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Effect of Deamidation on Stability for the Collagen to Gelatin Transition

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Deamidation of amide residues, Asn and Gln, in collagen occurs during the manufacture of B-type gelatin and could affect the performance of B-type gelatins as it may affect the refolding of triple-helical junctional domains that are formed during gelation. Host−guest peptides of the form acetyl-(Gly-Pro-Hyp)$_3$-Gly-Xaa-Yaa-(Gly-Pro-Hyp)$_4$-Gly-Gly-amide, where the X- and Y-positions of the guest peptide are varied, have been used to examine the effect of changing Asn to Asp and Gln to Glu on triple-helix stability. This paper reports the stability of host−guest peptides containing the guest triplets Gly-Ala-Asn, Gly-Asn-Ala, Gly-Asn-Lys, Gly-Gln-Ala, Gly-Glu-Glu, and Gly-Leu-Glu. In combination with previous data, these now provide 15 pairs of peptides in which the effect of deamidation can be compared. These comparisons show that the deamidation of Asn to Asp, regardless of whether it occurred in either the X- or Y-position, always gave a stabilizing effect; deamidation of Gln in the X-position also led to an increase in stability. In contrast, deamidation of Gln in the Y-position was quite distinct, leading to destabilization. The higher observed frequency of Gln in the Y-position compared with other amides may account for the slight destabilization of collagen following deamidation.

KEYWORDS: Gelatin; peptide models; thermal stability; deamidation; triple helix; collagen

INTRODUCTION

Collagen is the most abundant animal protein, being the principal component of all connective tissues including skin, bone, tendon, and cartilage. As a consequence, it is important in the manufacture of a range of products, including gelatin production. Collagens are characterized by their triple-helical structure, in which three extended left-handed polyproline II-like helical chains are supercoiled into a right-handed triple helix (*1*, *2*). This triple-helical conformation results in distinctive amino acid sequence, with Gly as every third residue, to accommodate the close packing of the three chains. This requirement leads to a characteristic repeating sequence, (Gly-Xaa-Yaa)$_n$, where Xaa and Yaa can be any amino acid but are frequently Pro in the X-position and Hyp, from secondary modification of Pro, in the Y-position (*3*). The high imino acid content, ∼20% in mammalian collagens, is also needed for collagen stability (*4*).

In mature tissues, individual collagen molecules are cross-linked into an extended network that provides tissue with strength and dimensional stability. The cross-linking arises from specific, enzyme-mediated modification of Lys residues in non-triple-helical ends of the collagen to aldehydes. These aldehyde groups then participate in the formation of initial bifunctional cross-links that further react to form a range of multifunctional mature cross-links (*5*). In addition, in older tissues nonspecific cross-links accumulate due to a nonenzymatic glycosylation-based mechanism (*6*, *7*).

In gelatin production the collagenous tissue is "conditioned" to break the cross-linking network. It is then extracted by hot water, which denatures the triple-helical structure into soluble chains and chain fragments (*8*). On cooling, rewinding of the individual chains into new triple-helical structures, not necessarily in the same register as the native collagen structure, is a key step in gel formation (*8*, *9*). The re-forming of triple-helical segments leads to the junction zones that are necessary for gelation (*9*).

For bovine hide raw material, an extensive lime/alkali process was commonly used for conditioning, followed by an acid treatment prior to extraction. More recently, other approaches have been established, for example, an enzyme/alkaline conditioning process of shorter duration (*10*). Bovine hides, particularly hide splits, give a gelatin that is derived almost entirely from type I collagen (*11*). The alkali used in conditioning leads to a polydisperse gelatin product, containing cross-linked polymeric components as well as many small fragments. For other raw materials, for example, porcine skin, an alkaline

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treatment may not be required, and a less cross-linked material (*8*), which is potentially less polydisperse (*12*) and with a higher proportion of intact collagen chains, is obtained (*8*).

