

Molecular stability of chemically modified collagen triple helices

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Abstract Ionic residues influence the stability of collagen triple helices and play a relevant role in the spontaneous aggregation of fibrillar collagens. Collagen types I and II and some of their CNBr peptides were chemically modified in mild conditions with two different protocols. Primary amino groups of Lys and Hyl were *N*-methylated by formaldehyde in reducing conditions or *N*-acetylated by sulfosuccinimidyl acetate. The positive charge of amino groups at physiological pH was maintained after the former modification, whereas it was lost after the latter. These chemical derivatizations did not significantly alter the stability of the triple helical conformation of peptide trimeric species. Also the enthalpic change on denaturation was largely unaffected by derivatizations. This implies that no significant variation of weak bonds, either in number or overall strength, and of entropy occur on modification. These properties can probably be explained by the fact that chemically modified groups maintain the ability to form hydrogen bonds.

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Key words: Collagen; Chemical modification; Molecular stability

1. Introduction

Fibrillar collagens, abundant and essential in the extracellular matrix of connective tissues, are characterized by the assembly of three polypeptide chains (α chains) to form trimers with triple helical conformation and by the supramolecular assembly of trimers to fibrils and fibers [1,2].

The triple helix is formed by three interwoven α chains, each adopting a polyproline II-like helix. This assembly is the direct consequence of the primary structure characterized by the repetition of Gly-X-Y triplets. The side-chain hydrogen atom of Gly is hidden in the core of the triple helix, whereas the side chains of residues X and Y are on the surface and accessible to solvent and other molecules, X residues more than Y residues [3]. The sources of stability in the mammalian triple helix are interchain hydrogen bonds involving the backbone and a large content of cyclic amino acids proline and

hydroxyproline, with a particular relevance for the Hyp hydroxyl group (review in Privalov [4]; recent findings in [5,6]). The other, non-cyclic residues modulate the stability. A complete scale of amino acid propensity for triple helix formation has recently been determined in a system where each amino acid occurs in X or Y position of a X-Hyp-Gly or Pro-Y-Gly guest triplet in the core of the (Gly-Pro-Hyp)₈ host peptide; a moderate correlation of this scale with frequency of occurrence in collagens was found [7]. Ionic residues are abundant in fibrillar collagens, about 17% of all residues; in collagens I–III, Arg and Lys occur preferentially in Y position (more than 80% and 65%, respectively), Glu in X position (~90%) [8]. When compared to triplet Pro-Hyp-Gly in the model host peptide cited above, all ionic residues in X position of the guest triplet X-Hyp-Gly are among the less destabilizing amino acids; Arg in Y position of Pro-Arg-Gly is stable as Pro-Hyp-Gly [7]. As a consequence, variation of stability along the triple helix is expected. This was empirically calculated by Bächinger and Davis [9]. Recent findings indicate that type I collagen is thermally unstable at body temperature and suggest that micro-unfolding of the least stable domains would trigger self-assembly of fibers that protect collagen helices from unfolding [10].

Recent studies on crystals of two collagenous peptides (named EKG and T3-785) showed that the ionic groups of Glu and Lys in EKG peptide display direct interactions with backbone carbonyl (and eventually with other groups such as Hyp hydroxyl) or interactions mediated by water molecules instead of forming ion pairs, notwithstanding these are stereochemically possible [6]; arginine residues in T3-785 peptide have similar characteristics [11]. At present it is unknown if these structural characteristics also hold in aqueous solution. A preliminary indication suggests that, in some cases, interactions in solution between ionic side chains within the triple helix do not occur [12].

This demonstrates that ionic residues play relevant roles in collagen structure. They are also responsible for organization at higher levels, such as for spontaneous fibril formation and periodicity of fibrillar collagens [13], or for interactions of collagens with at least some of their ligands. We might therefore expect that chemical modifications of charged residues influence the stability of the triple helical conformation and collagen interactions. Here we report the determination in physiological and acidic conditions of the effects on triple helical stability of two types of derivatization performed with mild protocols, namely *N*-methylation and *N*-acetylation of Lys/Hyl ϵ -amino group of collagens I and II and some of their CNBr peptides. We have recently shown that decorin–collagen interactions are influenced by the same modifications [14].

