Amino Acid Sequence Environment Modulates the Disruption by Osteogenesis Imperfecta Glycine Substitutions in Collagen-like Peptides†

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ABSTRACT: Ostoegenesis imperfecta (OI) or "brittle bone" disease is associated with mutations in the genes for type I collagen chains and produces variable phenotypes, ranging from lethal cases at birth to mild cases with increased bone fractures. The most common OI mutations are single base substitutions leading to replacement of Gly by another residue, breaking the typical $(Gly\text{-}X\text{-}Y)_n$ repeating sequence pattern of the collagen triple-helix. Triple-helical peptides were designed to focus on residues 892−921 of the α1 chain of type I collagen, where two OI Gly→Ser mutations are found in close proximity, a mild mutation at site 901 and a lethal mutation at site 913. Peptides were designed to include amino acid sequences around these mutation sites, and were synthesized with the normal sequence or with the Gly→Ser mutated sequence. The peptide including the normal sequence residues 892−909 with four Gly-Pro-Hyp triplets at the C-terminus formed a stable triple-helix, and introduction of a Ser residue for Gly at the 901 mutation site led to a 50% loss of triple-helix content and a decrease in thermal stability, with little effect on folding. A peptide including residues 904−921 again formed a stable triple-helix, but the introduction of the Gly→Ser substitution at site 913 led to a much greater decrease in thermal stability. These studies demonstrate the impact of local sequences flanking the Gly substitution on structural consequences and support the concept of variability and regional effects along the collagen molecule.

Collagens are triple-helix-containing structural proteins in the extracellular matrix, and more than 19 different genetic types of collagens have been defined (*1*−*3*). The most well characterized and common are the five fibril-forming collagens, which are found in $D = 67$ nm periodic fibrils in connective tissues (*4*). For instance, type I collagen, a heterotrimer composed of two αl(I) chains and one α2(I) chain, forms D-periodic fibrils in bone, providing the matrix for mineralization. All collagen molecules include a domain with a triple-helix conformation, consisting of three extended polypeptide chains supercoiled about a common axis (*5*−*7*). The close packing of the three chains generates the requirement for Gly as every third residue, and a high content of the imino acids proline (Pro) and hydroxyproline (Hyp)[1] is necessary to stabilize the individual extended polyproline II-like helices.

Type I collagen has a $(Gly\text{-}X\text{-}Y)_{338}$ triple-helix domain, with Gly-Pro-Hyp as the most frequent triplet unit. This triple-helical domain is capable of self-association to form fibrils and of binding to integrins, proteoglycans, matrix metalloproteinases, and many other molecules (*4*, *8*). Along the uninterrupted triple-helix of type I collagen, there must be recognizable variations to specify binding sites, as well as regions which undergo local microunfolding (*9*) or form cooperative folding units (*10*).

Mutations which interrupt the repeating $(Gly\text{-}X\text{-}Y)_n$ pattern in the triple-helix of collagen have been associated with a variety of hereditable connective tissue disorders (*2*, *11*). The most well characterized of these disorders is osteogenesis imperfecta (OI), which is characterized by bone fragility and is associated with defects in the αl or α2 chain of type I collagen (*12*). The most common causes of OI are single point mutations that result in the change of Gly to another residue, most frequently Cys, Ser, Arg, and Asp. These substitutions can occur at many different locations covering the full range of the αl(I) and α2(I) chains and can result in clinical severity that ranges from mild to lethal phenotypes (*12*). Lethal OI mutations are usually located in the C-terminal half of the chains, while nonlethal mutations cluster near the N-terminus, an observation that relates to the C- to N-terminal folding direction of the collagen triple-helix (*13*). However, the severity of the phenotype resulting from Gly→Cys and Gly→Ser substitutions shows exceptions to this trend, in both α1(I) and α2(I) chains (*14*−*16*).

Peptides with Gly as every third residue and a high imino acid content will adopt a triple-helical conformation (*17*−*20*) and provide an approach for elucidating sequence-dependent variations in triple-helix features as well as defining the impact of Gly mutations. In this work, peptides were designed to focus on a local region of the αl(I) chain encompassing the sites of a nonlethal Gly→Ser substitution at residue 901 (*21*) and the lethal Gly→Ser substitution at residue 913 (*22*). The stability and folding of homologous peptides containing sequences around each of these two mutation sites were compared.