The functional properties of gelatins are affected by the nature and extent of the changes that occur during the conditioning and subsequent manufacturing processes. Changes, such as racemization and deamidation, which could interfere with re-forming of the triple helix, may reduce the rate of gel formation and also the resulting gel strength. Alkaline-processed gelatins typically exhibit poorer functional properties than gelatins processed according to other methods (*8*). A major modification that occurs during the alkaline treatment is the deamidation of all Asn and Gln residues to the corresponding Asp and Glu residues.

In the present study, we have used a peptide model to examine the effect of deamidation on the stability of the triple helix. A "host−guest" peptide approach, in which selected guest Gly-Xaa-Yaa triplets were included in a common acetyl-(Gly-Pro-Hyp)$_3$-Gly-Xaa-Yaa-(Gly-Pro-Hyp)$_4$-Gly-Gly-amide peptide framework, has demonstrated the effects deamidation on triple-helix stability (melting temperature, T_m). These data showed that deamidation led to variable changes in stability, depending on whether the Gln or Asn occurs in the X- or Y-position and depending on the neighboring residue.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized on an Applied Biosystems 430A synthesizer using the standard FastMoc (Applied Biosystems) method on Fmoc-RINK resin. Peptides had the general formula acetyl-(Gly-Pro-Hyp)$_3$-Gly-Xaa-Yaa-(Gly-Pro-Hyp)$_4$-Gly-Gly-amide with various guest Gly-Xaa-Yaa triplets located in an acetyl-(Gly-Pro-Hyp)$_8$-Gly-Gly-amide host peptide. Side-chain protection during synthesis was *tert*-butyl for Hyp, *tert*-butyl ester for Asp and Glu, trityl for Asn and Gln, benzyloxycarbonyl for Lys, and pentamethylchroman-sulfonyl for Arg. Acetylation was by acetic anhydride and triethylamine in dimethylformamide. Peptides were purified to >90% purity using a Shimadzu reverse-phase HPLC system on a C-18 column and eluted in 0.1% (v/v) trifluoroacetic acid with a binary gradient of 0−40% (v/v) water/acetonitrile. Amino acid analysis and matrix-assisted laser desorption ionization (MALDI) mass spectrometry confirmed peptide identity. The characterization of certain peptides has been previously described (*13*−*15*).

Bovine Collagens. Bovine hide types I and III collagen and bovine articular cartilage type II collagen were prepared by pepsin digestion followed by purification by fractional salt precipitation (*16*). Deamidation was by incubation in saturated Na_2SO_4 in 5% w/v NaOH for 120 h (*17*). This method had previously been shown to give full deamidation of collagen (*17*). Samples were dialyzed against 25 mM acetic acid and freeze-dried.

Sample Preparation. Peptides were dried in vacuo over P_2O_5 for 48 h prior to the preparation of solutions for circular dichroism (CD) spectroscopy. Solutions of concentrations of 1.0 mg/mL were made by weight in 10 mM sodium phosphate buffer with 0.15 M NaCl, pH 7.0 (PBS). The concentration for each peptide was determined by absorbance at 214 nm ($\epsilon_{214} = 2.02 \times 10^3$) as determined by related peptides containing tyrosine. All solutions were held at 4 °C for at least 48 h to allow triple-helix formation and then for 2 h at 2 °C before CD measurements. Bovine collagens were dissolved in 0.1 M acetic acid, pH 2.9, at 0.5 mg/mL and held at 4 °C before CD measurements.

Circular Dichroism Spectroscopy. Peptide CD spectra were recorded at 5 °C on an Aviv model 62DS spectropolarimeter equipped with a Hewlett-Packard Peltier thermoelectric temperature controller. Spectra were recorded from 210 to 260 nm using cells of 1 mm path length. For thermal equilibrium studies, the wavelength was kept constant at 225 nm (near the maximum ellipticity), while the temperature was increased from 5 to 80 °C in increments of 0.3 °C followed by a 2 min equilibration at each temperature; data were collected for 10 s at each point. For bovine collagens the temperature was increased from 15 to 60 °C in increments of 2 °C/min.

