units (Fig. 3A). In contrast with this, unfolding of DELβgalcoll 123 with urea is not detectable below 3 M urea, even though unfolding of DELβgalcoll 2 is practically complete at this concentration (Fig. 3B). It seems likely that interactions in DELβgalcoll 123 increase the stability of the second type II domain to urea-induced unfolding.

Thermal unfolding was also monitored by changes in the CD spectra of type II modules at 224 nm. These studies have shown that the three type II modules show marked differences in their thermal stability (Fig. 4 and Table I). The least stable is the second type II module with a Tm value of 44 °C, the most stable is the third module with a Tm of 80 °C. Differences in their stability are retained in the recombinant proteins that contain two or three type II domains, permitting the resolution of the thermal transitions into distinct components (cf. Fig. 4 and Table I). From a comparison of recombinant proteins containing the N-terminal fusion peptide (DELβgalcoll series) with those lacking this segment (coll series), it is also clear that the N-terminal peptide does not have a major effect on the thermal transition of type II modules (Table I).

When the Tm values of the second type II module of different recombinant proteins are compared (Table I), it is clear that neighboring modules have a marked influence on its thermal stability. This is most obvious in the comparison of the Tm value (44 °C) of type II unit 2 of coll 2 with its Tm value (54 °C) in full-length collagen-binding domain (coll 123): the presence of module 1 and module 3 increases its Tm value by 10 °C. The presence of module 1 or module 3 alone (in coll 12 or coll 23) causes a smaller but significant increase in thermal stability of module 2 (Table I). It thus seems likely that the second type II
Differential scanning calorimetry studies (e.g. Fig. 5) yielded \( T_m \) values similar to those obtained by monitoring changes in CD spectra (Table I). The melting curves of the individual domains are each well described by single two-state transitions, consistent with their \( \Delta H_{cal} / \Delta H_{vH} \) ratios close to unity (data not shown). Comparison of the enthalpy changes of the unfolding of type II domain 2 in coll 123 (\( \Delta H_{cal} = 58 \text{ kcal/mol} \)) and coll 2 (\( \Delta H_{cal} = 49 \text{ kcal/mol} \)) indicates that domain-domain interactions in coll 123 significantly stabilize the folded state of domain 2.

**DISCUSSION**

In the present investigation we have shown by analysis of the CD spectra of recombinant proteins that the type II domains of the gelatin-binding site of type IV collagenase have a protein-fold characterized by a high content of \( \beta \)-structures, with no \( \alpha \)-helix. This observation is in harmony with structural information obtained on a related type II domain, the second unit of the bovine seminal fluid protein PDC-109 (Constantine et al., 1992). Secondary structure prediction from multiple alignments of type II units also supports the presence of \( \beta \)-structures but no \( \alpha \)-helix (Patthy et al. (1984) and the present study).

CD spectroscopy and differential scanning microcalorimetry of the collagen-binding domain has shown that the thermal stability of the second type II module is significantly increased by its interactions with the first and third modules: its \( T_m \) value is increased from 44 °C to 54 °C. The fact that the stability of the second type II domain to urea-induced unfolding is increased by the presence of the other two type II units also suggests that these units are involved in tight interactions. It seems likely that these interactions between the tandem modules permit little flexibility in their relative orientation at physiological temperatures. In view of the fact that all three
type II units contribute to gelatin binding (Bányai et al., 1994), we propose that their fixed arrangement facilitates the tight binding of a single substrate molecule.

The binding of the substrate by the type II domains may play a crucial role in the proper positioning of these substrates relative to the active site cleft of gelatinase A. Although the three-dimensional structure of gelatinase A has not yet been determined, structures of proteins homologous to its three constituent domains: the catalytic domain (Lovejoy et al., 1994a, 1994b; Borkakoti et al., 1994; Gooley et al., 1994; Stams et al., 1994; Bode et al., 1994), hemopexin-like domain (Faber et al., 1995; Li et al., 1995), and fibronectin type II domain (Constantine et al., 1992) have been determined recently.

The catalytic domains of other members of the matrixin family of metzincins MMP-1, MMP-3, and MMP-8 (Lovejoy et al., 1994a, 1994b; Borkakoti et al., 1994; Gooley et al., 1994; Stams et al., 1994; Bode et al., 1994; Stöcker and Bode, 1995) have been shown to have very similar three-dimensional structures. They all comprise a regularly folded “upper” subdomain consisting of a twisted five-stranded β-sheet flanked by two α helices and connecting loops, and a smaller, less regularly folded, “lower” subdomain comprising two open loops and the C-terminal helix. The catalytic zinc ion residing at the bottom of the active-site cleft between these subdomains is bound by a characteristic helix-bend-loop structure comprising the HEXX-HXXGGXHH consensus sequence. The catalytic zinc ion is coordinated by the imidazolyl N2 atoms of the three consensus histidine residues and by a water molecule that is also bound to a glutamate (Bode et al., 1994; Stöcker and Bode, 1995).

On the basis of the close homology of gelatinase A (MMP-2) with other matrix metalloproteinases, it is safe to assume that its three-dimensional structure is also similar to these enzymes, except that three fibronectin-related type II units are inserted at the N-terminal boundary of the active site helix of gelatinase A, seven residues upstream of the HEXX-HXXGGXHH consensus sequence motif. Homology modeling of the catalytic domain of gelatinase A indicates that the type II domains are inserted at the right-hand end of the active-site cleft, close to the S1 pocket of the enzyme (Fig. 6). In view of the proximity of the collagen-binding type II units to the active site cleft, it is conceivable that they are crucial for properly orienting the substrate relative to the catalytic site, presenting the scissile bond to the active site of the enzyme. Such a role of the collagen-binding domain is supported by the observation that the cleavage pattern of type IV collagen is altered in a mutant gelatinase A lacking this domain (Murphy et al., 1994).

In accordance with the experimental data, we suggest a model in which the three type II units form an extension of the substrate binding cleft of gelatinase A (Fig. 6).

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