The C-terminal Domain of Matrilin-2 Assembles into a Three-stranded α-Helical Coiled Coil*

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Matrilin-2 is a member of von Willebrand factor A containing extracellular matrix proteins in which the cDNA-derived sequence shows similar domain organization to cartilage matrix protein/matrilin-1, but information on the protein structure is limited. Here we studied the oligomerization potential of a synthetic peptide NH$_2$-ENLILFQNVANEEVRKLTQRLEEMTQRMEALE-NRLKYR-COOH corresponding to the C-terminal sequence of mouse matrilin-2. The central portion of this sequence shows a periodicity of hydrophobic residues occupying positions *a* and *d* of a heptad pattern (*abcdefg*)$_n$, which is characteristic for α-helical coiled-coil proteins. Circular dichroism spectroscopy revealed a high α-helical content, and the shape of the spectra is indicative for a coiled-coil conformation. Chemical cross-linking and size exclusion chromatography suggest a homotrimeric configuration. Thermal denaturation in benign buffer shows a single cooperative transition with $\Delta H^0 = -375$ kJ/mol. Melting temperatures T_m varied from 38 to 51 °C within a concentration range of 10 to 85 μM, which is about 35 °C lower than determined for a peptide corresponding to the C-terminal domain of matrilin-1. The data suggest that despite the low sequence identity within this region, matrilin-2 will form a homotrimer as matrilin-1 does.

Matrilins form a subfamily of extracellular matrix proteins containing von Willebrand factor A-like domains. Its prototype member, matrilin-1 (also known as cartilage matrix protein), is specifically localized in some types of the hyaline cartilage (1) where it can interact with aggrecan and collagen type II fibrils (2–4), but it can also form a filamentous network by itself (5). Its primary structure contains two N-terminal sequence segments with similarities to the von Willebrand factor A domain separated by an epidermal growth factor-like domain and a short unique C-terminal domain (6–9) that was recently found to be responsible for the oligomerization into homotrimers by assembling into a three-stranded α-helical coiled coil (4, 10, 11). Meanwhile, cDNA-derived sequences of two different gene products have been established that show a similar domain structure and are consequently named matrilin-2 and matrilin-3 (12–14). Within the matrilin-2 sequence, the two von Willebrand factor domains are separated by 10 epidermal growth factor-like domains, whereas matrilin-3 lacks the sec-

ond von Willebrand factor domain and contains four epidermal growth factor-like tandem repeats. In contrast to matrilin-1, matrilin-2 is absent from epiphyseal and other cartilages, but is found in high abundance in the limbs, calvaria, uterus, and heart and in lower amounts in skeletal muscle, brain, and skin (12). Expression studies suggest a similar tissue distribution for matrilin-3 as found for matrilin-1. It mainly co-localizes with collagen type II, especially in the periphery of cartilage in sternum, femur, and trachea (13, 14).

Currently the oligomerization state of the new matrilins is unknown. Preliminary data from SDS-polyacrylamide gel electrophoresis run under nonreducing conditions revealed only that matrilin-2 is not a monomeric protein (12). Although the overall sequence similarity between the different matrilins suggests a common evolutionary origin, it is lowest for the C-terminal domain. Only 11 and 13 of the 38 C-terminal residues of matrilin-2 and -3, respectively, are identical with those of matrilin-1 of the same species, and only two residues are identical in all known matrilin sequences in corresponding positions. To elucidate the oligomerization potential of matrilin-2, we synthesized a peptide corresponding to the 38 C-terminal residues of mouse matrilin-2 and analyzed it by circular dichroism (CD)[1] spectroscopy, chemical cross-linking, and gel filtration chromatography. Our data indicate that this sequence domain can assemble into a homotrimeric α-helical coiled coil similar to matrilin-1 although with a lower thermal stability.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Peptide synthesis was performed by solid-phase chemistry on a Milligen/Biosearch model 9050 synthesizer using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. The five arginine residues were protected by pentamethylchromansulfonyl. Cleavage from the resin and deprotection were carried out applying a two-step procedure as described (11). Purification was performed by reversed phase HPLC using a YMC C18 column (20 × 250 mm) eluted with a linear binary gradient of acetonitrile/water from 25 to 50% containing 0.1% trifluoroacetic acid (7 ml/min) where the peptide eluted at around 45% acetonitrile/water. The purified peptide gave a single absorption peak at 220 nm when analyzed on an analytical C18 column. Peptide identity was confirmed by laser desorption mass spectrometry performed by the Protein and Carbohydrate Structure Facility of the University of Michigan (Ann Arbor, MI). Concentrations were determined spectrophotometrically assuming $A_{1\,cm}^{1\%} = 3.32$ at 276 nm as calculated from the amino acid composition (15).