Table 1. Observed Thermal Stabilities for the New Asn and Gln Host–Guest Containing Peptides Examined in the Present Study[a]

triplet	T_m (°C)	triplet	T_m (°C)
Gly-Gln-Ala	33.3	Gly-Ala-Asn	30.4
Gly-Glu-Glu	31.0	Gly-Asn-Ala	30.3
Gly-Leu-Glu	30.9	Gly-Asn-Lys	27.6

[a] The estimate of error in determination of the T_m measurements is less than ±0.4 °C (*13*).

Data Analysis. The melting temperature (T_m) for peptides was derived from the melting curves, after correction for the slope before and after the thermal transition, and was defined as the temperature at which the fraction of peptide folded, *F*, was 0.5. Thermal transition curves were normalized to the fraction of triple-helical peptide *F* with $F = [\Theta - \Theta_u(T)]/[\Theta_n(T) - \Theta_u(T)]$, where Θ_n and Θ_u represent ellipticities of the fully folded and unfolded species, respectively, corrected for their temperature dependence by linear extrapolation of the low- and high-temperature baselines and Θ is the observed ellipticity.

RESULTS

The aim of this study was to quantitate the effect of changing Asn to Asp and Gln to Glu on triple-helix stability. This was done through the use of host−guest peptides of the form acetyl-(Gly-Pro-Hyp)$_3$-Gly-Xaa-Yaa-(Gly-Pro-Hyp)$_4$-Gly-Gly-amide, where the X- and Y-positions of the guest peptide are varied within the highly stabilizing Gly-Pro-Hyp environment. Previous studies on more than 80 peptides with different L-amino acids in the X- and Y-positions have shown that all formed stable triple helices with melting temperatures in the 20−47 °C range, with the host peptide containing a Gly-Pro-Hyp triplet being the most stable (T_m = 47.3 °C) (*13*, *15*). Peptides were selected so that the effect of a change from amide to acid could be examined both in the X-position, using pairs of peptides of the form Gly-Asn-Yaa to Gly-Asp-Yaa and Gly-Gln-Yaa to Gly-Glu-Yaa, and also in the Y-position, using pairs of peptides Gly-Xaa-Asn to Gly-Xaa-Asp and Gly-Xaa-Gln to Gly-Xaa-Glu. Here, we report the thermal stability of six new host−guest peptides containing the guest triplets Gly-Ala-Asn, Gly-Asn-Ala, Gly-Asn-Lys, Gly-Gln-Ala, Gly-Glu-Glu, and Gly-Leu-Glu (**Table 1**). Repeat determinations on the T_m data for these and other peptides (*13*) suggest that the estimate of error in determination of the T_m measurements is less than ±0.4 °C. Together with the stabilities of similar peptides that have been previously reported (*13*−*15*), this has enabled comparisons to be made on the effects of changing an amide to an acid residue for 15 pairs of peptides (**Table 2**). The pairs of peptides that have been examined include the majority of the most frequently found amide residues in bovine type I collagen (**Table 3**), the main source of B-type gelatin.

The CD spectra of these six new peptides all showed a single maximum at ∼225 nm at 4 °C that is characteristic of triple-helical peptides (**Figure 1A**). This maximum typically had a mean residue ellipticity (deg cm^2 dmol^{-1}) of ∼4200. This value was greatly reduced by heating to 60 °C (data not shown), indicating that all had formed triple-helical, collagen-like structures at 4 °C. For example, the CD spectrum and the thermal transition are shown for peptide Gly-Glu-Glu and are compared with those of peptide Gly-Glu-Gln (**Figure 1B**). In this case the change from an amide to acid residue had little effect on the mean residue ellipticity, but decreased the T_m by ∼7 °C.