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[1] Abbreviations: abbreviations used for standard amino acids follow the rules of the IUPAC−IUB Commission of Biochemical Nomenclature [(1972) *J. Biol. Chem. 247*, 977−983], and hydroxyproline is designated by Hyp (three-letter code) or O (one-letter code); OI, osteogenesis imperfecta; CD, circular dichroism; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-RINK amino resin, 4-(2,4-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin; PBS, phosphate-buffered saline; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; tBu, *tert*-butyl; HPLC, high-performance liquid chromatography.

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MATERIALS AND METHODS

Peptides. The peptides were synthesized by SynPep Corp. (Dublin, CA) on an Fmoc-RINK amino resin (*23*) manually using solid-phase peptide synthesis procedures. The side chain protecting groups were tBu for Hyp, Ser, Thr, and Asp; Pmc for Arg; Boc for Lys; and trityl for Gln. The identity of each peptide was confirmed using electrospray ionization mass spectroscopy. The purity of the peptides was greater than 95% as judged by reversed-phase analytical HPLC using a linear gradient of CH_3CN (0.075% TFA) and H_2O (0.1% TFA) as eluent.

Circular Dichroism. Circular dichroism (CD) spectra were recorded on an Aviv Model 62DS spectrometer. Cells of path length 0.1 cm were used, and the temperature in the cell was controlled using a Hewlett-Packard Peltier thermoelectric temperature controller. Samples were prepared at a concentration of 1 mg/mL, with peptides dried in vacuo over P_2O_5 for 48 h prior to weighing. Peptide solutions in either 0.1 M acetic acid, pH 2.7, or 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.1 (PBS), were equilibrated at 4 °C for more than 48 h prior to analysis.

For wavelength scans, the signal was collected from 210 to 260 nm at 0.5 nm intervals at 2 °C. For equilibrium melting temperature transitions, the ellipticity at 225 nm was monitored while the temperature in the cell was increased at a rate of 0.15 °C/min. The equilibration time at each point was set to 2 min.

Calculation of Thermodynamic Parameters. The equilibrium melting transitions were fit to a two-state monomer to trimer transition. A two-state model is supported by previous studies on peptides of similar design (*24*) and by the good fit of the melting curve. The fraction folded (F) was calculated using eq 1:

$$F = (\theta_{\text{observed}} - \theta_{\text{monomer}})/(\theta_{\text{trimer}} - \theta_{\text{monomer}}) \quad (1)$$

In this equation, F is the fraction folded, θ_{observed} is the observed ellipticity, θ_{trimer} is calculated from the best fit equation for the trimeric part of the melting curve, and θ_{monomer} is calculated from the best fit equation for the monomeric part of the melting curve.

The van't Hoff enthalpy (ΔH) was determined by curve-fitting with eq 2 (*18, 24*−*26*):

$$\ln K = (\Delta H/RT)[(T/T_m) - 1] - \ln (0.75c^2) \quad (2)$$

In this equation, K is the equilibrium constant between the monomer and trimer ($3M \rightleftarrows T$); T_m is the melting temperature in Kelvin where the peptide was 50% folded ($F = 0.5$); and c is the peptide concentration in moles.

Refolding Experiments. All the peptides were heated to dissociate the trimers to monomers, and then cooled to 2 °C to initiate refolding. About 0.4 mL of sample in a small glass tube was denatured in a 50 °C water bath for 15 min. The solution in the glass test tube was rapidly quenched in an iced KCl salt solution (30% KCl and ice; temperature between −5.8 and −6.7 °C) before being transferred into the CD cell, which had been equilibrated at 2 °C in the instrument. The quenching time to reach 2 °C was estimated to be 5.5 s using a blank solution with a thermal probe, and this time interval was used for quenching in the actual experiment. The ellipticity at 225 nm was monitored immediately afterward. The dead time associated with sample transfer for each experiment was between 10 and 15 s. The data were collected every 3 s with an averaging time of 1 s over 6 h, or were collected every 1 s with an averaging time of 1 s over 30 min.