Circular Dichroism Spectroscopy and Thermodynamic Analysis—CD spectra were recorded on an Aviv model 62DS spectrophotometer equipped with a five-cell holder and a Hewlett-Packard Peltier temperature controller. Spectra are normalized to mean residue ellipticities ([Θ]$_{MRW}$) with $M_r = 124$ as derived from the sequence. Thermal tran-

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[1] The abbreviations used are: CD, circular dichroism; BS^3, bis(sulfosuccinimidyl)suberate; MTR2-C38, peptide corresponding to the 38 C-terminal residues of mouse matrilin-2; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

sition curves were recorded at 222 nm from 0 to 85 °C in 0.2 °C intervals with a 0.2-min equilibration time. They were normalized to the fraction of folded peptide F with $F = (\Theta - \Theta_u(T))/(\Theta_n(T) - \Theta_u(T))$ where Θ_n and Θ_u represent ellipticities of the fully folded and unfolded species, respectively, corrected for their temperature dependence by linear extrapolation of the low and high temperature baselines, and Θ is the observed ellipticity.

Transition curves were interpreted assuming a two-state mechanism in which three unfolded chains u combine to a native α-helical coiled-coil trimer n (16). With the total peptide concentration $c_0 = c_u + 3c_n$ and the degree of conversion to the coiled-coil $F = 3c_n/c_0$, the equilibrium constant K follows as $K = c_n/c_u^3 = F/3c_0^2(1-F)^3$. With $\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0$, it follows that at the transition midpoint ($F = 0.5$ and $T = T_m$) (Equation 1),

$$\frac{1}{T_m} = \frac{\Delta S^0 + R\ln(0.75 c_0^2)}{\Delta H^0} \qquad \text{(Eq. 1)}$$

where ΔG^0, ΔH^0, ΔS^0, R, and T are the standard free energy, enthalpy, entropy, gas constant, and absolute temperature, respectively. Thus, ΔH^0 was calculated from the slope of $1/T_m$ *versus* $\ln(0.75 c_0^2)$ for T_m determined at different peptide concentrations. From single transition curves, T_m and ΔH^0 were evaluated by a nonlinear least-squares Marquardt-Levenberg fitting algorithm (Equation 2) of

$$\ln K(T) = \frac{\Delta H^0}{RT}\left(\frac{T}{T_m} - 1\right) - \ln(0.75 c_0^2) \qquad \text{(Eq. 2)}$$

with ΔH^0 and T_m as variables.

Crosslinking, Gel Electrophoresis, and Gel Filtration—Chemical cross-linking was performed at different ionic strengths adjusted by NaCl using bis(sulfosuccinimidyl)suberate BS^3 and disuccinimidyl glutarate (Pierce), which are homobifunctional *N*-hydroxysuccinimide ester analogs with a spacer arm length of 1.14 and 0.77 nm, respectively. The peptide ($c_{\text{final}} = 100 \ \mu\text{M}$, in 20 mM KH$_2PO_4$/NaOH, pH 7.2, plus NaCl) was incubated at various cross-linker concentrations for 1 h at 25 °C. The reaction was stopped by adding a 10-fold excess of Tris-HCl contained in the sample buffer, and the aggregation state was analyzed by Tris/Tricine SDS gel electrophoresis (17) using 16% acrylamide gels containing 5 M urea and a 3% stacking gel. Gels were stained with Coomassie Brilliant Blue G250 in 5% formaldehyde added to keep the peptide in the gel (18).