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Abbreviations: N⁰, original unmodified sample; N^m, *N*-methylated sample; N^a and N^{aa}, samples *N*-acetylated with sulfosuccinimidyl acetate and acetic anhydride, respectively; CD, circular dichroism; NHSAc, sulfosuccinimidyl acetate

2. Materials and methods

2.1. Collagens and their CNBr peptides

Acid-soluble type I collagen from bovine skin and its CNBr peptides were prepared and characterized, as described in [15–18]. Pepsinized type II collagen was purified from bovine nasal septum, essentially as described in [19]. CNBr peptides from collagen II were purified essentially following the procedures used for peptides from type I collagen, by means of a combination of gel filtration chromatography followed by ion-exchange chromatography or by reverse-phase chromatography for the two smaller peptides.

All collagenous samples were analyzed by means of a quantitative Hyp assay [20] after acid hydrolysis in 6 M HCl at 106–108°C for 22–24 h, gel electrophoresis in denaturing conditions [21] and gel staining with Coomassie brilliant blue R250, and N-terminal sequencing for some peptides.

Analytical gel filtration chromatography in denaturing conditions was performed, essentially as described in [15], on a Superose 6 column equilibrated and eluted at room temperature and low flow rate (0.2 ml min⁻¹) with 50 mM acetic acid, 500 mM NaCl. Peptides were dissolved in 0.1 M acetic acid at 4°C. Aliquots containing 50–100 µg were supplemented with one-tenth volume of 0.1 M acetic acid, 5 M NaCl, and denatured for 3 min at 60°C just before the analysis.

Circular dichroism (CD) spectroscopy and determination of melting profiles were performed in a neutral buffer composed of 132 mM NaCl, 1.5 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 1 mM EDTA, 1.5 mM NaN₃, pH 7.5, with the procedure we have used in the past [18]. Briefly, solutions of collagens and peptides were prepared by dissolving dry samples in 0.1 M acetic acid at 1–1.5 mg ml⁻¹ and equilibrated at 4–5°C for ≥7 days; after clarification by centrifugation, the concentration was determined by means of a Hyp assay [20]. Aliquots of the acidic solutions were freeze-dried, redissolved in the neutral solution reported above (at 80 µg ml⁻¹ for spectra recording or 500 µg ml⁻¹ for the determination of melting profiles) and equilibrated for ≥7 days at 4–5°C. All solutions were clarified by centrifugation immediately before CD analysis. Melting profiles were determined on collagenous samples by following the CD signal at 221 nm, starting at 0–4°C and increasing the temperature in steps of 1°C every 8 min (the average heating rate is therefore 7.5°C h⁻¹). Data points at each temperature were taken from the average CD signal over the last 1 min of the 8 min period. In some instances, 0.1 M acetic acid was used as solvent. The sample concentration was again checked after CD analysis by means of the cited quantitative Hyp assay. Thermodynamic data relative to denaturation profiles were determined, within the framework of the two-state model for trimer → monomer transition [4], with a classical van't Hoff treatment of the denaturation curve. Melting temperatures and van't Hoff enthalpies (ΔH^0) were obtained by best fitting of experimental data, ΔS^0 and ΔG^0 calculated from the two former parameters, with the mathematical procedure of Engel et al. [22]. In some detail, CD data at each temperature and total molar concentration of chains in the collagen sample (c_0 in the equations below) were utilized to sequentially compute: (i) mean residue ellipticities at each temperature $\theta(T)$; (ii) the contribution to total ellipticity by the trimeric and monomeric forms, θ_{trimer} and $\theta_{\text{monomer}}(T)$ respectively; the former is constant, whereas the latter is temperature-dependent; (iii) the fraction folded as the fraction of collagen chains in the folded triple-helical state; the fraction folded at each temperature $F(T)$ was calculated with the equation:

$$F(T) = \frac{\theta(T) - \theta_{\text{monomer}}(T)}{\theta_{\text{trimer}} - \theta_{\text{monomer}}(T)}$$

(iv) the melting temperature T_m , as the temperature at fraction folded = 0.5; (v) $K_{\text{eq}}(T)$, the equilibrium constant of the transition '1 mol of trimer → 3 mol of monomer', computed at each temperature with the equation:

$$K_{\text{eq}}(T) = \frac{[\text{monomer}]^3}{[\text{trimer}]} = \frac{3c_0^2[1-F(T)]^3}{F(T)}$$

(vi) van't Hoff enthalpy, ΔH^0 , obtained by curve-fitting from the van't Hoff equation:

$$K_{\text{eq}}(T) = \exp\left[\frac{\Delta H^0}{RT}\left(\frac{T}{T_m} - 1\right) - \ln\left(\frac{3c_0^2}{4}\right)\right]$$

(vii) ΔS^0 , computed with the equation:

$$T_m = \frac{\Delta H^0}{\Delta S^0 + R \ln(3c_0/4)}$$

(viii) ΔG^0 , computed at 298 K with the equation: $\Delta G^0 = \Delta H^0 - T\Delta S^0$.