Table 2. Comparisons of the Thermal Stabilities (T_m) of Host–Guest Peptides Containing Amide Residues with Those Containing the Corresponding Carboxylic Acid[a]

Asn in X-position			Asn in Y-position			Gln in X-position			Gln in Y-position		
peptide	T_m (°C)	ΔT_m	peptide	T_m (°C)	ΔT_m	peptide	T_m (°C)	ΔT_m	peptide	T_m (°C)	ΔT_m
Gly-Asn-Hyp Gly-Asp-Hyp	38.3 40.1	+1.8	Gly-Pro-Asn Gly-Pro-Asp	31.7 34.0	+2.3	Gly-Gln-Hyp Gly-Glu-Hyp	40.4 42.9	+2.5	Gly-Pro-Gln Gly-Pro-Glu	41.3 39.7	–1.6
Gly-Asn-Ala Gly-Asp-Ala	30.3 31.6	+1.3	**Gly-Ala-Asn** Gly-Ala-Asp	30.4 33.0	+2.6	**Gly-Gln-Ala** Gly-Glu-Ala	33.3 34.6	+1.3	Gly-Leu-Gln **Gly-Leu-Glu**	35.7 30.9	-4.8
Gly-Asn-Lys Gly-Asp-Lys	27.6 30.9	+3.3	Gly-Lys-Asn Gly-Lys-Asp	31.7 35.8	+4.1	Gly-Gln-Lys Gly-Glu-Lys	32.6 35.0	+2.4	Gly-Lys-Gln Gly-Lys-Glu	38.9 35.3	–3.6
			Gly-Glu-Asn Gly-Glu-Asp	29.5 29.7	+0.2	Gly-Gln-Arg Gly-Glu-Arg	39.5 40.4	+0.9	Gly-Glu-Gln **Gly-Glu-Glu**	37.7 31.0	–6.7

[a] The estimate of error in determination of the T_m measurements is less than ±0.4 °C (*13*).

Table 3. Occurrence of Amide Residues in Bovine Collagen α1[I] and α2[I] Chains[a]

occurrence	α1[I] chain	α2[I] chain
7	Gly-Pro-Gln	
6		
5		Gly-Asn-Hyp, Gly-Pro-Gln, Gly-Gln-Hyp
4	Gly-Glu-Gln	
3		Gly-Pro-Asn
2	Gly-Ala-Asn, Gly-Phe-Gln, Gly-Leu-Gln, Gly-Asn-Asp, Gly-Gln-Hyp, Gly-Gln-Arg	Gly-Ala-Asn, Gly-Asp-Gln, Gly-Glu-Asn, Gly-Phe-Gln, Gly-His-Asn, Gly-Leu-Gln, Gly-Asn-Lys, Gly-Asn-Ile, Gly-Gln-Thr, Gly-Ser-Gln
1	Gly-Ala-Gln, Gly-Glu-Asn, Gly-Ile-Gln, Gly-Lys-Asn, Gly-Lys-Gln, Gly-Leu-Asn, Gly-Asn-Ala, Gly-Asn-Lys, Gly-Asn-Hyp, Gly-Asn-Ser, Gly-Asn-Val, Gly-Gln-Ala, Gly-Gln-Met, Gly-Gln-Asn, Gly-Ser-Gln	Gly-Ala-Gln, Gly-Glu-Gln, Gly-Asn-Ala, Gly-Asn-Asp, Gly-Asn-Asn, Gly-Asn-Arg, Gly-Asn-Val, Gly-Val-Asn, Gly-Val-Gln
0	Gly-Asp-Gln, Gly-His-Asn, Gly-Asn-Ile, Gly-Asn-Asn, Gly-Asn-Arg, Gly-Pro-Asn, Gly-Gln-Thr, Gly-Val-Asn, Gly-Val-Gln	Gly-Ile-Gln, Gly-Lys-Asn, Gly-Lys-Gln, Gly-Leu-Asn, Gly-Asn-Ser, Gly-Gln-Ala, Gly-Gln-Met, Gly-Gln-Asn, Gly-Gln-Arg

[a] The sequence data for bovine collagen α1[I] and α2[I] chains were taken from Kadler (*17*) and the SWISS-PROT annotated protein sequence database (http://www.expasy.ch/sprot/). For the α1[I] chain, there were four potential amide residues undetermined in the protein sequence data. These positions were assigned on the basis of homology to the human sequence.