Gel filtration was performed using a Superdex 75 prep grade column (1×12 cm; Amersham Pharmacia Biotech) equilibrated in 0.25 M NaCl, 20 mM KH$_2$PO$_4$/NaOH, pH 7.2, at 4 °C. The column was eluted at 12 ml/h and calibrated using standard proteins of known viscosity radius, and results were analyzed as described (11, 19).

RESULTS

Peptide Design and Coiled-coil Prediction—Although the overall domain structure of matrilin-2 is similar to that of matrilin-1, the low sequence similarity within its C-terminal domain (Fig. 1) makes it uncertain whether the oligomerization state is the same. The common 3-4-3-4 spacing of hydrophobic residues allows to predict that in matrilin-2 this sequence region will also form an α-helical coiled coil. When analyzed by different algorithms, the probability for coiled-coil formation is similarly high for both protein domains. The MultiCoil program (20), aimed to differentiate between dimeric and trimeric coiled coils, assigns the highest probabilities for a two-stranded conformation.

To determine the secondary structure, stability, and oligomerization state of matrilin-2, we synthesized a peptide, designated MTR2-C38, corresponding to the 38 C-terminal residues of the mouse sequence (Fig. 1). Within the mature protein, the first peptide residue is adjacent to Cys-Lys-Cys-918, the cysteines of which might form interchain disulfide bridges as it was shown for the corresponding residues of chicken matrilin-1 (10, 23). The sequence differs from that of human matrilin-2 by three structurally related residues (L923M, V927L, K954R). All four coiled-coil predictions agree in their assignment of heptad positions $(abcdefg)_n$ to each residue where positions a and d are occupied by hydrophobic amino acids except for Gln-25 (Fig. 1). Within an α-helical coiled coil, these
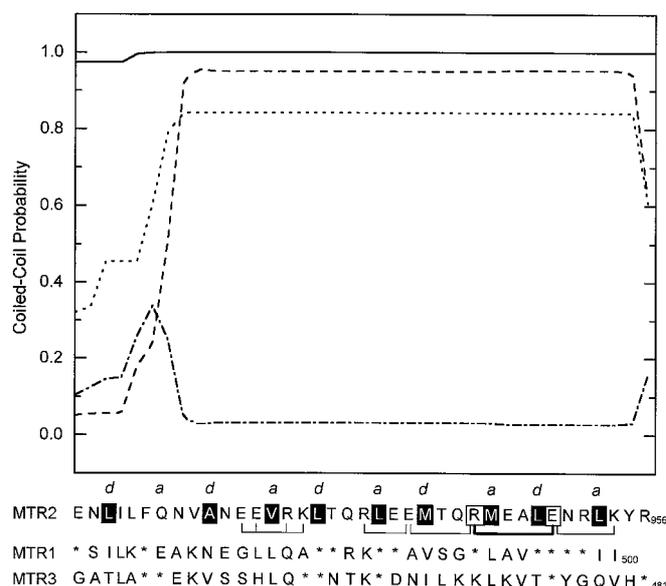
FIG. 1. **Coiled-coil probability of the C-terminal domain of mouse matrilin-2.** The sequence of the 38 C-terminal residues was analyzed for its coiled-coil-forming potential using the programs Coils (*solid line*) (21), version 2.2, applying the MTIDK matrix and using a 2.5 weight on residues in heptad positions a and d, PairCoil (*dashed line*) (22), and MultiCoil (20), which differentiates between the possibilities of forming a two-stranded (*dotted line*) or three-stranded (*dashed-dotted line*) conformation. In each case, a window size of 28 residues was used, and all algorithms agreed in the assignment of uninterrupted heptad positions as indicated above the sequence corresponding to peptide MTR2-C38. The first peptide residue corresponds to position 919 of the full-length sequence (12). Putative intrachain (spacing $i \to i + 3$; $i \to i + 4$, *brackets*) and interchain (heptad positions g-$2d'$, *boxed*, *thick bracket*) ionic interactions are indicated. Hydrophobic residues in heptad positions a and d are printed in *reverse font*. For comparison, the 36 and 38 C-terminal residues of mouse matrilin-1/cartilage matrix protein (MTR1, Ref. 9) and matrilin-3 (MTR3, Ref. 14), respectively, are included. *Asterisks* denote residues identical with MTR2, highlighting the low sequence identity within this region.

residues come into close contact as knobs filling holes in the center and stabilize the oligomer by hydrophobic interactions (for details, see *e.g.* Ref. 24). Further stabilization can arise from intrahelical ionic interactions between oppositely charged side chains of the type $i \to i + 3$ and $i \to i + 4$ (Fig. 1) (25). The single putative interchain ionic pair Arg-945/Asp-950 might be crucial for oligomerization specificity and chain orientation as it was found for a corresponding peptide resembling the matrilin-1 C-terminal domain (26).