Estimated uncertainties are 0.3–0.4°C for melting temperature [18,7], 15% for ΔH^0 and ΔS^0 , 5% for ΔG^0 [22].

2.2. Chemical modification of collagens and CNBr peptides

Chemical derivatizations have been performed with three different methods, all involving the primary ε-amino group of lysine and hydroxylysine side chains (Fig. 1). All derivatized samples have been analyzed for purity and conformation as described above.

N-methylation. A literature protocol involving formaldehyde in reducing conditions for the presence of NaBH₃CN was used [23]. The incubation with HCHO/NaBH₃CN was performed for 2 h at room temperature followed by 12–18 h at 4°C. The samples were then dialyzed against 0.1 M NaCl and then against 0.1 M acetic acid, clarified by centrifugation, freeze-dried and stored at -80°C.

N-acetylation with acetic anhydride. The protocol of Davis et al. [24] was used. Briefly, 5–10 mg of collagenous samples was suspended in 10 ml of 0.5 M borate buffer, pH 8.5, by stirring overnight. The derivatization was performed in an ice bath by adding 330 µl of acetic anhydride. The addition of acetic anhydride was repeated three times at intervals of 30 min. The pH was maintained constant under the pH meter by additions of aliquots of 5 M NaOH. These harsh conditions introduce artifacts (see Section 3). After derivatization, the samples were dialyzed against 0.1 M acetic acid, centrifuged and freeze-dried as *N*-methylated samples.

N-acetylation with sulfo-succinimidyl acetate (SNHSAc). The reaction conditions with SNHSAc are mild. Operating at 4°C, collagenous samples (5–15 mg) were suspended overnight in 10 ml of 0.5 M borate buffer, pH 8.5. Solid SNHSAc (Pierce) was quickly dissolved at 10.4 mg ml⁻¹ (40 mM) in 10 mM acetate buffer, pH 5.4–5.6, immediately before its subsequent use. SNHSAc solution was added under vigorous stirring to the collagen samples in order to have a 10:1 molar ratio between SNHSAc and primary amino groups. Derivatization was allowed to proceed overnight. Derivatives were then dialyzed, centrifuged and freeze-dried as above.

The degree of Lys/Hyl modification was determined by a colorimetric method involving sodium trinitrobenzenesulfonate, essentially as described in [25], using *N*α-acetyl-L-lysine as standard.

3. Results

3.1. Modification and analysis of collagens and peptides

Type I and type II collagens, as well as some of their CNBr peptides, were chemically derivatized with different protocols involving the primary amino group of Lys and Hyl side chains and of the N-terminal end. *N*-methylation was performed in a

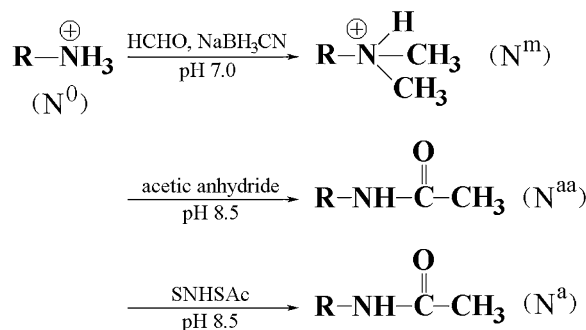


Fig. 1. Scheme of chemical modifications. Primary amino groups of collagen types I and II and of some of their CNBr peptides were derivatized as outlined. The scheme reports the forms prevailing at physiological pH. A mixture of *N*-mono- and *N*-di-methyl derivatives is formed with the top procedure (only the latter is shown). Loss of the positive charge occurs on *N*-acetylation. Symbols for the original and modified samples are reported in parentheses.