Calculation of Rate Constants. The base-line-corrected ellipticities were plotted against the corrected times (taking into account the dead time of each experiment). The fraction folded (F) was calculated using eq 1. In this case, θ_{trimer} was the ellipticity of the equilibrated peptide solution at 2 °C, and the θ_{monomer} was extrapolated to time zero using polynomial curve fitting of the folding data.

The concentration of monomer at any given time, [A], was calculated using eq 3, where $[A]_0$ is the initial monomeric peptide concentration:

$$[A] = (1 - F)[A]_0 \quad (3)$$

The folding data were fit to first-order, second-order, and third-order rate equations (eqs 4, 5, and 6 respectively):

$$\ln ([A]/[A]_0) = -kt \quad (4)$$

$$1/[A] - 1/[A]_0 = 2kt \quad (5)$$

$$1/[A]^2 - 1/[A]_0^2 = 6kt \quad (6)$$

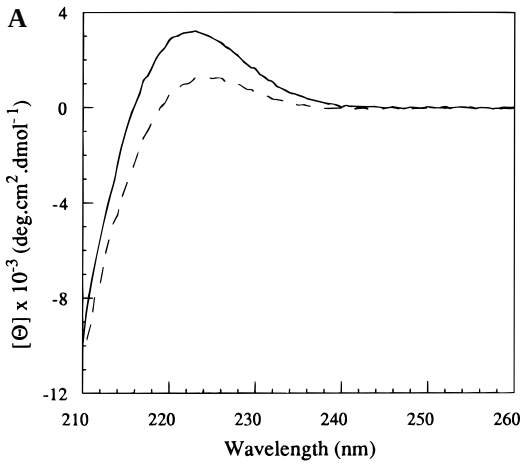
The rate constant was calculated from the slope for the best fit line. The correlation coefficient and the residual plot were used to evaluate which rate equation best fit the data.

RESULTS

Effect of Gly Substitution at a Nonlethal OI Site: Peptides 892 and 892A

Peptide Design. A set of two homologous peptides was designed to investigate the effect of a Gly to Ser substitution at site 901 of the α1 chain of type I collagen, where a nonlethal OI mutation has been reported (*21*). Peptides included 18 residues from the sequence surrounding the 901 site, together with 4 Gly-Pro-Hyp tripeptide units added to the C-terminus to promote triple-helix formation. A C-terminal Gly-Val was included to eliminate diketopiperazine formation during peptide synthesis. Both ends of the peptide were blocked, with acetylation of the N-terminus and amidation of the C-terminus, to prevent end effects. This design was previously shown to yield peptides with a stable triple-helical conformation (*19, 20, 27*). One peptide includes Gly at residue 901, as found in the normal sequence, while the second peptide includes Ser at position 901, as found in the OI mutated chain. Each peptide is designated by its N-terminal residue, using the residue number in the α1(I) chain triple-helix sequence. Thus, the two peptides studied are T1−892, with the normal sequence, and T1−892A (G901S), which has the Gly→Ser substitution (Figure 1).

Conformation and Stability. The CD spectrum of peptide T1−892 showed characteristic triple-helical features at 2 °C, with a maximum near 225 nm (Figure 1, panel A). Increasing temperature led to disappearance of this maximum, with a sharp thermal transition at $T_m = 27.5$ °C (pH 7) (Figure 1, panel B). Previous reports indicate that this corresponds to the trimer→monomer transition (*17, 24*). Peptide T1−892A, with the Gly→Ser replacement at position 901, also had a



