

Materials

Amino acids, amino acid derivatives, and resins were from Novabiochem. Gel filtration chromatography was performed with Sephadex G-50 resin from Sigma.

Instrumentation

Analytical HPLC was performed with an Aligent C8 column or a Varian Dynamax C18 column with linear gradients. Mass spectrometry was performed with an Applied Biosystems Voyager DE-Pro MALDI–TOF mass spectrometer with sinapinic acid (Fluka) as the matrix at the University of Wisconsin–Madison Biophysics Instrumentation Facility (www.biochem.wisc.edu/bif). Ultraviolet/visible spectra were recorded with either a Cary 50 Bio or a Cary 3 spectrometer.

Strand and Fragment Synthesis

Synthesis of (Pro-Pro-Gly)₈-Cys(StBu)-Gly, Pro- α 1(StBu). Fmoc-ProProGly-OH (Fmoc = fluorenylmethoxycarbonyl) was synthesized as described (1). Pro- α 1(StBu) (StBu = *tert*-butylthio) was then synthesized on Fmoc-Gly-PEG-PS resin (Applied Biosystems; PEG-PS = polyethyleneglycol-polystyrene copolymer): 1 × Fmoc-Cys(StBu)-OH, 8 × Fmoc-ProProGly-OH, cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH (8 ml, 2 h; TFA = trifluoroacetic acid, iPr = isopropyl) and precipitated from *tert*-butylmethyl ether: 58% yield; MS (MALDI–TOF) [M+H]⁺ calculated for C₁₀₅H₁₅₅N₂₆O₂₇S₂ 2277.6, found 2277.6; HPLC t_R 17.79 min [Aligent C8 column, 85:15–15:85 H₂O/CH₃CN containing TFA (0.1% vol/vol) over 50 min].

Synthesis of (Pro-Hyp-Gly)₈-Cys(StBu)-Gly, Hyp- α 1(StBu). FmocProHypGly was synthesized as described (1), but with Hyp in the Yaa position (no *tert*-butyl protection was necessary). Hyp- α 1(StBu) was then synthesized on Fmoc-Gly-PEG-PS resin: 1 × Fmoc-Cys(StBu)-OH, 8 × Fmoc-ProHypGly-OH, cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH (8 ml, 2 h) and precipitated from *tert*-butylmethyl ether: 64% yield; MS (MALDI–TOF) [M+H]⁺ calculated for C₁₀₅H₁₅₅N₂₆O₃₅S₂ 2405.6, found 2406.2; HPLC t_R 14.89 min [Aligent C8 column, 85:15–15:85 H₂O/CH₃CN containing TFA (0.1% vol/vol) over 50 min]. The sample was heated at 80°C for 5 min prior to injection onto the column; otherwise a peak for folded trimers (t_R ~22 min) was observed.

Attachment of Fmoc-Gly-OH to HMPA–PEGA resin, Fmoc-Gly-PEGA. HMPA–PEGA resin was obtained from Novabiochem already preswollen in MeOH with an approximate wet loading of 0.072 mmol/g. Preswollen HMPA–PEGA resin (1.545 g, 0.111 mmol; HMPA-PEGA = 4-hydroxymethylphenoxyacetyl-polyethyleneglycol-polyacrylamide copolymer) was washed with CH₂Cl₂ (3 × 5 ml) and dried under high vacuum for 1 h to remove all MeOH. The resin was then swollen in DMF (N,N-dimethylformamide) for Fmoc-Gly-OH attachment.

In a flame-dried flask under Ar(g) atmosphere, Fmoc-Gly-OH (331 mg, 1.11 mmol) was dissolved in CH₂Cl₂ (10 ml) and DMF (0.5 ml). The clear solution was cooled to 0°C, diisopropylcarbodiimide (87 ml, 0.56 mmol) was added and the mixture was stirred at 0°C for 30 min to generate the symmetrical anhydride. The solution was concentrated to remove CH₂Cl₂, DMF (3 ml) was added, and the anhydride solution was filtered through cotton (to remove the insoluble urea) into the swelled resin suspension. A solution of dimethylaminopyridine (1.4 mg,

0.11 mmol) in DMF (0.1 ml) was added, the vessel was purged with Ar(g), and the suspension was agitated gently for 1.5 h. The resin was then drained, washed with DMF (7×10 ml) and CH₂Cl₂ (7×10 ml), and dried under high vacuum to give Fmoc-Gly-PEGA. Resin-loading was measured by ultraviolet spectroscopy to be 0.25 mmol/g.