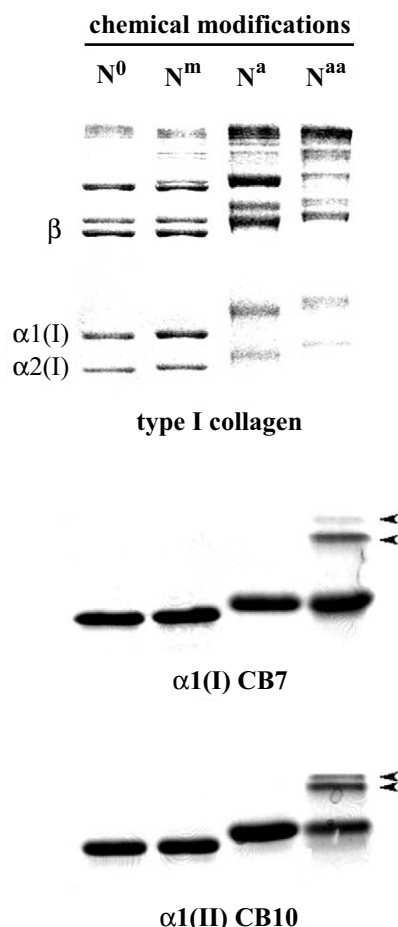


Fig. 2. Electrophoretic analysis of modified collagen samples. Representative SDS-PAGE for underivatized and derivatized type I collagen in 6% gels and two peptides in 15% gels. See Fig. 1 for the symbology of chemical derivatives. The position of bands for α and β chains (β : two cross-linked α chains) are indicated for type I collagen. Bands due to artifacts introduced by the procedure of *N*-acetylation with acetic anhydride are indicated by arrowheads. The amount analyzed was about 1.5 μ g for N^0 and N^m samples, whereas it was two to three times higher for N^a and N^{aa} samples because of their low affinity for Coomassie brilliant blue R250.

mild procedure by HCHO and a reducing agent (NaBH_3CN), with the formation of mono- and di-methyl derivatives that maintain the positive charge at physiological pH. *N*-acetylation was performed with acetic anhydride or with a milder procedure using SNHSAC; *N*-acetylation eliminates the positive charge (Fig. 1).

A quantitative assay of primary amino groups with sodium trinitrobenzenesulfonate showed a derivatization extent higher than 80% for most samples. A lower percentage was found for type I and II collagens when derivatized with SNHSAC (70 and 76%, respectively) and for two peptides from type II collagen when treated with acetic anhydride (56% for CB8 and 65% for CB10).

The electrophoretic analysis in denaturing conditions showed that *N*-methylated samples have a mobility similar to their unmodified counterparts, whereas *N*-acetylated samples have a slower electrophoretic migration (Fig. 2). The protocol involving acetic anhydride introduces artifacts, as seen by the appearance of new bands of larger size, particularly evident for peptides.

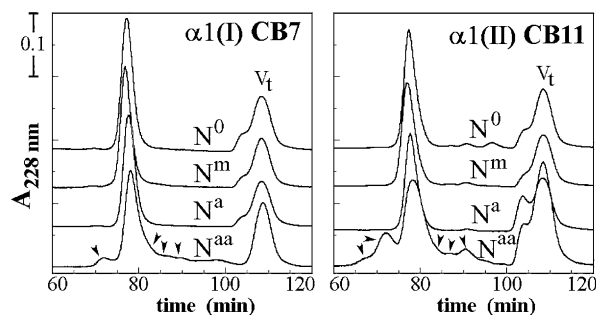


Fig. 3. Analytical gel filtration in denaturing conditions. Elution profiles from a Superose 6 column are shown for modified and unmodified samples of two peptides. For the symbology of chemical derivatives see Fig. 1. For clarity, the four traces in each panel are offset by 0.15 absorbance units. Peaks due to artifacts introduced by the procedure of *N*-acetylation with acetic anhydride are indicated by arrowheads. V_t indicates the total elution time or volume.

Analytical gel filtration chromatography in denaturing conditions on heat-denatured samples demonstrated slight differences of hydrodynamic size between underivatized and modified samples (Fig. 3). Again, samples treated with acetic anhydride showed a different pattern: with respect to the main peak, there is the presence of molecules of larger and lower size. This demonstrates that covalent chemical bonds were broken and also formed during the derivatization, probably due to the addition of concentrated NaOH in order to maintain constant pH.