Secondary Structure of Peptide MTR2-C38—When analyzed by far ultraviolet CD spectroscopy, MTR2-C38 shows a spectrum characteristic for a high α-helical content with extrema around 192, 208, and 222 nm (Fig. 2), although the amplitudes are slightly lower than observed for a corresponding peptide resembling the matrilin-1 C-terminal domain (11). In 50% trifluoroethanol, which disrupts the tertiary and quaternary structure and stabilizes single α-helices (27), the amplitudes increase, specifically at 208 nm. The relatively small increase indicates that the peptide is almost fully α-helical in benign buffer. As the n-π^* transition (222 nm) is mainly indicative for the α-helical content, whereas the π-π^* transition (208 nm) polarizes parallel to the helix axis, the ellipticity ratio $[\Theta]_{222}/[\Theta]_{208}$ reflects whether the α-helix is monomeric or forms a coiled coil (28, 29). For MTR2-C38, this ratio significantly depends on the concentration and is greater than 0.98 at peptide concentrations above 20 μM, which is compatible with the assumption that MTR2-C38 forms an α-helical coiled coil (Fig. 2, *inset*). The lower ratio of 0.87 observed at 5 μM might indicate some dissociation that is also reflected in the lower ellipticities
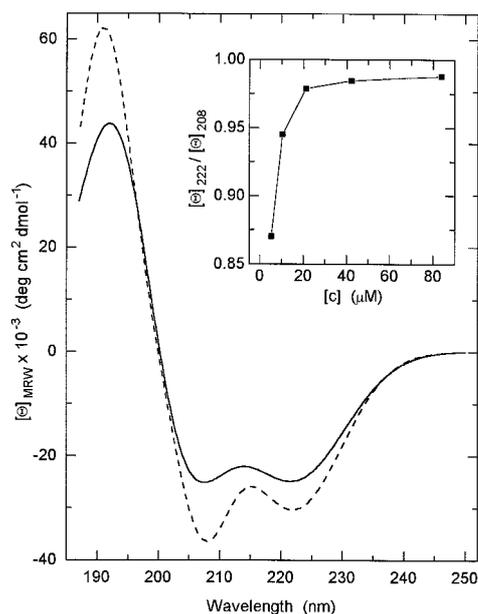
FIG. 2. **CD spectra of peptide MTR2-C38.** Spectra were recorded at 0 °C in the absence (*solid line*) and presence (*dashed line*) of 50% trifluoroethanol (peptide concentration: 20 μM in 150 mM NaF, 20 mM KH$_2$PO$_4$/NaOH, pH 7.2). The *inset* shows the ellipticity ratios $[\Theta]_{222}/[\Theta]_{208}$ determined at different peptide concentrations in the same buffer without trifluoroethanol.

observed (data not shown).

Oligomerization State of Peptide MTR2-C38—The specific homotrimeric association of matrilin-1 has been shown by electron microscopy of the reduced and unreduced native protein (4), site-directed mutagenesis on recombinantly overexpressed mini-gene matrilin-1 proteins (10), and analysis of peptides by analytical ultracentrifugation, size exclusion chromatography, and chemical cross-linking (11, 26). To test whether despite the low sequence identity (Fig. 1), matrilin-2 can also assemble into a homotrimer, we performed cross-linking studies on MTR2-C38 using BS^3 and disuccinimidyl glutarate, which differ in their spacer arm length. Fig. 3A shows the reaction products analyzed by SDS gel electrophoresis. At all cross-linker concentrations, major bands corresponding to a monomeric, dimeric, and trimeric state appeared, but small amounts of higher oligomerization states were detected at increased cross-linker concentrations. The migration pattern is essentially uneffected from the ionic strength in the 100–750 mM NaCl range (not shown).