B

Sequence	
T1-892	Ac-GPAGPAGPV<u>G</u>PAGARGPA(GPO)4GV-NH2
T1-892A(G901S)	Ac-GPAGPAGPV<u>S</u>PAGARGPA(GPO)4GV-NH2

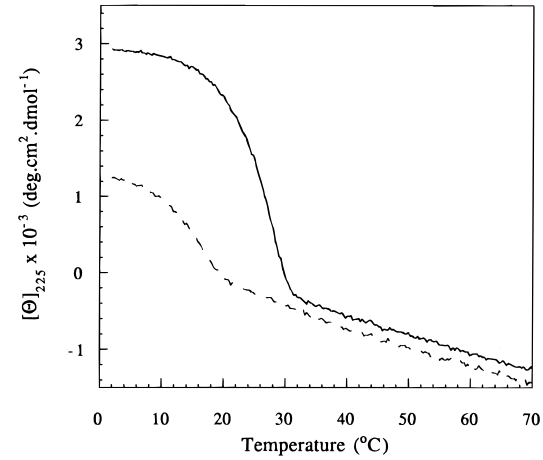


FIGURE 1: (A) CD spectra of peptide T1−892 (—) and peptide T1−892A (- - -) at 2 °C, pH 7. (B) Thermal equilibrium curves for T1−892 (—) and T1−892A (- - -) as monitored by CD at 225 nm. The peptide sequences for the two peptides are shown. Both peptides contain residues 892−909 of the α(I) chain of type I collagen, and one has the Gly→Ser substitution at position 901 (underlined residue).

characteristic triple-helical CD spectrum, but the magnitude of the mean residue ellipticity at the 225 nm peak was decreased from $[\theta]_{225} = +2900$ to $[\theta]_{225} = +1200$ deg·cm^2·dmol^{-1}. A sharp trimer to monomer thermal transition was observed for peptide T1−892A, but there was a substantial decrease in the thermal stability, dropping from $T_m = 27.5$ °C (T1−892) to $T_m = 15.8$ °C (T1−892A). These results suggest a loss of triple-helix content and a reduced thermal stability as a result of the Gly to Ser substitution. Changing from pH 7 to pH 2 had no effect on either the mean residue ellipticity at 225 nm or the thermal stability for both peptides (Table 1). This is expected since there are no ionizable side chains in these peptides and both ends are blocked.

The van't Hoff enthalpy was calculated for peptide T1−892 from its equilibrium melting curve, assuming a two-state trimer to monomer transition (Table 1). A similar calculation for peptide T1−892A showed a less favorable enthalpy value, which is likely to be a factor in its decreased thermal stability.

Table 1: Melting Temperatures, Enthalpies, and Kinetic Parameters for Peptides T1−892, T1−892A, T1−904, and T1−904A

peptide	T_m[b] (°C) pH 7	T_m[b] (°C) pH 2	ΔH (kcal/mol), pH 7	second-order rate constant at 2 °C (M^{-1} s^{-1})
T1−892	27.5	27.5	−150	0.76
T1−892A(G901S)	15.8	16.1	−114[c]	0.58
Δ[(T1−892)−(T1−892A)][a]	11.7	11.4	−36	0.18
T1−904	30.8	22.1	−173	0.28
T1−904A(G913S)	8.9	<2	nd[d]	0.31[d]
Δ[(T1−904)−(T1−904A)][a]	21.9	>20	−	not significant

[a] The difference in values resulting from Gly→Ser substitution. [b] T_m values are obtained from the first derivative of thermal equilibrium melting curves. [c] This value is an estimate since the calculated curve for the two-state model did not fit the experimental data well at both ends of the transition. [d] Peptide T1−904A is not completely trimeric at 2 °C. It was not possible to get a reasonable estimate of ΔH without the trimer portion of the curve. Based on the similar shapes between the melting curves for T1−904A and T1−892A, the triple-helix content at 2 °C can be estimated to be 80−90%. Using this assumption, the kinetic rate was calculated from the folding curve.

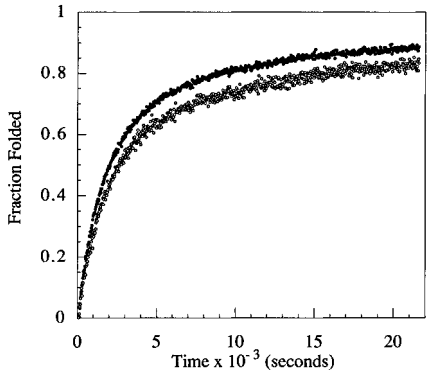


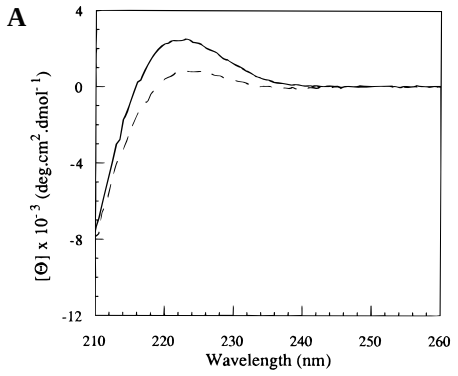
FIGURE 2: Folding kinetics of peptides T1−892 (filled circles) and T1−892A (open circles) at 2 °C, pH 7, following heat denaturation. The change in CD ellipticity was monitored at 225 nm.

