Type I Collagen CNBr Peptides: Species and Behavior in Solution†

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Received August 4, 1995; Revised Manuscript Received February 6, 1996⊗

ABSTRACT: The properties of type I collagen CNBr peptides in solution were studied to investigate the molecular species formed, their conformation, and factors influencing equilibria between peptide species. Peptides formed homologous trimers, even though the native parent protein is heterotrimeric, $[\alpha1(I)]_2\alpha2$-(I). Their triple-helical content was found to be high (>75% for most peptides). Full helical content was not reached mainly because of the presence of monomer species; chain misalignment, if present, and trimer unraveling at terminal ends appeared to play a minor role in reducing helicity. Circular dichroism spectra and resistance to trypsin digestion at 4 and 20 °C demonstrated that the conformation of trimers was very similar to the collagen triple-helical conformation. Rotary shadowing of peptide α1(I) CB7 supported this finding. Analytical gel filtration in nondenaturing conditions showed that the trimers of some peptides have the ability to autoaggregate. In the case of peptides α1(I) CB8 and α2(I) CB4, most of the intermolecular interactions between trimeric molecules were disrupted by 0.5 M NaCl, demonstrating that their ionic character is important. Changes in ionic strength also altered the hydrodynamic size of single- and triple-stranded molecules. The different molecular species are in equilibrium. The kinetics of the conversion of trimer to monomer species was determined in a time course experiment using trypsin digestion and found to be a relatively slow process (trimer half-life is a few days at 4 °C, about one order of magnitude lower at 20 °C) with an activation energy of roughly 4−9 kcal/mol. The circular dichroism profile at increasing temperatures showed that the melting temperature for triple-helical peptides is about 6−10 °C lower than that of the parent native type I collagen. The folding of peptides is a spontaneous process (exothermic but with unfavourable entropy change), and the triple-helical conformation originates solely as the result of the collagen sequence because it forms from heat-denatured samples.

Collagens are major components of connective tissues, with type I collagen being the most abundant protein of the body. The main biomechanical function of fibrillar collagen types I, II, and III is to oppose tensile forces, due to the action of the spontaneously self-assembled fibrils and fibers in the extracellular space of connective tissues. Other collagens have specific functions in specialized areas, e.g., collagen type IV in basement membranes and type VII in anchoring fibrils [for reviews on collagen structures, see Kielty et al. (1993) and van der Rest and Garrone (1991)]. Other connective tissue macromolecules (fibronectin, proteoglycans, elastin, etc.) perform specific tasks, some of them specifically interacting with some collagen type(s) (Heinegård & Oldberg, 1993; von der Mark & Goodman, 1993).

There are increasing reports of mutations in collagens which give rise to diverse pathologies, such as osteogenesis imperfecta, some subtypes of Ehlers-Danlos syndrome, chondrodysplasias, epidermolysis bullosa, etc., where connective tissue morphology and biomechanical function are affected [for recent reviews, see Royce and Steinmann, Eds. (1993)]. In osteogenesis imperfecta, for example, several hypotheses have been made to try to correlate defects in type I collagen with clinical severity [see, e.g., Byers (1993), Tenni et al. (1993), and Lightfoot et al. (1992, 1994)]. However, the exact structural effects of the mutations at the molecular level still need to be fully elucidated.

Collagens are also involved in biological processes such as interactions with diverse cell types (fibroblasts, chondrocytes, endothelial cells, platelets, etc.); these interactions appear to be very specific and important in cellular adhesion, migration, growth and differentiation, or even specific interactions with bacterial receptors [see, e.g., Tuckwell et al. (1994) and Visai et al. (1995) and literature cited therein]. The relevant domain in the collagen molecule or structure involved in these interactions is known in only a few of these processes, and much less is known about structural details at the molecular level (see Discussion for some examples).

Studies using collagen peptides with natural sequences or model collagen-like peptides may be useful for investigating effects of mutations and collagen interactions. Relevant examples are already reported in the literature, such as the studies on platelet adhesion to type I collagen (Santoro et al., 1994), melanoma cell adhesion and spreading on type IV collagen (Fields et al., 1993), integrin-type II collagen binding for chondrocytic cells (Tuckwell et al., 1994), and peptides modeling epitopes of type II collagen in the murine model of rheumatoid arthritis (Lambert & Berling, 1994; Michaëlsson et al., 1992; Myers et al., 1992, 1995). We

† This work was supported by grants from italian MURST, Consiglio Nazionale delle Ricerche and Regione Lombardia.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

S0006-2960(95)01815-0 CCC: $12.00

purified CNBr peptides from type I collagen and then analyzed them by physicochemical and biochemical methods to study the molecular species present in solution, their properties, and equilibria. The aim of this study was to determine the best conditions required for use of collagen fragments for duplicating or mimicking collagen processes.

MATERIALS AND METHODS

Materials, Analytical Procedures, and Sequence Data. Bio-Gel A 0.5 m, A 1.5 m, and P2 were purchased from Bio-Rad Labs. Source 15S was from Pharmacia; prepacked columns of MonoS (0.5 × 5 cm), Superose 6, and Superose 12 (both 1 × 30 cm) were from Pharmacia. All reagents were of analytical grade. SDS−PAGE[1] and hydroxyproline analyses were performed according to Laemmli (1970) and Huszar et al. (1980), respectively.

The hydroxyproline content, molecular mass, and mean residue molecular mass for type I collagen CNBr peptides were calculated from sequence data of bovine α1(I) and α2-(I) chains, where known. Otherwise, corresponding human sequences were used (Galloway, 1982; Phillips et al., 1992). Numerical values used for these parameters are reported in Figure 1A.

Preparation of Acid-Soluble Collagen. Acid-soluble type I collagen, i.e., collagen molecules with intact telopeptides at both ends of the triple helix, was prepared according to Na et al. (1986), with some modifications. Briefly, calf skin was obtained from a local slaughterhouse immediately after sacrifice and kept in ice. Within 2 h, it was freed from hair, fat, and subcutaneous tissues and delipidized for about 6 h at 4 °C with several changes of chloroform−methanol (1:1 v/v). The skin was then dried under vacuum before being finely minced and then sequentially treated with two changes (30 mL/g of original wet tissue) of 0.15 M NaCl and 50 mM Tris-HCl, pH 7.4, in the presence of protease inhibitors (20 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine) at 4 °C for 48 h. The residue was then treated with two changes of 0.5 M acetic acid (30 mL/g of original wet skin) for a further 48 h. The extracted acid-soluble collagen was precipitated by adding solid NaCl to a final concentration of 20% (w/v), and the precipitate was redissolved in 0.5 M acetic acid and then dialyzed exhaustively against 0.1 M acetic acid. The retentate was clarified by centrifugation and freeze-dried. The yield of acid-soluble collagen was about 3% of the original amount of wet skin. When analyzed by SDS−PAGE, the purified collagen showed a characteristic type I collagen pattern as well as some bands due to cross-linked species which were larger than trimers.

Cleavage of Collagen with CNBr. Collagen samples (10−15 mg/mL) were suspended in 10% (v/v) 2-mercaptoethanol and 10 mM NH_4HCO_3 and kept for 4 h at 45 °C, to reduce oxidized methionines. After freeze-drying, CNBr cleavage was performed under nitrogen for 5 h at 30 °C, at 10 mg/mL in 70% (v/v) HCOOH containing 10 mg/mL CNBr. Digestion was terminated by freeze-drying.