To test whether the different gel bands observed after cross-linking reflect a heterogeneous population of oligomers in solution, the noncross-linked peptide was analyzed by size exclusion chromatography. A relatively short column was used to diminish dilution effects. When injected at a concentration of 85 or 8.5 μM, the peptide eluted as a single sharp symmetric peak; in the former case, it eluted between the positions of carbonic anhydrase and myoglobin, and in the latter case, between those of cytochrome *c* and acetone (Fig. 3B). These results indicate that at the lower concentration, the peptide dissociates into monomeric chains of random-coil conformation, whereas at the higher concentration, they elute as an oligomer of extended structure with a hydrodynamic radius greater than that of a protein of globular shape as myoglobin ($M_r = 17,200$). When a similar peptide corresponding to the 36 C-terminal residues of matrilin-1 (11) was chromatographed under the same conditions, it eluted in the position of carbonic anhydrase.

The combined data from cross-linking and gel chromatography are most consistent with the assumption that MTR2-C38
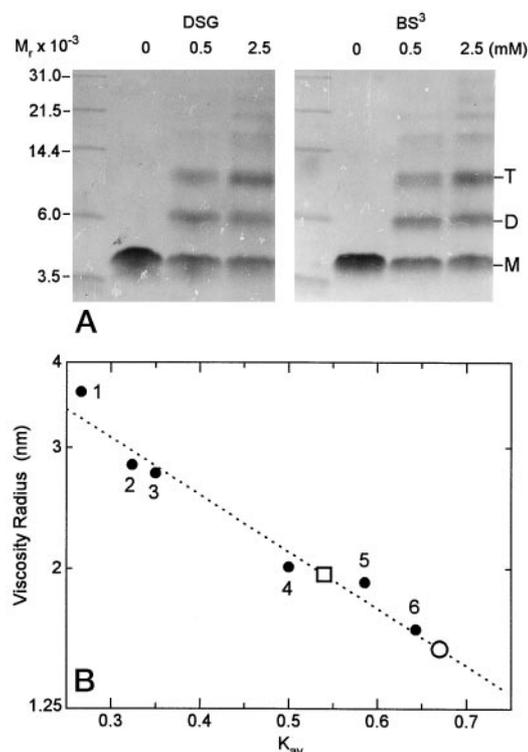

FIG. 3. **Oligomerization state of peptide MTR2-C38.** *A*, MTR2-C38 (100 μM) was incubated with various concentrations (*top row*, in mM) of the cross-linkers disuccinimidyl glutarate (DSG) (*left*) and BS^3 (*right*) for 1 h and analyzed by SDS Tris-Tricine gel electrophoresis. The position of molecular weight marker proteins are shown on the *left*, and the assignment of monomer (*M*), dimer (*D*) and trimer (*T*) positions are on the right. *B*, upon gel filtration on a Superdex 75 column, the MTR2-C38 elution position corresponds to a viscosity radius of 2.0 nm (□) when injected at high concentration ($c = 85$ μM), whereas at low concentration, it elutes corresponding to a radius of 1.5 nm (○, $c = 8.5$ μM). The calibration standards (Ref. 19) are bovine serum albumin (1), ovalbumin (2), trypsin inhibitor (3), carbonic anhydrase (4), myoglobin (5), and cytochrome *c* (6). The reduced retention volume $K_{av} = (V_e - V_0)/(V_t - V_0)$ was determined assuming that the elution positions V_e of laminin and acetone indicate the void volume V_0 and total volume V_t of the column, respectively.