CD spectroscopy in a neutral buffer showed that the spectra of peptides derivatized in mild conditions (samples *N*-methylated or *N*-acetylated with SNHSAC) have an identical profile with respect to the spectrum of the underivatized counterpart, differences concerning only the intensity of the positive peak (Fig. 4); therefore, the derivatized samples are trimers in triple helical conformation, independently of the maintenance or loss, on derivatization, of the positive charge. On the con-

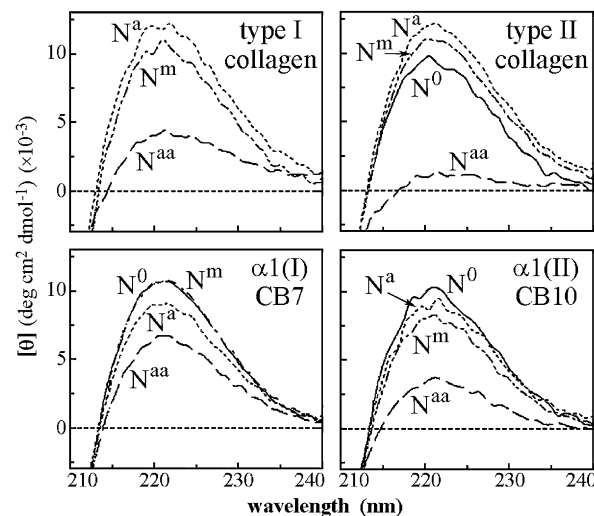


Fig. 4. CD spectroscopy. CD spectra were recorded in a neutral buffer (pH 7.5) at 30°C for collagens or at 20°C for peptides. For the symbology of chemical derivatives see Fig. 1. Underivatized type I collagen was not soluble in the buffer; no spectrum for it is therefore present in the top left panel. For each sample, only a portion of the spectrum is shown, centered on the positive peak near 221 nm. This positive peak in collagen samples is the index for triple helical conformation and disappears on denaturation.

trary, the protocol with acetic anhydride caused a drop of the 221 nm signal (Fig. 4), indicating a loss of triple helical conformation. For all these reasons, samples *N*-acetylated with acetic anhydride were not further used.

3.2. Thermal stability of trimeric species of derivatized peptides

Melting transitions were determined in neutral conditions and physiological ionic strength for five CNBr peptides, by measuring the 221 nm CD signal at increasing temperatures; melting profiles allowed us to calculate the melting temperature of peptide trimers and the standard change of van't Hoff enthalpy, entropy and free energy of the transition.

The results obtained, in agreement with the literature, show that the denaturation of the triple helix (trimer → monomer) is endothermic and endergonic both in neutral and acidic conditions (Table 1); also ΔS^0 is positive (data not shown).

For each peptide analyzed, melting curves of *N*-methylated or *N*-acetylated samples are largely superimposable with the profile of the original unmodified peptide, both for total ellipticity and fraction of folded molecules (for an example, see Fig. 5). Thermodynamic data calculated from melting transitions clearly show for most samples that chemical derivatizations do not significantly change the thermal stability of peptide trimeric species in physiological conditions, measured by

melting temperature or by ΔG^0 (Table 1, part A). The largest variation is shown by $\alpha 1(\text{II})$ CB10. Furthermore, the enthalpic change on denaturation also is not significantly affected by chemical derivatizations for almost all samples (Table 1, part A). As noted in the past by others and us, T_m is correlated with ΔG^0 and ΔH^0 is correlated with ΔS^0 [18,7].

Collagens type I and II, and other peptides, such as $\alpha 1(\text{I})$ CB8 and $\alpha 2(\text{I})$ CB4, were not analyzed because of their low solubility in the neutral buffer we used [18].

We determined the stability of peptides and their derivatives also at acidic pH in 0.1 M acetic acid. In this condition there is also no or a low influence of chemical derivatizations on the stability of peptide trimers (Table 1, part B). Furthermore, all samples have melting temperature and ΔG^0 lower than at neutral pH; it is therefore worth noting that the ionization status of carboxylic groups influences the stability more than the modifications of primary amino groups.

4. Discussion

In the present work we report the influence of two different chemical derivatizations of collagen Lys/Hyl ϵ -amino group on the stability of the triple helical conformation. Both modifications have been performed in mild conditions. One mod-

Table 1

Thermodynamic parameters calculated from the melting transition of collagen peptide trimers to monomers, in neutral (A) and acidic conditions (B)^a