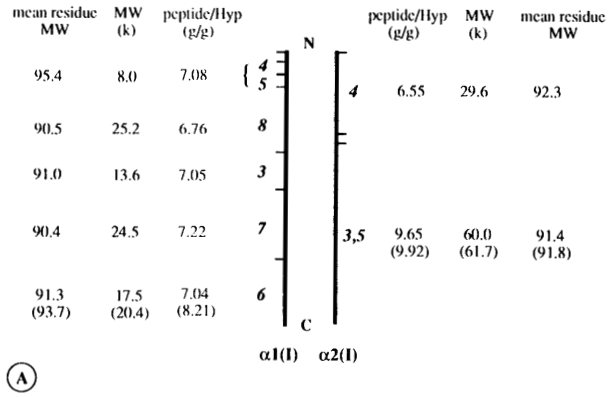
Purification of Peptides. CNBr peptides were purified by a combination of molecular-sieve chromatography followed by cation-exchange chromatography.

Molecular-Sieve Chromatography. The procedure of Piez (1968) was followed, with one major variation concerning the resin used. Briefly, the collagen CNBr peptide mixture was chromatographed on a column (2.6 × 100 cm) equilibrated and eluted in 1 M $CaCl_2$ and 50 mM Tris-HCl, pH 7.5, at room temperature in denaturing conditions (flow rate 13 mL/h, 20-min fractions). Samples, 5−15 mg/mL in the same buffer, were denatured (20 min, 55 °C) and clarified by centrifugation (20 min at 20000*g* and 20 °C) immediately before loading. The effluent was continuously monitored at 228 nm, and relevant peaks were desalted on Bio-Gel P2 in 0.6% (v/v) acetic acid and freeze-dried. The purity of each fraction was checked by SDS−PAGE and hydroxyproline analysis, as stated above. We used the resin Bio-Gel A 0.5 m instead of Bio-Gel A 1.5 m because we did not obtain a satisfactory resolution using the latter resin. For CNBr peptides from type I collagen α1(I) chain, we found that Bio-Gel A 0.5 m has a lower exclusion limit than A 1.5 m (80 versus 430 kDa) and a shallower slope in the linear relation log MW versus K_{av} (1.80 versus 2.41). Variations between different runs were within ±2.5%. Type II collagen CNBr peptides behaved similarly in the Bio-Gel A 0.5 m medium.

Cation-Exchange Chromatography. The procedure reported by Bateman et al. (1986) was followed on a pilot scale using a MonoS column (1-mL bed volume) and then a HR10 column packed with Source 15S resin (1 × 10 cm, 8-mL bed volume) eluted at 1 mL/min. Buffer (2 M urea, 20 mM formiate, pH 3.8) and gradient were as reported by Bateman with minor variations concerning gradient slope and total volume (about 60 mL total volume of about 70−260 mM NaCl in buffer) for different peptides. Elution was continuously monitored at 214 nm; relevant fractions were desalted and freeze-dried as above. Each fraction was checked by electrophoresis and hydroxyproline analysis. The purest fractions of every peptide (Figure 1B) were used for all subsequent procedures. We also obtained and analyzed a peptide with a molecular mass of about 8.0 kDa (with reference to α1(I) CNBr peptides); this was probably α1(I) CB4,5. In this work we also report data regarding this peptide, but we stress that the attribution of this peptide to a particular region of the triple helix of one or other α chain does not affect any of our conclusions.

Nondenaturing Analytical Gel Filtration. Peptides were dissolved in 0.1 M acetic acid, at 0.5−2 mg/mL, left at least 2 days at 4 °C, and clarified by centrifugation at the same temperature. Aliquots (60−200 μL containing 50−210 μg) were injected into Superose 6 or Superose 12 columns. Some samples were heat-denatured (10 min at 60 °C) immediately before the analysis. The columns were equilibrated and eluted at a low flow rate (0.2 mL/min) at room temperature (18−20 °C) with 50 mM acetic acid and 50 mM NaCl. The presence of sodium chloride was essential because the peptides stick to the column and/or to the resin on using 50 or 100 mM acetic acid alone. The chromatographic equipment was a Pharmacia FPLC system with two P-500 pumps, a LCC 500 controller, and an ultraviolet UV-M monitor set at 214 nm. Peak areas were integrated by the controller software. After each run, the column was washed with 200−500 μL of 1 M acetic acid and equilibrated with at least one column volume of the eluent in order to avoid any possible contamination of the following sample. In a further set of analyses (see Results), the salt concentration

[1] Abbreviations: ASC, acid-soluble type I collagen, i.e., collagen bearing telopeptides; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Hyp, hydroxyproline; CD, circular dichroism; T_m, melting temperature.



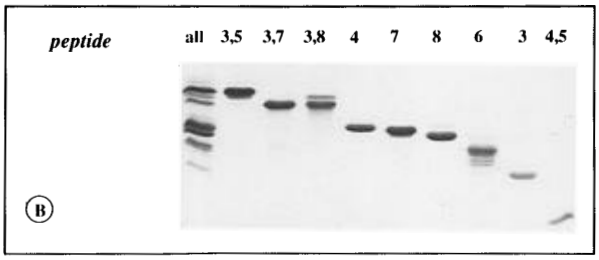


FIGURE 1: Peptide symbology and electrophoretic analysis of pure samples. (A) Symbology (in bold italics) and position of CNBr peptides along the triple-helical domain of type I collagen α chains; chain N- and C-terminal ends and numerical values used are indicated. For C-terminal peptides, values in brackets refer to specimens bearing intact telopeptides. The following composite peptides with uncleaved methionine are not reported in the panel: α1(I) CB3,7: 7.16 g of peptide/g of Hyp, 38.0K MW, 90.6 mean residue MW; α1(I) CB3,8: 6.86, 38.8K, 90.7. (B) SDS−PAGE analysis of purified peptides used in this work. The preparations are ≥95% pure, with the exception of α1(I) CB3,8 (≥90%). The presence of two minor bands for α1 CB6 is due to partial or total proteolysis of the C-telopeptide during purification of collagen and peptide.

in the eluent was increased to 500 mM NaCl. In this case and at least 3 h before the analysis, 5 M NaCl/0.1 M acetic acid was added to the samples to bring the NaCl concentration to 500 mM.

Circular Dichroism. Circular dichroic spectra were recorded on a Jasco J-710 spectropolarimeter, and data were handled through the manufacturer's software (J700 series spectropolarimeter system, ver. 1.20c). Samples of peptides and collagen were dissolved in 0.1 M acetic acid and their concentration was adjusted to 55−75 μg/mL and left to equilibrate at 4 °C for several days (≥7) and finally clarified by centrifugation at 4 °C. Heat denaturation, when necessary, was performed immediately before the analysis. Spectra were recorded at 10 °C in thermostated cells of 1 mm path length. Scans were performed at 20 nm/min, collecting data points every 0.05 nm, and averaging the data at least over three scans. After the scan, Hyp analysis was performed on each sample to have a direct measure of the peptide concentration.