Folding. The refolding of the triple-helix in peptide T1−892 at 2 °C following thermal denaturation was monitored by measuring the ellipticity at 225 nm (Figure 2). The triple-helix folds slowly, taking about 20 min to regain 50% of the original ellipticity and 6 h to reach 90% of this value. The initial folding data, within 30 min, were fit to first-, second-, and third-order rate equations, and the second-order equations was found to be the best fit in terms of correlation coefficient and a residue plot (Table 1) (*28*). The folding of peptide T1−892A was also studied, and again indicated second-order kinetics, with a rate constant about 25% lower than seen for T1−892 (Figure 2; Table 1).

Effect of Gly Substitution at a Lethal OI Site: Peptides 904 and 904A

Peptide Design. Using a similar peptide design strategy, a set of two homologous peptides was synthesized to investigate the effect of a Gly to Ser substitution at site 913 of the α1 chain of type I collagen, where a lethal OI mutation has been reported (*22*). Peptide T1−904 has a Gly at residue 913, as found in the normal sequence, while peptide T1−904A includes Ser at position 913, as found in the mutated chain (Figure 3).

Conformation and Stability. A characteristic triple-helical CD spectrum was observed for peptide T1−904 at pH 7, and its melting temperature was 30.8 °C with a sharp thermal transition (Figure 3; Table 1). The CD spectrum of peptide



B

Sequence	
T1-904	Ac-GARGPAGPQ**G**PRGDKGET(GPO)$_4$GV-NH$_2$
T1-904A(G913S)	Ac-GARGPAGPQ**S**PRGDKGET(GPO)$_4$GV-NH$_2$

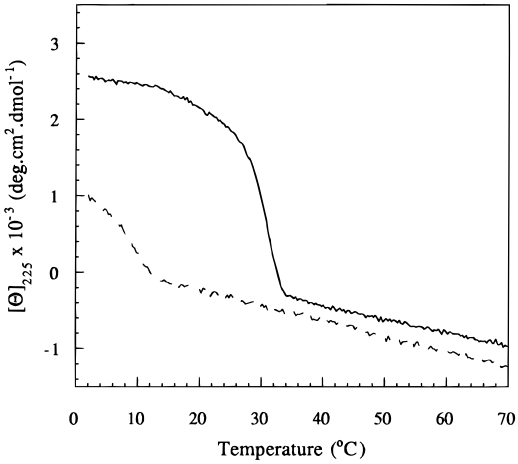


FIGURE 3: (A) CD spectra of peptide T1−904 (—) and peptide T1−904A (- - -) at 2 °C, pH 7. (B) Thermal equilibrium curves for T1−904 (—) and T1−904A (- - -) as monitored by CD spectroscopy at 225 nm. The peptide sequences for the two peptides are shown. Both peptides contain residues 904−921 of the α(I) chain of type I collagen, and one has the Gly→Ser substitution at position 913 (underlined residue).

T1−904A, with the Gly→Ser replacement at position 913, also has a maximum at 225 nm, but its magnitude was decreased from $[\theta]_{225} = +2600$ to $+1000$ deg·cm^2·dmol^{-1}. As the temperature is increased, there is a sharp decrease in ellipticity at 225 nm. It is clear from the curve, that even at 2 °C, the peptide is not fully associated as trimers. Even though it is not a complete transition, the first derivative of the transition is easily measured and indicates a T_m of 8.9 °C for peptide T1−904A. Thus, the Gly to Ser substitution at position 913 resulted in a loss of triple-helix content and a dramatic decrease in thermal stability.

The effect of pH on the thermal stability of peptides T1−904 and T1−904A was investigated. At pH 2, both peptides showed a decreased stability compared with their values at pH 7 (Table 1), suggesting a stabilizing effect of ionization of the basic and acidic groups at neutral pH, possibly due to ion pair formation.