Synthesis of (Pro-Pro-Gly)₃-Gly-Cys(Acm)-Cys(StBu)-Gly-(Pro-Pro-Gly)₅-(HMPA-PEGA), Pro- α 2(Acm,StBu)-PEGA. Pro- α 2(Acm,StBu)-PEGA (Acm = acetamidomethyl) was synthesized starting with Fmoc-Gly-(HMPA-PEGA) resin (0.33 g, 80 μ mol): 2 \times Fmoc-Pro-OH, 4 \times Fmoc-ProProGly-OH, 1 \times Fmoc-Gly-OH, 1 \times Fmoc-Cys(StBu)-OH, 1 \times Fmoc-Cys(Acm)-OH, 1 \times Fmoc-Gly-OH, 3 \times Fmoc-ProProGly-OH. A small amount of peptide was cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH for analysis: MS (MALDI-TOF) [M+H]⁺ calculated for C₁₁₃H₁₆₈N₂₉O₃₀S₃ 2507.2, found 2508.0.

Synthesis of (Pro-Hyp-Gly)₃-Gly-Cys(Acm)-Cys(StBu)-Gly-(Pro-Hyp-Gly)₅-(HMPA-PEGA), Hyp- α 2(Acm,StBu)-PEGA. Hyp- α 2(Acm,StBu)-PEGA was synthesized by starting with Fmoc-Gly-(HMPA-PEGA) resin (0.25 g, 61 μ mol): 1 \times Fmoc-Hyp(tBu)-OH, 1 \times Fmoc-Pro-OH, 4 \times Fmoc-ProHypGly-OH, 1 \times Fmoc-Gly-OH, 1 \times Fmoc-Cys(StBu)-OH, 1 \times Fmoc-Cys(Acm)-OH, 1 \times Fmoc-Gly-OH, 3 \times Fmoc-ProHypGly-OH. A small amount of peptide was cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH for analysis: MS (MALDI-TOF) [M+H]⁺ calculated for C₁₁₃H₁₆₈N₂₉O₃₈S₃ 2635.1, found 2635.6.

Disulfide Bond Formation

Pro- α 2(*p*-Npys,Acm)-PEGA. Pro- α 2(Acm,StBu)-PEGA (7.7 μ mol) was swollen in 95:5 TFE/H₂O (1 ml, 5 min), Bu₃P (38 μ L, 150 μ mol) was added, and the suspension was agitated gently for 2 h. The resin was drained, washed with TFE (trifluoroethanol, 3 \times 1 ml), DMF (8 \times 1 ml) and CH₂Cl₂ (8 \times 1 ml), and dried under high vacuum for 30 min. The resin was then swollen in CH₂Cl₂ (0.6 ml, 5 min). A solution of *p*-Npys₂ (2,2'-dithiobis(5-nitropyridine), 24 mg, 77 μ mol) in CH₂Cl₂ (0.4 ml) was added to the swelled resin, the vessel was purged with Ar(g) and the suspension was agitated gently for 2 h. The resin was drained, washed with CH₂Cl₂ (3 \times 1 ml), DMF (20 \times 1 ml), and CH₂Cl₂ (8 \times 1 ml), and dried under high vacuum. A small amount of peptide was cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH for analysis: MS (MALDI-TOF) [M+H]⁺ calculated for C₁₁₄H₁₆₂N₃₁O₃₂S₃ 2573.1, found 2573.6.

Pro- α 1. A solution of Pro- α 1(StBu) (28 mg, 12 μ mol) and Bu₃P (61 μ L, 250 μ mol) in 95:5 TFE/H₂O (1 ml) was agitated gently for 2 h. The peptide was precipitated from *tert*-butylmethyl ether (15 ml) to give Pro- α 1: MS (MALDI-TOF) [M+H]⁺ calculated for C₁₀₁H₁₄₇N₂₆O₂₇S 2188.1, found 2188.8.