Thermal transition was determined on samples dissolved in 0.1 M acetic acid and equilibrated as above at a concentration close to 0.5 mg/mL (0.32 mg/mL for CB6) (18−61 μM). Melting profiles were followed by CD at 221 nm in 1 mm path length cells, starting at 4 °C and increasing the temperature in steps of 1 °C every 8 min. Data points at each temperature were taken from the average CD signal over the last 1 min of the 8-min period.

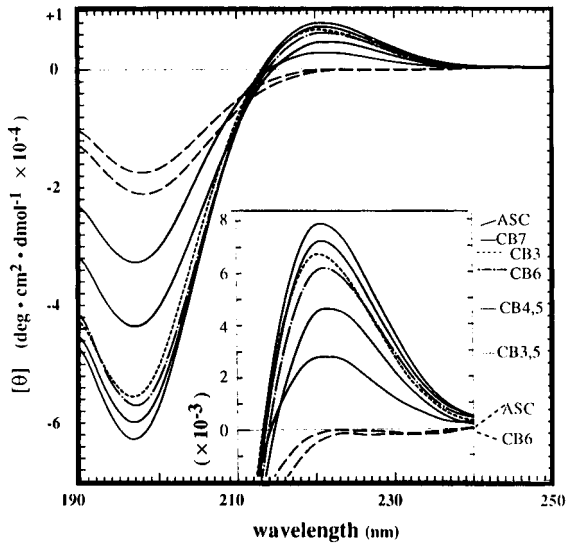


FIGURE 2: Circular dichroism spectroscopy. CD spectra for acid-soluble type I collagen (ASC) and some representative peptides in native conditions and for two heat-denaturated samples (collagen and one peptide; dashed lines). All spectra were recorded at 10 °C on samples dissolved in 0.1 M acetic acid and at a similar concentration (55−75 μg/mL). The inset is an enlargement of the positive peak centered at ∼221 nm. The figure and inset share the same abscissa. Attribution of spectra is indicated in the lower right; usually the intensities of positive and negative peaks are directly correlated; the only exceptions in the figure are for peptides CB6 and CB3, indicated by a different line style. Quantitative CD data are reported in Table 1.

Susceptibility to Trypsin. Peptides were dissolved in 5 mM acetic acid at a concentration of about 0.5 mg/mL. After standing 24 h at 4 °C, one-third volume of 1.6 M NaCl and 0.4 M Tris-HCl, pH 7.4, was added (final concentration 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4). After centrifugation at 4 °C for 15 min in a microcentrifuge, Hyp analysis was performed on the supernatant for direct measurement of the peptide concentration. Trypsin (1mg/mL in 1 mM HCl) was added to have a substrate/enzyme ratio of 10:1 (w/w). Aliquots of the solution were incubated for 0, 2, 5, 10, 20, 40 min and 1, 2, and 4 h at either 20 or 4 °C and at 4 °C also for 6 and 24 h. After each incubation period, 5 μL of soybean trypsin inhibitor (4 mg/mL in 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4) and 20 μL of 5× concentrated Laemmli sample buffer were added to each 80 μL aliquot containing 10−30 μg of peptide, and the sample was denatured (100 °C for 3 min). Each set of samples was submitted to SDS−PAGE, and gels were stained with Coomassie Brilliant Blue R250 (Stephano et al., 1986) and scanned with an LKB Ultroscan XL laser densitometer.

Rotary Shadowing. Samples of peptide α1(I) CB7 and its parent type I acid-soluble collagen were dissolved in 0.1 M acetic acid and equilibrated for several days at 4 °C. They were mixed to a final concentration of 7 μg/mL with ammonium acetate and glycerol (final concentration 0.3 M and 50% (v/v), respectively). The samples were prepared following the mica sandwich technique (Mould et al., 1985) and shadowed with platinum at 8° and with carbon at 90° in an Edwards Auto 306 coating system. The replicas were floated on distilled water and collected on 200-mesh copper grids. Electron micrographs were taken in a Zeiss 902 electron microscope using the energy filter to increase the contrast. The length and thickness of at least 145 well resolved molecules were measured with NIH image 1.55

Table 1: Type I Collagen CNBr Peptides: Percent Helical Form in Native Conditions and Melting Temperatures

	circular dichroism[a]			analytical gel filtration: % helical form[b]		trypsin susceptibility: % surviving molecules[c]		
peptide	λ_{max} (nm)	[θ] at λ_{max} (deg·cm^2·dmol^{-1})	% helicity[e]	50 mM NaCl	500 mM NaCl	4 °C	20 °C	melting temperature[d] (°C)
acid-soluble type I collagen	220.8	7873 (± 6.0%)[f]						33.8
α2(I) CB3,5	221.4	2786	35	5	11	nd (*42)	nd (*6)	nd
α1(I) CB3,7	220.8	6524	83	79	79	nd (*67)	nd (*59)	nd
α1(I) CB3,8	221.0	6342	81	73	72	nd (*67)	nd (*59)	nd
α2(I) CB4	221.2	6041	77	84	75	76 [37]	57 [28]	23.6
α1(I) CB7	220.8	7222	92	95	82	85 [47]	73 [19]	28.1
α1(I) CB8	220.9	6121	78	86	92	88 [61]	97 [19]	28.0
α1(I) CB6	221.2	6178	78	74	67	74 [43]	81 [44]	26.7
α1(I) CB3	220.4	6712	85	86	61	85 [31]	85 [<6]	23.9
α1(I) CB4,5	221.4	4618	59	63	66	nd	nd	27.7

[a] Spectral quantitative data at 10 °C (λ_{max}, wavelength at the maximum of the positive peak; [θ], mean residue ellipticity at λ_{max}) and percent helicity compared to parent native type I collagen. [b] Percent helical form at lower and higher ionic strength (data from Superose 6). [c] Fraction of molecules surviving rapid degradation; in square brackets, fraction of molecules surviving trypsin treatment after either 24 h (4 °C) or 4 h (20 °C). [d] See text for the limitations on this parameter. [e] [θ] for peptide/[θ] for acid-soluble type I collagen, at the respective maximum of the positive peak. nd, not determined ("*", fraction of molecules surviving the rapid degradation for peptide preparations less pure than those for the other determinations; α1 CB3,7 and CB3,8 were present in an about equimolecular mixture). [f] Mean ± standard deviation (as % of mean) of four determinations. At neutral pH we obtained [θ] = 8408 deg·cm^2·dmol^{-1} (n = 1). For three pepsinized type I collagen samples from bovine tissues other than skin, [θ] = 7456 ± 3.2% deg·cm^2·dmol^{-1}.

software. Magnification of the electron microscope was calibrated using negatively stained catalase crystals.

RESULTS

The nomenclature of the type I collagen CNBr peptides, their position along the triple-helical domain of α1(I) and α2(I) chains and electrophoretic analysis of our preparations are reported in Figure 1.