Assuming a two-state model, the van't Hoff enthalpy for peptide T1−904 is calculated to be −173 kcal/mol (Table 1), but the low-temperature region of the transition did not fit the two-state model well. It was not possible to calculate the enthalpy for peptide T1−904A because its transition to trimer was not complete even at 2 °C.

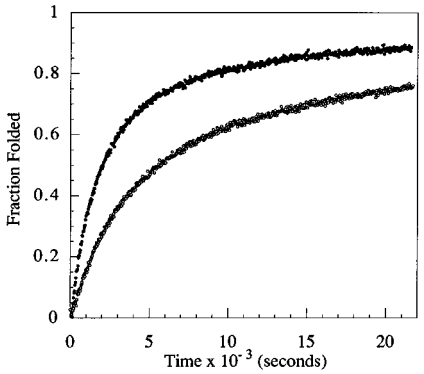


FIGURE 4: Folding kinetics of peptide T1−892 (filled circles) and peptide T1−904 (open circles) at 2 °C, pH 7, as monitored by CD spectroscopy at 225 nm. Both peptides were heat denatured, followed by rapid cooling.

Folding. The triple-helix folding of peptide T1−904 at pH 7 was monitored, following thermal denaturation and quenching to 2 °C. The folding followed second order kinetics (Table 1; Figure 4). Folding at pH 2 (rate constant of 0.29 M^{-1} s^{-1}) was indistinguishable from the results at pH 7 (rate constant of 0.28 M^{-1} s^{-1}). Folding studies were also carried out for peptide T1−904A, even though the thermal equilibrium measurements indicate that the peptide reaches at most 80−90% triple-helix content at 2 °C. Analysis of the data for T1−904A indicated a second-order rate constant with a value estimated to be similar to that seen for T1−904 (Table 1).

DISCUSSION

Approaches to clarifying amino acid sequence dependent properties of the collagen triple-helix include experimental studies on a "host-guest" triple-helical peptide set (*26*), proposal of a stability scale for different triplets (*29, 30*), and stability, folding, and dynamic studies on peptides incorporating collagen sequences (*31*−*33*). In the study reported here, a comparison is made of two peptides, T1−892 and T1−904, differing only in their N-terminal 18 residues, which are taken from adjacent and partially overlapping regions of the collagen α1(I) chain (Figure 5). Differences in the stability and folding of these peptides appear to relate, in part, to the ionizable residues and imino acid content in the distinctive 18-residue collagen sequence. For example, at pH 2, peptide T1−892 has a higher melting temperature than T1−904, consistent with the higher imino acid content of T1−892. At pH 7, peptide T1−904 is more stable than T1−892 (Table 1), and the substantial increase in stability of peptide T1−904 on going from pH 2 to pH 7 is likely to relate to its high content of ionizable residues, which stabilize the triple-helix, both individually (*34*) and through potential ion pairs (*19, 35, 36*).

The folding of peptides T1−892 and T1−904 is best fit by second-order kinetics, suggesting that nucleation is the rate-limiting step in their folding (*28*). These peptides have a (Gly-Pro-Hyp)$_4$ sequence at the C-terminus, which can promote nucleation and model the C-terminal nucleation of collagen folding (*28, 37, 38*). Peptide T1−892 folds much faster than T1−904 at pH 7, even though it is the less stable peptide at neutral pH (Figure 4; Table 1). This is likely to

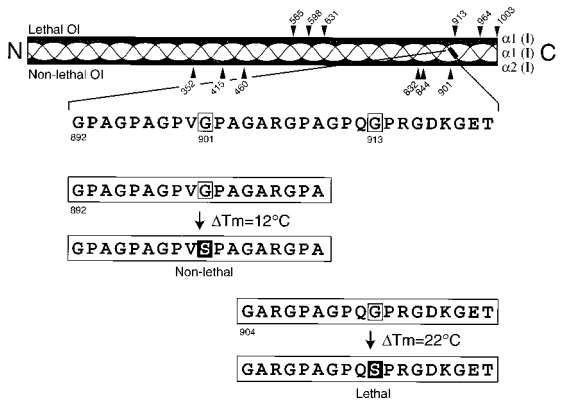


FIGURE 5: Triple-helix of type I collagen is represented at the top. For illustration, several sites of OI Gly→Ser mutations are indicated, with lethal sites at the top and nonlethal sites on the bottom. The region around mutations at residue 901 (nonlethal) and residue 913 (lethal) is expanded, showing the amino acid sequence 892−921 of the α1 of type I collagen (single-letter amino acid code, with O designating hydroxyproline). The amino acid sequences incorporated in the peptides studied (residues 892−909 and residues 904−921) are shown in the boxes below, to illustrate their relative location in the chain. The decrease in thermal stability resulting from the Gly→Ser substitution in both peptides is indicated.