Pro- α 1 α 2(Acm)-PEGA. Pro- α 2(Acm,*p*-Npys)-PEGA (7.7 μ mol) was swollen in buffer (0.4 ml, 5 min). A solution of Pro- α 1 (12 μ mol) in buffer (1 ml) was added to the swelled resin, the vessel was purged with Ar(g), and the suspension was agitated gently for 12 h [buffer: 50 mM NH₄OAc, 2 mM EDTA, pH 5.4, degassed and sparged with Ar(g)]. The resin was drained, washed with H₂O (7 \times 1 ml), DMF (7 \times 1 ml), and CH₂Cl₂ (7 \times 1 ml), and dried under high vacuum. A small amount of peptide was cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH for analysis: MS (MALDI-TOF) [M+H]⁺ (average) calculated for C₂₁₀H₃₀₄N₅₅O₅₇S₃ 4607.2, found 4607.9.

Pro- α 1 α 2(*o*-Npys)-PEGA. Pro- α 1 α 2(Acm)-PEGA was swollen in DMF (0.4 ml, 5 min), a solution of *o*-Npys-Cl (3-nitro-2-pyridinesulfenyl chloride, 2.9 mg, 15 μ mol) in DMF (0.6 ml) was added to the swelled resin, and the suspension was agitated gently for 2 h. The resin was drained, washed with DMF (8 \times 1 ml) and CH₂Cl₂ (8 \times 1 ml) and dried under high vacuum. A small amount of peptide was cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH for analysis: MS (MALDI-TOF) [M+H]⁺ (average) calculated for C₂₁₂H₃₀₁N₅₆O₅₈S₄ 4690.2, found 4688.9.

Fragment 1 (Pro- α 1 α 2 α 1'). Pro- α 1 α 2(*o*-Npys)-PEGA (7.7 μ mol) was swollen in buffer (0.4 ml, 5 min). A solution of Pro- α 1 (12 μ mol) in buffer (1 ml) was added to the swelled resin, the vessel was purged with Ar(g), and the suspension was agitated gently for 12 h [buffer: 50 mM NH₄OAc, 2 mM EDTA, pH 5.4, degassed and sparged with Ar(g)]. The resin was drained, washed with H₂O (7 \times 1 ml), DMF (7 \times 1 ml), and CH₂Cl₂ (7 \times 1 ml), and dried under high vacuum. The fragment was then cleaved from the resin with 95:2.5:2.5 TFA/H₂O/iPr₃SiH (8 ml, 2 h) and precipitated from *tert*-butylmethyl. Crude **1** was dissolved in 20 mM NH₄CO₃ (pH 6.0, 1 ml), introduced onto a Sephadex G-50 column (21 cm \times 2.5 cm) and eluted with the same 20 mM NH₄CO₃ buffer to give **1** (6.4 mg, 12% overall): MS (MALDI-TOF) [M+H]⁺ (average) calculated for C₃₀₈H₄₄₃N₈₀O₈₃S₄ 6722.6, found 6723.3; HPLC *t*_R 18.75 min [Aligent C8 column, 90:10–10:90 H₂O/CH₃CN containing TFA (0.1% vol/vol) over 60 min].

Hyp- α 2(*p*-Npys,Acm)-PEGA. Hyp- α 2(*p*-Npys,Acm)-PEGA was prepared as described for Pro- α 2(*p*-Npys,Acm)-PEGA starting with Hyp- α 2(Acm,StBu)-PEGA (8.3 μ mol): MS (MALDI-TOF) [M+H]⁺ calculated for C₁₁₄H₁₆₂N₃₁O₄₀S₃ 2701.1, found 2701.1.

Hyp- α 1. Hyp- α 1 was prepared as described for Pro- α 1 starting with Hyp- α 1(StBu) (32 mg, 13 μ mol): MS (MALDI-TOF) [M+H]⁺ calculated for C₁₀₁H₁₄₇N₂₆O₃₅S 2316.0, found 2316.3.