Circular dichroism analysis at 10 °C showed that peptide spectra are qualitatively identical to the typical spectrum obtained for the intact molecule, i.e., with a positive peak centered at about 221 nm and a more pronounced negative peak at 197 nm (Figure 2). On denaturation, the positive peak disappears and the negative peak becomes less profound and red-shifted by about 1 nm. Quantitatively, differences in the absolute values of mean residue ellipticity were found for peptides and collagen (Table 1). These data, together with the results described below, strongly indicate that type I collagen CNBr peptides assume a triple-helical, collagen-like conformation in solution. An equilibrium monomer = trimer is thus expected to exist, the prevailing form, at any one given temperature, depending on the peptide, but the trimeric form giving the greatest contribution to the CD signal at ~221 nm. Peptides derived from either α1(I) or α2(I) chain and formed homotrimers, whereas their parent molecule is a heterotrimeric protein, [α1(I)]$_2$α2(I). CNBr peptides from type II collagen (a homotrimeric species) also showed very similar CD spectral characteristics (data not shown). We can consider that percent helicity, calculated from CD data (Table 1), is an appoximation of the percentage of molecules that assume a triple-helical conformation. All peptides, apart from the largest and the smallest analyzed, showed a high value (75% or more) with respect to that of parent collagen. Synthetic peptides such as (Pro-Hyp-Gly)$_{10}$ (which are probably shorter than an entire turn of triple helix) show very similar CD spectra, the positive peak being at about 225 nm, but with an intensity of about 4000−4500 deg·cm^2·dmol^{-1} (Long et al., 1992, 1993), even though a higher concentration was used and the fact that more than 99% of the peptide was associated to form a triple helix (Long et al., 1993; Li et al., 1993).

Analytical gel filtration chromatography on Superose 6 and Superose 12 was performed under nondenaturing conditions with quantitative recovery of the injected peptide. Peptide preparations eluted as a single peak when heat-denatured immediately prior to analysis. Undenatured samples showed two or three molecular species: single-stranded molecules, trimers, and aggregates (see two representative examples in Figure 3A,B). Only large peptides (α2(I) CB4, α1(I) CB8, and CB3,7 and CB3,8) formed aggregates which were the prevailing species and which eluted in the void volume with both Superose 12 and Superose 6. For our peptides, the latter medium showed an exclusion limit about 6−10 times larger than Superose 12 for single-stranded species and about 2 times larger for trimers (data not shown). SDS−PAGE analysis of peak fractions showed only one band corresponding to single-stranded species (Figure 3C). This confirmed that only weak intermolecular noncovalent bonds are involved in trimer formation and their aggregation. Different slopes for monomers and trimers were obtained when log MW was plotted versus K_{av} (Figure 3D). One obvious explanation is that the different behavior on chromatography is due to the differing conformation. Table 1 (analytical gel filtration section) reports the percentage of molecules eluting earlier than monomers, i.e., the percent helical form for each peptide; the values are similar to those obtained by CD.

To determine whether the largest oligomeric species described above are due to the presence of a mixture of (monomer)$_n$ with a different n, or the result of ionic interactions between trimers, gel filtration analysis of peptides was repeated on Superose 6 using a higher concentration of NaCl in the eluent (500 mM). Both monomeric and trimeric species eluted earlier in the presence of the higher NaCl concentration, demonstrating a greater hydrodynamic size (Figure 3A,B) and percent helical form changed somewhat, but not significantly (α1(I) CB3 showed the greatest variation, from 86% to 61%) (Table 1). More importantly, there

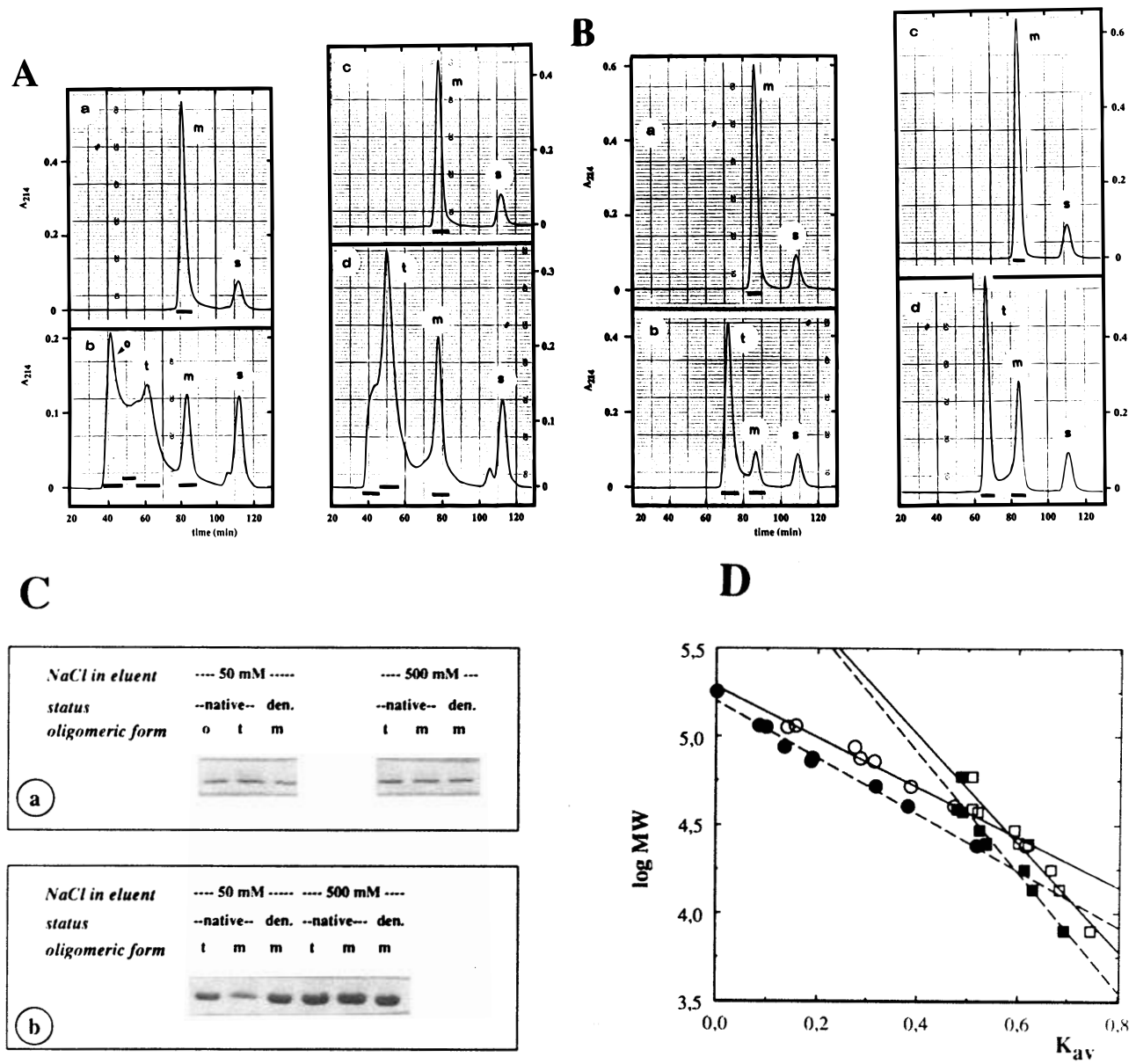


FIGURE 3: Analytical gel filtration chromatography. Elution profiles [$A_{214\ nm}$ versus time (min)] for two representative peptides: α2 CB4 (panel A) and α1 CB3 (panel B). Subpanels refer to denatured samples (a and c) or native ones (b and d). NaCl in eluent is 50 mM (a and b) or 500 mM (c and d) Peaks for monomers, trimers, other species and solvent are indicated by m, t, o, and s, respectively. Bars on the bottom of peaks are the fractions collected for analysis on SDS−PAGE. (C) SDS−PAGE of some selected peaks from analyses shown in panels A and B (a for CB4 and b for CB3). NaCl in eluent, native or denatured status, and molecular forms (as stated for panels A and B) are indicated. The panel shows the only band seen, which corresponds to single-stranded species. This clearly demonstrates that only noncovalent weak intermolecular bonds are present in trimers and aggregates. (D) Plot for all peptides of chromatographic parameters (log MW versus K_{av}) from gel filtration analysis on Superose 6. Monomeric (squares) and trimeric species (circles) are plotted; open and filled symbols are from 50 and 500 mM NaCl in eluent, respectively.