Peptide	Derivatization	T_m (°C)	ΔH^{0b} (kJ mol ⁻¹)	ΔG^{0b} (kJ mol ⁻¹)
(A) In neutral conditions				
$\alpha 1(\text{I})$ CB3	none	31.5	557	64
	<i>N</i> -methylation	31.0	511	62
	<i>N</i> -acetylation	29.3	461	58
$\alpha 1(\text{I})$ CB7	none	33.4	508	69
	<i>N</i> -methylation	33.0	537	69
	<i>N</i> -acetylation	31.7	490	66
$\alpha 1(\text{II})$ CB11	none	27.7	574	63
	<i>N</i> -methylation	26.8	590	59
	<i>N</i> -acetylation	28.0	606	62
$\alpha 1(\text{II})$ CB8	none	27.3	643	57
	<i>N</i> -methylation	26.9	642	56
	<i>N</i> -acetylation	27.0	682	56
$\alpha 1(\text{II})$ CB10	none	30.0	511	69
	<i>N</i> -methylation	27.3	478	61
	<i>N</i> -acetylation	27.2	514	60
(B) In acidic conditions				
$\alpha 1(\text{I})$ CB3	none	27.2	553	56
	<i>N</i> -methylation	27.5	552	56
	<i>N</i> -acetylation	25.7	613	53
$\alpha 1(\text{I})$ CB7	none	29.4	594	63
	<i>N</i> -methylation	29.6	605	64
	<i>N</i> -acetylation	29.7	545	63
$\alpha 1(\text{II})$ CB11	none	25.5	446	56
	<i>N</i> -methylation	26.0	509	57
	<i>N</i> -acetylation	27.1	650	60
$\alpha 1(\text{II})$ CB8	none	24.9	593	51
	<i>N</i> -methylation	24.5	655	51
	<i>N</i> -acetylation	24.4	667	50
$\alpha 1(\text{II})$ CB10	none	25.2	538	57
	<i>N</i> -methylation	24.7	518	56
	<i>N</i> -acetylation	26.2	502	58

^aMelting transitions were determined from the 221 nm CD signal at increasing temperatures. The concentration of peptides was 0.5 mg ml⁻¹; a lower solubility was found in neutral conditions for underivatized $\alpha 1(\text{II})$ CB11, underivatized and *N*-methylated $\alpha 1(\text{II})$ CB10, namely 0.27, 0.19 and 0.38 mg ml⁻¹, respectively. For each peptide the table reports the type of derivatization, the value of melting temperature of trimeric species, the standard van't Hoff enthalpic and free energy changes. The thermodynamic parameters were calculated within the framework of the two-state model for trimer → monomer transition [4], with a classical van't Hoff treatment of the denaturation curve and the mathematical procedure of Engel et al. [22]. Estimated uncertainties are 0.3–0.4°C for melting temperature [7,18], 15% for ΔH^0 and ΔS^0 , 5% for ΔG^0 [22].

^b ΔH^0 and ΔG^0 are in kJ (mol of trimer)⁻¹.

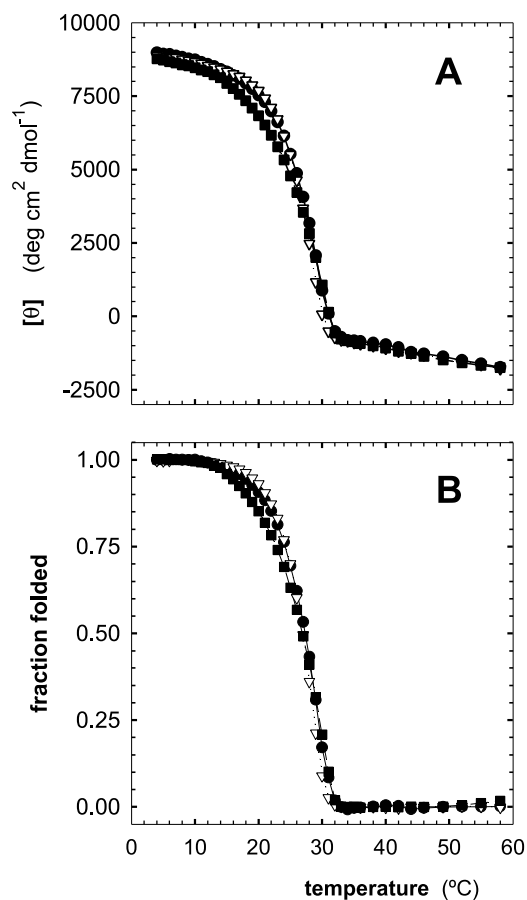


Fig. 5. Melting transitions. A: CD signal at 221 nm, recorded at increasing temperatures and plotted as mean residue ellipticity (θ). B: Fraction of chains in the folded triple helical state (fraction folded), calculated as described in Section 2. The examples reported in both panels are from melting transitions determined in physiological conditions for peptide $\alpha 1(\text{II})$ CB8, before and after *N*-methylation or *N*-acetylation (filled circles, filled squares and open triangles, respectively). It is worth noting that the three curves show only slight differences.

ification, *N*-methylation, preserves the positive ionic charge, whereas the other, *N*-acetylation, eliminates it. *N*-acetylation was also obtained with acetic anhydride, but in this case the artifactual products precluded any use.