relate to differences between T1−892 and T1−904 in the Gly-X-Y units adjacent to the $(Gly\text{-}Pro\text{-}Hyp)_4$ region that may form part of the nucleation domain. It is possible that the presence of a high density of charged residues adjacent to the $(Gly\text{-}Pro\text{-}Hyp)_4$ strong nucleation site in T1−904 slows down association or nucleation of multiple chains.

Studies on OI collagens with Gly substitutions at different sites along the αl(I) or α2(I) chains have indicated the position-dependent nature of various structural, biological, and clinical features (*12*, *30*). Some features that differ depending on the mutation site include: (1) the decrease in collagen thermal stability, which most often is a few degrees, but is 20 °C in one case (*39*); (2) the time of delay in folding (*40*); and (3) the degree of clinical severity, ranging from lethal to mild (*12*). In addition, a kinking of the molecule occurs when there is a Gly→Cys substitution (*41*, *42*), which may also lead to abnormal fibril branching (*43*). The nonequivalence of different mutation sites appears to be strongly influenced by the nature of collagen triple-helix folding, which is nucleated at the C-terminus followed by propagation in a C- to N-terminal manner (*13*). However, adherence to this general pattern appears to be modulated for Gly→Ser and Gly→Cys mutations by localized sequence features, resulting in more regional groupings of lethal and nonlethal mutations (*14*−*16*).

Previously, studies on a simple $(Gly\text{-}Pro\text{-}Hyp)_{10}$ peptide with a single Gly→Ala substitution indicated that in a very stable environment, a Gly substitution leads to destabilization, untwisting, and local disruption of direct intramolecular hydrogen bonding (*7*, *24*). The peptides T1−892 and T1−904 provide more realistic collagen sequence environments and allow comparison of the effect of the Gly→Ser substitutions in different local environments. One limitation of these studies is the homotrimeric nature of these peptides, in contrast to the heterotrimeric composition of type I collagen and the heterozygous nature of the dominant OI mutations. It is only recently that synthetic procedures to make heterotrimers of reasonable purity are being developed (*44*, *45*). However, conclusions based on studies of these homotrimeric peptides offer insights that are valuable in considering heterotrimers.

The introduction of Gly→Ser substitutions in peptides T1−892 and T1−904 resulted in very different effects on thermal stability, demonstrating the nonequivalence of Gly sites in different collagen sequence environments. The Gly→Ser substitution led to a 22 °C drop in thermal stability in peptide T1−904, and only a 11 °C drop in stability in T1−892 (Table 1; Figure 5). This observation is consistent with the hypothesis of Bächinger et al. (*30*) that substitutions in stable regions may be more disruptive than those in less stable regions. Both peptides T1−892 and T1−904 showed a decreased magnitude of their 225 nm CD maximum, suggesting a 50% loss of triple-helix as a result of the substitution. The large loss of triple-helix content resulting from the Gly substitution in these peptides suggests renucleation N-terminal to the substitution site is not occurring, probably due to the short peptide length available. This contrasts with OI collagens, which can apparently renucleate and form full-length triple-helical molecules (*12*, *30*). The Gly→Ser substitutions had relatively little effect on the folding rates of the peptides (Table 1). This suggests that the nucleation event including the $(Gly\text{-}Pro\text{-}Hyp)_4$ domain does not extend into the substitution site, which is consistent with an experimentally estimated nucleation size of six tripeptide units (*42*). In summary, these peptide studies demonstrate that the introduction of a Gly→Ser substitution leads to a loss of triple-helix content and a decreased stability, and that the degree of destabilization depends on the local amino acid sequence surrounding the substitution site. These results indicate that the sequence environment of a mutation site has the potential to play a role in the degree of clinical severity in OI and other collagen diseases.

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