The characterization of these additional host−guest peptides has provided 15 pairs of peptides for which the effect of changing from an amide to an acidic residue can be examined (**Table 2**). These data show that the change of Asn to Asp, regardless of whether it occurs in either the X- or Y-position, always gave a stabilizing effect (+0.2−4.1 °C). When Asn was in the Y-position, even Gly-Glu-Asn → Gly-Glu-Asp, where potential for new electrostatic repulsion arises from the change, showed no destabilization. On the other hand, the highest stabilizing effect (+4.1 °C) was seen for Gly-Lys-Asn → Gly-Lys-Asp, where an increased potential for electrostatic attraction is likely. Similarly, for Asn in the X-position, the greatest stabilizing effect arising from the change of an amide to an acidic residue was observed for Gly-Asn-Lys → Gly-Asp-Lys, where an increased potential for electrostatic attraction is again likely. Overall, comparison of the effects of a change of Asn to Asp in the X- or Y-position for pairs with equivalent compositions, for example, Gly-Asn-Ala → Gly-Asp-Ala compared with Gly-Ala-Asn → Gly-Ala-Asp, suggests that change of an Asn amide in the Y-position leads to a greater stabilization than a similar change in the X-position by ∼1.3 °C.

Changing of Gln in the X-position to Glu in four different pairs of Gly-Gln-Yaa sequences again leads to a small increase in stability (+0.9−2.5 °C). The changes in stabilization appear to be similar to those arising from changing of Asn to Asp residues in the X-position. When similar residues are present in the Y-position, for example, Gly-Asn-Ala → Gly-Asp-Ala compared with Gly-Gln-Ala → Gly-Glu-Ala, both give ∼1.3 °C stabilization. Also, when the change for Gly-Asn-Lys → Gly-Asp-Lys is compared with that for Gly-Gln-Lys → Gly-Glu-Lys, +3.3 and +2.4 °C stabilizations are observed, respectively, showing that slightly greater stabilization is found when the potential for electrostatic attraction is likely.

Deamidation of Gln in the Y-position, however, is quite distinct, leading to clear destabilization (−1.6 to −6.7 °C) in each of four different Gly-Xaa-Gln → Gly-Xaa-Glu pairs. The extent of this destabilization generally appears to be larger than the stabilization observed for the other three changes. The greatest destabilization (−6.7 °C) was observed for Gly-Glu-Gln → Gly-Glu-Glu, where the additional electrostatic repulsion is likely to play a role.

In the present study, we have also examined the effect of deamidation on the thermal stability of types I, II, and III collagens. In all three cases a similar extent of destabilization was observed. Thus, the stability of type I collagen decreased by −4.6 °C, that of type II collagen by −6.3 °C, and that of type III collagen by −5.6 °C.

DISCUSSION

In the present study, the use of peptide models has enabled the effect of deamidation, a significant chemical change to collagen that occurs during its processing into B-type gelatin, to be examined. The deamidation gives a product with an acidic