forms a rod-shaped homotrimer in solution. The heterogeneity observed upon cross-linking is probably due to the reaction mechanisms of BS^3 and disuccinimidyl glutarate. Their principal targets are primary amines, such as the amino group at the N terminus of the peptide chains and the ϵ-amino group of lysine residues (30). The human matrilin-1 domain has four lysines. According to their optimized positioning into heptads, one is located near the center of the coiled coil (heptad positions *g*). Within MTR2-C38, the two lysines are probably more exposed to the surface of the coiled coil in heptad positions *b* and *c* (Fig. 1). Thus the probability for an intermolecular cross-link between them is substantially lower, and besides one cross-link between the N-terminal amino groups covalently stabilizing a dimer, at least one further cross link is necessary to observe a trimer on SDS gels. The population of peptides running in the dimer position (Fig. 3A) might include intermolecular cross links between both the N termini and one or two of the lysines, which excludes their native third chain partner from participating in a covalent structure and lets this chain run in the monomeric position. A similar heterogeneity of bands on SDS gels upon cross-linking with BS^3, where the majority of the material runs in the monomer position, has also been observed for a rat mannose-binding protein fragment, the crystal structure of which shows a three-stranded α-helical coiled coil containing four lysine residues (31).
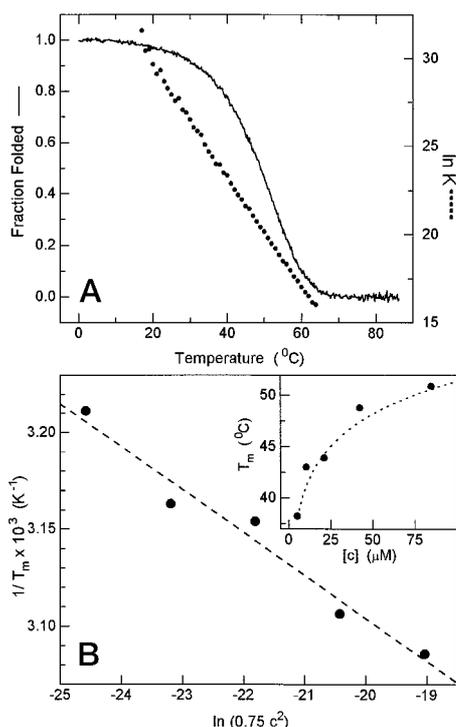
Fig. 4. **Thermal denaturation of peptide MTR2-C38.** *A*, the thermal transition was recorded by following the CD signal change at 222 nm and normalized to the fraction of folded peptide (*left ordinate*, *straight line*; (c) = 42 μM). The *dotted line* represents the same curve plotted as $ln\ K(T)$ (*right ordinate*) derived from the fraction folded, assuming a two-state transition from a three-stranded α-helical coiled coil to random-coil monomers. ΔH^0 and T_m values were derived from such curves by least-squares fitting as described under "Experimental Procedures" (Equation 2). *B*, from the slope of the straight line of the reciprocal transition temperature *versus* ln (0.75 c^2) results ΔH^0 = −375 kJ/mol (Equation 1). The *inset* shows the same values plotted as $T_m(c)$.

DISCUSSION

The α-helical coiled coil is a ubiquitous structural domain found in many intra- and extracellular proteins within which it specifically serves as a region for polypeptide chain recognition and oligomerization (for recent reviews, see Refs. 24 and 33). Despite its relatively simple heptad sequence motif where hydrophobic residues are predominently located in a 3-4-3-4 spacing, it is difficult to predict the correct oligomerization state. Thus the multidimensional scoring approach for identifying and distinguishing between dimeric and trimeric coiled coils implemented in the MultiCoil program (20) favors a dimer assembly for matrilin-2 (Fig. 1) as well as for matrilin-1 (data not shown). For matrilin-1, different approaches have conclusively shown that it forms a homotrimeric α-helical coiled coil (4, 10, 11), and the data presented here make this the most likely assembly state also for matrilin-2. Some important rules for predicting the association state of coiled coils were derived from the systematic exchange of hydrophobic residues in heptad positions *a* and *d* within the yeast transcription factor GCN4. The geometry of the Cα-Cβ bond of the core residues is parallel to the Cα-Cβ vector of the opposing hole in heptad position *a* and perpendicular in position *d* for the dimeric variant, whereas the trimeric variant shows acute geometry of these residues, and in the tetrameric one, *a* is perpendicular, and *d* is parallel (34). Although the periodicity of hydrophobic residues is less regular within the matrilin C-terminal domains, the predominance of leucine residues assigned to positions *a* and *d* according to these rules is consistent with a trimeric state. It has been shown, however, that a single residue, Arg-487 of the human matrilin-1 sequence, which is probably involved in an interchain ionic interaction with Glu-492, is crucial for trimer formation; when replaced by Gln within a peptide resembling the C-terminal domain, tetramer formation was observed at physiological pH and ionic strength (26). Interestingly, the positively charged character of Arg-487 is conserved within all matrilin sequences. The corresponding residues of matrilin-2 studied here are Arg-945/Glu-950 (Fig. 1, *thick bracket*) and are identical both in the mouse and human sequence (12). The matrilin-3 sequences contain a lysine instead of arginine in this position (13, 14).