Hyp- α 1 α 2(Acm)-PEGA. Hyp- α 1 α 2(Acm)-PEGA was prepared as described for Pro- α 1 α 2(Acm)-PEGA starting with Hyp- α 2(*p*-Npys, Acm)-PEGA (8.3 μ mol) and Hyp- α 1 (13 μ mol): MS (MALDI-TOF) [M+H]⁺ (average) calculated for C₂₁₀H₃₀₄N₅₅O₇₃S₃ 4863.2, found 4864.6.

Hyp- α 1 α 2(*o*-Npys)-PEGA. Hyp- α 1 α 2(*o*-Npys)-PEGA was prepared as described for Pro- α 1 α 2(*o*-Npys)-PEGA starting with Hyp- α 1 α 2(Acm)-PEGA (8.3 μ mol): MS (MALDI-TOF) [M+H]⁺ (average) calculated for C₂₁₂H₃₀₁N₅₆O₇₄S₄ 4946.2, found 4948.6.

Fragment 2 (Hyp- α 1 α 2 α 1). Fragment **2** was prepared and purified as described for fragment **1**, starting with Hyp- α 1 α 2(*o*-Npys)-PEGA (8.3 μ mol) and Hyp- α 1 (13 μ mol) to give **2** (16.5 mg, 28% overall): MS (MALDI-TOF) [M+H]⁺ (average) calculated for C₃₀₈H₄₄₃N₈₀O₁₀₇S₄ 7106.5, found 7109.0; HPLC *t*_R 13.88 min (Aligent C8 column, 90:10–10:90 H₂O/CH₃CN containing TFA (0.1% vol/vol) over 60 min).

Estimation of Assembly Length from R_h

The lengths of assemblies (**1**)_n and (**2**)_n were estimated from the R_h values measured with DLS by using the Broersma relations (Eq. 1) (2–4) and Tirado and Garcia de la Torre relations (Eq. 2) (5, 6), as described for collagen (7).

Broersma (2–4) relations:

$$\begin{aligned} 0 &= 2R_h[\delta - (\frac{1}{2})(\gamma_{\parallel} + \gamma_{\perp})] - L \\ \delta &= \ln(2L/d) \\ \gamma_{\parallel} &= 0.807 + 0.15/\delta + 13.5/\delta^2 - 37/\delta^3 + 22/\delta^4 \\ \gamma_{\perp} &= -0.193 + 0.15/\delta + 8.1/\delta^2 - 18/\delta^3 + 9/\delta^4 \end{aligned} \quad (1)$$

Tirado and Garcia de la Torre (5–6) relations:

$$\begin{aligned} 0 &= 2R_h[(\ln(p) + v)] - L \\ p &= L/d \\ v &= 0.312 + 0.565p^{-1} - 0.1000p^{-2} \end{aligned} \quad (2)$$

In Eqs. 1 and 2, L is the rod length and d is the rod diameter. After assuming that $d = 1.36$ nm for collagen (8, 9), Eqs. 1 and 2 were solved for L with the FindRoots algorithm of Igor Pro (Wavemetrics).

References

1. Jenkins, C. L., Vasbinder, M. M., Miller, S. J. & Raines, R. T. (2005) *Org. Lett.* **7**, 2619–2622.
2. Broersma, S. (1960) *J. Chem. Phys.* **32**, 1626–1631.
3. Broersma, S. (1960) *J. Chem. Phys.* **32**, 1632–1635.
4. Broersma, S. (1981) *J. Chem. Phys.* **74**, 6989–6990.
5. Tirado, M. M. & Garcia de la Torre, J. (1979) *J. Chem. Phys.* **71**, 2581–2587.
6. Tirado, M. M. & Garcia de la Torre, J. (1980) *J. Chem. Phys.* **73**, 1986–1993.
7. Claire, K. & Pecora, R. (1997) *J. Phys. Chem. B* **101**, 746–753.
8. Boedtker, H. & Doty, P. (1956) *J. Am. Chem. Soc.* **78**, 4267–4280.
9. Boudko, S., Frank, S., Kammerer, R. A., Stetefeld, J., Schulthess, T., Landwehr, R., Lustig, A., Bächinger, H. P. & Engel, J. (2002) *J. Mol. Biol.* **317**, 459–470.