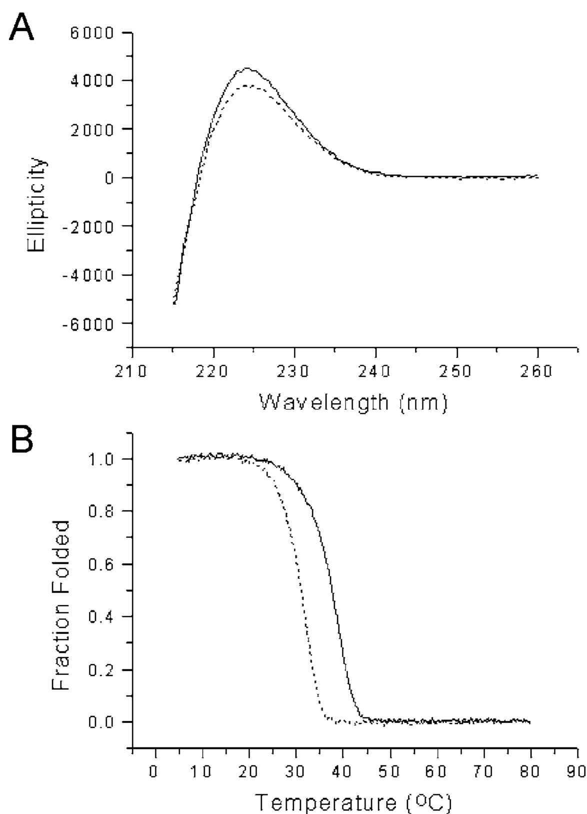

Figure 1. (**A**) CD spectra for the peptides containing guest triplets Gly-Glu-Gln (—) and Gly-Glu-Glu (•••) at 4 °C showing mean residue ellipticity (deg cm^2 dmol^{-1}) against wavelength (nm). (**B**) Thermal equilibrium curves for these peptides, Gly-Glu-Gln (—) and Gly-Glu-Glu (•••), showing fraction folded (F) against temperature (°C).

p*I* of ~4−5, compared to the basic p*I* of ~9 that is observed for native collagen (*18*). The use of peptide models of triple-helical structure is valuable as models allow insights into the structure and stability of the triple helix that would not be accessible through studies on intact collagen. For example, peptide models have previously shown that amino acid racemization, which would most likely occur during the high-temperature extraction step of gelatin production, would be particularly detrimental to the thermal stability of gelatin, as the presence of a racemized amino acid is highly inhibitory to triple-helix formation (*19*). However, it is likely that changes due to racemization would affect both A- and B-type gelatins and, although detrimental to gelatin properties, would not account fully for the poorer functional performance of B-type gelatins.

The re-forming of triple-helical structure from gelatin chains is known to be a critical part of gel formation (*8*, *9*) as the re-formed triple-helical elements form junctional domains that are key to the gel network. The deamidation that accompanies B-type gelatin production has been considered as potentially affecting gelatin properties, by effecting the efficiency and stability of the re-formed triple-helical domains.

The changes in the thermal stability, T_m, that would be caused by deamidation of amide residues during gelatin processing (Asn → Asp, Gln → Glu) appear to be small from comparisons of guest peptide pairs, with some changes favoring stability. Thus, Asn → Asp and Gln → Glu changes in the X-position would both lead to an increase in triple-helix stability, with both amide-containing triplets having moderate abundance (**Table 3**). Also, in the Y-position an Asn → Asp leads to a slight gain of stability for the triple helix, although this may not have a major effect on gelatin properties as Gly-X-Asn triplets are not particularly abundant (**Table 3**).

These changes contrast with those of Gly-Xaa-Gln peptides, where change of Gln to Glu leads to significant destabilization of the triple helix. This is particularly significant for the Gly-Pro-Gln triplet, as this has a significant abundance, ~2.5% (*20*), and is among the most stable of the host−guest peptides (*13*, *15*). Thus, the change from stable Gly-Pro-Gln triplets to the lower stability Gly-Pro-Glu triplets appears to be the dominant effect, leading to the small observed destabilization of collagens by deamidation.