The main conclusion that can be drawn is that the mild modifications do not significantly alter triple helical stability

at physiological pH for most cases and have only a small effect for the others. This conclusion also holds at acidic pH. The effect of chemical modifications on triple helical stability is qualitatively based on the similarity of CD spectra and melting profiles of modified collagens and peptides with respect to their unmodified counterparts (Fig. 4 and 5), showing that modified samples are in triple helical conformation; this effect is quantitatively demonstrated by thermodynamic data from melting profiles of five CNBr peptides (Table 1). Chemical derivatizations of Lys/Hyl amino groups have a low or null effect on the stability of modified peptide trimers, as indicated by melting temperatures and ΔG^0 . Furthermore, the enthalpic change on denaturation is also largely unaffected for almost all cases. Derivatizations therefore cause no or only slight variations of weak bonds, either in number or strength, since only weak bonds and no covalent bonds are involved in the melting of triple helical trimers. It turns out that ΔS^0 is also unaffected by modifications, since in our set of data ΔH^0 is highly correlated with ΔS^0 . The determinations at acidic pH confirm these results. Interestingly, the variation of ionization status for carboxylic groups on decreasing the pH has a larger effect on stability than chemical modifications.

These findings are the outcome of structural characteristics. We think that a basic reason explaining our results might be found in the fact that chemical derivatives maintain the ability to form hydrogen bonds: potentially, *N*-methyl derivatives act as H donors, *N*-acetyl derivatives are H donors and acceptors with their NH group and H acceptors also for their C=O group. Structural data on the crystals of the synthetic peptide (Pro-Hyp-Gly)₄-Glu-Lys-Gly-(Pro-Hyp-Gly)₅ showed that Glu and Lys, instead of forming stereochemically allowed ion pairs, display direct interactions with backbone carbonyl or Hyp hydroxyl groups or interactions mediated by water molecules [6]. In the crystal of a different peptide (T3-785), Arg guanidinium groups make direct contacts with backbone C=O and water-mediated H bonds with other groups [11]. If this also occurs in solution, chemically modified groups can participate in direct or water-mediated H bonding, without any large difference, with respect to the underivatized samples, for number or overall strength of weak bonds and for entropy.

Reviewing and discussing earlier work, Privalov [4] reported that the process of denaturational transition of collagen structure is relatively slow as compared with denaturation of other proteins, and, more importantly, a real equilibrium cannot be achieved. The apparent transition temperature strongly de-

Table 2

Comparison between different chemical derivatizations: effect on thermal stability of the collagen triple helical conformation

Modification: reaction and consequences	T_m (variation on derivatization)	
	neutral pH	acidic pH
CNBr peptides from collagens I and II (this work) ^a		
<i>N</i> -methylation: $-\text{NH}_2 \rightarrow -\text{NHCH}_3$ and $-\text{N}(\text{CH}_3)_2$, decreased content of $-\text{NH}_2$	from -0.4 to -2.7°C	from $+0.5$ to -0.5°C
<i>N</i> -acetylation (with SNHSAc): $-\text{NH}_2 \rightarrow -\text{NHCOCH}_3$, decreased content of $-\text{NH}_2$	from $+0.3$ to -2.8°C	from $+1.6$ to -1.5°C
Type I collagen (Rauterberg and Kühn [30]) ^b		
Esterification: $-\text{COOH} \rightarrow \text{COOCH}_3$, decreased content of $-\text{COOH}$	-8.6°C	-5.6°C
Deamidation: $-\text{CONH}_2 \rightarrow -\text{COOH}$, increased content of $-\text{COOH}$	-4.1°C	-3.1°C
<i>N</i> -succinylation: $-\text{NH}_2 \rightarrow -\text{NHCOCH}_2\text{CH}_2\text{COOH}$, decreased/increased content of $-\text{NH}_2/-\text{COOH}$	-4°C^c	–

^aNeutral solvent: 132 mM NaCl, 1.5 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4 , 1 mM EDTA, 1.5 mM NaN_3 , pH 7.5. Acidic solvent: 0.1 M acetic acid, pH 2.9–3.0.

^bNeutral solvent: 0.05 M Tris-HCl containing 0.1 or 0.5 M CaCl_2 , pH 7.4. Acidic solvent: 0.225 M citrate buffer, pH 3.7.