was an increase in the amount of the trimers for peptides α2(I) CB4 (Figure 3A) and α1(I) CB8 at the expense of more massive species (aggregates). Thus, ionic interactions play an important role in the formation of peptide aggregates.

Digestion with trypsin was exploited as a further approach to study peptide conformation and equilibria. Heat-denatured peptides were very quickly degraded, i.e., more than 80% within 2 min (data not shown). However, only a small fraction of molecules were quickly degraded when samples were not denatured. On comparing undigested samples with samples digested for a very short time (time 0; see Materials and Methods), the fraction of peptides resistant to degradation is similar to the fraction of peptides in helical form as determined by circular dichroism or analytical gel filtration (compare in Figure 4A, lanes nt and 0; see Table 1, trypsin susceptibility section, for quantitative data). We conclude that single-stranded peptides have a structure which is accessible to the proteolytic enzyme, whereas oligomeric species are resistant or much less susceptible to trypsin. This was confirmed by the time course of trypsin digestion up to 4 h at 20 °C and 24 h at 4 °C (see Figure 4A for representative electrophoretograms and Table 1), even though some peptides were converted by trypsin to slightly shorter but more enzyme-resistant peptides. In particular, α1(I) CB7 was partly digested at both 4 and 20 °C to species about 40, 60, and 90 residues shorter than the parent peptide; α1(I) CB8 and α2(I) CB4 were digested at 20 °C only forming trypsin-resistant fragments about 30 and 25 residues shorter, respectively. The behavior of peptide α1(I) CB6 could be due to the fact that the C-telopeptide is more sensitive to

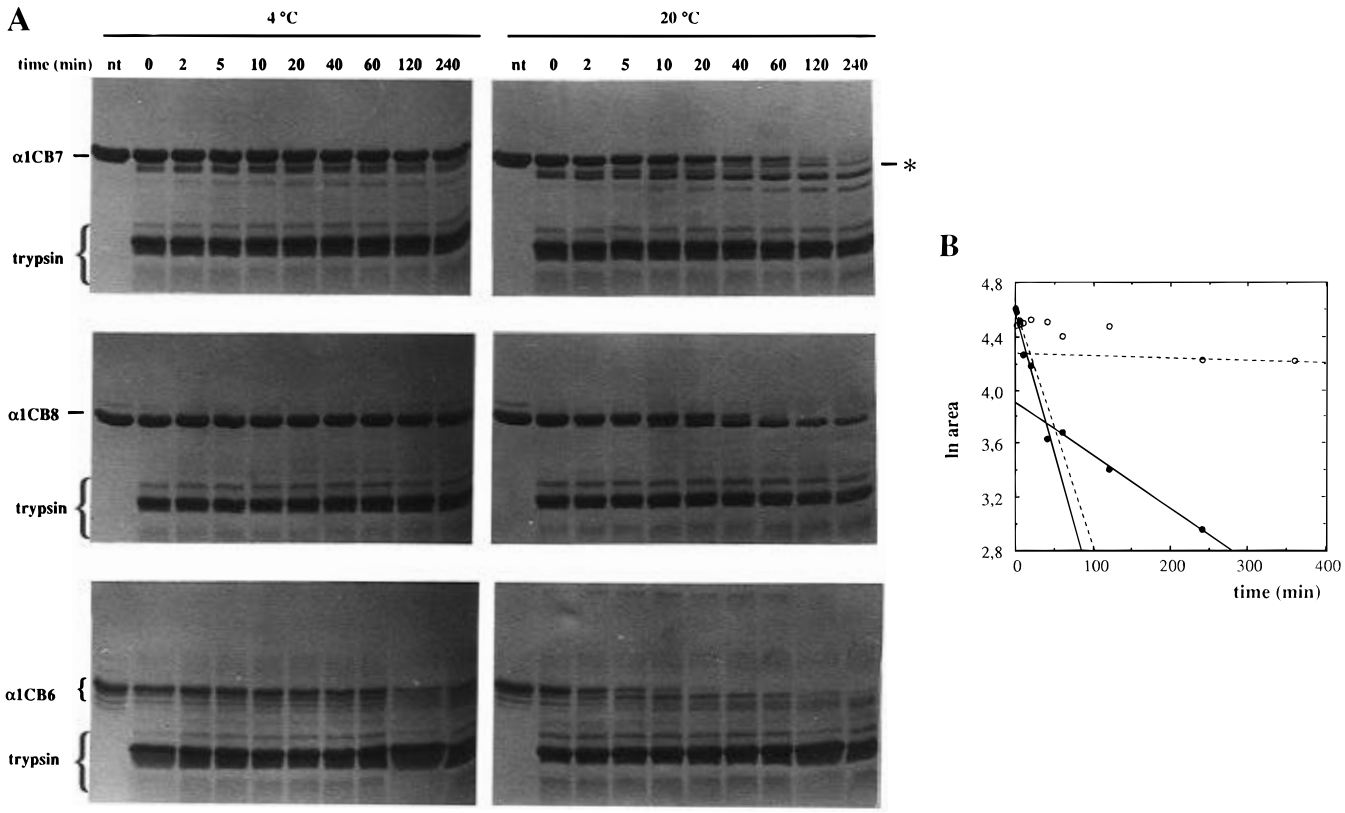


FIGURE 4: Time course of trypsin digestion and half-life of trimeric species. (A) Stained gels after SDS−PAGE showing the time course of trypsin digestion at 4 (left) and 20 °C (right) for peptides α1(I) CB7 (top row), CB8 (center), and CB6 (bottom) (trypsin incubation times are indicated at top; lanes labeled nt refer to the control peptide with no trypsin added). Bands for peptides and trypsin are indicated. In the analysis of CB7 at 20 °C the asterisk refers to a band due to a small contamination by CB8. (B) Example for calculating the half-life of trimers. Densitometry of gels (such as those shown in panel A) gave band areas that were used to construct plots by following a first-order kinetic law: natural logarithm of band area versus time (exactly: ln [100 × (band area/trypsin area)$_{\text{time } t}$/(band area/trypsin area)$_{\text{time } 0}$], i.e., band areas are normalized by using trypsin area and this ratio at time 0 is set at 100% as the reference value). Reported are experimental data for CB7 only and linear interpolations for times 0−5 min and for late times, at 4 °C (filled circles and continuous lines) and at 20 °C (open circles and dashed lines). For clarity, the experimental data point for 24 h at 4 °C is not included in the graph. The half-life of species surviving trypsin digestion was calculated from the kinetic constant k, equal to the slope of interpolating lines, using the formula $t_{1/2}\ k = \ln 2$. The half-life is less than 40 min in early digestion times, about a few hours at 20 °C, and about 1.5−4 days at 4 °C in late trypsin digestion times. Under our experimental conditions, the half-life at late times can be considered a measure of the time of trimer to monomer conversion.

trypsin than the portion bearing the Gly-X-Y repeat.