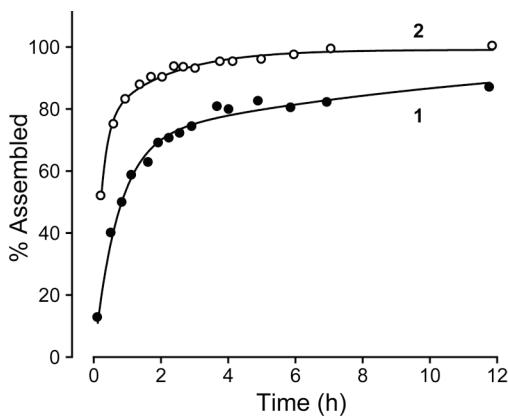


Fig. 5. Rate of assembly of fragments **1** and **2** (200 μ M) over 12 h, monitored at 226 nm after a rapid transition from 90 to 10°C. The maximal change in ellipticity ($[\Theta]_{\max}$) for each sample was calculated as the difference between the ellipticity prior to denaturation (100% assembled) and the first data point at 10°C, thus omitting the initial jump in ellipticity (which was not resolvable) (9). The value of “% assembled” was then calculated as the ratio of the observed recovery to $[\Theta]_{\max}$. Data were from solutions in 50 mM HOAc(aq) at pH 2.9.

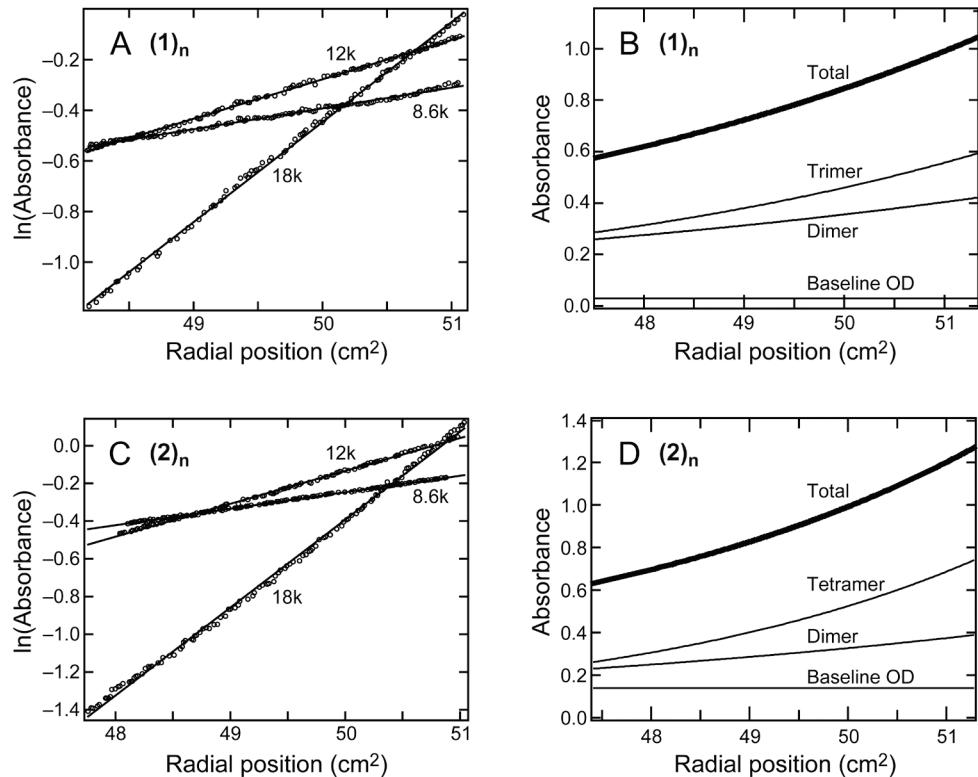


Fig. 6. Sedimentation equilibrium data for assemblies of fragments **1** and **2**. Data were obtained by analytical ultracentrifugation of fragments (30 μM) in 50 mM potassium phosphate buffer at pH 2.9. (A),(C) \ln plots of data for $(1)_n$ and $(2)_n$, respectively, recorded at three speeds at 4°C. (B),(D) Species plots of $(1)_n$ and $(2)_n$ data, respectively, derived from analyzing the 12k data as a combination of species from monomer to hexamer.

