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 NH₂-ENLILFQNVANEEVRKLTLQRLEMTQRMEEAL-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 (abc-defg)n, which is characteristic for \( \alpha \)-helical coiled-coil proteins. Circular dichroism spectroscopy revealed a high \( \alpha \)-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 \( \Delta T_m = -375 \, \text{kJ/mol} \). Melting temperatures \( T_m \) varied from 38 to 51 °C within a concentration range of 10 to 85 \( \mu \text{M} \), 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 unique C-terminal domain (6–9) that was recently found to be responsible for the oligomerization into homotrimers by assembling into a three-stranded \( \alpha \)-helical coiled coil (4, 10, 11).

**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-fluorenylmethoxycarbonyl)) chemistry. The five arginine residues were protected by pentamethylchroman-sulfonyl. 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 100% 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 a 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 \( A_{1%}^{1\%} = 3.32 \) 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 (\( \Theta_{\text{MRELL}} \)) with \( M_r = 124 \) as derived from the sequence. Thermal tran-
transition curves were interpreted assuming a two-state mechanism in which three unfolded chains combine to a native α-helical coiled-coil trimer \( n \) (16). With the total peptide concentration \( c_0 = c_u + 3c_c \) and the degree of conversion to the coiled-coil \( F = c_c/c_0 \), the equilibrium constant \( K \) follows as \( K = c_u/c_c \sim F/3c_c (1 - F)^3 \). With \( \Delta G_0^\circ = -RT \ln K \), it follows that at the transition midpoint \( (F = 0.5 \) and \( T = T_m) \) (Equation 1),

\[
\frac{1}{T_m} = \frac{\Delta S^0 + R \ln(0.75c_0^2)}{\Delta H^0}
\]

where \( \Delta G_0^\circ, \Delta H^0, R, \) and \( T \) are the standard free energy, enthalpy, entropy, gas constant, and absolute temperature, respectively. Thus, \( \Delta H^0 \) 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 \( \Delta H^0 \) were evaluated by a nonlinear least-squares Marquardt-Levenberg fitting algorithm (Equation 2) of

\[
\ln(K(T)) = \frac{\Delta H^0}{RT} \left( \frac{T}{T_m} - 1 \right) - \ln(0.75 \, c_0^2)
\]

with \( \Delta H^0 \) 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(sulfosuccinimidyldisulfate BS \(^3\)) and disuccinimidyl glutarate (Pierce), which are homobifunctional N-hydroxysuccimide ester analogs with a spacer arm length of 1.14 and 0.77 nm, respectively. The peptide \( c_{\text{sample}} = 100 \mu M \), in 20 mM KH\(_2\)PO\(_4\)/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 μm urea and a 3% stacking gel. Gels were stained with Coomasie 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 mM 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, K945R). All four coiled-coil predictions agree in their assignment of heptad positions (abcdefgij), 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 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 interactions between oppositely charged side chains of the type \( i \rightarrow i + 3 \) and \( i \rightarrow i + 4 \) (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 \( \pi^\alpha \) transition (222 nm) is mainly indicative for the \( \alpha \)-helical content, whereas the \( \pi^\sigma \) transition (208 nm) polarizes parallel to the helix axis, the ellipticity ratio \( [\theta]_{222} / [\theta]_{208} \) reflects whether the α-helix is in 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. 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 \( i \rightarrow i + 3 \); \( i \rightarrow i + 4 \), brackets) and interchain (heptad positions g-2id, 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.
observed (data not shown).

**Oligomerization State of Peptide MTR2-C38**—The specific homotrimeric association of matrin-1 has been shown by electron microscopy of the reduced and unreduced native protein (4), site-directed mutagenesis on recombinantly overexpressed mini-gene matrin-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), matrin-2 can also assemble into a homotrimer, we performed cross-linking studies on MTR2-C38 using BS3 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 unaffected 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 cytochrome c 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, it elutes as an oligomer of extended structure with a hydrodynamic radius greater than that of a protein of globular shape as myoglobin (Mr = 17,200). When a similar peptide corresponding to the 36 C-terminal residues of matrin-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 forms a rod-shaped homotrimer in solution. The heterogeneity observed upon cross-linking is probably due to the reaction mechanisms of BS3 and disuccinimidyl glutarate. Their principal targets are primary amines, such as the amino group at the N terminus of the peptide chains and the e-amino group of lysine residues (30). The human matrin-1 domain has four lysines. According to their optimized positioning into heptads, observed (data not shown).

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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° and Tm 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°) results ΔH° = −375 kJ/mol (Equation 1). The inset shows the same values plotted as Tm(c).

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 do/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. 4A). 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. 4B). These temperatures are about 35 °C lower than those of a corresponding noncovalently associated peptide, the matrilin-1 C-terminal domain containing three peptide chains in a coiled-coil conformation into single random-coil chains (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).

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 predominantly 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 Ca-Cα bond of the core residues is parallel to the Ca-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 Tm 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 ΔΔG° ≈ −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-
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 $\Delta G_u = -4 \text{ kJ/mol}$. 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 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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