When compared with the peptide corresponding to the C terminus of matrilin-1, MTR2-C38 is considerably less stable with T_m values about 35 °C lower, and the enthalpy change differs by ~130 kJ/mol (11). This is most probably due to the weakening in the stabilizing interactions between hydrophobic core residues. Matrilin-2 residues Ala-928, Met-942, and Met-946, which are assigned to heptad positions *a* and *d* (Fig. 1), are considerably less hydrophobic than the corresponding amino acids (Val-470, Val-484, Leu-488) of matrilin-1. Based on model peptides, it was found that depending on its position, an Ala-Ala interaction can decrease the stability by $\Delta\Delta G_u$ ~ 13 kJ/mol when compared with a Leu-Leu interaction (29). Although methionine residues are relatively rare in coiled-coil domains, their preference to occupy heptad positions *a* or *d* is less pronounced than observed for other hydrophobic amino acids (21, 35), which might indicate that their contribution to stability is weak.

The most conserved residue pair within the currently known C-terminal sequences of matrilins is Phe-Gln-925 of matrilin-2 (exceptions: Phe-Glu in mouse matrilin-1; Leu-Gln in chicken matrilin-3). Interestingly, Gln is specifically found near the N terminus of many trimeric in contrast to dimeric coiled coils (36). Indeed, Gln residues in the heptad position *a* are thought to specify a trimer assembly (37). Heteronuclear NMR assignments for the chicken matrilin-1 C-terminal domain containing the two cysteine residues preceding the coiled-coil region indi-

Thermal Stability of Peptide MTR2-C38—To measure the thermal stability and evaluate the nature of the folding/unfolding transition of MTR2-C38, the CD signal at 222 nm was monitored at peptide concentrations ranging from 5 to 85 μM upon raising the temperature. The melting curves showed a sigmoidal shape indicating a cooperative unfolding from a coiled-coil to random-coil conformation. The first derivatives $d\Theta/dt$ exhibit a single minimum that is compatible with the assumption of a single transition (data not shown). Transition curves were converted to the fraction of folded protein (Fig. 4*A*). Assuming a first order transition reflecting the unfolding of three peptide chains in a coiled-coil conformation into single random-coil chains, the data were analyzed for the melting temperature and transition enthalpies by nonlinear least-squares fitting to Equation 2. As expected for the unfolding of noncovalently associated peptide chains, the melting temperature varied with concentration ranging from 38.2 °C determined at 5 μM to 50.9 °C at 85 μM (Fig. 4*B*). These temperatures are about 35 °C lower than those of a corresponding peptide of matrilin-1 at similar concentrations (11). From the slope of $1/T_m$ *versus* ln (0.75 c_0^2), the enthalpy change results to −375 kJ/mol, which is consistent with ΔH^0 values resulting from fitting the single transition curves but significantly lower in its amount than the −508 kJ/mol reported for the matrilin-1 peptide (11). Assuming that the entire peptide chain participates in the coiled-coil conformation, a mean value of −3.3 kJ/mol/residue results that is within the range of −1.8 to −4.6 kJ/mol/residue measured calorimetrically for a large number of coiled-coil proteins (32).

cate that they form symmetric disulfide bonds in which the first cysteine of one chain is linked to the second cysteine of a neighboring chain, contributing a stabilizing energy of $\Delta\Delta G_u \sim$ 4 kJ/mol. They are incompatible with a coiled-coil conformation, but this starts at the following residue and lasts up to near the C terminus (23). In the reduced form, however, the N termini of this domain are less ordered (23). These results suggest for MTR2-C38 that the $\sim$10 N-terminal residues are not well ordered and thus account for the less than fully α-helical structure as determined by CD spectroscopy (Fig. 2).

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