^cSolvent not specified, but probably at neutral pH since the sample was reported to precipitate as fibrils in acidic medium.

depends on the heating rate. Most of the collagen melting experiments have been performed with a temperature gradient at or near $0.1^{\circ}\text{C min}^{-1}$. Recently, Leikin and coworkers stated that the thermal denaturation of type I collagen is a fully reversible process and demonstrated an extremely slow equilibrium kinetics between denatured and native type I collagen; equilibrium was not reached in scanning calorimetric experiments even at ultra-slow heating rate ($0.004^{\circ}\text{C min}^{-1}$) [10].

In agreement with this, earlier results we obtained on CNBr peptides from collagens I and II have shown that: (a) a high percentage of single-stranded monomeric peptides is able to form trimeric species, after long standing at low temperature [15,16]; (b) repeated denaturation of the trimeric species of peptide $\alpha 1(\text{I})$ CB2 was fully reversible at acidic pH after long standing at $4\text{--}5^{\circ}\text{C}$, as seen by nuclear magnetic resonance (NMR) spectroscopy; furthermore, the melting transition curves determined at the level of peptide bonds by CD measurements and for an amino acid side chain by NMR were superimposable [17]; (c) by using trypsin at neutral pH, the trimeric species of CNBr peptides have a half-life of 1.5–4 days at 4°C and a few hours at 20°C [15].

On this basis, a true equilibrium was not reached during the melting experiments described in this article, notwithstanding the ‘low’ heating rate (an average of $0.125^{\circ}\text{C min}^{-1}$), as in literature experiments. Nevertheless, we think that the low or null effect of chemical derivatization on thermal stability is a real effect: on the basis of the above-discussed null or slight variation on derivatizations of weak bonds in the trimers, it seems improbable to us that the equilibrium kinetics is significantly slower or faster with respect to the unmodified counterparts. Moreover, we can compare our data with literature data on the effect of chemical modifications on triple helical stability.

Several papers described preparation and use of chemically modified collagens and peptides for functional studies (see e.g. [24,26–29]), but none reported the influence of chemical modifications on thermal stability of the triple helical conformation, with the exception of a paper by Rauterberg and Kühn [30]. Some direct comparison with our data is thus possible. As can be seen in Table 2, the modifications described by Rauterberg and Kühn involved a variation for the content of carboxylic groups, whereas our derivatizations involved primary amino groups. Other relevant differences in the protocols used by Rauterberg and Kühn with respect to ours are: (a) the conditions used in the derivatization, which cannot be considered mild because they involved either very low or very high pH (HCl or NaOH at 0.1 M or more); (b) the presence of calcium ions at 0.1 or 0.5 M in the solvent used for the melting experiments at neutral pH. Von Hippel and Wong [31,32] have demonstrated that calcium chloride decreases the stability of calf skin collagen by 12.8°C/mol of added CaCl_2 per liter. Furthermore, Ca^{2+} is a hard acid preferentially interacting with hard bases, such as O- or N-containing groups. It follows that the decrease of T_m at neutral pH might be due not only to the modification itself but also to a contribution brought by the presence of Ca^{2+} ; the latter contribution may be different for derivatized samples with respect to the unmodified ones because of the altered content of carboxylate groups. From this comparison between the two derivatization types, it follows that, to our knowledge, the data we present here are the first reported concerning the direct determination in physiological conditions (for pH and ionic

strength) of the influence of chemical modifications on collagen molecular stability. It also follows that the variation of the $-\text{COOH}$ content has probably a greater influence on thermal stability than the modification of primary amino groups.

Diamond et al. [33] reported a drop of shrinkage temperature of total insoluble collagen from bovine dermis after chemical derivatizations modifying the $-\text{COOH}$ content. However, the protocols used and the thermal transition analyzed are not directly comparable to ours. Again, it is possible that the quantitative differences between our and Diamond’s data depend on the variation of the $-\text{COOH}$ content.

Here, we do not report any binding studies of derivatized collagens and peptides with self or other extracellular matrix components, but recent determinations of our group have shown that decorin–collagen interactions are influenced by the same modifications described here, and that *N*-methylation has a diversified effect on different collagen samples [14].

In conclusion, our determinations and data indicate that chemical modifications of amino groups performed with mild procedures have no or little influence on collagen at the level of triple helical stability. They also show that reliable functional studies concerning the effect of derivatizations on the binding of collagen with self or other macromolecules need the knowledge of the properties of the samples used; biochemical control experiments are thus required, such as verification of side reactions in the modification protocols with the formation of unwanted molecules, and direct analysis of triple helical stability.

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