In the present study we have examined pairs of peptides that represent the most frequent amide-containing triplets and the corresponding triplets with acidic residues. In collagens, only ~20% of the possible 400 triplets are observed at a significant frequency. Only 24 triplets are found with a frequency of >1% (*20*), with only 2 containing amide residues, Gly-Pro-Gln and Gly-Gln-Hyp, with frequencies of 2.5 and 1.1%, respectively. In theory, there is a range of Gly-Xaa-Yaa tripeptide sequences that include Gln—20 of the form Gly-Gln-Yaa and 20 of the form Gly-Xaa-Gln. Similarly, there are 20 Asn-containing peptides of the form Gly-Asn-Yaa and 20 Gly-Xaa-Asn sequences. This gives 76 potential triplets, because some contain sequences containing two amide residues, either Asn or Gln or both (Gly-Gln-Gln, Gly-Asn-Asn, Gly-Asn-Gln, and Gly-Gln-Asn). For all of these possible amide-containing combinations, examination of the observed collagen sequences (*21*) shows that the distribution of Gly-Xaa-Yaa sequences is not uniform, so that only 31 of the possible 76 triplets are found in bovine type I collagen, and of these, only 20 are found more than twice (**Table 3**). It has been recently shown, however, that it is possible that the present data could be used for estimating the changes for triplet pairs where no experimental data are available by use of an "additivity model" (*13*); for example, the stabilities for Gly-Pro-Gln to Gly-Pro-Glu could be used to predict a value for the magnitude of the change from Gly-Phe-Gln to Gly-Phe-Glu. This approach would allow stabilities for amide to acid changes where no experimental data are available to be predicted.

The differences in properties between A- and B-type gelatins may depend in part on the change in triple-helical stability arising from deamidation. Previous data have also shown that nucleation and folding rates can be reduced by deamidation, particularly amides in the Y-position (*22*). For the formation of gelatin gels, the rate of association and nucleation may be important for the initial setting rate and may also be important in the longer term for the development of gel strength as the gel structure reorganizes during maturation (*8*). The present study has examined the effects of changing an amino acid from an amide to an acidic residue in single triplets in a constant (Gly-Pro-Hyp)$_n$ background and has not examined the role of adjacent triplets with other sequences. In the present study, examples of the potential change in stability from newly formed electrostatic interactions within a triple helix have been observed. In the collagen triple helix, electrostatic interactions are also possible involving adjacent triplets. Recent studies (*23*) have shown that peptides containing Gly-Pro-Lys-Gly-Asp-Hyp and Gly-Pro-Lys-Gly-Glu-Hyp sequences provide triple helices with much greater triple-helical stability (>15 °C) than expected from individual triplet stabilities (*23*). This stabilization is Lys-specific, because it is not seen for Arg-Gly-Asp/Glu sequences (*23*), and requires participation from residues in more than one chain. Examination of the amino acid sequences for bovine type I collagen chains shows that a Lys-Gly-Asn sequence is present

once in the α1 chain and that Lys-Gly-Gln is not present in either chain. Hence, additional stability to refolded, deamidated triple helix in gelatin would not be significant by this mechanism. On the other hand, the significant occurrence of Lys-Gly-Asp and Lys-Gly-Glu sequences in the bovine α1[I] chain (six and four times, respectively) and in the Lys-Gly-Glu in the α2[I] chain (five times) means that loss of native chain registration on triple-helix refolding could lower the triple-helical stability and could be important if the chain registration on refolding was less in B-type than in A-type gelatins due to the greater polydispersity that is present in B-type gelatins. This higher polydispersity in B-type gelatins, with the presence of higher molecular weight branched components derived from interchain cross-linking, could affect gelling rate and gel strength by steric hindrance of gel formation, whereas the greater amount of low molecular weight fragments could affect gel strength by limiting the extent of network formation and lowering the stability of refolded triple-helix segments.

ABBREVIATIONS USED

CD, circular dichroism; T_m, melting temperature; standard three-letter abbreviations are used for the common amino acids, with hydroxyproline denoted Hyp.

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