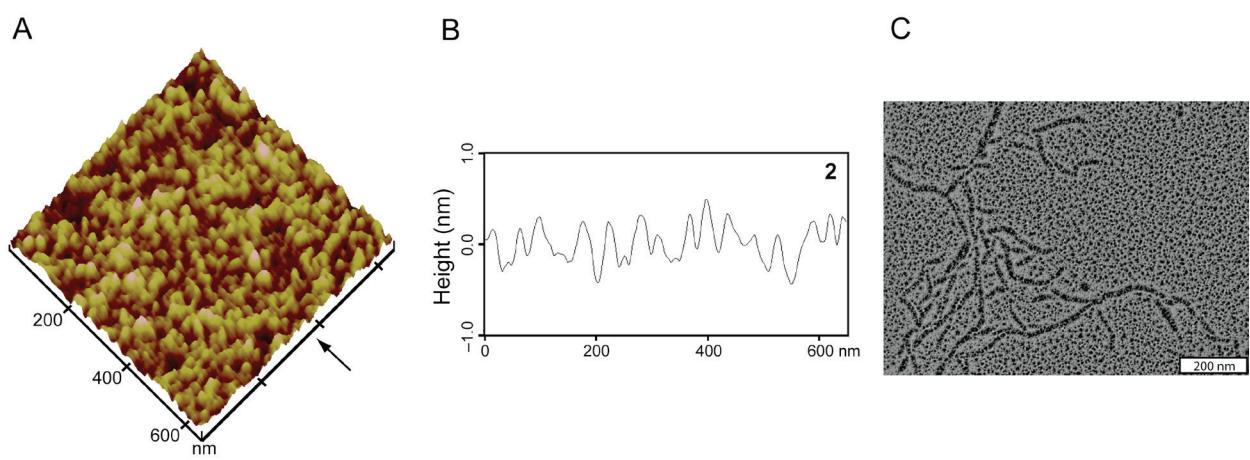


Fig. 7. AFM and TEM images of natural type I collagen. (A) AFM height image. (B) Section analysis of data along the direction indicated by the arrow in Panel A. (C) TEM image after rotary shadowing with platinum.

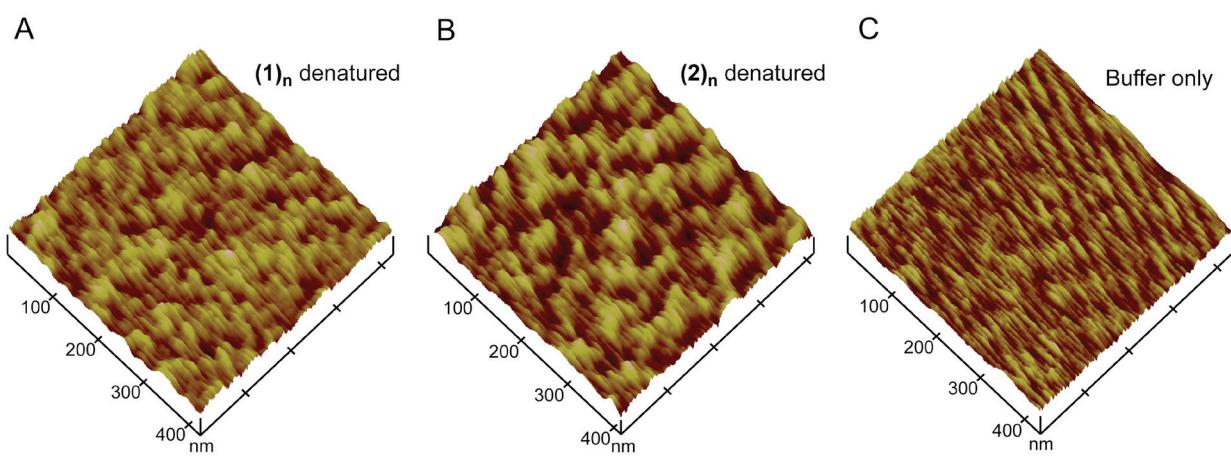


Fig. 8. AFM images of control samples. (A) Height image of **1** after assembly denaturation (90°C for 15 min). (B) Height image of **2** after assembly denaturation (90°C for 15 min). (C) Height image of 50 mM HOAc(aq) without **1** or **2**.