The data obtained by densitometry of gels (those shown in Figure 4A and similar ones for the other peptides) can be explained by the following scheme (v are the reaction rates; v_1 and v_2 are the rates for the conversion of trimer to monomer and of monomer to trimer, respectively):

$$\text{peptide trimer} \underset{v_2}{\overset{v_1}{\rightleftharpoons}} \text{peptide monomer} \xrightarrow[v_3]{\text{trypsin}} \text{degraded products}$$

We considered that the rate-limiting step is the conversion of trimer to monomer ($v_3 \gg v_1, v_2$) and that $v_1 > v_2$ because monomer concentration is maintained very low by trypsin action. Therefore, during early stages of digestion the variation with time of the amount of species surviving trypsin digestion is a kinetic measure of trypsin action. More importantly, the variation occurring at late digestion times is a measure of the conversion of trimer to monomer and thus an estimate of trimer half-life can be obtained.

We used a linear interpolation in a plot that follows first-order kinetics (natural logarithm of band area versus time) and sectioning the shorter times (0−5 min of trypsin treatment) from late digestion times (Figure 4B). The densitometric data are affected by some uncertainties, due both to the procedure and to the approximations that had to be made in the case of peptides showing the presence of smaller fragments. It was, however, evident that in the first stages of proteolytic digestion the half-life of dissolved intact molecules is short (3−39 min) for all peptides (except α1-(I) CB4,5, not tested). At late times of trypsin treatment, the half-life of the surviving species is about one order of magnitude higher at 20 °C (a few hours) and about two orders of magnitude higher at 4 °C (about 1.5−4 days). This determination of the half-life of trimeric species is useful also because it justifies the need for long equilibration times of the peptide solution before any analysis. Due to the above mentioned qualitative and quantitative limitations of the procedure, we did not perform any further experiments at different temperatures to obtain an accurate estimation of the activation barrier for what we consider the trimer−monomer conversion. However, the trimer half-life for all peptides was about 5−30 times higher at 20 °C than at 4 °C; the kinetic constant is correspondingly lower, and therefore an activation energy of roughly 4−9 kcal/mol can be calculated by using the Arrhenius equation.

Rotary shadowing studies on native α1(I) CB7 (the peptide that showed the largest fraction of trimers in analytical gel filtration) clearly demonstrated the presence of thin rod-like molecules 88 ± 13 nm long (mean ± standard deviation; n

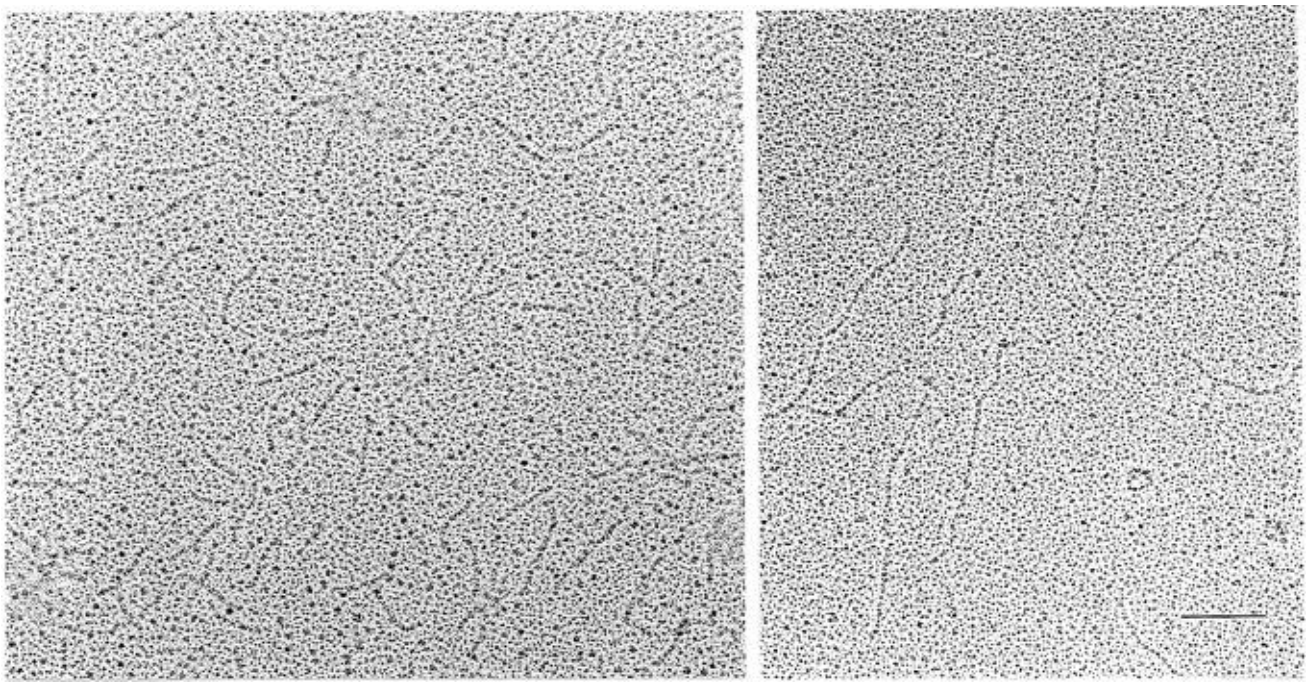


FIGURE 5: Rotary shadowing. Rotary shadowing of peptide α1(I) CB7 (left) and of its parent acid-soluble type I collagen (right). Bar, 100 nm.

= 145) and 3.0 nm thick, in comparison to 293 ± 18 nm (*n* = 181) and 2.7 nm for parent acid-soluble type I collagen molecules (Figure 5). Terminal unraveling was seen only for a few molecules, when all three unraveled monomer portions are adjacent (data not shown). As expected, shadowing of heat-denatured samples did not show any molecules (Engel, 1994), with the exception of very few trimeric molecules than can re-form in between the denaturation and the solvent evaporation steps, because their formation is favored by thermodynamic and kinetic factors. The measured peptide length is therefore 30% of collagen length, slightly longer than that expected from the primary sequence (CB7 is 271 residues out of 1014 of total triple-helical domain). This discrepancy should not be attributed to misalignment of monomers in most trimers, because this is inconsistent both with data from the trypsin experiment and with the fact that single α chains or portions are undetectable, as stated above. Rather, this discrepancy could be explained by the high standard deviations caused by the effect of drying onto the mica, as shown for type I collagen by Fleischmajer et al. (1991).

The trimer−monomer transition was also followed by circular dichroism at 221 nm at increasing temperatures. The melting temperatures for the peptides were found to be in the range 23.6−28.0 °C, i.e., 6−10 °C less than the value of 33.8 °C found for intact type I collagen analyzed under the same conditions (0.49 mg/mL, 5.31 μM) (Figure 6 and Table 1). We stress that our procedure for T_m determination is not an equilibrium method [see Privalov (1982) and our previous data on long trimer half-lives, as deduced in the trypsin experiment]. For some α1(I) CNBr peptides, Saygin et al. (1978) showed hysteresis of CD signal as a function of temperature because equilibrim is not reached at each temperature. Therefore, melting temperatures we determined are only approximate, probably overestimated for the trimer inertia to melting.

The value of collagen T_m is compatible with the solvent used and its pH [see Dick and Nordwig (1966)]. For comparison, the fragment B, obtained by collagenase digestion of human type I collagen and covering the N-terminal two-thirds of the triple-helical domain, melts at about 4−5 °C less than type I collagen (values measured with a different procedure, i.e., trypsin susceptibility) (Lightfoot et al., 1994). Dick and Nordwig (1966) correlated the nonlinear dependence of collagen T_m on the pH with the pK_a values and the ionization status for the carboxyl groups. At neutral pH a higher T_m could also be expected for peptides. We also deduced that the trimer formation is enthalpy-driven, entropy having an opposite effect, as already reported for some type I collagen α1(I) CNBr peptides (Saygin et al., 1978) or collagen-like model peptides (Long et al., 1993). This experiment also shows that 8−20% of trimers convert to monomers when the temperature is raised from 4 to 20 °C.

Monomer CD signal at 221 nm has a pronounced linear temperature dependence seen over a range of 12−27 °C, as already noted for model peptides by Engel et al. (1977) and Long et al. (1993). Type I collagen and its CNBr peptides showed a monomer drift at increasing temperatures, d[θ]/dT, in the range −23 to −34 deg·cm^2·dmol^{-1}·K^{-1}, with the exception of α1(I) CB3 which showed a value of about −6.5, for unknown reasons. Independent confirmation of these d[θ]/dT values has been obtained over a range of 40 °C for all peptides from spectra of denatured samples at 10 and 50 °C (data not shown). Furthermore, the intercept *at 0 K* of the linear drift has a similar value (18−46% higher) to that of the mean residue ellipticity of folded molecules reported in Table 1.

DISCUSSION

There is renewed attention concerning studies on collagen fragments or peptides which bear either natural or model collagen-like sequences, especially those with imino acid-rich sequences such as (Pro-Pro-Gly)$_n$ or (Pro-Hyp-Gly)$_n$.

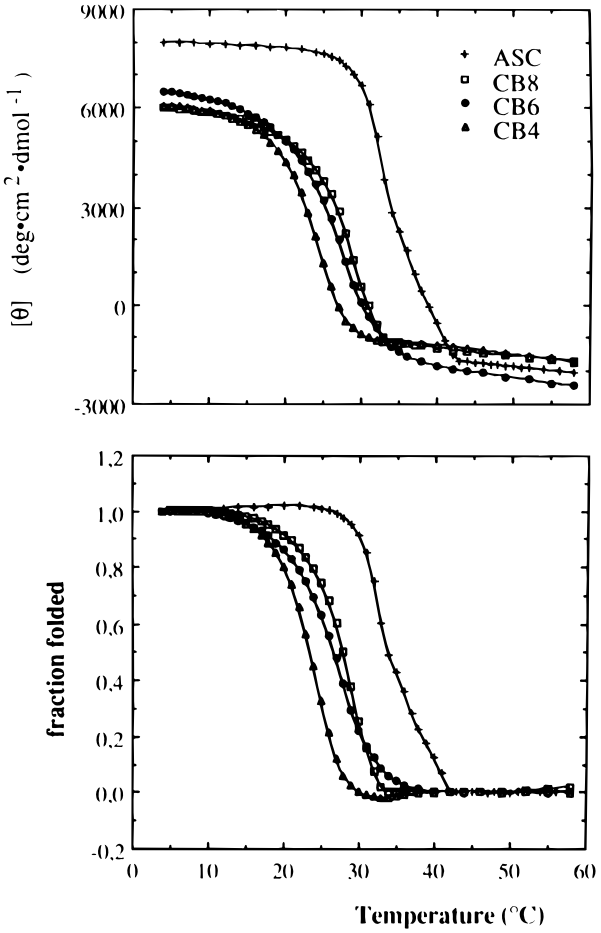


FIGURE 6: Melting curves and temperatures. (Top panel) Variation of the CD signal at 221 nm was followed at increasing temperatures (4−58 °C) for peptides dissolved in 0.1 M acetic acid at 0.5 mg/mL (0.32 mg/mL for CB6), i.e., 18−61 μM. For clarity, the panel reports the profile for some peptides only. (Bottom panel) Fraction of folded molecules was computed according to Engel et al. (1977). Melting temperature values (fraction folded = 0.5) are reported in Table 1.

This is because of recent advances in studies on diseases due to mutations in the genes coding for collagen α chains and studies on collagen interactions with other extracellular matrix macromolecules and with cell receptors.

In the late sixties and the seventies studies were performed on collagen peptides mainly to elucidate their physicochemical properties. Examples are the studies on type I collagen CNBr peptides [see, e.g., Piez and Sherman (1970) and Saygin et al. (1978)], on imino acid-rich model peptides, such as the aforementioned or similar peptides of differing length and composition [see Engel et al. (1977) and Brown et al. (1969, 1972)]. Studies were also performed on proteins and their fragments with a collagen-like domain such as complement subcomponent C1q (Brodsky-Doyle et al., 1976). The availability of powerful peptide synthetic procedures and analytical techniques, such as high-resolution NMR, have enabled studies on model peptides which either incorporate natural sequences or bear modifications or interruptions of the repeating Gly-X-Y sequence (Long et al., 1992, 1993; Li et al., 1993; Fan et al., 1993; Venugopal et al., 1994). The crystal and molecular structure was recently determined for one of these peptides giving detailed information concerning the structural effect of a single glycine substitution and the hydration structure (Bella et al., 1994, 1995).

The aim of this investigation was to study the properties of type I collagen peptides in solution, namely, the molecular species they form, their conformation and factors influencing equilibria between peptide species. Knowledge of these properties is important to determine the best conditions for the use of peptides as substrates or inhibitors in investigations on collagen properties and its interactions with other connective tissue macromolecules and cellular or bacterial receptors. The data we obtained for all type I collagen fragments analyzed with different biochemical and physicochemical tools (circular dichroism, analytical gel filtration, susceptibility to trypsin, rotary shadowing) showed that trimeric species are present in solution and that they are in equilibrium with the monomer forms. This part of our work confirms previous results (Piez & Sherman, 1970; Saygin et al., 1978). Thanks to the collagen sequence and, in particular, to the constraints of the imino acid cyclic structure, peptides spontaneously folded into a conformation very similar to that of the native parent molecule, even though they were denatured at least three times during purification.

The fact that type I collagen CNBr peptides do not reach 100% helical form (Table 1) could be due to several factors: (i) a fraction of the peptide remains in a random conformation, as stated previously; (ii) destabilization occurs because of unraveling at trimer terminal ends due to charge repulsion (Venugopal et al., 1994); (iii) there is misalignment of chains in the trimers (Saygin et al., 1978). Since we obtained a consistent value of percent helical form with three different methods, it is likely that the first factor plays a major role (see, in particular, gel filtration results). Terminal unraveling is almost certainly present and chain misalignment cannot be ruled out by the trypsin experiment. Also in view of the rotary shadowing results, we think that misalignment occurs in a small fraction of trimers and/or there is only a small monomer shift.

Our experiments provided additional relevant information about the physicochemical properties of the peptide species. Some type I collagen CNBr peptides interact to form aggregates which are in equilibrium with triple-stranded species. Since this equilibrium is sensitive to the ionic strength of the medium, the ionic character of interactions in forming the aggregates is important. We are unable to say whether the specificity of trimer self-association is the same as that of the native type I collagen in fibril formation. At higher neutral salt concentrations there is also an increase in hydrodynamic size of monomers and trimers for all peptides. Because of these effects exerted by neutral salt, one wonders whether ionic strength differences in differing tissues, e.g., due to different concentrations of polyanionic proteoglycans and their counterions, could exert any influence on the packing mode of collagens. In fact, type I collagen molecules form fibrils with a molecular packing mode that is tissue-specific (Katz & David, 1992). Furthermore, Burjanadze and Bezhitadze (1992) recently described the effect of neutral salt on the renaturation of partially denatured type I collagen that occurs as the result of the presence of a thermostable domain, probably at the very C-terminal end of the triple helix. This is because the water shell, whose structure is influenced by the concentration of salt, plays an active role not only in stabilization of collagen structure but also in the denaturation−renaturation mechanism. The presence of a thermostable domain cannot be assumed for type I collagen CNBr peptides to justify their

trimer formation with a collagen conformation.

As regards factors influencing equilibria between peptide species, we estimated some thermodynamic and kinetic parameters of the trimer−monomer transition for type I collagen CNBr peptides, i.e., melting temperatures (see Results for theoretical limitations on our data) and the fraction folded in the range 4−58 °C, trimer half-life, which is relatively high at 4 and 20 °C, and the activation energy for trimer conversion to monomer. Careful consideration of these parameters is necessary in studies using peptides when different species and conformation are thought to behave differently. It is also worth noting that mammalian type I collagen (and also its CNBr peptides) spontaneously forms triple-helical species because favorable enthalpic factors overwhelm an unfavourable entropy change [see Privalov (1982)], whereas the opposite happens for the spontaneity of fibril formation (Kadler et al., 1988).

The CD temperature-dependent linear drift at 221 nm seen for collagen and peptide monomers could perhaps be interpreted as the consequence of increasing conformational freedom. As cited in Result, the drift intercept at 0 K has a value similar to the value shown by folded species. If extrapolation to much lower temperatures than those tested is valid, the decrease of freedom with decreasing temperatures could be read as a modification toward a stable conformation. In other words, it is tempting to speculate that the preferred conformation of a collagen α chain could be similar (or the same) either assumed by a single-stranded molecule at 0 K and in the form of a triple helix at much higher temperatures (i.e., at body temperature) because of many weak interchain bond constraints.

At present, knowledge at the molecular level of interactions occurring between collagen and other connective tissue macromolecules and cells is insufficient. There are a few studies whereby the type I collagen binding domain for collagenase and fibronectin (Dzamba et al., 1993) or cellular integrins (Staatz et al., 1991) or proteoglycans like decorin (Scott & Glanville, 1993) has been investigated or postulated.

The structure of peptide species, whether single-stranded or triple-helical, is a relevant factor when considering their interactions with other extracellular matrix components or with cell receptors. In fact, when the inhibitory effect of a peptide on platelet adhesion to collagen was studied, it was found that inhibition was greater when the peptide is in a triple-helical conformation rather than when single-stranded (Santoro et al., 1994). Peptide structure was also found to be an important factor in melanoma cell adhesion and spreading on a substratum of a synthetic peptide that incorporates a natural type IV collagen sequence (Fields et al., 1993). A further example is the conformation dependence of integrin-type II collagen binding: integrin $\alpha_2\beta_1$ is the direct and dominant receptor for native collagen, whereas the binding to denatured collagen, with a major site within peptide α1(II) CB10, is mediated by an integrin $\alpha_5\beta_1$−fibronectin bridge. Denatured CNBr peptides from type II collagen are unable to reproduce the native collagen binding (Tuckwell et al., 1994). Unfortunately, no studies with renatured peptides were reported in this work. Studies on type II collagen fragments that model collagen epitopes in the investigations of murine type II collagen-induced arthritis, an animal model for autoimmune human rheumatoid arthritis, are also worth considering. In particular, two determinants within α1(II) CB11 and their critical residues have been identified as being important for tolerance and suppression of collagen-induced arthritis in mice (Lambert & Berling, 1994; Michaëlsson et al., 1992; Myers et al., 1992, 1995). It is, however, difficult to infer from the original papers whether fragment conformation (single- or triple-standed) is important for activity, even though we can reasonably think that fragments are mostly single-stranded species when used *in vivo* (injected in mice as tolerogens), for melting temperature being very probably lower than body temperature, as we determined for type I collagen CNBr peptides.

A final point is worth discussing. To date, two routes for obtaining fragments with a collagen sequence are used, i.e., from natural sources or by synthesis. It is now also possible to synthesize peptides with a triple-helical conformation ("minicollagens"; Fields et al., 1993). Many collagen types are heterotrimers, but, to our knowledge, there is still no procedure available that can yield heterotrimeric triple-helical collagen-like peptide species. In principle, if kinetic and thermodynamic factors are favorable, heterotrimeric species could form by mixing stoichiometric amounts of relevant peptides. However, in these conditions, we would also expect a heterogeneous population of molecular species to form which would quite difficult to deal with.

All side chains of X and Y residues of the Gly-X-Y repeat from all three α chains protrude outward, and all atoms create characteristic superficial features [for a recent brief description, see Kühn, (1994)]. It is obvious that *homo*trimeric peptides from *hetero*trimeric collagens could match only some of the superficial features exactly but could also mimic some other features, due to frequent similarity or identity of sequence in corresponding stretches of genetically different α chains in a heterotrimer. The relevance of surface features can be understood, for instance, from studies from a few cases of osteogenesis imperfecta where a phase shift in the folding of the three helices has indirectly been inferred (Wallis et al., 1992; Lightfoot et al., 1992, 1994). The phase shift may have a profound effect on collagen fibril formation, on collagen interactions, and thus on the phenotype. Which molecular features are involved and which consequences at a molecular level are relevant to explain the phenotype and phenotypic differences remain unanswered questions. We think that proper use of collagenous peptides will increasingly be of great value in collagen studies for solving the biological problems mentioned. For more than a century collagen has been known as a chemical and biological individuality, and after the seventies it became better known as a family of related individuals. However, it has still not revealed all its secrets.

ACKNOWLEDGMENT

We thank Profs. Luigi Casella and Severino Ronchi for helpful suggestions, discussions, and criticism. Our thanks also to Angelo Gallanti for technical assistance.

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BI9518151